VASCULAR REMODELING

Dengue virus NS1 triggers endothelial permeability and vascular leak that is prevented by NS1 vaccination

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The four dengue virus serotypes (DENV1 to DENV4) are mosquito-borne flaviviruses that cause up to ~100 million cases of dengue annually worldwide. Severe disease is thought to result from immunopathogenic processes involving serotype cross-reactive antibodies and T cells that together induce vasoactive cytokines, causing vascular leakage that leads to shock. However, no viral proteins have been directly implicated in triggering endothelial permeability, which results in vascular leakage. DENV nonstructural protein 1 (NS1) is secreted and circulates in patients' blood during acute infection; high levels of NS1 are associated with severe disease. We show that inoculation of mice with DENV NS1 alone induces both vascular leakage and production of key inflammatory cytokines. Furthermore, simultaneous administration of NS1 with a sublethal dose of DENV2 results in a lethal vascular leak syndrome. We also demonstrate that NS1 from DENV1, DENV2, DENV3, and DENV4 triggers endothelial barrier dysfunction, causing increased permeability of human endothelial cell monolayers in vitro. These pathogenic effects of physiologically relevant amounts of NS1 in vivo and in vitro were blocked by NS1-immune polyclonal mouse serum or monoclonal antibodies to NS1, and immunization of mice with NS1 from DENV1 to DENV4 protected against lethal DENV2 challenge. These findings add an important and previously overlooked component to the causes of dengue vascular leak, identify a new potential target for dengue therapeutics, and support inclusion of NS1 in dengue vaccines.

INTRODUCTION

Dengue virus (DENV) is a mosquito-borne flavivirus that is estimated to cause up to 390 million infections, 96 million cases of dengue, and ~500,000 hospitalizations annually (*1*). Infection with any of the four DENV serotypes (DENV1, DENV2, DENV3, and DENV4) results in a range of syndromes from inapparent infection to classic dengue fever (DF) to dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS), which is characterized by vascular leakage and shock (*2*). Most primary DENV infections caused by any of the four serotypes are asymptomatic or lead to the self-limiting but debilitating DF; however, secondary infections with a different (heterologous) DENV serotype can lead to increased risk of severe dengue (*3*). Immune responses after primary DENV infection lead to protective immunity to homologous secondary infection but may either protect against or cause increased disease severity in a subsequent DENV infection with a different serotype. The latter is thought to be mediated by serotype cross-reactive T cells or antibody-dependent enhancement (ADE), whereby cross-reactive antibodies that target viral structural proteins facilitate DENV infection of Fcγ receptor–bearing cells, leading to increased viral load (*4*, *5*). ADE and cross-reactive T cells are thought to trigger an exaggerated and skewed immune response to a previously infecting serotype, resulting in a "cytokine storm"—rapid-onset, high-level production of proinflammatory cytokines, including tumor necrosis factor–α (TNF-α) and interleukin-6 (IL-6), in the blood that leads to endothelial permeability and vascular leak (*6*). However, the potential role of viral proteins in mediating vascular leakage has not been demonstrated.

The DENV 10.7-kb, positive-strand RNA genome encodes a polyprotein that is posttranslationally cleaved by host and viral proteases into three structural proteins [capsid, membrane, and envelope (E)] and seven nonstructural proteins. DENV nonstructural protein 1 (NS1) is a glycosylated 48-kD protein that plays a role in both viral replication and immune evasion (*7*, *8*). NS1 is initially translated as a monomer that is glycosylated in the endoplasmic reticulum (ER) but rapidly forms a dimer that can have four fates: association with the viral replication complex on the surface of the ER membrane, plasma membrane association via a glycophosphatidylinositol linkage on infected cells, formation of a soluble lipophilic hexamer secreted by infected cells, or binding back of the hexameric NS1 to the surface of uninfected cells via glycosaminoglycan interactions (*9*, *10*).

Secreted soluble DENV NS1 (sNS1) is found in patient serum during acute illness and is used as a diagnostic indicator of acute DENV infection. High levels of sNS1 are associated with increased disease severity, although it is unclear whether this measure has functional significance or is just a marker of higher viremia in severe cases. sNS1 can bind host complement components directly and inhibit complement activation in solution and on the cell surface, whereas sNS1-antibody complexes can consume complement in the context of secondary human DENV infection (*11*–*14*). Although sNS1 binds to endothelial cells (*12*), the functional consequences of this interaction for disease pathogenesis are poorly understood. Antibodies to NS1 cross-react with platelets, endothelial cells, and thrombin and are postulated to contribute to pathogenesis (*15*–*17*). However, vaccination with NS1 or passive transfer of monoclonal antibodies to NS1 (anti-NS1 mAbs) can at least partially protect against DENV infection in mice that are injected intracranially (*18*–*21*) or intraperitoneally (*22*) with DENV, indicating that anti-NS1 antibodies can be protective and not pathogenic (*23*). NS1 is considered a promising vaccine candidate (*23*–*26*) because it is highly conserved across DENV serotypes and because it should not generate disease-enhancing antibodies.

Although the levels of sNS1 correlate with disease severity, a direct role for NS1 in triggering endothelial disruption has not yet been established; therefore, we evaluated whether NS1 compromises endothelial

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cell integrity and contributes to vascular leakage in vitro and in vivo, respectively, and whether the immune response to NS1 after vaccination provides protection in a lethal mouse model of DENV vascular leak syndrome.

RESULTS

NS1 triggers vascular leak–mediated pathogenesis in vivo

We hypothesized that NS1 might elicit direct pathogenic effects in DENV disease and sought to test its effect in both the absence and presence of viral replication. For all experiments, we used commercially obtained recombinant NS1 produced in human embryonic kidney (HEK) 293 cells or in S2 cells. These NS1 preparations are highly purified (fig. S1A) and reported to be hexameric in structure; we confirmed that almost all the proteins are in the oligomeric form (fig. S1B). The NS1 preparation is reported to be endotoxin-free, which we confirmed using the Limulus Amebocyte Lysate assay [<0.1 EU/ml in 25 μg of NS1 from DENV1 to DENV4 NS1 (HEK 293) or DENV2 NS1 (S2)]. C57BL/6 mice deficient in the interferon-α/β receptor (*Ifnar*$^{-/-}$) inoculated with DENV2 NS1 (10 or 20 mg/kg) in the absence of DENV infection exhibited morbidity on days 2 to 4 post-inoculation (p.i.), although they recovered by day 5 p.i. (fig. S2A). Moreover, 100% of naïve mice inoculated intravenously with DENV2 NS1 (10 mg/kg) combined with a sublethal dose [10^6 plaque-forming units (PFU)] of DENV2 strain D220 (hereafter referred to as DENV2) succumbed 3 to 4 days p.i., equivalent to mice receiving a lethal dose (10^7 PFU) of DENV2 (Fig. 1A). In contrast, mice receiving a sublethal dose of DENV2 alone or ovalbumin (OVA) (10 mg/kg) with a sublethal dose of DENV2 exhibited 100% survival and little morbidity. Mice injected with less DENV2 NS1 (5 mg/kg) combined with a sublethal dose of DENV2 all exhibited signs of disease starting day 2 p.i., and two of six mice succumbed on day 3 p.i. (Fig. 1A), suggesting a

