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Article

Enhanced Infection of Liver Sinusoidal Endothelial Cells in a Mouse Model of Antibody-Induced Severe Dengue Disease

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SUMMARY

Dengue virus (DENV) causes disease ranging from dengue fever (DF), a self-limited febrile illness, to the potentially lethal dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS). DHF/DSS usually occurs in patients who have acquired DENV-reactive antibodies prior to infection, either from a previous infection with a heterologous DENV serotype or from an immune mother. Hence, it has been hypothesized that subneutralizing levels of antibodies exacerbate disease, a phenomenon termed antibody-dependent enhancement (ADE). However, given the lack of suitable animal models for DENV infection, the mechanism of ADE and its contribution to pathology remain elusive. Here we demonstrate in mice that DENV-specific antibodies can sufficiently increase severity of disease so that a mostly nonlethal illness becomes a fatal disease resembling human DHF/DSS. Antibodies promote massive infection of liver sinusoidal endothelial cells (LSECs), resulting in increased systemic levels of virus. Thus, a subprotective humoral response may, under some circumstances, have pathological consequences.

INTRODUCTION

The four serotypes of dengue virus (DENV1–4), a flavivirus transmitted to humans by *Aedes* mosquitoes, induce a spectrum of disease ranging from dengue fever (DF), an acute, self-limited febrile illness, to the life-threatening dengue hemorrhagic fever and dengue shock syndrome (DHF/DSS), characterized by plasma leakage, low platelet counts, liver damage, elevated cytokine levels ("cytokine storm"), and, in the most severe cases, death due to shock (Halstead, 2007). Two and a half billion people in tropical and subtropical regions are at risk of infection, and it is estimated that 50–100 million cases occur annually, of which 500,000 are severe and 15,000 are fatal (Rico-Hesse, 2007). Epidemiological studies suggest that DHF/DSS occurs predominantly during either secondary infection with a heterologous serotype (Halstead et al., 1967) or primary infection in 6- to 9-month-old infants of DENV-immune mothers (Halstead, 1982). Therefore, it appears that nearly all severe dengue cases occur in patients who have acquired DENV-reactive antibody prior to infection, either actively from a previous infection or passively from an immune mother. Accordingly, it has been hypothesized that subneutralizing levels of DENV-specific antibodies exacerbate disease by increasing infection of cells bearing Fc-γ receptors (FcγRs), a phenomenon termed antibody-dependent enhancement of infection (ADE) (Halstead, 2003). However, little is known about the mechanism of ADE in vivo and its contribution to pathology, as increased disease severity due to antibodies has never been demonstrated in vivo.

Here we demonstrate that administering anti-DENV antibodies to DENV-infected mice can sufficiently exacerbate disease so that a mostly nonlethal illness turns into a fatal disease resembling human DHF/DSS. Massively enhanced infection of liver sinusoidal endothelial cells (LSECs) occurred in mice treated with DENV-specific antibodies. Following enhanced infection of LSECs, mice exhibited increased levels of virus in other tissues, cytokine storm, low platelet counts, increased vascular permeability, intestinal hemorrhage, and ultimately death.

RESULTS

Subprotective Levels of Antibody Enhance Severity of Dengue Disease in Mice

Epidemiological observations and in vitro studies have provided the majority of the evidence for the occurrence of ADE. In vitro, subneutralizing concentrations of DENV-specific antibodies enhanced viral replication in mononuclear phagocytes (Halstead et al., 1977). In vivo, passive transfer of immune serum (Halstead, 1979) or antibodies (Goncalvez et al., 2007) into DENV-infected rhesus monkeys resulted in increased viremia, although signs of disease were not apparent. However, to date, it has not been determined whether antibodies alone can exacerbate DENV-induced disease.

To investigate the effect of DENV-specific antibodies in vivo, type I and II interferon (IFN) receptor-deficient 129/Sv mice (AG129) were administered 200 μl of DENV1-, 2-, 3-, or 4-immune serum (obtained from AG129 mice infected with 1.5×10^6 FACS infectious units [FIUs] of DENV) or naive serum 1 day before and 1 day after i.v. infection with 5×10^8 genomic equivalents (GE) (approximately 10^4 PFU) of the DENV2 strain S221, a triple-plaque-purified clone isolated from a mouse-passaged DENV2 strain (Shresta et al., 2006). Mice administered

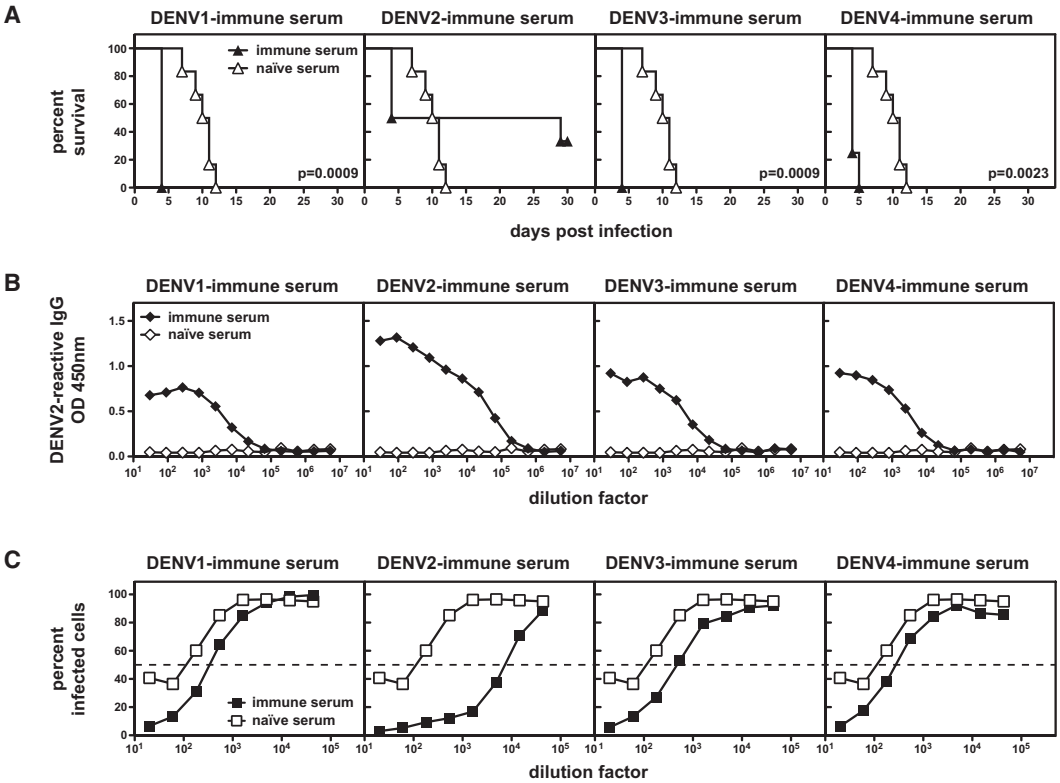


Figure 1. Heterologous Immune Serum Decreases Mean Survival Time of DENV-Infected Mice, Homologous Serum Protects 50% of the Mice from Disease

(A) Survival of DENV-infected AG129 mice (5×10^8 GE S221 i.v.) in the presence of heterologous DENV1-, 3-, or 4-immune serum, homologous DENV2-immune serum (black triangles), or naive serum (white triangles); 200 μl of serum was administered i.p. on days −1 and 1. P values from Gehan-Breslow-Wilcoxon test, n = 4–6 mice per group.

(B) DENV2-reactive IgG in the sera used in (A) was measured by ELISA on plates coated with sucrose gradient-purified S221.

(C) Biological activity of the sera used in (A) was assessed by measuring their ability to reduce infection of C6/36 cells by DENV2. For clarity, a dotted line has been added at 50% infection.

For comparison purposes, the naive serum curve is depicted in all panels.

