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ORIGINAL INVESTIGATION

Subversion of early innate antiviral responses during antibody-dependent enhancement of *Dengue virus* infection induces severe disease in immunocompetent mice

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Abstract Dengue is a mosquito-borne disease caused by one of four serotypes of *Dengue virus* (DENV-1–4). Epidemiologic and observational studies demonstrate that the majority of severe dengue cases, dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS), occurs predominantly in either individuals with cross-reactive immunity following a secondary heterologous infection or in infants with primary DENV infections born from dengue-immune mothers, suggesting that B-cell-mediated and antibody responses impact on disease evolution. We demonstrate here that B cells play a pivotal role in host responses against primary DENV infection in mice. After infection, $\mu MT^{-/-}$ mice showed increased viral loads followed by severe disease manifestation characterized by intense thrombocytopenia, hemoconcentration, cytokine production and massive liver damage that culminated in death. In addition, we show that poly and monoclonal anti-DENV-specific antibodies can sufficiently increase viral replication through a suppression of early innate antiviral responses and enhance disease manifestation, so that a mostly non-lethal illness becomes a fatal disease resembling human DHF/DSS. Finally, treatment with intravenous immunoglobulin containing anti-DENV antibodies confirmed the potential enhancing capacity of

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subneutralizing antibodies to mediate virus infection and replication and induce severe disease manifestation of DENV-infected mice. Thus, our results show that humoral responses unleashed during DENV infections can exert protective or pathological outcomes and provide insight into the pathogenesis of this important human pathogen.

Keywords *Dengue virus* · Antibody-dependent enhancement · DHF/DSS · Type I interferons · Humoral response against DENV

Introduction

Dengue is an important emerging infectious disease, consisting in a major public health concern. It is defined as an acute systemic viral infection transmitted to humans by *Aedes* mosquitoes, with a wide spectrum of clinical presentations, varying from an asymptomatic or a self-limited illness, called dengue fever (DF), to the potentially lethal forms, dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS) [1–3]. Bhatt and colleagues showed using updated cartographic approaches that there are approximately 390 million dengue infectious cases per year around the world, of which 96 million manifest some level of disease severity [4]. These numbers are even higher that those related previously, which suggest the occurrence of 230 million human infections annually, of which 500,000 are severe and 25,000 are fatal, across approximately 100 endemic countries [3, 5, 6]. Recently, a new dengue serotype, DENV-5, was discovered and characterized from samples collected during an outbreak in Malaysia's Sarawak state in 2007 [7]. This discovery brings even more challenges in the search for new targeted therapies and vaccines against this important human disease.

The pathogenesis of DENV infection remains poorly understood and involves a complex interplay between viral and host factors [1, 2, 8]. Severe forms of disease can be seen during primary infections [9, 10]; however, epidemiological and observational studies demonstrate that the majority of DHF/DSS cases occur predominantly in either individuals with secondary heterologous DENV infections or in infants born from DENV-immune mothers [5, 11–14]. While the exact mechanism of this phenomenon remains to be fully elucidated, several hypotheses have been raised to explain the reason for the exacerbated pathology found in these patients. The antibody-dependent enhancement of infection (ADE) theory postulates that after an initial period of cross-reactive protection, antibodies from the first infection remain cross-reactive with other DENV serotypes, but have waned to subneutralizing or non-neutralizing levels. These non-neutralizing antibodies could then lead to viral internalization via the Fc portion of immunoglobulin G receptors (FcγRs) and increase virus replication into phagocytic cells, in which is accompanied by massive release of soluble factors that could account for the increased vascular permeability and hemostatic disorder found in severe cases [2, 15, 16].

Interestingly, it has been demonstrated that ADE does not only facilitate the process of viral entry into monocytes and macrophages but can also modify innate and adaptive intracellular antiviral mechanisms, through suppression of intracellular antiviral immune responses, a phenomenon defined as "intrinsic ADE" (iADE) [17]. These phenomena suggest that the elevated intracellular DENV production is a result of idiosyncratic Fc-receptor signaling. DENV immunocomplexes activate suppressive antiviral pathways, and the final outcome is a marked decrease in the production of type I IFNs as well as the interferon-activated antiviral molecules (e.g., ISGs). Concomitantly, there is upregulation of anti-inflammatory mediators, including the cytokine IL-10 [18, 19]. Similar responses have been observed in samples of DHF patients as compared to DF or dengue-like syndrome individuals [19, 20]. Overall, these events contribute to the elevated intracellular production of the virus.

A distinct but complementary theory of immunopathology involves reactivation of cross-reactive memory B and T cells specific for the previous rather than the current DENV infection, resulting in delayed viral clearance and/or massive production of effectors mediators, a so-called cytokine storm [21, 22]. While human T-cell responses during acute DENV infections have been largely studied [16, 23–26], much less is known about the B cell and antibody responses to *Dengue viruses* infections. Early studies of Boonpucknavig et al. [27] have demonstrated that B lymphocytes are increased during the acute phase of illness after a secondary dengue infection. Another study has also found that DHF patients had significantly higher $CD19^{+}$ B cells than dengue-like syndrome patients on the day of defervescence and 1 day after defervescence [28]. Most recently, studies have focused on virus-specific plasmablast responses after primary and secondary DENV infections. These studies demonstrate that rapid and massive virus-specific plasmablast responses were found in patients with acute DENV infection, especially in individuals manifesting DHF [29, 30]. In experimental settings, B-cell responses were shown to be unnecessary for the control of primary DENV infection in AG129 mice [31]. However, protective cross-reactive antibodies secreted by both long-lived plasma cells and memory B cells and both cross-reactive B and T cells were shown to provide protection against a secondary heterotypic DENV infection [32]. From the later findings, it seems that B cells play negligible roles in host protection to primary DENV infection, but may be involved in protection or disease evolution during secondary infections. However, studies are still needed to pinpoint the circumstances

involved in the protective or disease-enhancing activities of B cells and their products during this important infectious disease.

In the present study, we first decided to investigate the role played by B cells and antibodies during primary and secondary *Dengue virus* infection in mice. We conducted experiments in mice infected with adapted strains of DENV-2 and DENV-3 that were previously shown to induce in immunocompetent mice a disease that resembles the severe dengue manifestations found in humans [33–35]. We demonstrate that, although B cells are pivotal for host resistance to primary DENV infection, passive transfer of anti-DENV-immune serum or subneutralizing monoclonal antibodies leads to the enhancement of DENV infection and replication as well as the occurrence of severe disease manifestations in mice. In addition, we show that these disease-enhancing activities were dependent on FcγRs activation and consequent suppression of early innate immune antiviral responses in host-infected tissues. Finally, we demonstrate the potential deleterious effects of IVIG during the course of DENV infection, once administration of IVIG containing anti-DENV antibodies to primarily infected mice led to increased lethality rates and to worsening of clinical signs of disease due to enhancement of virus replication.

Materials and methods

Ethics statement

This study was carried out in strict accordance with the Brazilian Government's ethical and animal experiments regulations (Law 11794/2008). The experimental protocol was approved by the Committee on the Ethics of Animal Experiments of the Universidade Federal de Minas Gerais (CETEA/UFMG, Permit Protocol Number 113/09). All surgeries were performed under ketamine/xylazine anesthesia, and all efforts were made to minimize animal suffering.

Animals

B-cell-deficient mice (congenic μMT mice, strain B6-Igh6-6^{tm1Cgn}) were kindly provided by Dr. Thiago Matar Cunha and Dr. João B. Calixto (FMRP-USP, Brazil), and mice with null mutations for the IFNα/β receptors ($A129^{-/-}$ mice) were kindly given by Dr. Adriana Aballen (UFMG, Brazil). Wild-type controls on the C57BL/6J (WT) genetic background (back-crossed at least 10 times) were provided by Centro de Bioterismo—CEBIO, UFMG, BH, Brazil, and 129/Sv/Ev (WT129) were kindly given by Dr. Adriana Aballen (UFMG, Brazil). All mice were bred and maintained at the Gnotobiology and Immunology Laboratory of Instituto de Ciências Biológicas (ICB–UFMG). For experiments, 7–10-week-old mice were kept under specific pathogen-free conditions, in filtered cages with autoclaved food and water available ad libitum.

Cell lines, monoclonal antibodies and viruses

Vero cells were cultured in RPMI 1640 medium (Cultilab) supplemented with 5 % inactivated fetal bovine serum (Cultilab). The hybridoma of mAb 4G2 was purchased from ATCC, grown in serum-free medium (Cultilab) and purified using the method of ammonium sulfate precipitation, followed by phosphate-buffered saline (PBS) dialysis and protein-A/G affinity chromatography as described in [36]. As control, a mouse IgG2a isotype control mAb from Acris Gmbh, Germany, was used. The hybridoma of mAb clone 2.4G2 from ATCC was purchased from the Rio de Janeiro cell Bank, grown in serum-free medium (Cultilab) and purified as described previously. For in vivo experiments, DENV-2 and DENV-3 strains previously adapted to mice [34, 35], respectively, were propagated in Vero cells, and the supernatants of infected cells were harvested, filtered, tittered by plaque assay in Vero cells and stored at −70 °C until use. All in vivo studies with the infectious viruses were performed in a BSL-2 facility of the Laboratório de Interação Microrganismo-Hospedeiro—ICB–UFMG.

Experimental procedure

Infection of mice

For primary infection experiments, the virus stocks or mock control were diluted in endotoxin-free PBS (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl) and the virus inoculum "sublethal" or "lethal" of the adapted DENV-2 or DENV-3 (100 or 1000PFU), depending of the experiment, were injected via i.p route. As an additional control in some experiments, the virus was heat-inactivated (56 °C, 1 h) before inoculation into mice. The results were pooled with the NI (non-infected) group just for easing representation of the data.

Generation of α-DENV-3 serum

Six- to eight-week-old C57BL/6 mice were inoculated with 100 PFU of the adapted DENV-3 i.p. Pre-infection serum was collected on day zero before the infection of mice. Blood was obtained by tail vein bleeding every 7 days until day 63 post-DENV-3 inoculation. Serum was separated from whole blood by centrifugation, heat-inactivated and frozen at −80 °C until use.

Enhancement of Dengue virus infection and FcRs blockade in vivo

Mice were injected intraperitoneally with mAbs (4G2, 15 or 400 μg per treatment) or with the anti-DENV-3 sera (obtained at 49 day post-infection) in a total volume of 200 μL on days −1, +1 and +3 after *Dengue virus* inoculation and then infected 24 h later with a sublethal inoculum of the DENV-2 or DENV-3 by i.p injection in a total volume of 100 μL. IgG2a mAb was used as an isotype control of the anti-dengue 4G2 mAb, and the Fc-blocker mAb, clone 2.4G2 from ATCC, at dose of 500 μg per/mouse given daily was used in some experiments.

IVIG therapy administration to mice

Mice were administered with different doses (30, 100 or 300 mg/kg) of the 5 % IVIG solution from pooled donors from an endemic area for *Dengue virus* circulation produced by HEMOPE, Recife, Brazil. The administration via i.v route in a final volume of 200 μL per mouse per treatment occurred on days −1, +1 and +3 after adapted DENV-3 inoculation to mice.