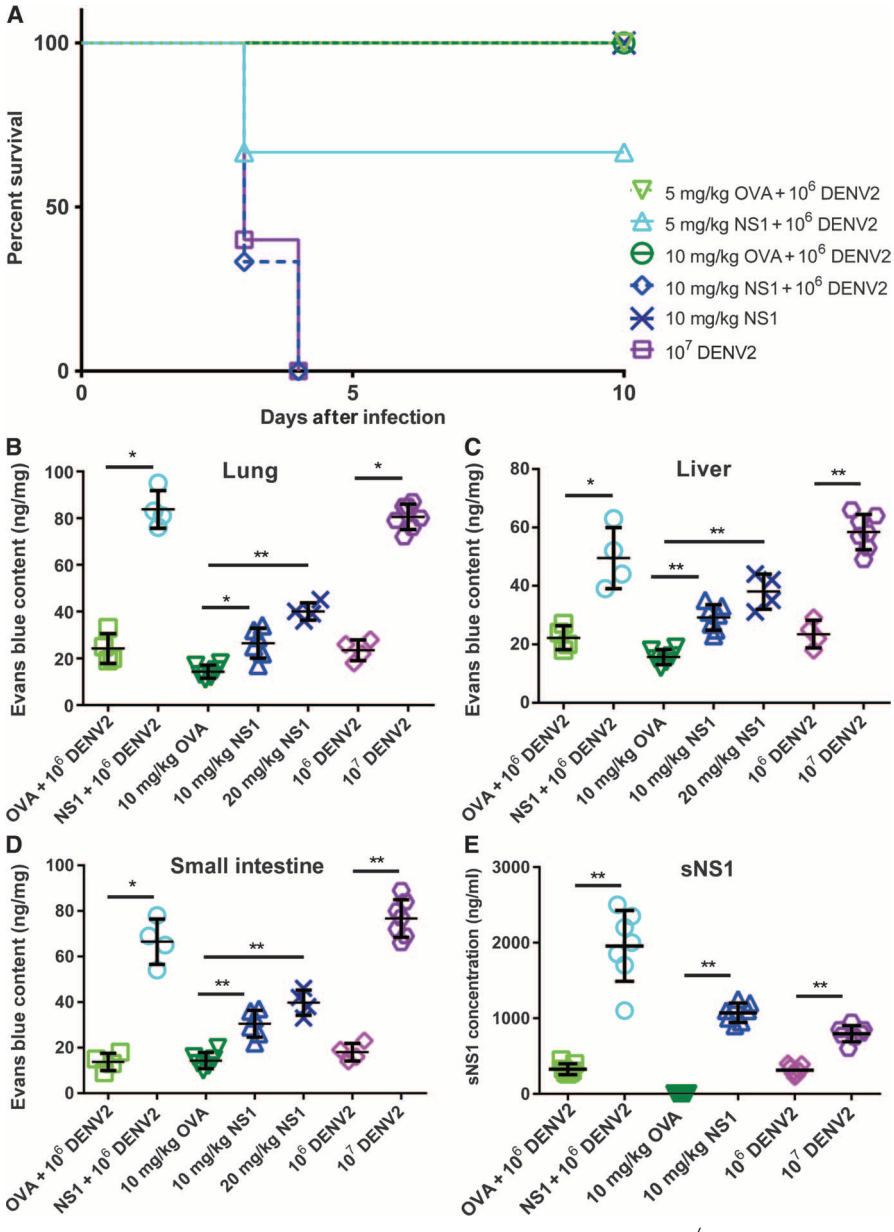

Fig. 1. NS1 triggers vascular leak–mediated pathogenesis in vivo. (**A**) *Ifnar*$^{-/-}$ mice were infected intravenously with a lethal dose of DENV2 (10^7 PFU) alone ($n = 7$), a sublethal dose of DENV2 (10^6 PFU) with DENV2 NS1 [5 mg/kg ($n = 6$) or 10 mg/kg ($n = 7$)], a sublethal dose of DENV2 with OVA [5 mg/kg ($n = 6$) or 10 mg/kg ($n = 7$)] as a negative control, or DENV2 NS1 (10 mg/kg) alone ($n = 7$). A Kaplan-Meier survival curve is shown, with data derived from two independent experiments. Mice given NS1 (10 mg/kg) alone or OVA (5 or 10 mg/kg) + 10^6 PFU of DENV2 exhibited statistically significant survival using a nonparametric Mantel-Cox log-rank test ($P = 0.022$) compared to mice given NS1 (10 mg/kg) + 10^6 PFU of DENV2. (**B** to **D**) Evans blue dye was injected intravenously into mice 3 days after treatment with DENV2 NS1 (10 mg/kg) ($n = 7$), DENV2 NS1 (20 mg/kg) ($n = 4$), OVA (10 mg/kg) ($n = 6$), NS1 (10 mg/kg) + 10^6 PFU of DENV2 ($n = 4$), OVA + 10^6 PFU of DENV2 ($n = 4$), or 10^6 ($n = 4$) or 10^7 PFU of DENV2 alone ($n = 7$), as indicated. The dye was allowed to circulate for 1 h before mice were euthanized, tissues were harvested, and Evans blue was extracted in formamide and quantified in (**B**) lung, (**C**) liver, and (**D**) small intestine tissue by measuring absorbance at OD_{610}. In all tissues, the mice given NS1 (20 mg/kg) or NS1 (10 mg/kg) alone or NS1 (10 mg/kg) + 10^6 PFU of DENV2 had significantly higher levels of Evans blue than the OVA controls. (**E**) *Ifnar*$^{-/-}$ mice were administered DENV2 NS1 (10 mg/kg) or OVA (10 mg/kg) alone, NS1 or OVA (10 mg/kg) plus 10^6 PFU of DENV2, or 10^6 or 10^7 PFU of DENV2 alone, as indicated ($n = 4$ per group). Plasma was collected 3 days after treatment, and the levels of NS1 were measured by NS1-specific capture enzyme-linked immunosorbent assay (ELISA). (**B** to **E**) Data were derived from two independent experiments and were analyzed by nonparametric Mann-Whitney analysis. *$P < 0.05$; **$P < 0.01$.

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dose-dependent effect of NS1 in mediating severe dengue disease.