DENV1-, 3-, or 4-immune serum died from severe disease 4–5 days after infection, without manifesting neurologic abnormalities, whereas mice treated with naive serum were sacrificed when they developed signs of paralysis 7–12 days after infection (Figure 1A). Half of the mice administered DENV2-immune serum were protected from disease and survived much longer than control mice (33% survived over 30 days), whereas the other half exhibited the early, severe disease phenotype. All immune sera contained DENV2-reactive antibodies (Figure 1B), although the DENV2-immune serum contained about ten times more than the others. The ability of the sera to reduce infection of C6/36 mosquito cells by the DENV2 strain S221 was measured (Figure 1C), and the DENV2-immune serum neutralized virus 15–20 times better than the DENV1-, 3-, or 4-immune sera. Naive serum at low dilutions was able to prevent infection of C6/36 cells; however, this effect is likely independent of antibodies, as similar observations were made with serum obtained from μMt mice, which lack antibodies (data not shown). Notably, DENV2-immune serum obtained from AG129 mice infected with a higher dose of virus (4.0×10^7 FIU, instead of 1.5×10^6 FIU as in Figure 1), completely protected against disease in recipient mice infected with 5×10^8 GE of S221 i.v. (data not shown). These results demonstrate that while heterologous immune serum exacerbates disease in DENV-infected mice, homologous serum can protect from disease.

To confirm that antibodies were responsible for the decreased survival time observed in mice treated with immune serum, the capacity of purified monoclonal antibodies (mAbs) to enhance disease was tested. AG129 mice were administered 15 μg of mouse monoclonal antibody 2H2 (IgG2a anti-membrane protein [prM/M], DENV1-4 reactive), 4G2 (IgG2a anti-envelope protein [E], pan-flavivirus-reactive), or 3H5 (IgG1 anti-E, DENV2-specific) and infected with S221 (anti-DENV-treated mice). Isotype-treated mice were administered an isotype-matched antibody of irrelevant specificity. Anti-DENV-treated mice exhibited severe disease and died 4–5 days after infection, whereas isotype-treated mice survived longer and were sacrificed when they developed neurological symptoms around 8–13 days after infection (Figure 2A), demonstrating that ADE is not restricted to one IgG subclass or DENV epitope.

To determine the relationship between disease outcome and the amount of antibody administered, doses ranging from 0.5 to 500 μg of the neutralizing mAb 4G2 were administered to S221-infected mice. While mice treated with 2 and 15 μg

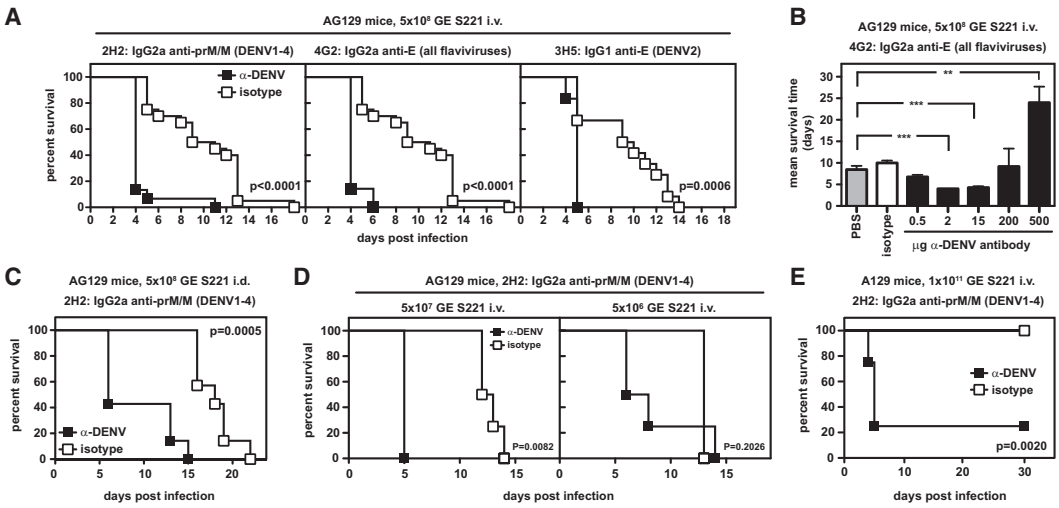


Figure 2. Subprotective Levels of Anti-DENV Monoclonal Antibodies Decrease Mean Survival Time of DENV-Infected Mice

(A) Survival of DENV-infected AG129 mice treated on days −1 and 1 with 15 μg of anti-DENV antibody (black squares, n = 7–12) or an isotype control (white squares, n = 12–20). Various anti-DENV antibodies were used, see figure and text for details. As the same isotype control was used for both IgG2a antibodies, the same isotype control curve is depicted in the left and middle panels.

(B) Mean survival time of DENV-infected AG129 mice treated with 0.5, 2, 15, 200, or 500 μg of IgG2a against DENV E (clone 4G2, black bars, n = 4–7), 500 μg of an isotype control (white bar, n = 7) or PBS (gray bar, n = 11). For simplicity, statistically significant differences are indicated only between the PBS-treated group and the anti-DENV-treated groups.

(C) Survival of AG129 mice infected intradermally (i.d.) with 5×10^8 GE S221 and treated with anti-DENV antibodies (black squares, n = 7) or isotype control (white squares, n = 7). IgG2a (15 μg) against DENV prM/M was administered i.p. on days −1 and 1.

(D) Survival of AG129 mice infected with 5×10^7 or 5×10^6 GE S221 i.v. and treated with anti-DENV antibodies (black squares, n = 4) or isotype control (white squares, n = 4). Antibody was administered as in (C).

(E) Survival of A129 mice infected on day 0 with 1×10^{11} GE S221 i.v. and treated on days −1, 1, 2, and 3 with 50 μg of IgG2a against DENV prM/M (black squares, n = 8) or an isotype control (white squares, n = 9).

P values from Gehan-Breslow-Wilcoxon test (A and C–E) or two-tailed unpaired t test with Welch's correction, c.i. 95% (B), *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001, error bars represent SEM, n is the number of mice per group.

exhibited severe disease and decreased survival time, mice administered 500 μg were protected from severe disease and survived longer than control mice (Figure 2B). These results show that, depending on the concentration, a neutralizing antibody can either increase or decrease disease severity.

Decreased survival following administration of DENV-reactive mAbs was also observed in AG129 mice infected intradermally (i.d.) with 5×10^8 GE S221 (Figure 2C), in AG129 mice infected i.v. with 5×10^7 or 5×10^6 GE S221 (Figure 2D), and in 129/Sv mice lacking only the type I IFN receptor (A129), although a higher amount of antibody and a 200-fold greater dose of virus were necessary to induce severe disease in these mice compared to AG129 (Figure 2E). Therefore, ADE-induced severe dengue disease occurs under various experimental conditions.

Further experiments were performed in AG129 mice infected with 5×10^8 GE S221 i.v. on day 0 and treated on days −1 and 1 with 15 μg 2H2 (anti-DENV-treated mice) or an isotype control unless stated otherwise. At this dose, it was possible to assess how antibodies turn a mostly nonlethal disease in isotype-treated mice into a lethal disease resembling DHF/DSS in anti-DENV-treated mice.

Disease Induced by Subprotective Levels of Antibodies in DENV-Infected Mice Resembles DHF/DSS

Elevated levels of various cytokines (IL-6, IL-10, IL-8, IFN-γ) (Chaturvedi et al., 2000), presence of TNF (Green et al., 1999), increased vascular permeability (Halstead, 2007), thrombocytopenia (Halstead, 2007), increased hematocrit (Kittigul et al., 2007), and gastrointestinal bleeding (Chiu et al., 2005) are hallmarks of DHF/DSS in humans. Anti-DENV-treated mice had significantly higher levels of TNF, IL-6, and IL-10 compared to isotype-treated mice 90 hr after infection (Figure 3A). In addition, IL-1β, IL-9, IL-12(p40), IL-17, KC, G-CSF, and RANTES were elevated in anti-DENV-treated mice compared to isotype-treated mice 72 and/or 90 hr after infection (see Figure S1 available online). Neither IL-6 neutralization nor IL-10 receptor blockade had any effect on the survival time of anti-DENV-treated mice, but neutralization of TNF prevented early death (Figure 3B). Shortly before succumbing, anti-DENV-treated mice exhibited low platelet counts (Figure 3C); elevated hematocrit (Figure 3D); increased vascular permeability in the liver, as measured by extravasation of Evans blue (Figure 3E); and gastrointestinal hemorrhage (Figure 3F), paralleling DHF/DSS symptoms.