Titration of virus by plaque assay

Mice were assayed for viral titers in blood, spleen and liver. Blood samples (50 μL) were collected in heparinized tubes, diluted in 450 μL of endotoxin-free PBS (3.2 mM Na_2HPO_4, 0.5 mM KH_2PO_4, 1.3 mM KCl, 135 mM NaCl) and stored at −70 °C. For virus recovery from spleen and liver, the organs were collected aseptically in different time points and stored at −70 °C until assayed for DENV-2 or DENV-3 presence. Tissue samples were weighed grounded using a pestle and mortar and prepared as 10 % (w/v) homogenates in RMPI 1640 medium without fetal bovine serum (FBS). Viral load in supernatants of tissue homogenates and blood samples were assessed by direct plaque assay using Vero cells as described in [35]. Results were measured as plaque-forming units (PFU) per gram of tissue weight or per mL of blood. The limit of detection of the assay was 100 PFU/g of tissue or per mL.

Plaque reduction neutralization test in Vero cells

PRNT assays were performed in duplicate based on the original protocol described by [37]. Briefly, complement was inactivated by incubating serum in a 56 °C water bath for 30 min; then, 5 serial twofold dilutions of serum were prepared, starting at 1:20, in RPMI medium (Cultilab) with 5 % fetal bovine serum (FBS; Cultilab) and 100U penicillin/100 μg streptomycin (P/S; Invitrogen). Working stocks of virus were prepared that yielded 15–30 plaques/well in a 24-well tissue culture plate. Viruses used for PRNT tests were the adapted DENV-2 and DENV-3 [34, 35], respectively. Sixty microliters of each serum dilution was combined with 60 μL of virus and incubated for 90 min at 37 °C with 5 % CO_2. After incubation, 100 μL of the virus–serum mixture was transferred to 80 % confluent Vero cells and processed as in a standard plaque assay. Fifty percent neutralization titer (PRNT50) values was determined as the highest serum dilution capable of neutralizing 50 % virus infection, as detected by the inhibition of plaque formation in cell cultures.

Evaluation of blood parameters

Blood was obtained from the cava vein in heparin-containing syringes at the indicated time points under ketamine and xylazine anesthesia (150 mg/Kg and 10 mg/Kg, respectively). The final concentration of heparin was 50 U/mL. Serum was obtained from tail vein bleed. Platelets were counted in a Neubauer chamber. Briefly, 10 μL of solution (blood and 1 % p/v ammonium oxalate in a dilution of 1:100) was placed in the chamber, and platelets were visualized in a Nikon XP-1000 microscope, magnification of 400×, using phase-contrast. Results are presented as number of platelets per μL of blood. For the determination of the hematocrit, a sample of blood was collected into heparinized capillary tubes (Perfecta) and centrifuged for 10 min in a hematocrit centrifuge (Fanem, São Paulo, Brazil).

Anti-dengue IgM and IgG quantification

Antibody quantification in serum from DENV-3-infected mice was performed by an indirect ELISA assay as adapted from [38]. Briefly, the same adapted DENV-3 used in infections was UV-inactivated (60’) and diluted in 0.01 M carbonate buffer (pH 9.6) in a concentration of 1×10^6 PFU per well of microtiter plates and incubated overnight. Plates were washed three times and blocked with bovine serum albumin 1 % for 2 h and subsequently washed. Then, four dilutions of each serum sample from 1:50 to 1:6,250 were plated in duplicates and incubated for 3 h. After another wash step, plates were incubated for 2 h with peroxidase-conjugated anti-mouse IgG or IgM (Southern Biotech). Toward, ortho-phenylenediamine was used as a substrate, and the reaction was stopped with 1 M sulfuric acid. The absorbance was measured at 492 nm. Samples of DENV-3-infected mice were considered positive in the first dilution in which mock samples were negative and were expressed as optical densities (O.D.).

Measurement of cytokines/chemokine concentrations

The concentration of cytokines TNF-α, IFN-γ in serum or IL-12p40, IL-10 and IFN-γ in spleen samples was measured using commercially available antibodies and according to the procedures supplied by the manufacturer (R&D

Systems, Minneapolis). Results are expressed as pg/mL or pg/100 mg of tissue. The detection limit of the ELISA assays was in the range of 4–8 pg/mL.

ALT transaminase activity

The activity of the transaminase alanine aminotransferase (ALT) was measured in individual serum samples, using a commercially colorimetric available kit (Bioclin, Quibasa, Belo Horizonte, Brazil). Results are expressed as the mean ± SEM of transaminase concentration in U/dL of plasma.

Evaluation of changes in vascular permeability

The extravasation of Evans blue dye into the tissue was used as an index of increased vascular permeability, as previously described in [39]. Briefly, Evans blue (20 mg kg^{-1}) was administered i.v. (1 mL kg^{-1}) via an eye vein 30 min prior to mice killing. The right ventricle was flushed with 10 mL of PBS to wash the intravascular Evans blue in the lungs. After that, one lobe of liver and the left lung were cut and allowed to dry in a Petri dish for 24 h at 37 °C. The dry weight of the tissue was calculated and Evans blue extracted using 1 mL of formamide (24 h at room temperature). The amount of Evans blue in the tissue was obtained by comparing the extracted absorbance with that of a standard Evans blue curve read at 620 nm in an ELISA plate reader. Results are presented as the amount of Evans blue per 100 mg of tissue.

Hemodynamic measurements

All mice were habituated to the blood pressure measurement device for 7 days. SBP was determined with tail-cuff plethysmography method in unanesthetized mice, as previously described in [40]. All data are expressed as mean ± SEM. Changes in SBP from baseline are expressed as absolute values as well as areas under the BP curves.

Real-time PCR

For the evaluation of IFN-α2, IFNα-4 and ISG15 mRNA expression, spleens were removed 4 and 7 days after DENV-3 inoculation into mice. Total RNA was isolated from tissues using a QIAGen RNEasy RNA isolation kit. The RNA obtained was resuspended in nuclease-free water (GIBCO) and stocked at −70 °C until use. Real-time RT–PCR was performed on a Applied Biosystems 7500 sequence-detection system (Applied Biosystems) using SYBR Green PCR Master Mix (Applied Biosystems) after a reverse transcription reaction of 2 μg of total RNA by using M-MLV reverse transcriptase (Promega) and 50 μM of Oligo dT(15) (Promega). The relative level of gene expression was determined by the comparative threshold cycle method as described by the manufacturer, whereby data for each sample were normalized by 18S ribosomal RNA and expressed as a fold change compared with non-infected controls. The following primer pairs were used: *18S ribosomal RNA*, 5′-CGTTCCACCAACTAAGAACG-3′ (forward) and 5′-CTCAACACGGGAAACCTCAC-3′ (reverse); and *IFNα2* 5′-GGACAGGCAGGACTTTGGATT-3′ (forward) 5′-GCCTTCTGGATCTGCTGGTAA 3′ *IFNα4* 5′-CCACAGCCCAGAGAGTGACCAGC-3′ (forward) 5′-AGGCCCTCTTGTTCCCGAGGTTA-3′ (reverse) *ISG15* 5′-CTGCAGCAATGGCCTGGGACCT-3′ (forward) 5′-AGTTTGGTGGGCCAGGCGCT-3′.

Histopathological and immunohistochemistry analysis

Liver samples from adult euthanized mice were obtained at the indicated time points. Afterward, they were immediately fixed in 10 % buffered formalin for 24 h and embedded in paraffin. Tissue sections (4 μm thicknesses) were stained with hematoxylin and eosin (H&E) and evaluated under a microscope Axioskop 40 (Carl Zeiss, Göttingen, Germany) adapted to a digital camera (PowerShot A620, Canon, Tokyo, Japan). Histopathology score was performed according to [35] evaluating hepatocyte swelling, degeneration, necrosis and hemorrhage, added to a five-point score (0, absent; 1, minimal; 2, slight; 3, moderate; 4, marked; and 5, severe) in each analysis. For easy interpretation, the overall score was taken into account and all the parameters totalized 20 points. A total of two sections for each animal were examined, and results were plotted as the media of damage values in each mouse. Immunohistochemistry analysis for detection and quantification of DENV-3-infected cells in liver was also performed as previously described in [35].

Statistical analysis

Results are shown as mean ± SEM percent inhibition was calculated by subtracting the background values obtained in non-infected animals. Differences were compared using analysis of variance (ANOVA) followed by Student–Newman–Keuls post hoc analysis. Differences between lethality curves were calculated using log-rank test (Graph Prism Software 4.0). Changes in SBP from baseline are expressed as absolute values as well as areas under the BP curves. Results with a $P < 0.05$ were considered significant.

Results

B cells are necessary for host resistance to primary *Dengue virus* infection

Humoral immune responses are thought to play a major role during DENV-induced disease in humans [30]. To

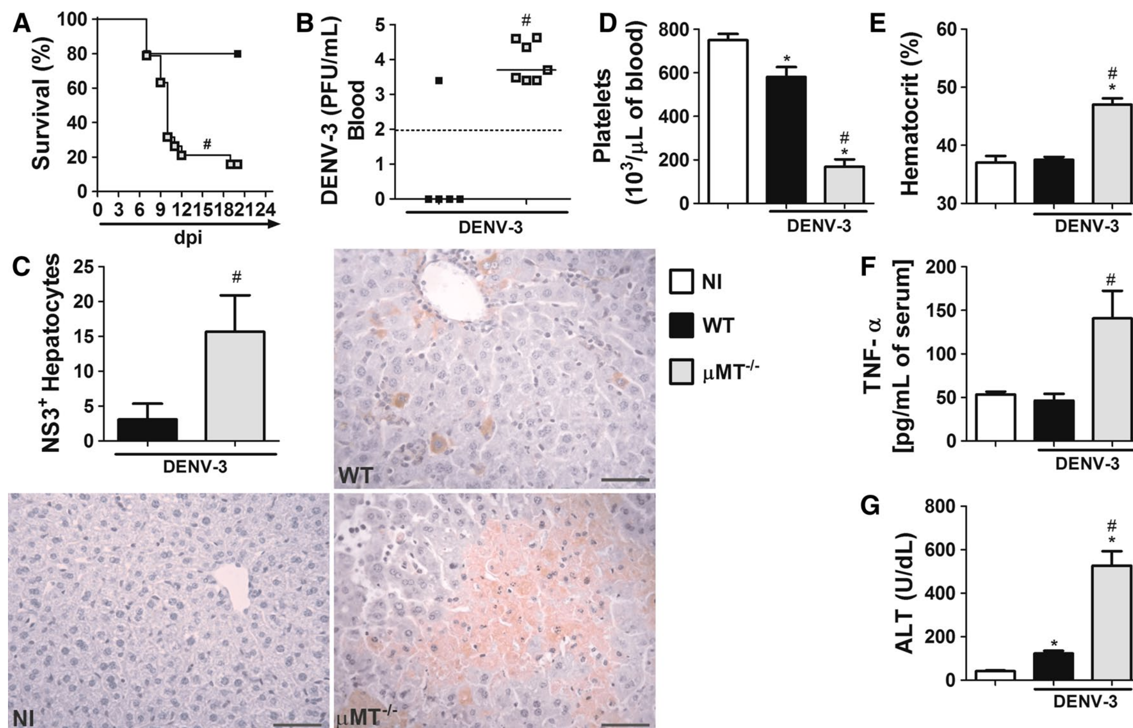

Fig. 1 B-cell-deficient ($\mu MT^{-/-}$) mice are more susceptible to primary *Dengue virus* infection. **a** WT and $\mu MT^{-/-}$ mice ($n = 8$ mice per group) were inoculated with 100PFU of DENV-3 (i.p), and lethality rates were evaluated every 12 h during 21 days. Results are expressed as % of survival. In **b–g,** WT ($n = 5$) and $\mu MT^{-/-}$ mice ($n = 7$) were inoculated with 100PFU of DENV-3 (i.p), and 7 days after infection, mice were culled and blood and tissue collected for the following analysis: **b** Viral loads recovered from blood by plaque assay in Vero cells. Results are shown as the log of PFU per mL of blood. **c** Quantification of NS3-positive hepatocytes in the liver of WT and $\mu MT^{-/-}$ mice. Liver was collected, formalin-fixed and processed into paraffin sections. Serial sections from each tissue were stained with anti-DV NS3 antibody E1D8 (NS3) or an isotype control mouse IgG2a, and multiple sections of each tissue type were thoroughly examined for staining. Positive staining for NS3 is *brown,* while hematoxylin counterstain is blue. **d** Number of platelets, shown as platelets $\times 10^3/\mu L$ of blood. **e** Hematocrit, shown as % volume occupied by red blood cells. **f** Concentrations of TNF-α in serum, quantified by ELISA. Results are shown as pg per mL (serum). **g** ALT activity determination in plasma of control and DENV-3-infected mice is shown as U/dL of plasma. All results are expressed as mean ± SEM (except for B, expressed as median) and are representative of at least two experiments. $^*P < 0.05$ when compared to control uninfected mice. $^\#P < 0.05$ when compared to WT-infected mice. *NI* non-infected, *dpi* days post-infection (color figure online)