We performed parallel experiments to measure vascular leakage as detected by tissue-associated Evans blue dye, which quantifies leakage out of the circulatory system when injected intravenously. We evaluated the vascular leakage on day 3 p.i. because this day was when we observed the greatest morbidity in the NS1-injected mice (fig. S2A). Mice receiving NS1 (10 mg/kg) plus a sublethal dose of DENV2 or a lethal dose of DENV2 displayed high levels of Evans blue dye in the lung, liver, spleen, and small and large intestines (Fig. 1, B to D, and fig. S2, B and C), indicating substantial vascular leakage. Mice receiving NS1 (10 or 20 mg/kg) alone contained significantly higher levels of Evans blue in all tissues than mice administered OVA as a negative control, whereas mice receiving sublethal doses of DENV2 (plus or minus OVA) had lower but measurable levels of Evans blue (Fig. 1, B to D, and fig. S2, B and C). These data demonstrate a role for NS1 alone in inducing vascular permeability in vivo.

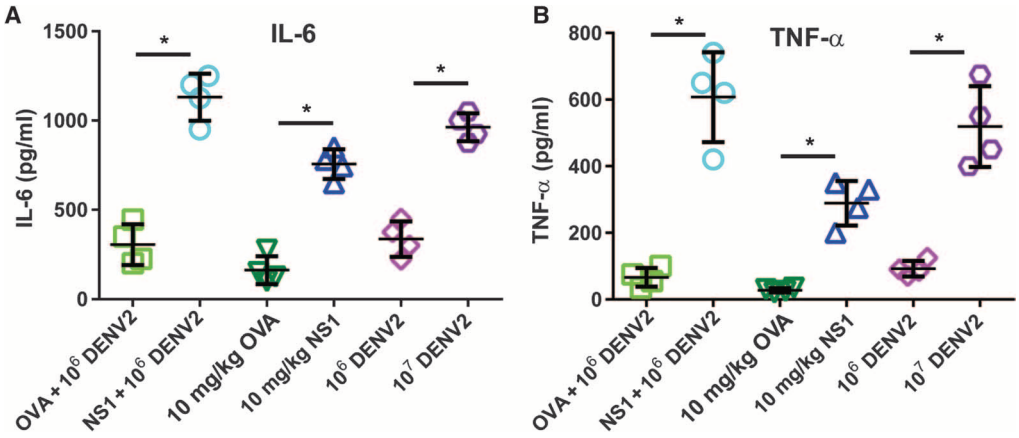

Fig. 2. NS1 causes increased production of inflammatory cytokines in vivo in the presence or absence of DENV2. *Ifnar*$^{-/-}$ mice were administered DENV2 NS1 (10 mg/kg) or OVA (10 mg/kg) alone, NS1 or OVA (10 mg/kg) plus 10^6 PFU of DENV2, or 10^6 or 10^7 PFU of DENV2 alone, as indicated ($n = 4$ per group). (**A** and **B**) Plasma was collected 3 days after treatment, and the levels of (**A**) IL-6 and (**B**) TNF-α were measured by ELISA. Mice given NS1 (10 mg/kg) + 10^6 PFU of DENV2 or NS1 (10 mg/kg) alone had significantly higher levels of both cytokines than controls receiving OVA. Data were derived from two independent experiments and were analyzed by nonparametric Mann-Whitney analysis. *$P < 0.05$.

In a separate set of mice, we evaluated the levels of circulating NS1, viremia, and selected cytokines in serum 3 days p.i., when maximum morbidity and mortality were observed, with or without NS1 or OVA as above. Injection of NS1 (10 mg/kg) alone resulted in ~1 μg/ml circulating NS1 in serum at day 3 p.i., only slightly higher than levels in mice infected with a lethal dose of DENV2 (Fig. 1E). Mice infected with a sublethal dose of DENV2 alone or combined with OVA (10 mg/kg) had ~0.25 μg/ml circulating NS1 at day 3 p.i., whereas mice infected with a sublethal dose of DENV2 and NS1 (10 mg/kg) yielded an average of ~2 μg/ml circulating NS1 at day 3 p.i. (Fig. 1E). We also evaluated the levels of NS1 every 12 h after inoculation with NS1 (10 mg/kg) and found that they decreased from ~15 μg/ml at 12 h p.i. to ~1 μg/ml at 72 h p.i. (fig. S2D); these levels are consistent with the range seen in humans with severe dengue (~1 to >10 μg/ml) (*27*, *28*). Viremia levels were comparable between mice receiving NS1 plus a sublethal dose of DENV2 and mice infected with a lethal dose of DENV2, and both were 10-fold greater than viremia levels in OVA plus a sublethal dose of DENV2 or a sublethal dose of DENV2 alone (fig. S2E). With respect to cytokines often associated with DHF/DSS, we found that inoculation of NS1 alone or addition of NS1 to a sublethal dose of DENV2 significantly increased levels of TNF-α and IL-6 compared to negative controls, to levels similar to those observed in mice experiencing a lethal DENV2 infection (Fig. 2, A and B). In sum, the simultaneous injection of NS1 with a sublethal dose of DENV2 resulted in a lethal infection associated with vascular leakage, and NS1 alone increased vascular leakage in vivo. Furthermore, injection of NS1 in both the presence or absence of viral infection led to increased levels of critical inflammatory cytokines.

NS1 causes endothelial permeability in vitro

To directly evaluate a possible role for NS1 in triggering endothelial cell dysfunction, NS1 alone was incubated with cultures of a human pulmonary microvascular endothelial cell (HPMEC) line. HPMECs were grown on Transwell permeable membranes as a model of barrier function in vitro and incubated with DENV2 NS1 (0.2 to 20 μg/ml). Endothelial cell permeability was examined by measuring the transendothelial electrical resistance (TEER) of the HPMECs at different time points after treatment with NS1. TNF-α, known to induce endothelial barrier dysfunction, was used as a positive control and resulted in decreased TEER values (Fig. 3A). DENV2 NS1 induced a significant ($P < 0.0001$) dose-dependent increase in endothelial permeability compared to TEER baseline values exhibited by untreated controls and treatment with control protein (20 μg/ml OVA), starting ~2 h post-treatment (hpt) and persisting for 12 to 24 hpt depending on the dose (Fig. 3A and fig. S3A). Similar results were observed in primary human umbilical vein endothelial cells (HUVEC; fig. S3B). We then tested NS1 from DENV1, DENV2, DENV3, and DENV4 at 5 or 20 μg/ml on HPMEC monolayers (Fig. 3B and fig. S3C). NS1 from all four serotypes resulted in a significant ($P < 0.0001$) decrease in TEER; DENV1 and DENV2 NS1 caused the greatest reduction, from 3 to 13 hpt, whereas DENV3 and DENV4 NS1 displayed similar decreases from 9 to 13 hpt. In contrast, NS1 from West Nile virus (WNV), a closely related flavivirus that causes encephalitis but no systemic vascular leak, did not trigger reduction in TEER, nor did another DENV protein (E) when administered at the same concentration (Fig. 3B and fig. S1A). Thus, NS1 alone from all four DENV serotypes, but not WNV, can directly trigger increased endothelial cell permeability.