Antibodies Mediate Increased Levels of Virus First in the Liver and Later in Other Organs

In the liver of anti-DENV-treated mice, levels of viral RNA were 8-fold, 42-fold, and 10-fold higher than isotype-treated mice 48, 72, and 90 hr after infection, respectively (Figure 4A). In the small intestine, levels of viral RNA were 3.3-fold higher at 72 hr and 10-fold higher at 90 hr (Figure 4A). In the spleen and serum, respectively, RNA levels were 4.2-fold and 2.8-fold higher at

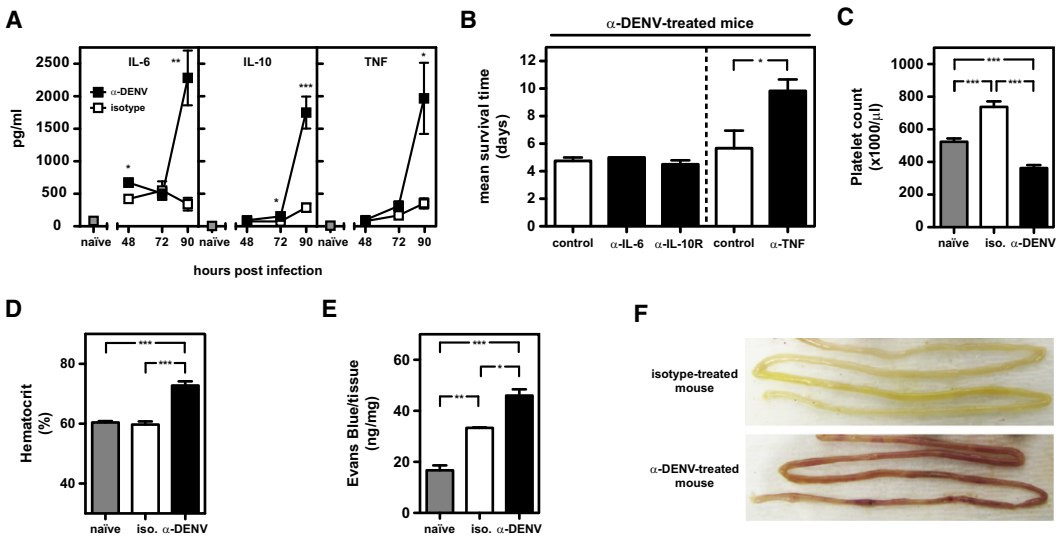


Figure 3. Administration of Anti-DENV Antibodies Induces a DHF/DSS-like Disease in DENV-Infected Mice

AG129 mice were infected with 5×10^8 GE S221 and administered 15 μg IgG2a against DENV prM/M on days −1 and 1.

(A) IL-6, IL-10, and TNF levels in the serum of DENV-infected mice 48, 72, and 90 hr after infection (n = 3–9).

(B) Mean survival time of anti-DENV-treated mice in which IL-6, IL-10R, or TNF was neutralized or blocked (black bars, n = 4–6); control groups were treated with the respective isotype controls of the IL-6, IL-10R, or TNF blocking antibodies (white bars, n = 4–6).

(C) Platelet counts 90 hr after infection (n = 6–7).

(D) Hematocrit 90 hr after infection (n = 6–7).

(E) Vascular permeability in the liver of naive mice and DENV-infected mice in the presence of isotype control or anti-DENV antibody 90 hr after infection (n = 3–4). Evans blue in tissues was extracted with formamide and quantified spectrophotometrically. The experiment was repeated 72 hr after infection and similar results were obtained.

(F) Gastrointestinal bleeding in the small intestine 84 hr after infection in the presence of anti-DENV antibodies. Mice were perfused with PBS before pictures were taken. Tissues from one representative animal per group are shown.

P values from two-tailed unpaired t test with Welch's correction, c.i. 95%, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, error bars represent SEM, n is the number of mice per group. See also Figure S1.

72 hr (Figure 4A). In all other organs examined, no significant differences between anti-DENV-treated and isotype-treated mice were observed until 90 hr after infection, at which time viral RNA levels were higher in the kidney, stomach, large intestine, and brain of anti-DENV-treated mice (Figure 4B). At 90 hr post-infection, infectious virus was detected in all the organs by plaque assay on BHK cells (Figure S2), and the differences observed in viral RNA levels were confirmed. In summary, viral RNA levels were first increased in the liver of anti-DENV-treated mice by 48 hr, followed by a general increase in other organs, most notably the small intestine.

Interactions between FcγRs and Virus-Bound Antibodies Mediate Increased Levels of Virus

In vitro, ADE of DENV infection requires the presence of FcγRs on target cells (Halstead et al., 1977), suggesting the involvement of the Fc portion of the antibody. To test this in vivo, mice were infected with 5×10^8 GE S221 and, 1 day later, treated with equimolar amounts of either an isotype control antibody, the anti-DENV antibody 2H2, the $F(ab')_2$ of 2H2, intact 2H2 and an FcγR-blocking antibody, or the $F(ab')_2$ of 2H2 chemically coupled to the isotype control. Viral RNA levels in the liver were elevated 72 hr after infection only in the mice treated with either intact anti-DENV antibody or the $F(ab')_2$ of 2H2 chemically coupled to the isotype control (Figure 4C). In addition, blocking FcγRs II and III prevented the increase in viral load in mice administered intact 2H2 (Figure 4C). These results demonstrate that ADE requires an interaction between the Fc portion of the virus-bound antibodies and FcγRs. The DENV reactivity and the presence (or absence) of the Fc portion of the isotype control, the anti-DENV antibody 2H2, the $F(ab')_2$ of 2H2, and the $F(ab')_2$ of 2H2 chemically coupled to the isotype control were confirmed by ELISA on DENV2-coated plates using a secondary antibody specific for either the Fc portion (Figure S3, left panel) or the $F(ab')_2$ portion (Figure S3, right panel).

Antibodies Enhance Infection of LSECs

The liver and small intestine were the tissues in which levels of viral RNA were most elevated in anti-DENV-treated mice relative to isotype-treated mice. To investigate infection of the cells in those tissues, total mononuclear cells were purified, stained for various surface molecules and intracellular DENV membrane protein (DENV prM, using clone 2H2), and analyzed by flow cytometry. In the liver, DENV prM^+ cells were $CD45^-$ and $CD31^+$, which is phenotypically consistent with LSECs (Katz et al., 2004) (Figure 5). A large percentage of LSECs expressed DENV prM at 48 and 72 hr in anti-DENV-treated mice, whereas the percentage of DENV prM^+ LSECs was low in isotype-treated mice (Figure 5, upper and lower left part). Expression of DENV prM was negligible in $CD45^+$ and $CD45^-CD31^-$ cell populations in both groups of mice. In the small intestine (Figure 6, upper and lower left part), the DENV prM^+ cells were MHC-II^+, $CD103^-$,