investigate in vivo the role played by B cells during DENV infection, wild-type (WT) and B-cell-deficient ($\mu MT^{-/-}$) mice were inoculated with 100 PFU of adapted DENV-3 i.p and mortality rates and disease parameters on day 7 after DENV inoculation (peak of disease) were evaluated. After infection, about 80 % of $\mu MT^{-/-}$ mice were dead at day 12 of infection, while only 20 % of WT mice had succumbed to infection after 21 days of inoculation of DENV-3 (Fig. 1a). The earlier lethality of $\mu MT^{-/-}$ mice was associated with increased DENV-3 replication in the spleen (WT: 3.3×10^7 PFU/g ($n = 5$); $\mu MT^{-/-}$: 2×10^8 PFU/g of spleen ($n = 7$), $P = 0.01$) and elevated viremia as assessed on day 7 after virus inoculation (Fig. 1b). Moreover, there was enhancement of $NS3^+$ staining of hepatocytes in the liver of infected $\mu MT^{-/-}$ mice when compared with WT (Fig. 1c). In addition to the increased lethality rates and to the enhanced viral replication, $\mu MT^{-/-}$ mice presented more severe manifestation of disease after infection (Fig. 1d–g and S1). After DENV-3 inoculation, WT mice presented only mild disease manifestation as demonstrated by slight drop in platelets numbers (Fig. 1d), absence of hemoconcentration (Fig. 1e), no increase in TNF-α levels in serum (Fig. 1f) and only a slight increase in ALT transaminase levels in serum (Fig. 1g) when compared to non-infected (NI) mice. However, DENV-inoculated $\mu MT^{-/-}$ mice had greater thrombocytopenia (Fig. 1d), increased hematocrit levels, a marker of hemoconcentration (Fig. 1e) and elevated levels of the proinflammatory cytokine TNF-α in the serum (Fig. 1f). Levels of IFN-γ were elevated to a similar extent in serum of infected $\mu MT^{-/-}$ and WT mice (NI: ND; WT: 1.851.8 ± 493.2; $\mu MT^{-/-}$: 2.048.7 ± 819.3), suggesting that the enhanced DENV replication in $\mu MT^{-/-}$ mice was not due to impairment of the production of this mediator. Finally, infection of $\mu MT^{-/-}$ mice resulted in significant increase in serum levels of ALT transaminase at day 7 after virus inoculation

(Fig. 1g), an indicative of liver damage. To confirm this, we performed histopathological analysis in the liver of WT and $\mu MT^{-/-}$ mice after DENV-3 inoculation, and the results revealed an elevated histopathological score in $\mu MT^{-/-}$ mice in comparison with the WT-infected controls (Figure S1). Thus, the data depicted here demonstrate a protective role of B cells during primary DENV infection.

Subneutralizing levels of anti-DENV antibodies enhance disease severity during *Dengue virus* infection of immunocompetent mice

We have demonstrated that B cells are necessary for host resistance against primary *Dengue virus* infection; however, it has been shown that people experiencing a secondary heterologous DENV infection have a much greater risk of developing a more severe disease manifestation (DHF/DSS), indicating that preexisting immunity could exacerbate disease [41]. Thus, B-cell activation and antibody production can exert a dual role in DENV pathogenesis, with potential to benefit or harm the host [11]. In order to evaluate whether antibodies would be involved in the protective role played by B cells during primary DENV infection, we have conducted experiments in mice injected with anti-DENV-immune serum. To this end, we have first performed a longitudinal analysis of the antibody response of mice primarily inoculated with DENV-3 starting on day 0 until day 63 after virus inoculation (Fig. 2a). WT mice ($n = 6$) were inoculated with a sublethal inoculum of DENV-3 (100 PFU) and bleeding was performed every 7 days after DENV-3 infection until day 63 post-DENV-3 inoculation. Our data demonstrated an increased DENV-specific IgM response at days 14 and 21 of infection. After day 28, the levels of IgM returned to basal values as found in day 0 of infection. Further, IgG responses raised on day 14 after DENV-3 inoculation and remained elevated until the last day evaluated (day 63 after *Dengue virus* inoculation) (Fig. 2a). These data demonstrated that WT mice inoculated with DENV-3 were capable of mounting a specific-α-DENV IgM and IgG responses at the course of DENV infection.

To investigate the role of these antibodies in the context of DENV infection, WT mice were passively transferred with 200μL of the DENV-3-immune serum (collected at day 49 of infection) at days −1, +1 and +3 after homologous (DENV-3) (Figure S2A) or heterologous (DENV-2) inoculation (Fig. 2b–h). Figure S2A and 2B shows that mice who received the DENV-3-immune serum and were challenged with either of DENV serotypes (DENV-3 or DENV-2, respectively) presented elevated lethality rates in comparison with mice that received only serum from naïve mice (Figure S2A and 2B) or serum from $\mu MT^{-/-}$ mice previously inoculated with a sublethal inoculum of DENV-3 (*data not shown*). After infection, only 25–30 % of the mice that had received the naïve serum (Fig. 2b and S2A) or serum from $\mu MT^{-/-}$ mice (*data not shown)* were dead until the day 14 of DENV-2 inoculation. However, on day 6 of infection, 75 % of the mice inoculated with the homologous serotype (DENV-3) (Figure S2A) and 100 % of the mice infected with the heterologous serotype (DENV-2) had succumbed to the infection (Fig. 2b). Of note, the remaining 25 % of mice that received the α-DENV-3 serum and were infected with the homologous serotype were protected from lethality (Figure S2A) and from disease manifestation *(data not shown)*. Since the transference of the DENV-3-immune serum was not protective from lethality of DENV-3-infected mice, we decided to evaluate the neutralizing capacity of this α-DENV-3 serum to neutralize DENV-3 or DENV-2 infections in vitro. Heat-inactivated α-DENV-3 serum exhibited low neutralizing titers after a plaque reduction assay in Vero cells *(data not shown)*. These data suggest the presence of non-neutralizing or subneutralizing levels of α-DENV-3 and α-DENV-2 antibodies, suggesting the occurrence of ADE after passive transfer of DENV-immune serum.

The hallmark of ADE-induced disease is an increase in systemic viral burden after infection. Hence, after DENV-2 infection of mice previously administered with α-DENV-3 serum, there was a remarkable increase in viral load in spleen (naïve serum = 2.2 × 10.6 PFU/g ($n = 4$) and α-DENV-3 serum = 6.1 × 10.7 PFU/g of spleen ($n = 5$) $P = 0.01$) and viremia (Fig. 2c) as assessed by plaque assay on the peak of disease manifestation after DENV-2 inoculation. In addition, Panel 2D shows elevated numbers of $NS3^{+}$ hepatocytes in the liver of mice that received DENV-3-immune serum and were challenged with the DENV-2 in comparison with mice treated with naïve serum and that were equally infected with 100 PFU of DENV-2 (Fig. 2d). Corroborating these findings, the increased lethality and the enhanced viral burden found in mice that received the α-DENV-3 serum was associated with more severe disease manifestation after DENV-2 challenge as demonstrated by the occurrence of marked thrombocytopenia (Fig. 2e), intense hemoconcentration (Fig. 2f), production of elevated levels of TNF-α (Fig. 2g) and higher levels of the ALT transaminase in serum (Fig. 2h). In accordance with the elevated levels of ALT, there was heightened liver damage in the liver sections of mice that received α-DENV-3 serum (Figure S2B). These results demonstrated that homologous or heterologous α-DENV serum transference, at subneutralizing levels, can enhance the viral burden of mice inoculated with a sublethal inoculum of DENV, resulting in increased lethality and more severe disease manifestation.

To test whether the levels of anti-DENV neutralizing antibodies would directly impact on severe dengue disease evolution during infection, we conducted experiments with