NS1 vaccination protects against lethal DENV–induced vascular leak syndrome

Given the ability of NS1 to induce vascular leak, we hypothesized that vaccination with NS1 might constitute a strategy for protecting against severe dengue disease. To investigate whether immune responses against NS1 could prevent lethal vascular leak–associated DENV disease, we immunized *Ifnar*$^{-/-}$ mice three times intraperitoneally with 20 μg of DENV2 NS1 and several different adjuvant systems over a 6-week period (days 1, 14, and 42) and challenged the mice with a lethal DENV2 infection 2 weeks after the last immunization (day 56). Mice immunized

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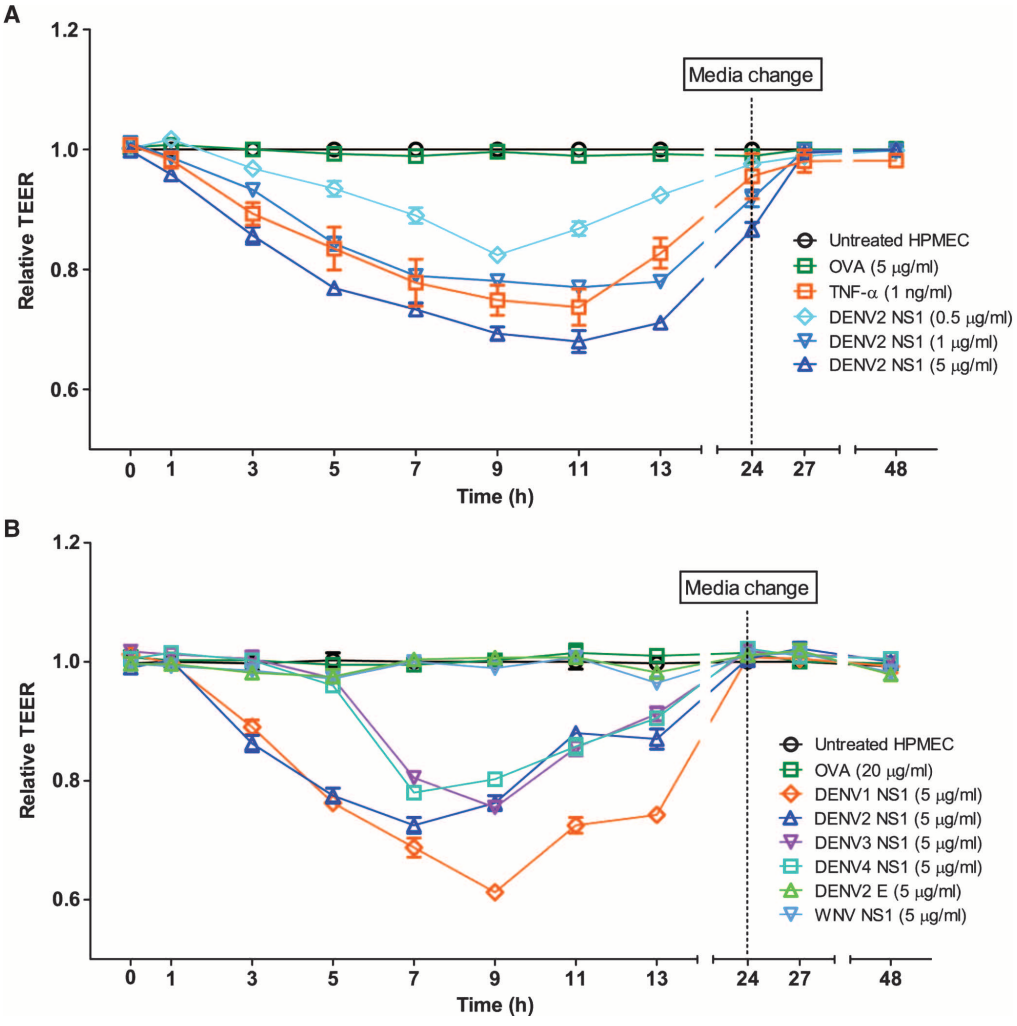

Fig. 3. NS1 induces endothelial permeability in human pulmonary endothelial cells in vitro. Confluent monolayers of HPMECs grown on Transwell inserts were incubated for 48 h with NS1, and TEER (ohm) was measured at indicated time points. Relative TEER (ratio of resistance values between experimental and untreated cells) was plotted. Data were normalized to inserts containing medium only. (**A**) Increasing concentrations of DENV2 NS1 (0.5 to 5 μg/ml) or TNF-α (1 ng/ml) were added to HPMEC monolayers, and TEER was measured at the indicated time points. (**B**) DENV1, DENV2, DENV3, DENV4 NS1, WNV NS1, or DENV2 E protein (5 μg/ml) was added to HPMEC monolayers, and TEER was analyzed at indicated time points. OVA (20 μg/ml) was used as a treatment control. Results are representative of three independent experiments using duplicate Transwells. Error bars indicate SEM. NS1, DENV2 NS1 (5 μg/ml).

with DENV2 NS1 and monophosphoryl lipid A (MPLA)/AddaVax were completely protected against a lethal systemic challenge (Fig. 4A) and exhibited little morbidity. Similarly, NS1/MPLA/AddaVax-immunized mice were protected against antibody-enhanced lethal challenge with DENV2 (*29*, *30*) (Fig. 4A). None of the OVA-immunized mice survived the high-dose or ADE lethal challenge. A low-dose (10^5 PFU) primary homologous infection with the parental DENV2 strain PL046 provided 100% protection against lethal challenge as a positive control (Fig. 4A). Thus, immune responses to adjuvanted NS1 protein alone were sufficient to provide complete protection, comparable to immunity induced by primary DENV infection. We then immunized mice with NS1, OVA, or DENV2 PL046, subjected them to lethal DENV2 challenge, and evaluated levels of NS1 in the serum. Sera from NS1-immune mice 3 days after the challenge contained almost no detectable NS1 compared to ~0.75 μg/ml in OVA-immunized mice, whereas NS1 levels in DENV2 PL046-immune mice were reduced to a lesser degree (~0.45 μg/ml) (Fig. 4B). NS1-immune and DENV-immune mice also displayed lower viremia in serum and lower viral load in bone marrow, spleen, and small intestine after challenge compared to OVA-immunized mice (Fig. 4, C and D, and fig. S4, A and B). These data suggest that the immune response to NS1 protected against mortality by greatly reducing circulating NS1 as well as decreasing the peripheral viral load.