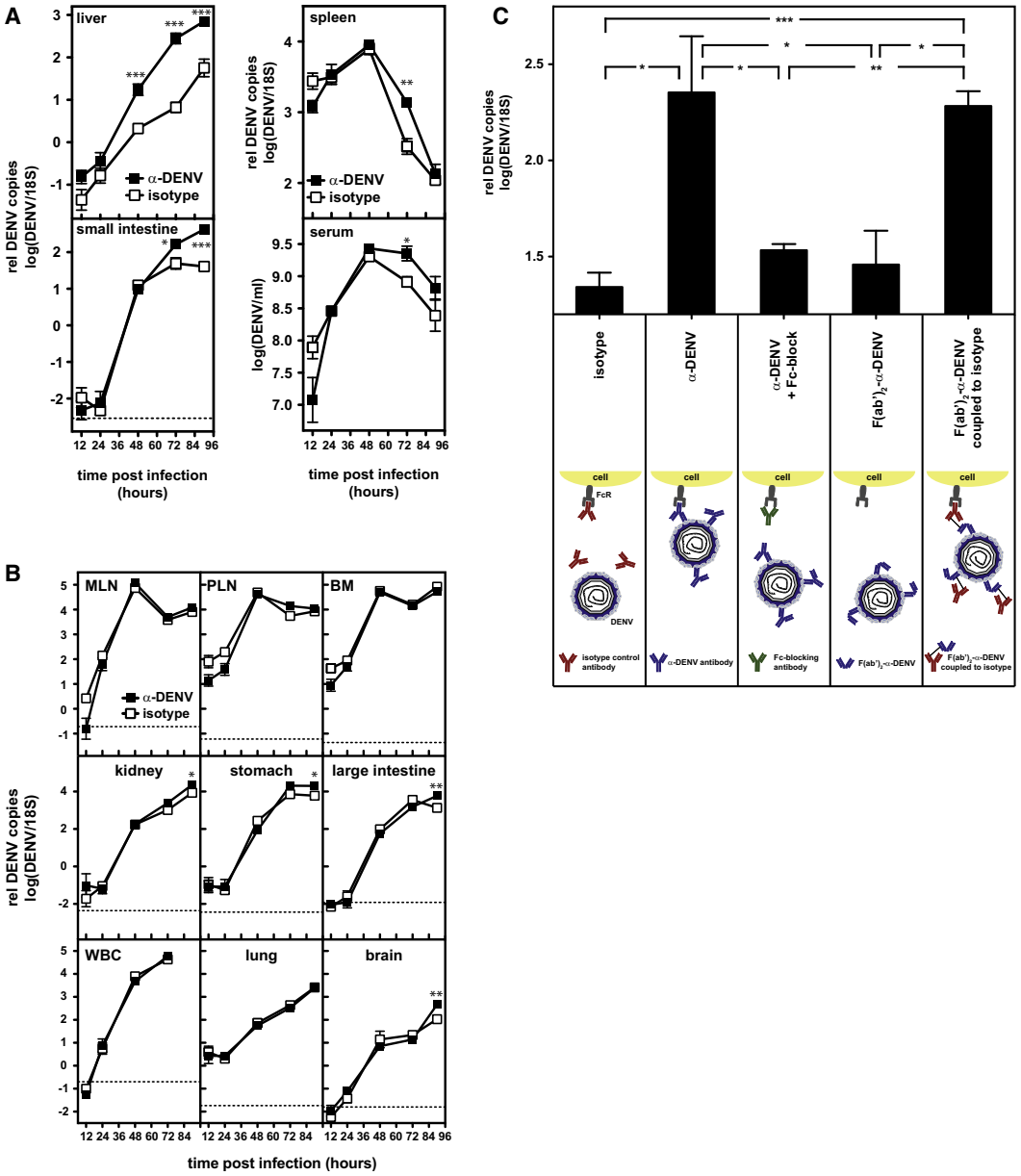


Figure 4. Administration of Anti-DENV Antibodies Results in an FcγR-Dependent Increase in Viral Load in DENV-Infected Mice

(A and B) Viral RNA levels were quantified by qRT-PCR 12, 24, 48, 72, and 90 hr after infection of AG129 mice (5×10^8 GE S221 i.v. on day 0) in the presence or absence of DENV-specific antibody (15 μg clone 2H2, administered on days −1 and 1). Symbols represent mean ± SEM, n = 3–6.

(C) DENV-infected AG129 mice (5×10^8 GE S221 i.v. on day 0) were treated on day 1 with equimolar amounts of intact anti-DENV antibody (15 μg, n = 8), intact anti-DENV antibody (15 μg) in the presence of FcγR-blocking antibody (500 μg clone 2.4G2 i.v. on day 0 and 1, n = 7), anti-DENV $F(ab')_2$ fragment (10 μg, n = 4), or anti-DENV $F(ab')_2$ fragment chemically coupled 1:1 to an isotype control (25 μg, n = 4). As a control, one group was treated with an isotype control (n = 4).

P values from two-tailed unpaired t test with Welch's correction, c.i. 95%, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$, n is the number of mice per group; MLN, mesenteric lymph node; PLN, peripheral lymph nodes (axillary, brachial and inguinal); BM,: bone marrow. See also Figures S2 and S3. Error bars represent SEM.

Ly-6C$^-$, CD11c$^{lo\text{-}int}$, CD11b$^+$, and F4/80$^+$, consistent with the phenotype of lamina propria macrophages (LPMs) (Uematsu et al., 2008) (MHC-II$^+$ cell populations in Figure 6 and MHC-II$^-$ cell populations in Figure S4). At 48 hr, a small percentage of LPMs expressed DENV prM in both isotype-treated and anti-DENV-treated mice. This percentage was similar in both groups, indicating that infection of LPMs is not directly affected by anti-DENV antibodies. By 72 hr, the percentage of prM$^+$ LPMs had increased in both groups but was three times higher in anti-DENV-treated mice.

To assess viral replication in situ, frozen tissue sections were stained for DENV nonstructural protein 3 (NS3), which is absent from the virion, but produced by the host cell during viral genome translation. In the liver, NS3 colocalized with cells expressing CD31, confirming productive infection of LSECs (Figure 5, lower right part). Colocalization of NS3 with CD68 was observed only