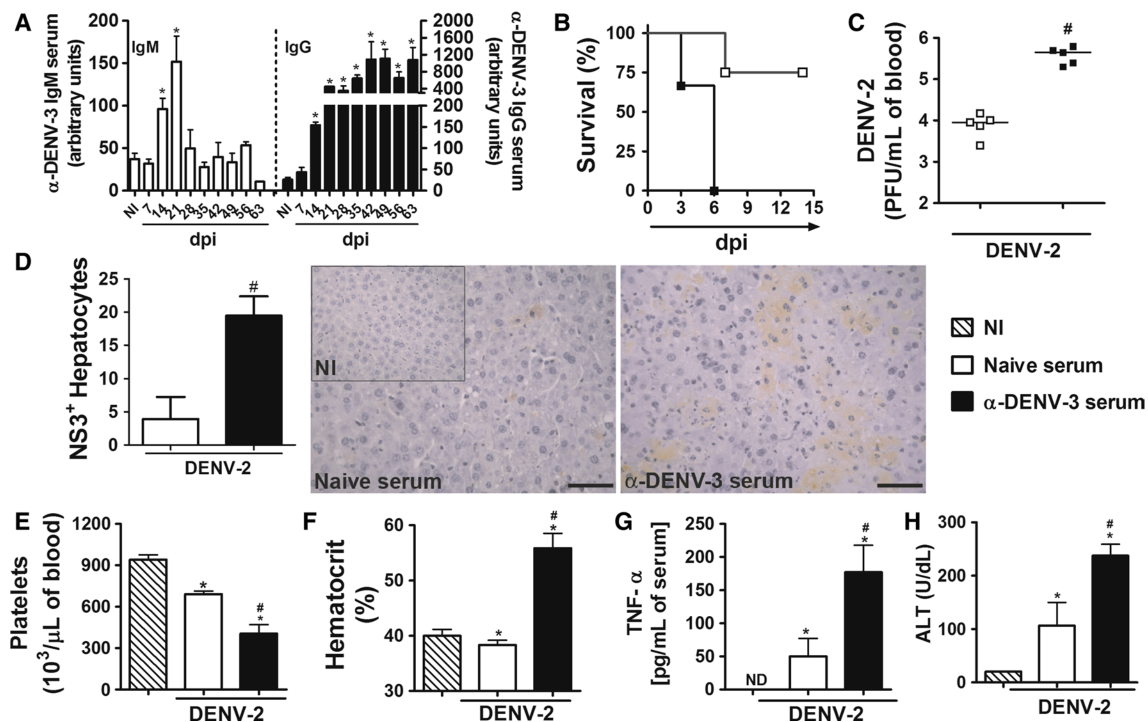

Fig. 2 Passive transference of DENV-3-immune serum decreases survival time and enhances *Dengue virus* replication and disease manifestation in DENV-2-inoculated mice. **a** WT mice ($n = 6$) were inoculated with 100PFU of DENV-3 (i.p) and anti-dengue IgM and IgG titers of pre- and post-infection serum samples, collected on day zero and every 7 days until day 63, were measured by ELISA. Results are expressed as arbitrary units. **b** WT mice ($n = 8$ mice per group) were administered with naïve serum or anti-DENV-3 serum (collected on day 49) and inoculated with 100PFU of DENV-2 (i.p), and lethality rates was evaluated every 12 h during 14 days. Results are expressed as % of survival. In **c–h,** WT mice ($n = 5$ per group) treated with naïve or anti-DENV-3 serum were inoculated with 100PFU of DENV-2 i.p), and seven days after infection, mice were culled and blood and tissue collected for the following analysis: **c** Viral loads recovered from blood by plaque assay in Vero cells. Results are shown as the log of PFU per mL of blood. **d** Quantification of NS3-positive hepatocytes in the liver of WT mice administered or not with anti-DENV-3 serum. Liver was collected, formalin-fixed and processed into paraffin sections. Serial sections from each tissue were stained with anti-DV NS3 antibody E1D8 (NS3) or an isotype control mouse IgG2a, and multiple sections of each tissue type were thoroughly examined for staining. Positive staining for NS3 is *brown,* while hematoxylin counterstain is *blue*. **e** Number of platelets, shown as platelets × 10^3/μL of blood. **f** Hematocrit, shown as % volume occupied by red blood cells. **g** Concentrations of TNF-α in serum, quantified by ELISA. Results are shown as pg per mL (serum). **h** ALT activity determination in plasma of control and DENV-3-infected mice is shown as U/dL of plasma. All results are expressed as mean ± SEM (except for **c**, expressed as median) and are representative of two experiments. $^{*}P < 0.05$ when compared to control uninfected mice. $^{\#}P < 0.05$ when compared to WT-infected naïve serum treated mice. *NI* non-infected, *dpi* days post-infection (color figure online)

commercial anti-DENV mAbs, assessing whether different antibody doses would impact differently in disease manifestation. First, plaque reduction neutralizing titer assays ($PRNT_{50}$) were performed in Vero cells. Mice were administered with 15 or 400 μg of mouse anti-DENV monoclonal antibody clone 4G2 (IgG2a anti-Envelope protein [E], pan-flavivirus-reactive) or with an isotype control antibody with irrelevant specificity at the same doses. Eighteen hours after antibody administration, mice were bled and the $PRNT_{50}$ titers of pre-infection serum samples were performed. Results demonstrate that serum from mice that received the low dose of 4G2 antibody (15 μg) was unable to neutralize the DENV-3 infection in vitro. However, neutralizing titers were found in the serum of mice that were administered with the high dose of 4G2 antibody (400 μg) (*data not shown*). To determine, in vivo, the relationship between disease manifestation and the administered dose of anti-DENV antibody, WT mice received the mouse monoclonal antibody 4G2 at concentration of 15 μg/treatment or 400 μg/treatment daily (day −1, day +1 and day +3) after DENV-3 inoculation. Control mice received an IgG2a isotype antibody of irrelevant specificity at similar concentrations and treatment schedule. Figure 3a, b shows that mice that received the lower dose of 4G2 antibody and that were challenged with a sublethal inoculum of DENV-3 presented elevated viral replication as demonstrated by the presence of viremia 7 days after DENV-3 inoculation (Fig. 3a), as well intense $NS3^{+}$ hepatocytes staining, indicative of active virus replication (Fig. 3b, c). However, opposite results were found when mice received higher 4G2 doses. These mice presented reversion of antibody enhancement of infection as demonstrated by the absence of viremia (Fig. 3a) and marked reduction in

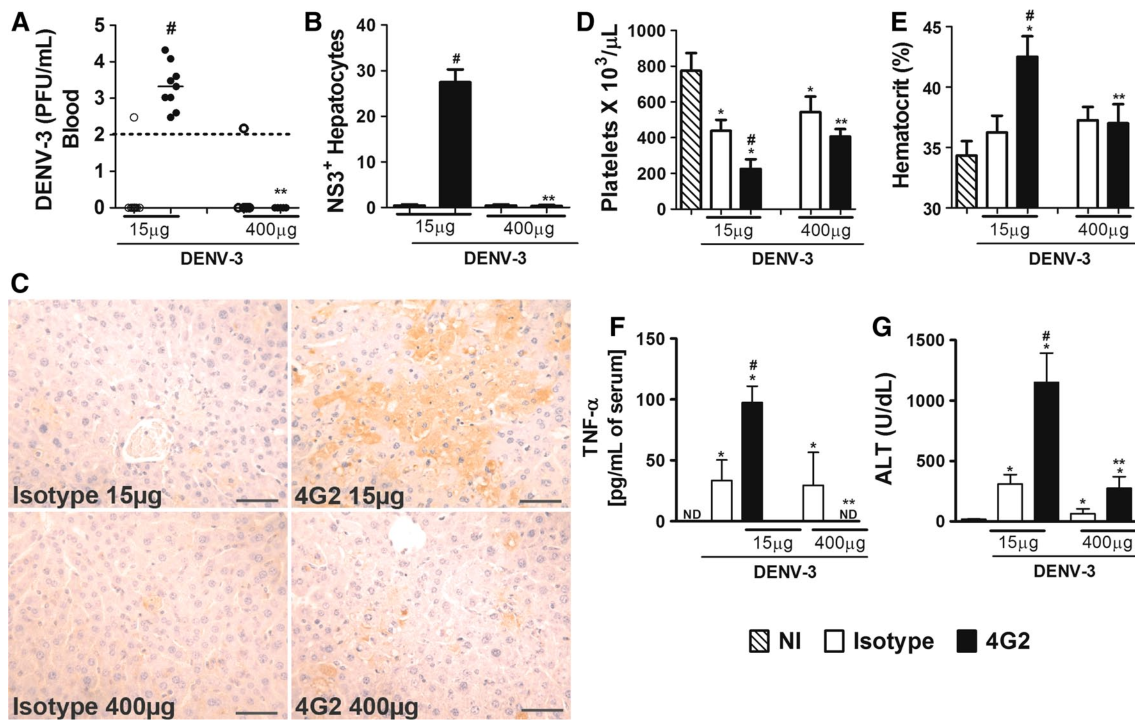

Fig. 3 Antibody-mediated enhancement of *Dengue virus* infection depends on antibody dose. WT mice ($n = 5$–9) were administered with (15 or 400 µg) of the mouse monoclonal antibody—4G2 (IgG2a anti-Envelope protein, pan-flavivirus-reactive) or with an isotype control antibody with irrelevant specificity followed by inoculation of 100PFU of DENV-3 (i.p). Seven days later, mice were culled and blood and tissue collected for the following analysis: **a** Viral loads recovered from blood by plaque assay in Vero cells. Results are shown as the log of PFU per mL of blood. **b** Quantification of NS3-positive hepatocytes in the liver of 4G2 or isotype-control-treated mice. Liver was collected, formalin-fixed and processed into paraffin sections. Serial sections from each tissue were stained with anti-DV NS3 antibody E1D8 (NS3) or an isotype control mouse IgG2a, and multiple sections of each tissue type were thoroughly examined for staining. **c** Representative pictures of positive staining for NS3$^+$ cells are expressed in *brown,* while hematoxylin counterstain is *blue*. **d** Number of platelets, shown as platelets × 10^3/µL of blood. **e** Hematocrit, shown as % volume occupied by red blood cells. **f** Concentrations of TNF-α in serum, quantified by ELISA. Results are shown as pg per mL (serum). **g** ALT activity determination in plasma of control and DENV-3-infected mice is shown as U/dL of plasma. All results are expressed as mean ± SEM (except for **a**, expressed as median) and are representative of two experiments. $*P < 0.05$ when compared to control uninfected mice. $^{\#}P < 0.05$ when compared to isotype control DENV-3-infected mice. $**P < 0.05$ when compared to 4G2 (15 µg) DENV-3-infected mice. *NI* non-infected (color figure online)

the numbers of NS3$^+$ hepatocytes (Fig. 3b, c). As expected, no viremia (Fig. 3a) or significant NS3$^+$ hepatocyte staining was detected in isotype-control-treated mice (15 or 400 µg) that received a small inoculum of the DENV-3 (Fig. 3b, c). In addition to enhanced viral replication, low-dose 4G2-treated mice presented more severe disease manifestation, as demonstrated by intense thrombocytopenia (Fig. 3d), occurrence of hemoconcentration (Fig. 3e), elevated levels of TNF-α and ALT in serum (Fig. 3f, g, respectively) when compared to low-dose isotype-control-treated mice. Accordingly, low dose of 4G2 administration into mice led to marked liver injury on day 7 after DENV-3 inoculation (Figure S3). All these findings were strikingly reduced in mice that received the higher doses of 4G2 or the isotype control antibodies (Fig. 3d–g and S3). The present data demonstrate that anti-DENV antibodies can worsen the disease outcome induced by DENV-3 infection in vivo, depending on their neutralizing circulating titers.

ADE-mediated severe disease manifestation resembles severe dengue disease induced by primary infection with higher viral inoculum in immunocompetent mice

Mice that receive subneutralizing amounts of anti-DENV antibodies presented higher viral burden during the course of infection. In order to evaluate whether enhanced viral loads would be the cause behind severe disease evolution, we conducted experiments comparing anti-DENV-treated mice infected with a lower DENV inoculum and mice infected only with a higher viral inoculum. For this, WT mice were administered with 15 µg of the 4G2 monoclonal antibody (mAb) in the previously described schedule and then inoculated with 100PFU of DENV-3 i.p. This group of mice was compared to another two groups: one inoculated only with 100PFU of DENV-3 who was called as "sublethal" and another which received an inoculum 10 times higher (1000PFU of DENV-3) that was designated