Because relatively high homology exists in the amino acid sequence of NS1 within and across the four DENV serotypes (*31*), we examined whether immunization with NS1 could protect against a heterologous, lethal DENV infection. Mice were immunized as above with DENV1, DENV2, DENV3, or DENV4 NS1, challenged 2 weeks after the third immunization with a lethal dose of DENV2, and monitored for morbidity and mortality. As expected, DENV2 NS1 provided 100% protection against lethal homologous DENV2 challenge (Fig. 4E). Immunization with DENV1 NS1 provided 75% protection, and DENV3 or DENV4 NS1 provided 60% protection against DENV2 lethal challenge (Fig. 4E), demonstrating sufficient immune cross-reactivity to NS1 epitopes to provide partial protection across serotypes. Serum was obtained from mice 1 week after the third immunization with NS1 or OVA, which coincided with 7 weeks after DENV2 PL046 primary infection. We tested the prechallenge immune serum by ELISA for reactivity to NS1 of all four DENV serotypes (fig. S5). DENV NS1-immune sera bound strongly to NS1 from the homologous serotype and also exhibited substantial cross-reactivity to NS1 from the other three serotypes. In addition, the level of anti-NS1 antibody binding and serotype cross-reactivity was greater in NS1-immunized mice than in natural infection with the different DENV serotypes (fig. S5).

NS1-immune serum and anti-NS1 mAbs block NS1-induced vascular leak

To determine whether antibodies from NS1-immunized mice could prevent the direct effect of NS1 on endothelial permeability, we tested NS1- or OVA-immune sera from vaccinated mice on HPMEC monolayers using the TEER assay. Intact NS1-immune sera inhibited the

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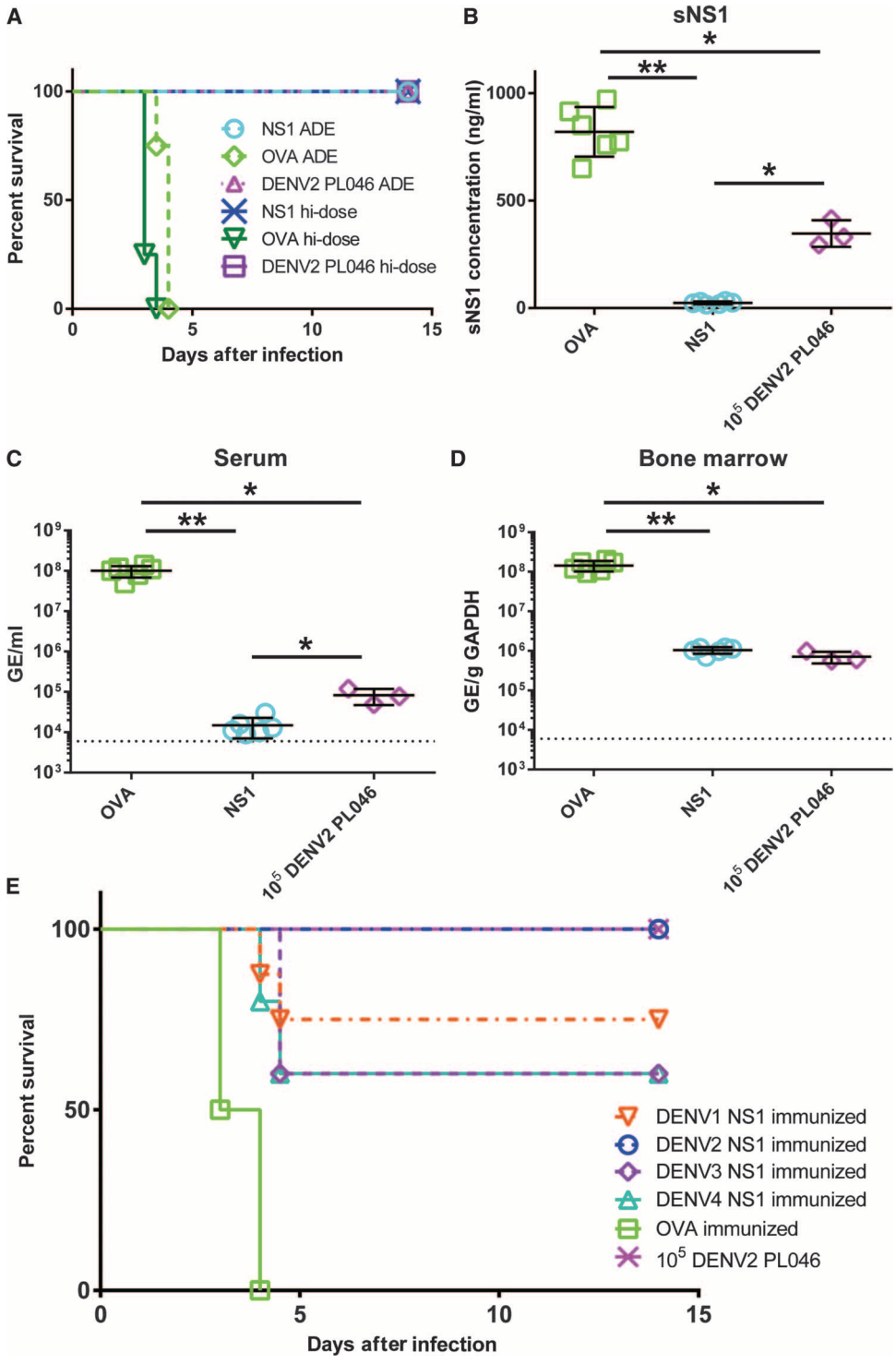