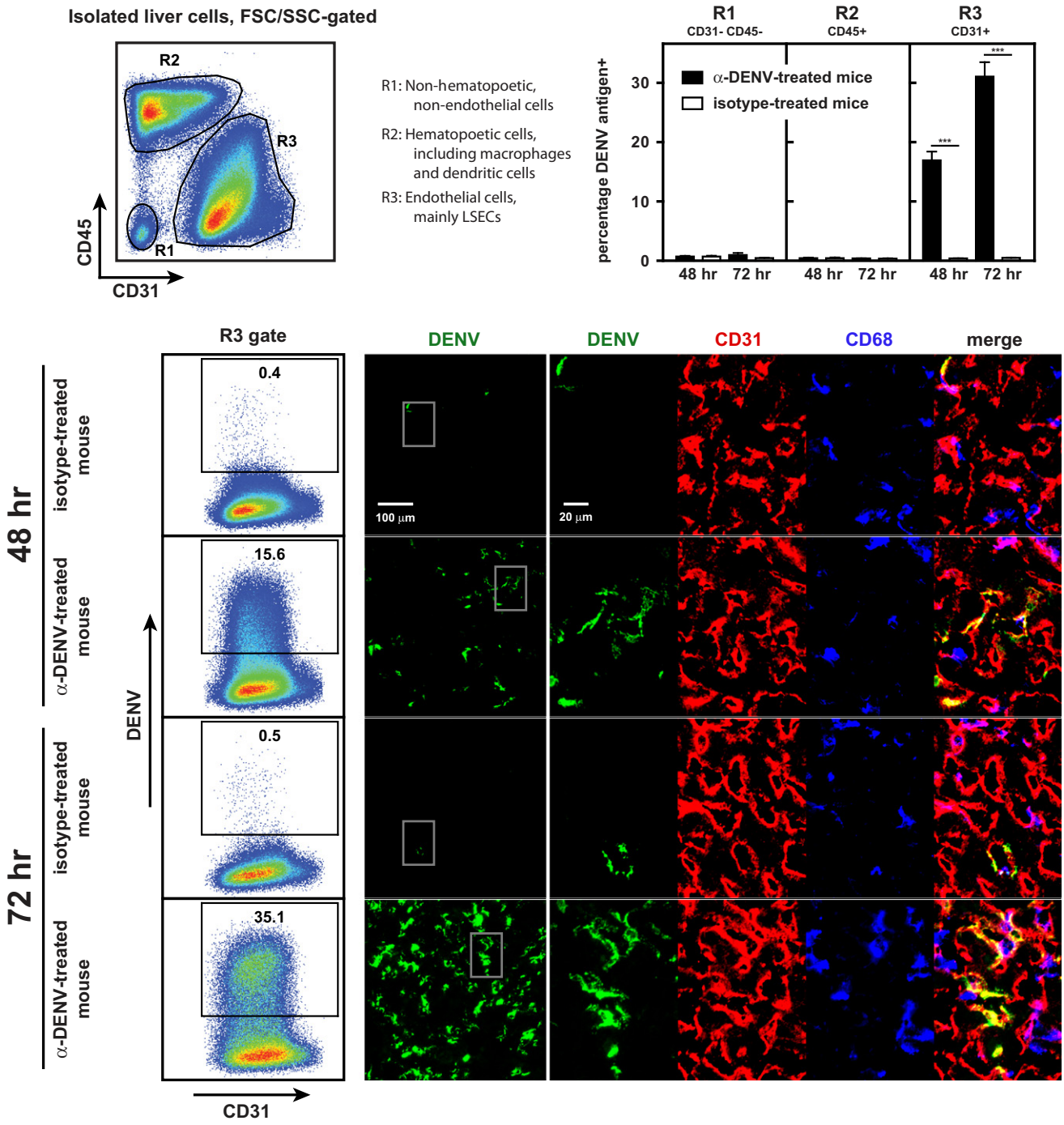
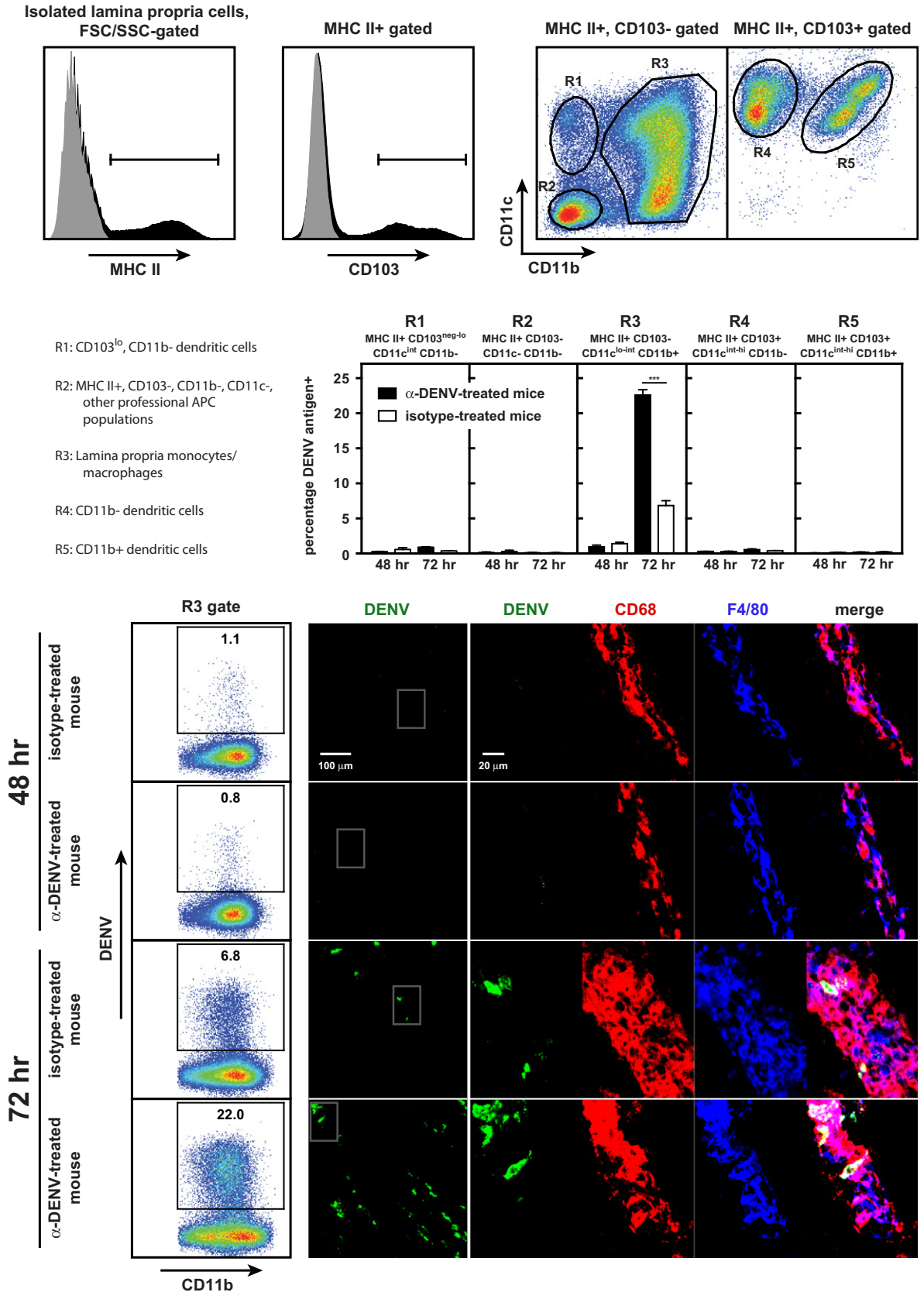


Figure 5. Anti-DENV Antibodies Increase Infection of LSECs in DENV-Infected Mice

Percentage of DENV antigen-positive cells in different liver cell populations. Gating is indicated on the left, population statistics are indicated in the bar graph on the right. Representative dot plots of DENV antigen staining of LSECs (R3 gate) and immunohistochemical staining of liver sections, stained for DENV NS3 (green), CD31 (red), and CD68 (blue) (low- and high-magnification images are on the left and right, respectively, and the size is indicated by the scale bars). n = 3 per group; representative FACS plots and immunohistochemistry images are shown; P values from two-tailed unpaired t test with Welch's correction, c.i. 95%: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, $^{***}p \leq 0.001$. Error bars represent SEM.

where CD31 was expressed as well, likely because $CD68^{+}$ macrophages are located in the liver sinusoids, surrounded by $CD31^{+}$ LSECs. In the small intestine, all NS3 localized to cells expressing F4/80 and CD68 (Figure 6, lower right part), confirming productive infection of LPMs. To determine the effect of anti-DENV antibodies on infection of LSECs by non-mouse-passaged DENV strains, immunofluorescent staining was performed on liver sections from anti-DENV-treated and


Isolated lamina propria cells, FSC/SSC-gated
MHC II+ gated
MHC II+, CD103- gated
MHC II+, CD103+ gated
R1
R2
R3
R4
R5
MHC II
CD103
CD11c
CD11b
R1: CD103lo, CD11b- dendritic cells
R2: MHC II+, CD103-, CD11b-, CD11c-, other professional APC populations
R3: Lamina propria monocytes/ macrophages
R4: CD11b- dendritic cells
R5: CD11b+ dendritic cells
R1 MHC II+ CD103neg-lo CD11cint CD11b-
R2 MHC II+ CD103- CD11c- CD11b-
R3 MHC II+ CD103- CD11clo-int CD11b+
R4 MHC II+ CD103+ CD11cint-hi CD11b-
R5 MHC II+ CD103+ CD11cint-hi CD11b+
percentage DENV antigen+
α-DENV-treated mice
isotype-treated mice
***
48 hr
72 hr
R3 gate
1.1
0.8
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α-DENV-treated mouse
DENV
CD11b
DENV
DENV
CD68
F4/80
merge
100 μm
20 μm