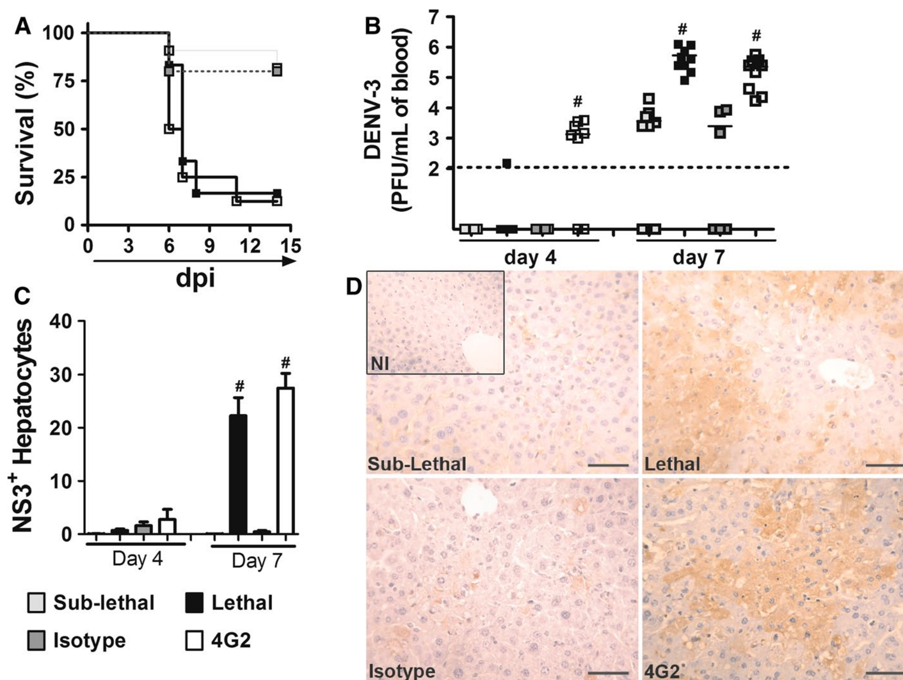

Fig. 4 Subneutralizing levels of anti-DENV mAbs increases lethality rates and viral burden to levels similar to those of mice infected with a higher viral inoculum. **a** WT mice ($n = 8$) were administered with 15 µg of the mouse monoclonal antibody—4G2 (IgG2a anti-Envelope protein, pan-flavivirus-reactive) or with an isotype control antibody with irrelevant specificity on days −1, +1 and +3 and were inoculated with 100PFU of DENV-3 (i.p). Another two groups were inoculated only with the DENV-3 (100 PFU "sublethal" or 1000PFU "lethal"), without any antibody treatment. **a** Lethality rates were evaluated every 12 h during 14 days. Results are expressed as % of survival. **b–d** WT mice ($n = 5–9$) were administered with 15 µg of the mouse monoclonal antibody—4G2 (IgG2a anti-Envelope protein [E], pan-flavivirus-reactive) or with an isotype control antibody with irrelevant specificity on days −1, +1 and +3 and were inoculated with 100PFU of DENV-3 (i.p). Four or seven days later, mice were culled and blood and tissue collected for the following analysis: **b** Viral loads recovered from blood by plaque assay in Vero cells. Results are shown as the log of PFU per mL of blood. **c** Quantification of NS3-positive hepatocytes in the liver of 4G2 or isotype-control-treated mice. Liver was collected, formalin-fixed and processed into paraffin sections. Serial sections from each tissue were stained with anti-DV NS3 antibody E1D8 (NS3) or an isotype control mouse IgG2a, and multiple sections of each tissue type were thoroughly examined for staining. **d** Representative pictures of positive staining for $NS3^+$ cells are shown where NS3 signal is *brown,* while hematoxylin counterstain is *blue*. *NI* non-infected, *dpi* days post-infection. $^{\#}P < 0.05$ when compared to sublethal or isotype control DENV-3-infected mice (color figure online)

as "lethal". As controls, isotype-treated mice were administered with the same dose and treatment schedule of an isotype-matched control with irrelevant specificity and then inoculated with 100 PFU of DENV-3. During the experiments, euthanasia was performed at two time points after infection; day 4 and day 7. Figure 4a shows the lethality rates of mice that received or not the mAbs treatment and were inoculated with DENV-3. Results show that only 25 % of the mice treated with the isotype control mAb or those who were infected only with the sublethal inoculum of the DENV-3 were dead after 14 days of infection. In contrast, almost 90 % of mice that were inoculated with the high inoculum of the virus (lethal group) had succumbed to infection by day 8 after DENV-3 inoculation. Similar results were found in the group of mice who received the 4G2 mAbs. Almost all mice were dead at day 11 after DENV-3 inoculation (Fig. 4a). In accordance with the theory of the "antibody-dependent enhancement of infection," the higher lethality rates found in the 4G2-treated group was associated with an early replication of the virus as shown by the presence of viremia in almost all mice already on day 4 of infection with even higher values at day 7 of infection (Fig. 4b). Interestingly, mice that received the higher inoculum of DENV-3 showed elevated viremia only at day 7 of infection, suggesting the role of antibodies in mediating the enhancement of infection by increasing viral replication in early times after infection (Fig. 4b). As expected, mice that received the isotype control or the sublethal inoculum of the virus showed smaller viremia levels on day 7 of infection when compared to the lethal and 4G2-treated groups (Fig. 4b). In accordance with the elevated viremia, marked virus replication was found in the liver of lethal-DENV-3-inoculated or 4G2-treated mice groups, as assessed by $NS3^+$ staining of hepatocytes (Fig. 4c). These data show that subneutralizing levels of anti-DENV antibodies enhance viral replication to similar extents found

in mice primarily infected with a higher DENV inoculum. Next, we analyzed the disease parameters on days 4 and 7 of infection using the same experimental design described before. Of note, there were no differences in any of the disease parameters evaluated among the several groups on the day 4 after DENV-3 inoculation (data not shown). On day 7 (peak of disease manifestation), our results show that mice that received only the sublethal inoculum of DENV-3 or were treated with the isotype control mAbs presented a slight reduction in the platelets counts (Fig. 5a); however, no alteration in the hematocrit levels was found after DENV-3 inoculation (Fig. 5b). Moderate plasma extravasation in liver (Fig. 5c) and lungs [NI = 4.1 ± 0.9 μg 100 mg lungs; sublethal = 7.9 ± 1.6 μg 100 mg lungs *; isotype control = 7.3 ± 1.3 μg 100 mg lungs *] as well as discrete reduction on systolic blood pressure (Fig. 5d) was found in these groups of DENV-3-inoculated mice, when compared to the NI controls. Beyond the hematological parameters, there was no increase in TNF-α levels (Fig. 5e) in the serum of these mice; however, IFN-γ values (Fig. 5f) were significantly high when compared to the NI controls. Finally, discrete liver damage was found in sublethal or isotype control DENV-3-inoculated mice, as shown by moderate elevation in ALT levels in the serum (Fig. 5g) and by the low scores found after the histopathological analysis of the H&E-stained sections in the liver (Fig. 5h, i). In sharp contrast, mice inoculated with the high inoculum of DENV-3 or those who received the 4G2 antibody followed by infection with a low inoculum of DENV-3 presented severe disease manifestation as demonstrated by the presence of markedly lower platelet counts (Fig. 5a), hemoconcentration (Fig. 5b), intense plasma extravasation in liver (Fig. 5c) and lungs [NI = 4.1 ± 0.9 μg/100 mg of lungs, lethal = 17 ± 2.3 μg 100 mg of lungs and 4G2 = 13.3 ± 2.5 μg 100 mg of lungs] and severe reduction in the systolic blood pressure (Fig. 5d). In addition, TNF-α (Fig. 5e) and IFN-γ (Fig. 5f) cytokine levels in serum were markedly high in comparison with NI controls. Of note, IL-10 levels in the spleen of lethal-DENV-3-inoculated and 4G2-treated mice were also markedly elevated in comparison with NI, sublethal- and isotype-control-treated mice [NI = 36 ± 72; sublethal = 58 ± 89; lethal = 187 ± 126*#; isotype = 60 ± 85; 4G2 = 187 ± 104*# pg/100 g of spleen], but similarly as saw in IFN-γ levels, equivalent high levels of IL-12p40 were found in the spleen of all DENV-3-inoculated mice (*data not shown*). In addition, there was massive release of ALT transaminase in the serum of lethal- or 4G2-treated mice (Fig. 5g), and histopathological analysis in the liver of these mice revealed that WT mice inoculated with 1000PFU of DENV-3 or those that received the 4G2 mAb showed a higher score, demonstrating a significant degree of liver injury in these groups (Fig. 5h, i). Therefore, treatment of mice with subneutralizing doses of anti-DENV monoclonal antibodies followed by challenge with a low inoculum of DENV led to an enhancement of disease manifestation in a similar way as found in mice that received an inoculum 10 times higher of the virus.

Anti-dengue immunocomplexes enhance viral replication in vivo through FcγR-mediated suppression of the type I IFN antiviral response

It has been proposed that interaction of DENV immunocomplexes with FcγRs in target cells (monocytes, macrophages and DCs) enhances the access of virions to these cells and contributes to the increased titers of virus found in the blood of DHF/DSS patients [15]. To confirm the involvement of FcγRs in mediating virus enhancement in our model of antibody-induced disease, we treated mice daily with an FcγR-blocking antibody (clone 2.4G2) starting on day -1 until day 5 post-DENV inoculation in mice in the presence or not of the anti-DENV (4G2) antibody. Our results show that mice administered only with the isotype control mAb or the group of mice treated with the isotype control + FcγR-blocking mAb that were inoculated with a low inoculum of DENV-3 presented only a mild disease manifestation as demonstrated by no detection of virus in the blood (Fig. 6a), low replication levels of virus in the liver (Fig. 6b, c), occurrence of discrete thrombocytopenia (Fig. 6d) and absence of hemoconcentration (Fig. 6e). In addition, only small levels of TNF-α (Fig. 6f) and ALT transaminase (Fig. 6g) were detected in serum of mice in these groups. Finally, histopathological scores in liver section of these mice were low (Figure S4). However, as demonstrated before in Figs. 4, 5 and here in Fig. 6a–g and S4, 4G2 treatment of mice with subneutralizing doses followed by a low DENV-3 inoculation led to an enhancement of virus replication and increase in values of all the disease parameters assessed (Fig. 6a–g and S4). In contrast, blockade of FcγRs by Fc-blocker treatment resulted in complete reversion of 4G2-induced ADE disease in mice (Fig. 6a–h and S4 [dark gray bars]). Therefore, the present data show an essential role for FcγRs in mediating enhanced viral burden and in inducing severe disease manifestation in DENV-infected mice that present subneutralizing levels of anti-DENV antibodies.

Evidences suggest that the mechanism of ADE-enhanced DENV infection is associated with both increase in the number of infected cells, a phenomenon called "Extrinsic ADE," and a subversion of the intracellular innate immune host responses through suppression of a the type I IFN and proinflammatory cytokines production—an event denominated as "Intrinsic ADE" [17]. The next set of experiments was conducted to evaluate whether FcγR activation by anti-DENV immunocomplexes would be associated with