Fig. 4. NS1 vaccination protects against lethal DENV–induced vascular-leak syndrome. (**A**) Mice were immunized intraperitoneally with 20 μg of DENV2 NS1 ($n = 12$) or OVA ($n = 8$) combined with MPLA/AddaVax adjuvants on days 0, 14, and 42 or infected with a sublethal dose (10^5 PFU) of DENV2 PL046 ($n = 12$) at day 0; on day 56, half were challenged intravenously with a lethal dose of 10^7 PFU of DENV2 and half with lethal antibody-enhanced DENV2 infection. A Kaplan-Meier survival curve is shown. Mice immunized with NS1 were significantly protected compared to OVA controls from both ADE ($P = 0.003$) and high-dose (hi-dose) ($P = 0.001$) lethal challenge. (**B**) NS1 levels in serum were measured by NS1-specific capture ELISA in NS1-immunized ($n = 6$) or OVA-immunized ($n = 6$) mice and DENV2 PL046–immune ($n = 3$) mice 3 days after DENV2 lethal challenge. Circulating NS1 was significantly higher in OVA-immunized compared to NS1-immunized mice after challenge; $^*P < 0.05$; $^{**}P < 0.01$. (**C** and **D**) The viral RNA copy number was measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR) in (**C**) serum and (**D**) bone marrow 3 days post-lethal challenge. OVA-immunized mice ($n = 6$) had higher levels of viral RNA in both tissues compared to NS1-immunized mice ($n = 6$); $^*P < 0.05$; $^{**}P < 0.01$. The limit of detection is indicated by a dashed line. GAPDH, glyceraldehyde phosphate dehydrogenase. GE, genome equivalent. (**E**) Mice were immunized with 20 μg of NS1 from DENV1 ($n = 8$), DENV2 ($n = 8$), DENV3 ($n = 5$), or DENV4 ($n = 5$) or OVA ($n = 10$) with MPLA/AddaVax adjuvants as above, or 10^5 PFU of DENV2 PL046 ($n = 10$) at day 0 and then challenged intravenously on day 56 with a lethal dose of DENV2. Mice immunized with the four DENV NS1 serotypes separately were significantly protected against lethal challenge compared to OVA-immunized groups (DENV1, $P = 0.002$; DENV2, $P = 0.008$; DENV3, $P = 0.004$; DENV4, $P = 0.014$). Data were derived from three independent experiments.

NS1-induced decrease in TEER, whereas OVA-immune sera provided no inhibition (Fig. 5A). Heat-inactivated NS1-immune sera also inhibited the NS1-induced decrease in TEER, demonstrating that the inhibition was not dependent on complement. Sera from DENV2 PL046-immune mice partially inhibited the reduction in TEER, consistent with the lower level of anti-NS1 antibodies observed in DENV2-immune sera compared to the level in NS1-immune sera (fig. S5). To further confirm the specificity of the antibody-mediated blocking of NS1 function in vitro, a panel of anti-NS1 mAbs was evaluated, and several anti-NS1 mAbs (for example, 2B7 and 1H7.4) were shown to inhibit the NS1-induced decrease in TEER (Fig. 5B). Several other anti-NS1 mAbs (for example, 2E9.E2) did not inhibit the reduction in TEER (Fig. 5B), demonstrating that blocking certain but not all epitopes leads to the loss of NS1 disruption of endothelial integrity. As expected, mAbs directed to another DENV protein, E (for example, 3H5), did not

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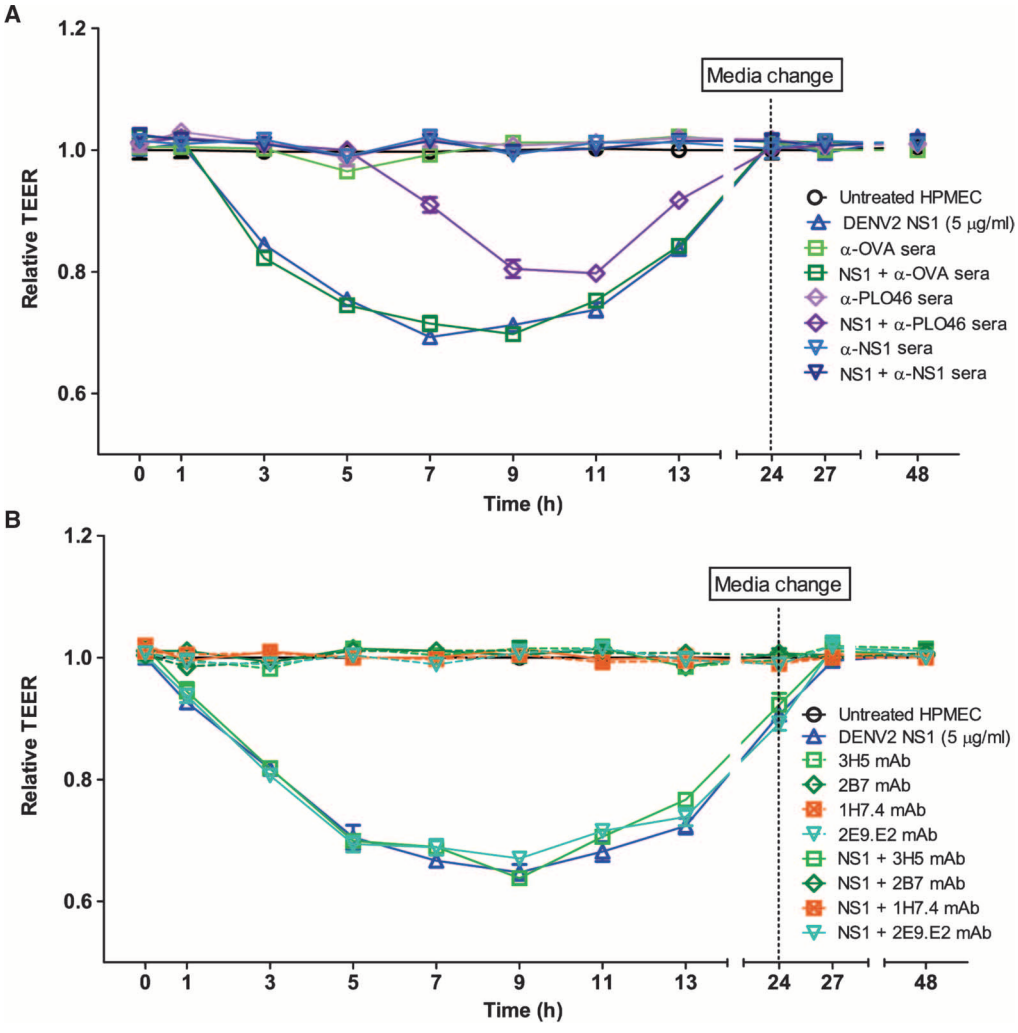

Fig. 5. NS1-immune serum and anti-NS1 mAbs block NS1-induced endothelial permeability in vitro. (**A**) Monolayers of HPMECs grown on Transwell inserts were incubated for 48 h with both NS1 (5 μg/ml) and anti-NS1 serum (1:10 dilution), NS1 alone, or anti-NS1 serum alone, and TEER (ohm) was measured at indicated time points. OVA (5 μg/ml) was used as a protein control. Anti-OVA and anti-DENV2 PL046 sera were also included as controls. Results are representative of two independent experiments with duplicate Transwells. (**B**) Monolayers of HPMECs grown on Transwell inserts were incubated for 48 h with both NS1 (5 μg/ml) and NS1-specific mAbs (10 μg/ml) or either NS1 or anti-NS1 mAbs alone, and TEER (ohm) was measured at indicated time points. A mAb specific to DENV E protein (3H5) was used as an isotype control. Results are representative of three experiments, each with duplicate Transwells. Error bars indicate SEM.

block NS1 function in the TEER assay, nor did the anti-NS1 mAbs alone have any effect on endothelial permeability (Fig. 5B).