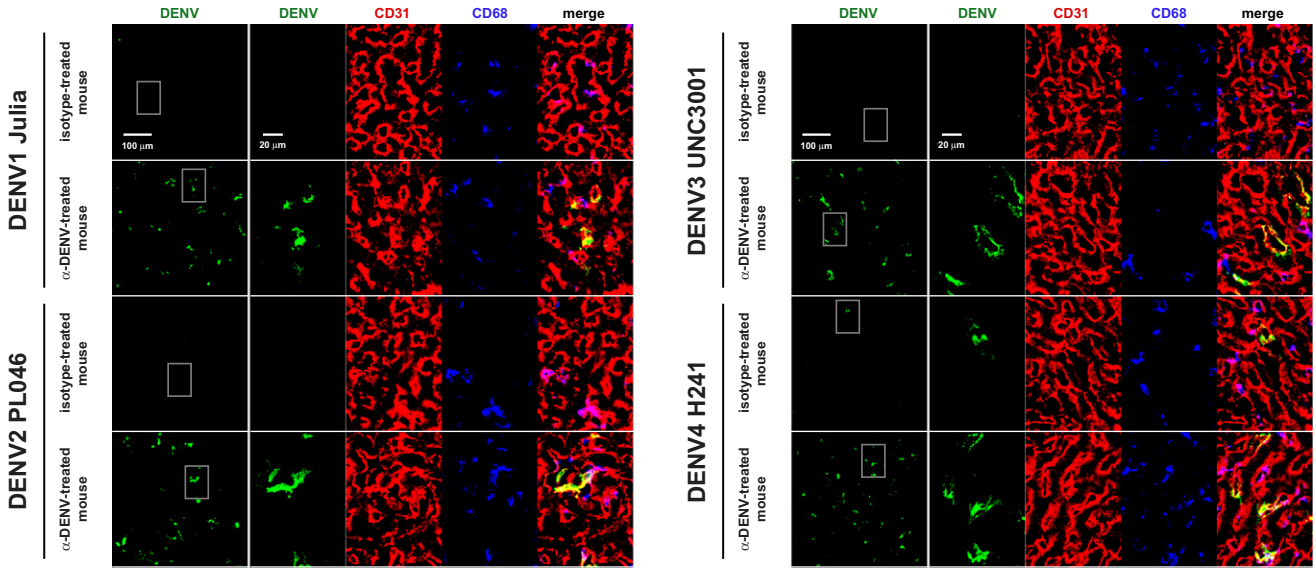

Figure 7. Antibodies Enhance Infection of LSECs by DENV Clinical Isolate Strains of Each Serotype

AG129 mice were infected with following clinical isolates: DENV1 Julia (5×10^6 FIU), DENV2 PL046 (1.5×10^7 FIU), DENV3 UNC3001 (2.5×10^5 FIU), or DENV4 H241 (3.5×10^6 FIU) on day 0, and 50 μg of anti-DENV antibody 2H2 was administered on days −1 and 1. On day 3, liver sections were stained for DENV NS3 (green), CD31 (red), and CD68 (blue). Low- and high-magnification images are on the left and right, respectively, and the size is indicated by the scale bars.

isotype-treated mice 72 hr after infection with four clinical isolates: DENV1 Julia (Nicaragua), DENV2 PL046 (Taiwan), DENV3 UNC3001 (Sri Lanka), or DENV4 H241 (Philippines). Anti-DENV antibodies greatly increased infection of LSECs by all clinical isolates (Figure 7).

DISCUSSION

Using an in vivo model for ADE-induced severe dengue disease, we have shown that anti-DENV antibodies enhance infection of LSECs, ultimately resulting in increased infection of other tissues, low platelet counts, elevated hematocrit, cytokine storm, intestinal hemorrhage, and early death, which was prevented by neutralizing TNF. As this model is based on passive transfer of anti-DENV antibodies, it is analogous to DHF/DSS in infants, which is believed to result from ADE mediated by DENV-specific maternal antibodies. With respect to secondary infections, this work separates the antibody component from other aspects of a secondary immune response, enabling the role of antibodies in DENV-induced disease to be studied in isolation.

Although many of the symptoms of human DHF/DSS were observed in the anti-DENV-treated mice in our study, the fact that we used IFN receptor-deficient mice must be taken into account when interpreting our results because of the potential role of IFN in both pathogenesis and protection. This study would ideally have been performed using immunocompetent mice, but wild-type mice did not permit detectable viral replication and did not exhibit signs of disease. A potential explanation for this is that although DENV is able to inhibit IFN signaling in human cells (Jones et al., 2005; Munoz-Jordan et al., 2003), viruses that are able to block IFN signaling in human cells have been shown to fail to do so in mouse cells (Young et al., 2001). Accordingly, it seems that the virus used in the present study is unable to sufficiently disrupt IFN signaling to allow replication in wild-type mice. Thus, although studying DENV infection in complete absence of IFN signaling has limitations, IFN receptor-deficient mice permit viral replication and develop disease much like that observed in humans, making these mice useful for studying DENV-induced disease. The use of a mouse-passaged virus for these studies could potentially be another limitation. However, increased infection of LSECs via ADE was not restricted to the mouse-passaged dengue strain, as Ab-mediated increased infection of LSECs was confirmed with all the DENV strains tested, including clinical isolates from each serotype. Interestingly, overt disease was only observed in mice infected with the mouse-passaged strain S221, likely due to its increased ability to replicate in mice relative to the clinical isolates.

The present study shows in vivo that anti-DENV antibodies specifically enhance infection of LSECs, as only the liver

Figure 6. An Increased Percentage of LPMs Are Infected Following Elevated Infection of LSECs in Mice Treated with Antibodies

Percentage of DENV antigen-positive cells in MHC-II$^+$ lamina propria cell populations. Gating is indicated on the top (the gray histogram depicts cells stained with an isotype control antibody), and population statistics are indicated in the bar graph below. Representative dot plots of DENV antigen staining of LPMs (R3 gate) and immunohistochemical staining of small intestine sections, stained for DENV NS3 (green), CD68 (red), and F4/80 (blue) (low- and high-magnification images are on the left and right, respectively, and the size is indicated by the scale bars). n = 3 per group; representative FACS plots and immunohistochemistry images are shown; P values from two-tailed unpaired t test with Welch's correction, c.i. 95%, *$p \leq 0.05$, **$p \leq 0.01$, ***$p \leq 0.001$. See also Figure S4. Error bars represent SEM.

exhibited increased viral loads during the first 48 hr after infection. Anti-DENV antibodies seem to directly and exclusively enhance infection of LSECs, subsequently resulting in the increased infection observed later in other organs. The finding that LPMs are infected to the same level at 48 hr regardless of the presence of antibody provides support for this scenario. Only after increased replication in LSECs has occurred is a higher level of infection in LPMs detected in anti-DENV-treated mice. Similarly, in other organs, higher viral loads in anti-DENV-treated mice were detected only after increased replication in LSECs. A role for LSECs in permitting ADE seems reasonable, considering that LSECs specialize in filtering small particles, including particles the size of DENV virions, out of the bloodstream (Elvevold et al., 2008), promote active antigen uptake via FcγRs (Elvevold et al., 2008), and constitutively express L-SIGN (Bashirova et al., 2001) and mannose receptors (Elvevold et al., 2008), which have been demonstrated to facilitate DENV infection (Miller et al., 2008; Tassaneetrithep et al., 2003).

ADE in vitro results from increased attachment of virus to the surface of cells through interaction of antibody-virus complexes with cellular FcγRs (Halstead et al., 1977), which subsequently results in enhanced infection. Cells that lack attachment factors, and consequently do not permit efficient attachment of virus in the absence of antibodies, are most susceptible to ADE (Boonnak et al., 2008). This likely occurs because, in the absence of attachment factors, binding of antibody-virus complexes with cell-surface FcγRs greatly increases virus-cell interactions. On cells that express high levels of attachment factors, it is likely that the virus-cell interactions that are antibody independent substantially exceed those that are antibody dependent and, therefore, the effect of antibodies becomes negligible. Accordingly, the effect of ADE in vitro is most pronounced on monocytes (Kou et al., 2008) and macrophages (Blackley et al., 2007), which exhibit very low susceptibility to antibody-independent infection in vitro (Diamond et al., 2000), and on DCs expressing low levels of attachment factors (Boonnak et al., 2008).