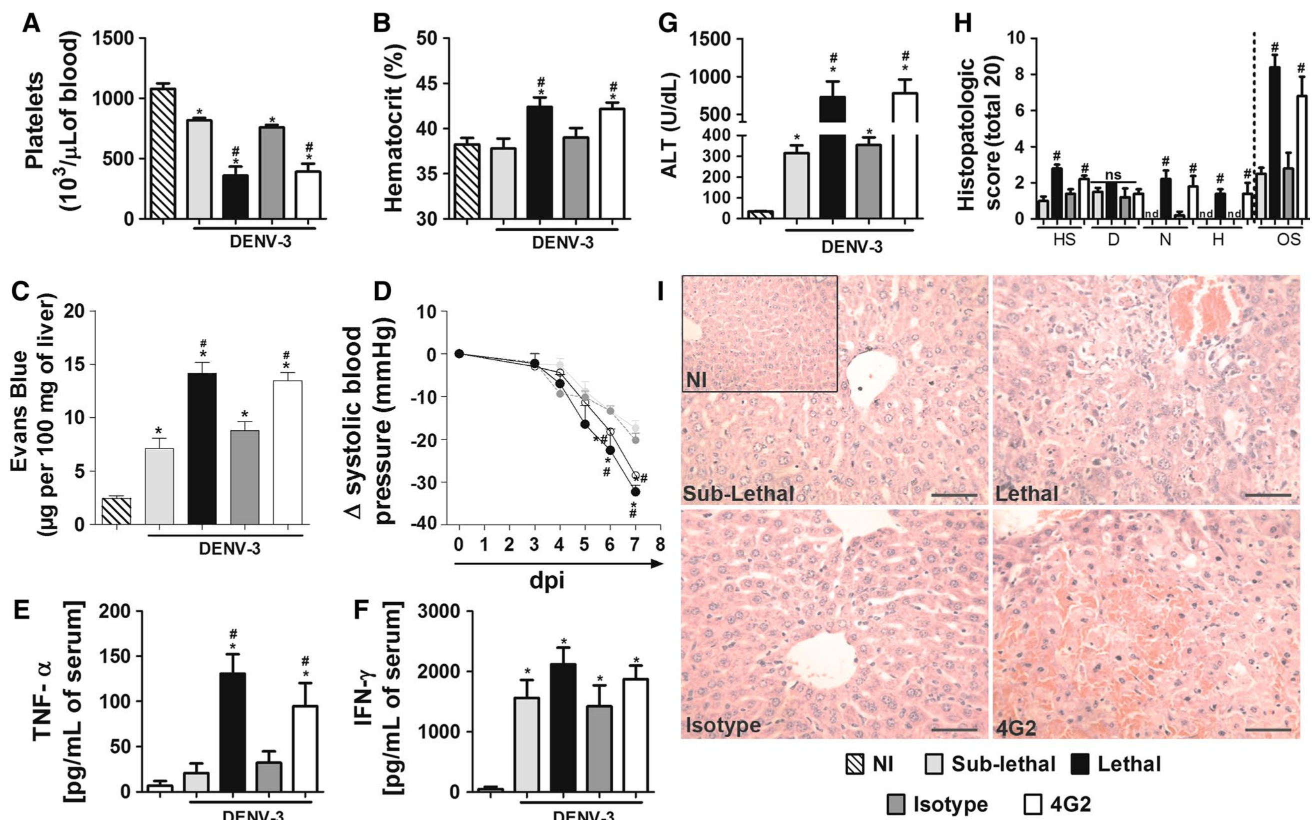

Fig. 5 Mice that received subneutralizing levels of anti-DENV mAbs develop a severe dengue disease resembling mice infected with a higher DENV inoculum. WT mice ($n = 5–9$) were administered with 15 µg of the mouse monoclonal antibody—4G2 (IgG2a anti-Envelope protein, pan-flavivirus-reactive) or with an isotype control antibody with irrelevant specificity on days −1, +1 and +3 and were inoculated with 100PFU of DENV-3 (i.p). Another two groups were inoculated only with the DENV-3 (100 PFU "sublethal" or 1000PFU "lethal"), without any antibody treatment. Euthanasia was conducted on day 7 after DENV-3 inoculation, and the following parameters were evaluated: **a** Number of platelets, shown as platelets × 10^3/µL of blood. **b** Hematocrit, shown as % volume occupied by red blood cells. **c** Changes in vascular permeability in the liver, shown as µg of Evans blue per 100 mg of tissue. In **d** changes in systolic blood pressure from baseline until day 7 after infection, expressed as Δ of blood pressure in mmHg. **e–f** Concentration of TNF-α and IFN-γ in serum, quantified by ELISA. Results are shown as pg per mL (serum). **g** ALT activity determination in plasma of control and DENV-3-infected mice is shown as U/dL of plasma. **h** Liver of control and DENV-3-infected mice were collected, formalin-fixed and processed into paraffin sections. Liver sections were stained with hematoxylin and eosin, and histopathological scores of each mouse were performed. **i** Representative images of each group of mice are shown (*Scale Bar* 400 µm). All results are expressed as mean ± SEM and are representative at least two experiments. *$P < 0.05$ when compared to control uninfected mice. #$P < 0.05$ when compared to sublethal or isotype control DENV-3-infected mice. *NI* non-infected, *dpi* days post-infection, *HS* hepatocyte swelling, *D* degeneration, *N* necrosis, *H* hemorrhage, *OS* overall score

impairment in host antiviral response. First, experiments conducted in mice with null mutations for the IFNα/β receptors (A129$^{-/-}$ mice) revealed the essential role of type I IFNs in host response to primary DENV infection in the present model (Figure S5). After infection, while 50 % of WT mice had succumbed to infection until day 12 (Figure S5A), almost 90 % of the type I-IFN-deficient mice were dead by day 7 of virus inoculation (Figure S5A). The increased lethality of A129$^{-/-}$ mice was associated with elevated virus replication in spleen already on day 5 of infection (Figure S5B) and presence of higher viremia on day 7 after DENV inoculation (Figure S5C). In addition, there was no difference in thrombocytopenia between WT- and A129$^{-/-}$-infected mice (Figure S5D); however, hemoconcentration (Figure S5E), higher levels of TNF-α (Figure S5F) and more intense ALT (Figure S5G) were detected in the serum of these knockout mice in comparison with the WT-infected controls.

Next, we assessed type I IFN expression in spleen of mice allocated on the same experimental design of the one previously depicted for the Fig. 6. Results show that there was a marked increase in mRNA expression of the antiviral genes; *IFNα2* (Fig. 7a), *IFNα4* (Fig. 7b) and the IFN-induced *ISG15* gene (Fig. 7c) in the spleen of mice that received the treatment with the isotype control mAb and were inoculated with the low inoculum of virus, in

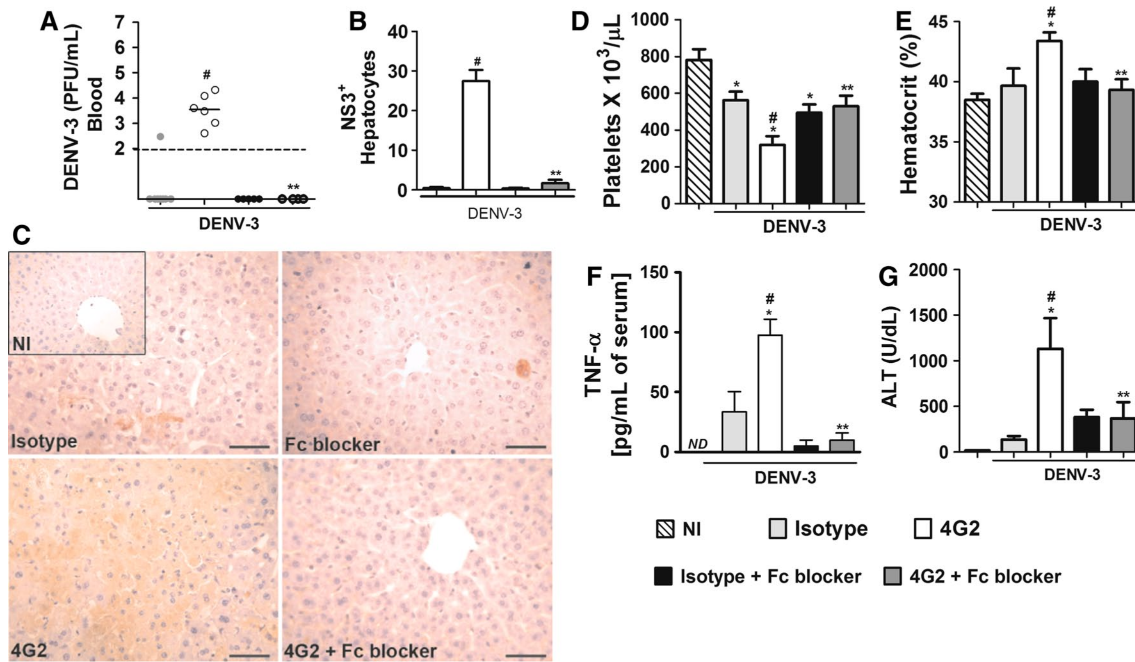

Fig. 6 FcγR blockade avoids ADE occurrence and dengue severe disease development in mice. WT mice ($n = 5$–8 per group) were administered with 15 μg of the mouse monoclonal antibody—4G2 (IgG2a anti-Envelope protein, pan-flavivirus-reactive) or with an isotype control antibody with irrelevant specificity followed by inoculation of 100PFU of DENV-3 (i.p). Another two groups received the anti-DENV 4G2 mAb together with the Fc-blocking antibody (clone 2.4G2) or were administered with the isotype control mAb and the Fc-blocker. Seven days later, mice were culled and blood and tissues collected for the following analysis: **a** Viral loads recovered from blood by plaque assay in Vero cells. Results are shown as the log of PFU per mL of blood. **b** Quantification of NS3-positive hepatocytes in the liver of mice. Liver was collected, formalin-fixed and processed into paraffin sections. Serial sections from each tissue were stained with anti-DV NS3 antibody E1D8 (NS3) or an isotype control mouse IgG2a, and multiple sections of each tissue type were thoroughly examined for staining. **c** Representative pictures of positive staining for NS3^{+} cells are shown where NS3 signal is *brown,* while hematoxylin counterstain is *blue*. **d** Number of platelets, shown as platelets × 10^3/μL of blood. **e** Hematocrit, shown as % volume occupied by red blood cells. **f** Concentrations of TNF-α in serum, quantified by ELISA. Results are shown as pg per mL (serum). **g** ALT activity determination in plasma of control and DENV-3-infected mice is shown as U/dL of plasma. All results are expressed as mean ± SEM (except for **a**, expressed as median) and are representative of two experiments. *$P < 0.05$ when compared to control uninfected mice. $^{\#}P < 0.05$ when compared to isotype control or isotype control aan 2.4G2 DENV-3-infected mice. **$P < 0.05$ when compared to 4G2 DENV-3-infected mice. *NI* non-infected, *ND* non-detected (color figure online)

both days 4 and 7 after DENV inoculation. Similar results were found in mice that received the isotype control mAb together with the Fc-blocker antibodies (Fig. 7a–c—black bars). Otherwise, in 4G2-treated mice equally infected with the same inoculum of DENV-3, a drastic suppression in the mRNA expression of all these evaluated genes was found (Fig. 7a–c). Of note, *IFNα2* suppression in 4G2-treated mice occurred as early as the day 4 of infection, supporting the higher viral replication found previously in this group of mice (Fig. 4b). Interestingly, the blockade of FcγRs signaling in 4G2-treated mice reversed this situation (Fig. 7a–c—dark gray bars). In addition, elevated IL-10 production [NI = 36 ± 72; sublethal = 58 ± 89; lethal = 187 ± 126*#; isotype = 60 ± 85; 4G2 = 187 ± 104*# pg/100 g of spleen] was detected in the spleen of 4G2-treated mice in comparison with isotype controls littermates. Of note, blockade of FcγRs in 4G2-treated mice reverted these phenotypes [NI = 36 ± 72; isotype = 60 ± 85; 4G2 = 187 ± 104*#; isotype + 2.4G2 = not detectable; 4G2 + 2.4G2 = 27 ± 54#]. Therefore, the data depicted here show that type I IFN responses play an essential role during primary and secondary DENV infections and during antibody-mediated enhancement of virus replication in mice; there is an early suppression of innate antiviral immune responses that results in enhanced *Dengue virus* replication and severe disease manifestation.