To investigate the role of anti-NS1 antibodies in the protective immunity observed against NS1 toxicity in vivo, we passively transferred sera from NS1- or OVA-immunized mice (both in combination with MPLA/AddaVax) concurrently with a lethal challenge of NS1 plus a sublethal dose of DENV2. The passive transfer of 300 μl of anti–NS1-immune serum provided 100% protection from lethality, whereas mice receiving 300 μl of anti-OVA sera succumbed (Fig. 6A). Therefore, antibodies from NS1-vaccinated mice were sufficient to inhibit NS1-mediated toxicity, suggesting that anti-NS1 antibodies provide a critical component of immune protection against lethal DENV2 challenge observed in NS1-vaccinated mice.

Finally, we tested the ability of an anti-NS1 mAb that blocked TEER function in vitro (Fig. 5B) to protect against NS1-induced toxicity in vivo. The anti-NS1 mAb was administered to mice immediately after inoculation with a sublethal viral dose plus NS1 (10 mg/kg), and the mice were monitored for morbidity and mortality for 10 days. Mice receiving the lethal virus inoculum as a positive control and the mice receiving a sublethal viral dose plus NS1 (10 mg/kg) and the isotype control succumbed to the vascular leak disease; however, the mice receiving the sublethal viral dose plus NS1 (10 mg/kg) and the anti-NS1 mAb demonstrated 100% survival (Fig. 6B). Therefore, both polyclonal and monoclonal anti-NS1 antibodies can inhibit lethal vascular permeability in vivo and can block the ability of NS1 to trigger endothelial cell dysfunction in vitro.

DISCUSSION

We have established that DENV NS1 causes vascular leak in vivo and induces increased permeability of human endothelial cells in vitro, both of which can be specifically blocked with NS1-immune polyclonal mouse sera or anti-NS1 mAbs. These results suggest that NS1 can cause endothelial dysfunction leading to vascular leakage during severe dengue disease. In addition, we demonstrated that immune responses elicited by vaccination with DENV2 NS1 protect against lethal challenge with high-dose or antibody-enhanced DENV2 infection, comparable to immunization with a primary sublethal DENV2 infection. Furthermore, vaccination with DENV1, DENV3, or DENV4 NS1 provided substantial protection against a heterologous DENV2 lethal challenge. Our findings, along with those in the accompanying paper by Modhiran *et al.* (*32*), add an important and previously overlooked component to the induction of vascular leak in severe dengue.

NS1 levels have been shown to correlate with disease severity (*11*, *28*), and although NS1 antigenemia peaks concurrently with viremia early in disease, cumulative effects of NS1 on the endothelium might occur after several days of antigenemia, during the "critical phase" on days 4 to 6 post-symptom onset when hypovolemic shock is observed. This would be consistent with our finding that mice displayed the greatest vascular leak and morbidity 3 days after injection with NS1 alone and experienced the most severe disease 3 days after inoculation with NS1 combined

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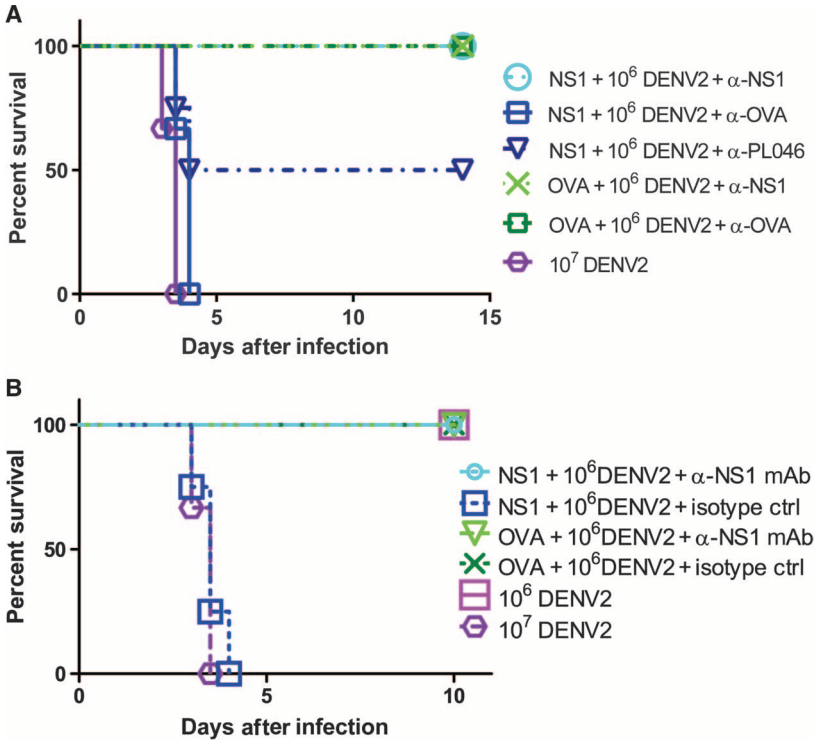

Fig. 6. NS1-immune serum and anti-NS1 mAb block NS1-induced lethality in vivo. (**A**) Pooled serum (300 μl) from NS1-immunized (n = 7), OVA-immunized (n = 7), or DENV2 PL046–immune (n = 4) mice was transferred intraperitoneally to naïve *Ifnar*$^{-/-}$ mice before intravenous injection of NS1 (10 mg/kg) + 10^6 PFU of DENV2. Anti-NS1 or anti-OVA serum was transferred to naïve mice before injection of OVA (10 mg/kg) + 10^6 PFU of DENV2 (n = 4 per group) as controls (ctrl). Mice were followed for survival for 14 days p.i. Mice administered NS1-immune serum followed by NS1 + 10^6 PFU of DENV2 were significantly protected (P = 0.014) against lethality compared to mice given OVA-immune serum (n = 7), using the log-rank Mantel-Cox test. Data were derived from two independent experiments. (**B**) *Ifnar*$^{-/-}$ mice were infected intravenously with 10^7 PFU of DENV2 alone (n = 3), 10^6 PFU of DENV2 alone (n = 3), 10^6 PFU of DENV2 with DENV2 NS1 (10 mg/kg), or 10^6 PFU of DENV2 with OVA (10 mg/kg), as indicated. Mice injected with DENV2 combined with DENV2 NS1 or OVA were concomitantly injected intraperitoneally with 200 μg of isotype control mAb or anti-NS1 mAb (1H7.4) (n = 4 for all groups). Kaplan-Meier survival curves are shown, where mice were monitored for morbidity and survival for 10 days p.i. Mice given anti-NS1 mAbs were significantly protected (P = 0.0069) against lethality compared to mice given the isotype control mAbs by log-rank Mantel-Cox test.