In contrast to the findings that, in vitro, macrophages and monocytes are not easily infected in the absence of antibodies, we have found that, in mice, macrophages of the small intestine lamina propria (as described above), as well as macrophages and DCs of many other tissues throughout the mouse (data not shown), are highly susceptible to antibody-independent infection but not subject to ADE. LSECs were the only cells supporting ADE in vivo, and they were, at the viral dose used in this study, only infected to a limited extent in the absence of antibody. It is unclear whether the discrepancy between our results and those obtained with isolated human cells is due to differences between mice and humans or due to differences in experiments performed in vitro versus in vivo. Regardless, our finding that, in mice, ADE occurs with LSECs, but not other cell populations, is in line with the observation that ADE occurs with cells that permit only low levels of antibody-independent infection.

An important point to consider is the influence of potential differences between mouse and human LSECs on our results. Attachment factors on human and mouse LSECs may differ in their expression level and ability to bind DENV, and therefore mouse and human LSECs may differ in their susceptibility to ADE. It could be argued that ADE is readily observed on mouse LSECs because they potentially lack attachment factors analogous to the ones binding DENV on human LSECs and, therefore, mouse LSECs could permit ADE even if human LSECs do not. We believe that this is not the case, because mouse LSECs, while infected only to low levels in the absence of antibodies at the dose used in the present study, permit much greater levels of antibody-independent infection at higher viral doses (data not shown). This suggests that mouse LSECs, like human LSECs, do express dengue attachment factors but, unlike macrophages, not at levels high enough to override the effect of antibodies.

The liver has been suggested to be an important site of replication for DENV in humans (Rosen et al., 1999), and the presence of DENV RNA or antigen in the liver of patients who succumbed to DHF/DSS has been reported (Balsitis et al., 2009; Jessie et al., 2004; Rosen et al., 1999). However, results from studies investigating the cellular localization of DENV antigen within the liver have been inconsistent. While Jessie et al. reported the presence of antigen in Kupffer and endothelial cells, Balsitis and colleagues found infection only in hepatocytes. Both experimental and methodological factors could account for differences in these findings. Studies have varied based on which viral proteins were detected, either structural or nonstructural, and whether polyclonal or monoclonal Abs were used. In our study, cellular tropism in the liver was assessed using two complementary methods: flow cytometry using a mAb against prM, and immunohistochemistry using polyclonal antibodies against NS3. In contrast to previous studies, the cell type permitting DENV infection was determined by both methods to be LSECs based on the expression of cellular markers rather than morphology. Additionally, increased infection of LSECs due to antibodies was observed with four clinical isolates (one of each serotype), further strengthening our results.

Our results confirm in vivo that even neutralizing antibodies have the potential to induce ADE (Mehlhop et al., 2007; Pierson et al., 2007), provided that the occupancy threshold required for neutralization is not reached (Burton, 2002). To be neutralizing, and therefore optimally protective, an antibody must be of high enough affinity for neutralizing epitopes on the surface of the virus, and it must be present in sufficient concentration (Pierson and Diamond, 2008). Failure to fulfill either of these requirements will prevent neutralization. This idea can be applied to sequential DENV infections to explain why antibodies induced by one DENV serotype, although protective against that serotype (Whitehead et al., 2007), may increase the risk of severe disease upon infection with a heterologous serotype due to enhanced infection of cells bearing FcγRs by subneutralized viral particles (Halstead, 2003). It seems likely that due to differences in the surface antigens between serotypes, only some of the antibodies raised against one will react with another, and, of the cross-reactive antibodies, some may have a reduced affinity for the second serotype. Therefore, during a secondary infection by a heterologous serotype, the threshold for neutralization is less likely to be reached and, consequently, ADE-mediated severe disease is more likely to occur. In our study, this is exemplified by the fact that the cross-reactive, neutralizing mAb 4G2 increased mean survival time at high doses but reduced mean survival time at low doses, demonstrating in vivo that neutralization and

enhancement are simply related by the stoichiometry of antibody binding to the surface of viral particles.

Multiple factors have been hypothesized to influence the severity of dengue disease, including subneutralizing levels of antibodies (Halstead, 1982), virus serotype and genotype (Rico-Hesse, 2007), and activation of serotype cross-reactive memory T cells (Rothman, 2003). Here we demonstrate that the presence of subneutralizing levels of DENV-specific antibodies can be sufficient to modify the course of infection, resulting in higher viral load, and ultimately severe disease characterized by increased cytokine release, increased vascular permeability, low platelet counts, elevated hematocrit, gastrointestinal hemorrhage, and early death. Beyond dengue disease, our findings have implications for the fields of humoral immunity and vaccine design, as they suggest a mechanism by which subprotective humoral responses may, under some circumstances, have pathological consequences.

EXPERIMENTAL PROCEDURES

Mice

Sv/129 mice deficient in type I (A129) or type I and II (AG129) IFN receptors, obtained from H. Virgin, and all other mice (purchased from The Jackson Laboratory) were housed under SPF conditions. All animal experiments were approved by the Animal Care Committee at La Jolla Institute for Allergy and Immunology (LIAI).

Antibodies

2H2 (IgG2a anti-DENV1-4 prM), 4G2 (IgG2a anti-all flavivirus E), 3H5 (IgG1 anti-DENV2 E), 15F3 (IgG1 anti-DENV1 NS1), and C44 (IgG2a anti-colchicine) hybridomas were purchased from ATCC. Hybridomas were grown in PFHM-II (GIBCO) with penicillin (100 U/ml), streptomycin (100 μg/ml), and 55 μM β-mercaptoethanol. Clarified supernatants were concentrated using Amicon 50,000 MWCO filter units (Millipore) and purified using protein G-coupled resin according to the manufacturer's instructions (Pierce). $F(ab')_2$ fragments were prepared using the $F(ab')_2$ Preparation Kit for mouse IgG2a (Pierce) by digesting purified IgG with immobilized pepsin at 37°C for 4 hr. The digest was incubated with protein A-coupled resin to remove large Fc fragments and undigested antibody. Unbound protein was dialyzed in 20,000 MWCO Slide-A-Lyzer cassettes (Pierce) to remove any remaining digested fragments. To couple 2H2 $F(ab')_2$ fragments to the isotype control antibodies, $F(ab')_2$ fragments were labeled with Sulfo-SMCC (Pierce) and mixed at an equimolar ratio with isotype control antibodies pretreated with SATA (Pierce). All antibodies were dialyzed against PBS, concentrated, and sterile filtered prior to use in experiments. The purity of antibody preparations was verified by SDS-PAGE, and binding to DENV was assessed by ELISA. Protein content was quantified using a BCA protein assay kit (Pierce).

Viruses

As previously described (Shresta et al., 2006), to generate D2S10, the C6/36 mosquito cell-adapted DENV2 isolate PL046, obtained from Dr. Huan-Yao Lei (National Cheng Kung University, Taiwan), was passaged ten times between the serum of AG129 mice and C6/36 mosquito cells. The biological clone S221 was obtained from D2S10 by growing virus from individual plaques on BHK monolayers three times serially. DENV1 strain Julia (Nicaraguan isolate) was obtained from Dr. Eva Harris, UC Berkeley. DENV3 strain UNC3001 (isolate from Sri Lanka) was obtained from Dr. Aravinda de Silva, UNC Chapel Hill. DENV4 strain H241 (isolate from the Philippines) was purchased from ATCC. Julia, PL046, UNC3001, and H241 were quantified using flow cytometry as described previously (Lambeth et al., 2005) and expressed as FIUs. Viral stocks were prepared as previously described (Prestwood et al., 2008). For ELISA, virus was purified over a sucrose gradient as previously described (Prestwood et al., 2008).