Passive intravenous immunoglobulin therapy (IVIG) containing subneutralizing titers of α-DENV antibodies enhances *Dengue virus* replication and induces severe disease manifestation in mice

Specific therapeutic approaches for severe thrombocytopenia and shock syndrome during severe dengue are still lacking. Intravenous immunoglobulin G (IVIg) therapy is widely used to treat inflammatory and autoimmune diseases,

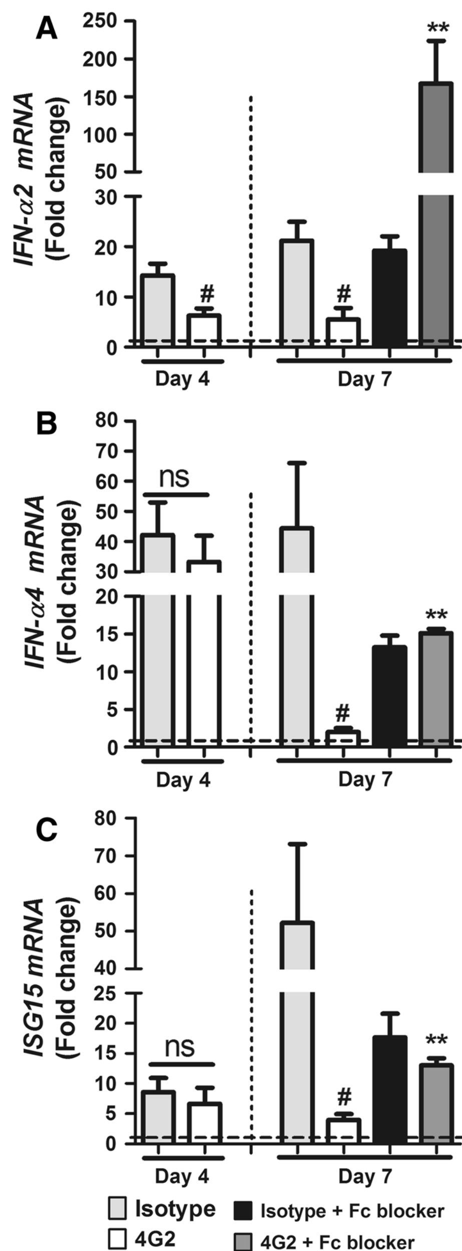

Fig. 7 Anti-dengue antibodies mediate suppression of early antiviral innate immune responses in spleen of mice infected with DENV-3 through FcgR activation. WT mice ($n = 5$ per group) were administered with 15 µg of the mouse monoclonal antibody—4G2 (IgG2a anti-Envelope protein, pan-flavivirus-reactive) or with an isotype control antibody with irrelevant specificity followed by inoculation of 100PFU of DENV-3 (i.p). Another two groups received the anti-DENV 4G2 mAb together with the Fc-blocking antibody (clone 2.4G2) or were administered with the isotype control mAb and the Fc-blocker and were infected with 100PFU of DENV-3. Four or seven days later, mice were culled and spleen was collected for the determination of IFN-α2 (**a**), IFN-α4 (**b**) and ISG15 (**c**) RNA expression by qPCR. Results are shown as mean ± SEM of fold increase over basal expression in naive mice. All results are representative of two experiments. $^{*}P < 0.05$ when compared to control uninfected mice. $^{\#}P < 0.05$ when compared to isotype control or isotype control and 2.4G2 DENV-3-infected mice. $^{**}P < 0.05$ when compared to 4G2 DENV-3-infected mice. *NI* non-infected

Table 1 Characteristics and composition of the 5 % human immune globulin intravenous solution produced by the HEMOPE, Recife, Brazil

Analysis[a]	Standard values	IVIG α-DENV
Protein concentration	Minimum 30 mg/mL	51.1 mg/mL
Protein composition (purity)	Minimum 95 %	96,80 %
pH	4.0–7.4	4.51
Quantification of aggregates	<3 %	1.8 %
Anti-HBs	Minimum 0.5 UI/g	8.7 UI/g
Immunoglobulin concentration		
IgG	–	44.4 mg/mIJ
IgA	–	l.25 mg/mL
IgM	–	0.25 mg/mL
IgE	–	0.03 mg/mL
Anticomplementary (CH50)	<1	<1
Anti-HIVl abs	Absent	Absent
Anti-HIV2 abs	Absent	Absent
Anti-HBSAg	Absent	Absent
Anti-Streptococcus abs	–	Present
Anti-measles	–	Present
Anti-DENGUE abs	_	Present
Stability at 57°/4 h	Do not freeze	Do not freeze

Formulation: 0.3M glycine

The isolation of the human immunoglobulin G (IVIG) from a pull of serum donors from an endemic region for *Dengue virus* was made by the method of Cohn–Oncley. The characteristics and composition of the IVIG are demonstrated above

[a] Using the methodologies recommended in the European Pharmacopoeia, VI edition

such as immunothrombocytopenia (ITP) [42]. However, the effects of IVIG therapy during *Dengue virus* infections are controversial [42–44]. In light of the previous results, we hypothesized that the prophylactic treatment of mice with human immunoglobulin (IVIG) from pooled blood from health donors from an endemic area for *Dengue virus* circulation could worsen DENV-induced disease in mice due to the presence of subneutralizing titers of anti-DENV antibodies. Table 1 shows the composition of the IVIG used in the present study. As highlighted, anti-DENV antibodies were detected by the Pan Bio qualitative anti-DENV ELISA in the IVIG used. In addition, plaque reduction neutralization tests of the IVIG sample showed that the utilized preparation presented subneutralizing titers of anti-DENV antibodies ($PRNT_{50}$ against the DENV-3 strain = 1:20). To verify the role of IVIG therapy during *Dengue virus* infection, WT mice were administered intravenously with three different doses of α-DENV-containing IVIG (30, 100 and 300 mg/kg) on days −1, +1 and +3 after DENV-3 inoculation. Figure 8a shows that only 25 % of mice that received the vehicle solution succumbed to infection until day 14 of DENV-3 inoculation. Interestingly, IVIG administration

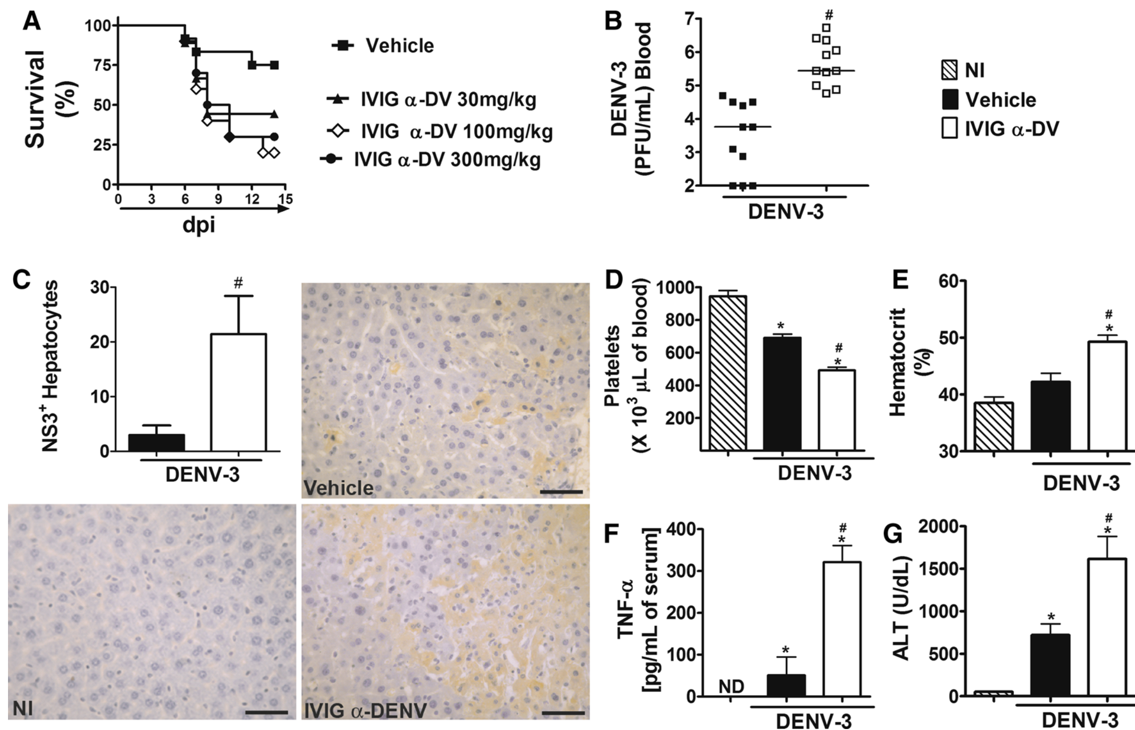

Fig. 8 Passive intravenous immunoglobulin therapy (IVIG) containing subneutralizing levels of α-DENV antibodies reduce survival rates, enhance *Dengue virus* replication and induce severe disease manifestation in DENV-3-inoculated mice. **a** WT mice ($n = 8$) were administered with different doses of the α-DENV IVIG (30, 100 or 300 mg/kg) or with the vehicle solution (i.v) on days −1, +1 and +3 and were inoculated with 100PFU of DENV-3 (i.p). **a** Lethality rates were evaluated every 12 h during 14 days. Results are expressed as % of survival. **b–g** WT mice ($n = 8$–11) were administered with the α-DENV IVIG (100 mg/kg) or with the vehicle solution (i.v) on days −1, +1 and +3 and 7 days after DENV-3 infection, mice were culled and blood and tissue collected for the following analysis: **b** Viral loads recovered from blood, assayed by plaque assay in Vero cells. Results are shown as the log of PFU per mL of blood. **c** Quantification of NS3-positive hepatocytes in the liver of 4G2 or isotype-control-treated mice. Liver was collected, formalin-fixed and processed into paraffin sections. Serial sections from each tissue were stained with anti-DV NS3 antibody E1D8 (NS3) or an isotype control mouse IgG2a, and multiple sections of each tissue type were thoroughly examined for staining. Representative pictures of positive staining for $NS3^+$ cells are shown, where NS3 signal is *brown,* while hematoxylin counterstain is *blue*. **d** Number of platelets, shown as platelets × 10^3/μL of blood. **e** Hematocrit, shown as % volume occupied by red blood cells. **f** Concentrations of TNF-α in serum, quantified by ELISA. Results are shown as pg per mL (serum). **g** ALT activity determination in plasma of control and DENV-3-infected mice is shown as U/dL of plasma. *$P < 0.05$ when compared to control uninfected mice. $^{\#}P < 0.05$ when compared to vehicle-treated and DENV-3-infected mice. *NI* non-infected, *dpi* days post-infection, *ND* non-detected (color figure online)

to DENV-3-inoculated mice led to an enhancement of the lethality rates varying between 50 % at dose of 30 mg/kg and 75 % at doses of 100 or 300 mg/kg per mouse/per treatment. Further analyses of disease parameters were done administering the dose of 100 mg/kg. In addition to elevated lethality rates, administration of α-DENV-containing IVIG led to severe disease manifestation and enhancement of virus replication (Fig. 8b–g). While vehicle-treated mice presented moderate viremia (Fig. 8b), viral load in blood of mice administered with α-DENV-containing IVIG was 2log higher at the same period analyzed. In accordance, the presence of $NS3^+$ hepatocytes was markedly increased in IVIG-treated mice in comparison with vehicle-treated ones (Fig. 8c). Hematological parameters were also aggravated in IVIG-treated mice as demonstrated by intense drop in platelets numbers (Fig. 8d), occurrence of hemoconcentration (Fig. 8e), heightened production of the pro-inflammatory cytokine TNF-α (Fig. 8f) and hepatic damage (Fig. 8g and S6). Next, we assessed the neutralizing titers of α-DENV antibodies in the serum of IVIG-treated mice in vitro. Plaque neutralization assays from serum of mice administered with 100 mg/kg of IVIG revealed non-neutralizing titers (*data not shown*), and these results corroborate the findings shown before that subneutralizing doses of α-DENV antibodies can enhance *Dengue virus* replication and disease manifestation in mice.

Discussion

The major findings of the present study can be summarized as follows: (1) B cells play a protective role in the

host response to primary *Dengue virus* infection in mice; (2) subneutralizing titers of anti-dengue antibodies enhance DENV infection and replication, leading to severe disease manifestation and increased lethality rates of immunocompetent mice in a similar way as found in DHF patients; (3) enhanced DENV replication mediated by subneutralizing levels of anti-DENV antibodies involves FcγR activation and subsequent impairment of host type I interferon responses and elevated production of the anti-inflammatory cytokine IL-10; (4) IVIG replacement therapy containing subneutralizing levels of anti-DENV antibodies led to more severe disease manifestation and increased lethality rates due to an enhancement of virus replication. These studies, therefore, indicate that B cells are pivotal for host resistance to primary *Dengue virus* infections although humoral responses unleashed during secondary infections may contribute to the enhancement of virus replication and severe disease manifestation in mice due to suppression of the early antiviral innate responses.

The adaptive immune response to *Dengue viruses* is thought to contribute to the resolution of infection and to play a major role in protection from reinfection [45]. Conversely, it is also believed to have a crucial role in the enhancement of disease severity seen in patients with DHF or DSS [46]. It has been previously demonstrated that there is early B-cell activation upon DENV infection of immunocompetent mice, with detectable levels of anti-DENV antibodies as early as 3 days post-infection. However, the same group showed that B-cell-deficient mice are as resistant to DENV-induced lethality as WT mice [31] although viral replication and disease parameters were not thoroughly addressed due to limitations of the experimental model utilized. Therefore, the data obtained so far have failed to properly define the role played by B cells during the course of DENV infection. Here, we have performed experiments in B-cell-deficient mice (μMT$^{-/-}$) using DENV strains shown to replicate in relevant host tissues and to induce a severe disease that resembles most of the parameters found in patients with DHF/DSS when primarily inoculated into immunocompetent mice [33–35]. Our data clearly show that μMT$^{-/-}$mice were more susceptible to DENV infection than WT-infected controls. DENV-3-inoculated μMT$^{-/-}$ presented elevated lethality rates, and importantly, lethality was associated with a systemic dissemination of the virus as well as with enhanced disease manifestation, as exemplified by the presence of marked thrombocytopenia, hemoconcentration, elevated levels of TNF-α and ALT in serum as well as an intense liver damage. These findings highlight the pivotal role played by early B-cell activation in controlling viral replication and disease evolution and are divergent from previous studies utilizing animal models of infection [31]. It is important to emphasize that such discrepancies may be justified by the use of different viral strains (serotypes/genotypes), as well as the different experimental protocols used in these studies. Thus, the reader should be cautious during the interpretation of such data. Also, the higher complexity of the human immune system in relation to the murine one should be taken into account when interpreting the current findings. But the data presented here strongly support a protective role for B cells during primary DENV infection and are in accordance with the role played by B-cell-mediated immunity during infection by other flaviviruses [47, 48].

The mechanisms involved in B-cell-mediated protection to DENV infection are still to be defined. Recently, it has been shown that B cells are important for proper innate antiviral responses by controlling macrophage maintenance and production of type I IFNs upon VSV infection [49]. Also, B cells seem to play a pivotal role during innate responses to systemic infections. Kelly-Scumpia et al. [49, 50] have demonstrated that during bacterial sepsis, type-I-IFN-mediated activation of marginal zone and follicular B cells contributed to early chemokine production and to improved survival rates. Thus, these two studies have identified a novel protective role for type I IFN and B cells during early immune responses to viral and systemic infections. Since type I IFN responses are essential during DENV infection and B-cell-deficient mice were markedly susceptible to primary DENV infection, similar mechanisms as described before could account to the severe disease manifestation observed in DENV-infected μMT$^{-/-}$ mice. However, future studies are needed to clarify these speculations.

In the previously cited work by Diamond and colleagues, it was found that μMT$^{-/-}$ mice developed increased viral burden and higher lethality rates upon WNV infection, similarly to what we have found in our system during the course of DENV infection [47]. Importantly, they have also shown that passive transfer of heat-inactivated serum from WT immune mice to μMT$^{-/-}$ mice protected them against morbidity and mortality induced by WNV inoculation, suggesting that antibodies are important for conferring protection to infection [47]. In order to evaluate whether antibodies would confer protection also to DENV infection, we have conducted experiments involving passive transfer of DENV-immune serum to mice, but we had strikingly different results. Hence, the administration of anti-DENV serum to immunocompetent mice can sufficiently increase severity of disease, so that a mostly non-lethal illness becomes a fatal disease. Similar results were found with the administration of subneutralizing titers of monoclonal anti-DENV antibodies. Of note, antibody-induced disease manifestation was completely reversed when higher doses of anti-DENV mAbs were administered to mice. These experiments suggested the occurrence of ADE phenomenon in vivo.

Findings in other experimental systems have previously suggested the occurrence of ADE of DENV infection in vivo. The first evidences came from experiments conducted in non-human primates [51, 52]. In vivo enhancement of *Dengue virus* infection in rhesus monkeys was detected after passive transfer of antibodies [52, 53]. However, despite the elevated viral titers detected, no signs of disease were apparent, which limits the study of *Dengue virus* pathogenesis in this system. Recently, studies conducted in AG129 mice have also brought support to this theory, showing the occurrence of many DHF/DSS signs of disease and symptoms after passive transfer of polyclonal-immune serum or anti-DENV mAbs [54, 55]. The fact that these studies were conducted in IFN receptor-deficient mice tempered further extrapolations to human situation, due to the essential role of IFN responses in both pathogenesis and protection against DENV infections. Of note, mice in the SV129 background are much more susceptible to infection by dengue than the C57BL/6 strain, which justify the difference in mortality rates found in both groups when infected with the same inoculums of dengue in this study. Our results in a immunocompetent system showed that after inoculation of a low inoculum of DENV-3 to mice treated with a low dose of the 4G2 antibody, there was increased viral burden together with marked thrombocytopenia, occurrence of hemoconcentration, increased vascular permeability, reduction in arterial blood pressure as well as elevated levels of TNF-α and hepatic transaminases in the serum, all parameters found in humans with DHF/DSS. In addition, intense liver damage was detected in anti-DENV mAbs-treated mice. The latter findings clearly show the occurrence of the ADE phenomenon in immunocompetent settings and demonstrate that ADE may impact in both viral burden and disease evolution in vivo. This system utilizing ADE-mediated DENV infection in immunocompetent mice may provide an important tool to study host–virus interactions during secondary DENV infection.

Of note, ADE-induced disease manifestation was completely reversed after the blockade of FcγRs. This is in accordance with previous findings by other groups, showing that antibody-induced disease could be prevented by blockade of interaction between the virus immunocomplexes to the FcγRs, through the use of anti-FcγR mAbs or by the administration of genetically modified antibodies that are incapable of interacting with the FcγRs [54, 55]. This FcγR-dependent antibody-enhanced infection can also be reproduced in cell culture models. In fact, in addition to enabling viral entry into target cells, it has been suggested that FcγR activation by DENV–antibodies complexes may interfere in cellular antiviral responses, in a mechanism called intrinsic ADE (iADE) [17, 18, 56, 57]. Hence, it was demonstrated that DENV–antibody complexes activated negative regulators of the RIG-I and MDA-5 cascade, resulting in suppression of type I interferon production and to impairment of the interferon-mediated antiviral responses. Modhiran et al. [58] have demonstrated in THP-1 cells that interaction between DENV–antibody complexes and FcγRs may also downregulate TLR gene expression and upregulate negative regulators of the NF-κB pathway, resulting in suppression of innate responses and increased viral production. Importantly, these alterations in gene expression were also described in cells of patients experiencing secondary DHF/DSS but not in DF patients [18, 58]. In the present study, we found that there was marked suppression in expression of the *IFNα2*, *IFNα4* and *ISG15* genes in the spleen of anti-dengue mAb-treated mice. Importantly, these effects were attenuated by blockade of Fcγ-receptors, suggesting the occurrence of the iADE phenomenon in the current experimental settings. To our knowledge, this is the first demonstration of the iADE phenomenon in vivo. These data also suggest that iADE occurrence impacts on severe disease manifestation during secondary dengue infection.

Another event shown to occur during iADE involves the production of the suppressive cytokine IL-10. It was previously shown that after internalization of DENV–antibody complexes in THP-1 cells, there is induction of high levels of IL-10 and expression of the regulatory molecule SOCS3, culminating in the inhibition of nitric oxide production and enhanced viral replication. Interestingly, we have found elevated IL-10 levels in spleen of anti-DENV mAb-treated mice on day 7 after DENV inoculation, suggesting that this mechanism may take place in vivo. This finding, in concert with the fact that IL-10 host genetic polymorphisms influence antibody-dependent enhancement of DENV infection, suggests that this pathway may be relevant to severe disease manifestation during DENV infection [59, 60]. Altogether, these findings show that there is suppression of early antiviral innate responses during antibody-mediated DENV infection, resulting in excessive virus replication and severe disease manifestation.

Specific therapeutic options for severe thrombocytopenia and shock syndrome, the main causes of mortality during severe dengue disease, are still lacking [1, 42]. The ADE phenomenon is considered a major bottleneck during the vaccine design and antibody therapeutic approaches development [15, 61, 62]. This seems to be the case for application of the IVIG therapy. This so-called intravenous immunoglobulin G therapy involves the application of pooled immunoglobulin G (IgG) preparations from thousands of donors to patients with a variety of hematological and immunological disorders [63]. Prophylactic and therapeutic efficacies of IVIG therapy have been reported during some *Flavivirus* infections, such as West Nile virus (WNV) and tick-borne encephalitis virus (TBEV) [44, 64]. However, in the context of *Dengue virus* infections, trials so far

have not shown any significant benefit in terms of survival or improvement in clinical parameters of patients submitted to the IVIG therapy [42, 43]. Our data clearly showed that replacement IVIG therapy containing subneutralizing titers of anti-DENV antibodies potentiated DENV infection and replication and induced severe disease manifestation in DENV-infected mice, suggesting that ADE may impact negatively the outcome upon IVIG therapy during DENV infection. These data suggest that different formulations of the IVIG should be developed in the case of any application of this therapy during the course of DENV infection. This assertion is supported by the finding that treatment with anti-D (Rh0 D IgG) immune globulin (WinRho® SDF), a preparation highly effective in producing Fc-receptor blockade, resulted in increase in platelet counts in DHF patients [65]. Therefore, proper formulations for IVIG preparations should be sought for application of this therapy to DENV-infected patients.

In conclusion, we have demonstrated that B cells play an essential role during protection of primary DENV infection in mice. However, our results also show that subneutralizing levels of anti-DENV-specific antibodies may aggravate the course of infection due to the enhancement of virus infection and replication in Fc-bearing cells through FcγR-mediated suppression of early antiviral innate responses. These data confirm the ADE phenomenon occurrence in vivo in mice with an intact immune system and highlight the great impact ADE-mediated infection exerts in severe dengue disease manifestation. Finally, we have demonstrated that these events involved in ADE occurrence may impact negatively in replacement IVIG therapy during the course of DENV infection. Therefore, the ADE phenomenon represents a major constraint for utilizing IVIG therapy in DENV-infected patients.

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Conflict of interest The authors have no conflicting financial interests.

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