Immune Serum

Immune serum was obtained from AG129 mice infected with 1.5×10^6 FIUs (as defined by infection of C6/36 cells) of DENV1 strain Julia, DENV2 strain E128-IC (Prestwood et al., 2008) (used due to its reduced capacity to cause paralysis in AG129 mice compared to the parental strain PL046), DENV3 strain UNC3001, DENV4 strain H241.

Administration of Virus and Antibody

If not stated otherwise, AG129 mice were inoculated i.v. with 5×10^8 GE of S221. Antibody or serum was administered intraperitoneally in 200 μl PBS 1 day before and 1 day after infection.

Viral RNA Quantification

As described previously (Prestwood et al., 2008), tissues were collected into RNAlater (QIAGEN) and subsequently homogenized. RNA was isolated and DENV and relative 18S were quantified using real-time qRT-PCR.

DENV ELISA

Sucrose gradient-purified S221 (10^9 GE per well) was used to coat 96-well plates. Next, virus on plates was UV inactivated, and plates were washed of unbound virus using 0.05% (v/v) Tween 20 (Sigma) in PBS (GIBCO). After blocking with 2% (w/v) BSA in PBS (1 hr, room temperature), purified monoclonal antibodies or $F(ab')_2$ fragments were titrated on virus and unbound antibody was removed by washing. Bound antibody was detected using HRP-conjugated goat anti-mouse IgG antibodies specific for either Fc or $F(ab')_2$ portions (Jackson Immunoresearch) and TMB (eBioscience).

Biological Activity of Sera

Three-fold serial dilutions of serum were incubated with 1.5×10^{10} GE of S221 for 1 hr at room temperature in a total volume of 250 μl PBS. Subsequently, 2×10^6 C6/36 cells were infected with 200 μl of the virus-antibody mix for 1 hr at 28°C. Cells were washed two times with 1 ml of PBS, and the cells were incubated at 28°C in 500 μl L-15 medium (GIBCO) containing 5% FCS, penicillin (100 U/ml) and streptomycin (100 μg/ml) for 23 hr. For each antibody dilution, the percent of infected cells was determined by flow cytometry as previously described (Lambeth et al., 2005) using 2H2-biotin and streptavidin-APC (Biolegend). The percent of infected cells was normalized to 100% (infection without antibody), and the average of two experiments is shown.

Measurement of Cytokines

Serum samples were analyzed by the Immune Reconstitution Facility, Duke University, Durham, NC, USA, using the Bio-Plex Multiplex Cytokine Assay (Bio-Rad). TNF in serum was measured using the Ready-SET-Go! TNF ELISA kit (eBioscience).

Hematologic Analysis

Platelet counts and hematocrit were measured on a Hemavet 950FS (Drew) according to the manufacturer's instructions.

Neutralization of TNF, IL-6, and Blocking of IL-10 Receptor and FcγRs II and III

Functional grade rat anti-TNF (clone MP6-XT22; eBioscience) was used to neutralize TNF as previously described (Shresta et al., 2006), except that 100 μg was injected i.p. each day for 4 days following infection, starting on day 1. As previously described, 500 μg of functional grade rat anti-IL-6 (Starnes et al., 1990) (clone MP5-20F3; BioXCell) or anti-IL10R (Brooks et al., 2006) (clone 1B1.3A; BioXCell) was administered i.p. on day 2 and 3. To block FcγRs II and III, 500 μg of mAb 2.4G2 (BioXCell) was administered i.v. on days 0 and 2.

Vascular Leakage Assay

Evans blue (Sigma), which binds avidly to albumin, is used to assess vascular permeability, as the extravasation of albumin into tissues indicates relative integrity of the vascular endothelium. Evans blue (0.2 ml, 0.5% [w/v] in PBS) was administered i.v. and, after 15 min, animals were sacrificed and extensively perfused with PBS. Livers were collected into formamide and, after

overnight extraction at 37°C, the dye was quantified by measuring absorbance at 610 nm.

Flow Cytometry

Tissues were collected into RPMI (GIBCO) containing 10% fetal calf serum (Gemini Bio-Products) and stored on ice. To isolate cells from the small intestine lamina propria, Peyer's patches were excised and the tissue was filleted longitudinally and washed in cold PBS. Epithelial cells were removed from segmented tissue by agitation for 30 min at 37°C in PBS containing 10% (v/v) FCS, EDTA (10 mM), HEPES (20 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), and polymyxin B (10 μg/ml; Calbiochem), followed by extensive washing with PBS. The small intestine and liver were minced and digested for 20 min at 37°C with agitation in RPMI containing 10% fetal calf serum and 1.5 mg/ml collagenase VIII (Sigma) in approximately 25 ml. Tissues were immediately washed in RPMI and digests were collected via centrifugation. Resuspended tissues were pressed through 70 μm strainers (BD) using the plunger of 3 ml syringes (BD). Liberated cells were collected and pelleted by centrifugation. Cells were resuspended in a 14.7% Optiprep (Sigma) solution diluted in 150 mM NaCl with 10 mM HEPES (pH 7.4) and layered over 22.2% Optiprep solution diluted in the same buffer. Cells were spun for 20 min at 750 × g at 25°C and washed immediately after with RPMI. 2×10^5 cells per well were plated in 96-well plates, and FcγRs were blocked with 1 μg of mAb 2.4G2. Cells from the small intestine were stained using FITC anti-Ly-6C (PharMingen), PE anti-CD103 (PharMingen), PE-Cy7 anti-CD11c (PharMingen), APC anti-CD11b (Biolegend), Pacific Blue anti-MHC II (Biolegend), Alexa-Fluor 700 anti-Gr-1 (PharMingen), and PerCP-Cy5.5 anti-F4/80 (Biolegend) or with appropriately labeled isotype controls. Cells from the liver were stained with PE anti-CD31 (PharMingen) and PE-Cy7 anti-CD45 (PharMingen). Cells were fixed and permeabilized using the Cytofix/Cytoperm Kit (BD). For intracellular staining, all steps were performed in 1× BD Perm/Wash. Cells were blocked briefly and stained for 30 min with biotinylated 2H2 or isotype control. After washing, cells were stained with streptavidin conjugated to either APC (eBioscience) for the liver cells or APC-Alexa Fluor 750 (Caltag Laboratories) for the small intestine cells. The gate for DENV antigen-positive cells was set at 0.1% of the isotype control staining. Data were collected using an LSR II (BD) and analyzed with FlowJo software (Tree Star).

Immunohistochemistry

Tissues were embedded in O.C.T. compound (Sakura). Sections (6 μm) were cut and stored at −80°C. Frozen sections were thawed and fixed for 10 min in acetone at 25°C, followed by 8 min in 1% paraformaldehyde (EMS) in 100 mM dibasic sodium phosphate containing 60 mM lysine and 7 mM sodium periodate at pH 7.4 on ice. Sections were blocked first using the Avidin/Biotin Blocking Kit (Vector Labs) followed by 5% normal goat serum (Caltag Laboratories) and 1% BSA (Sigma) in PBS. Sections were stained overnight with purified rabbit polyclonal anti-DENV NS3 (a generous gift from the Novartis Institute for Tropical Diseases, Singapore) and either rat anti-mouse CD31 (PharMingen) for liver sections or PE-labeled rat anti-mouse F4/80 (Caltag Laboratories) for small intestine sections. Sections were then washed and stained with DyLight 649-labeled goat anti-rabbit IgG (Jackson Immunoresearch) and biotinylated goat anti-rat IgG (PharMingen) and then with PE-labeled streptavidin (eBioscience). Sections were then blocked again with Avidin/Biotin Blocking Kit followed by 10% rat serum in PBS and finally stained with biotin anti-mouse CD68 (Serotec) and FITC-labeled streptavidin (PharMingen). Images were recorded using a Marianas deconvolution fluorescence microscope (3i) and prepared using Adobe Photoshop.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.chom.2010.01.004.

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