with a sublethal dose of DENV. We found evidence of vascular leak in the lung, liver, spleen, and lymph nodes of mice, which parallels the finding that most of the histopathological damage identified in fatal human cases of dengue disease occurs in these tissues as well (*33*, *34*). In terms of the physiological relevance of our findings, the in vitro and in vivo effects of NS1 occurred at concentrations similar to those reported in patients with DHF/DSS (*27*, *28*). We also observed a correlation between the amount of circulating NS1, the degree of vascular leak, and production of inflammatory cytokines (IL-6 and TNF-α) in vivo. This is consistent with NS1 triggering increased production of vasoactive cytokines by monocytes and macrophages, as shown in the accompanying paper (*32*). Finally, we studied the effects of NS1 on human endothelial cell monolayers (HPMECs and HUVECs) and demonstrated that DENV, but not WNV, NS1 can disrupt endothelial cell permeability in a dose- and time-dependent manner. These in vitro results indicate that NS1 can act directly on the endothelial barrier to increase vascular permeability. These findings are consistent with those of Modhiran *et al.* (*32*), which showed that NS1 triggers endothelial permeability directly in human dermal microvascular endothelial cells within several hours of addition. Several possible mechanisms could explain the direct contribution of NS1 to vascular leak, including disruption of components of the endothelial glycocalyx layer and extracellular matrix and mislocalization or degradation of intercellular-junction proteins triggered by activation of intracellular signaling pathways (*35*, *36*).

We show here that vaccination with NS1 protects against homologous and heterologous lethal challenge in *Ifnar*$^{-/-}$ mice, currently the in vivo model that best mimics features of severe human dengue (*30*). Previous studies using intracranial challenge demonstrated that immunization with DENV2 NS1 protected against homologous but not heterologous DENV serotypes (*18*). Earlier work also demonstrated that the passive transfer of NS1-specific mouse polyclonal antibodies or mAbs protected against intracranial homologous challenge with DENV2 (*20*, *21*). More recent work has shown that an NS1 DNA vaccine as well as NS1 combined with a heat-labile toxin adjuvant can protect against intracranial challenge (*23*, *25*, *26*) in BALB/c mice. Our data show that vaccination with NS1 protects against lethal DENV–induced vascular leakage syndrome in mice and that anti–NS1-immune sera or anti-NS1 mAbs can prevent NS1-mediated vascular leakage both in vivo and in vitro. The antibody-derived immunity to NS1 could be mediated via an Fc-dependent pathway, complement-dependent pathways (*37*), or inhibition of the pathogenic effects of NS1. We suggest that antibodies to NS1 generated from vaccination can increase virus clearance as well as neutralize the vasoactive effects of NS1. Together, our data indicate that immunization with NS1 could provide critical protection against severe DENV disease without the risk of ADE and argue for the inclusion of DENV NS1 in dengue vaccines.

Although anti-NS1 antibodies can have a potential pathogenic effect because of cross-reactivity with human endothelial cells, platelets, or thrombin (*15*–*17*), we observed a protective rather than a pathogenic effect of anti-NS1 polyclonal antibodies or mAbs on endothelial cells. Heat-inactivated anti-NS1 sera lacking active complement components blocked NS1-mediated endothelial permeability, as did anti-NS1 mAbs added to HPMECs in the absence of complement. Our results demonstrate that DENV NS1 has a direct effect on endothelial cells and suggest that NS1 triggers vascular leakage in the absence of antibodies or complement. Finally, although antibodies to NS1 are reported more frequently after secondary DENV infection (*38*), it is possible that the repertoire of anti-NS1 antibodies and the amount of free NS1 (*27*, *28*) may differ in patients who develop severe as compared to mild disease, a topic of future investigation.

In addition, we found that exogenous NS1 resulted in increased viral burden in vivo, which may also exacerbate disease. This is consistent with data showing increased viral output in DENV-infected cells exposed to exogenous NS1 (*39*). It also could result from the ability of NS1 to antagonize activation of complement pathways (*8*, *13*), which can directly neutralize DENV in plasma (*13*, *14*, *40*) and control virus replication

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in vivo. We also noted that mice receiving a lethal dose of DENV2 alone displayed high levels of vascular leakage and inflammatory cytokines with only a partial increase in circulating NS1. This suggests that the increase in vascular leakage leading to mortality in the high-dose infection with DENV2 is partially attributable to virus-induced damage in addition to the pathogenic effects of NS1. This is consistent with a report by Watanabe *et al.* (*41*), which demonstrated differences among DENV2 strains in relation to mortality that were independent of the NS1 levels in a mouse model of DENV infection. It is also possible that strain-related differences in NS1 can modulate the pathogenic properties of NS1.

One of the limitations of this study was our ability to explore different kinetics of NS1 delivery in vivo. We delivered the NS1 in vivo in a single dose, which resulted in circulating levels initially 15 μg/ml at 12 h (on the high end of what has been seen in humans) that decrease to ~1 μg/ml at 72 h (more often the range seen in severe dengue cases); the rapid decline in NS1 levels may be attributable to protein degradation or clearance from the bloodstream. However, another paper that examined levels of NS1 in a mouse model of DENV2 infection reported circulating levels of NS1 in mice of 8 to 30 μg/ml (*41*). It will be interesting to explore different kinetics of delivery of NS1 in vivo. This study was also limited by our ability to differentiate, in the mouse model, between NS1 triggering endothelial permeability directly versus NS1 triggering mediators that may be acting on endothelial cells. Evaluating the precise role of signaling molecules and inflammatory mediators, including cytokines, in the mechanisms of NS1-induced vascular leak in vivo requires study in genetically deficient mice and the use of various inhibitors and/or antibodies; these studies are currently underway. In separate in vitro experiments, we have identified cytokine-independent, endothelial cell–intrinsic mechanisms through which NS1 leads to increased endothelial permeability, in addition to the cytokine-dependent pathways triggered by NS1 described by Modhiran *et al.* (*32*).

To date, the dominant theory to explain dengue shock invokes immunopathogenic processes thought to lead to endothelial dysfunction; here, we propose that DENV NS1 protein by itself is a key component in triggering the severe vascular leakage associated with life-threatening dengue. This work opens the door to evaluating potential drug therapeutics that can inhibit NS1-mediated disease. Likewise, our NS1 vaccination data showing protection against DENV lethal vascular leak syndrome points to the importance of including NS1 in dengue vaccine formulations.

MATERIALS AND METHODS

Study design

We designed these studies to show that DENV NS1 plays a role in causing vascular leakage and that immunization with NS1 could prevent DENV-induced systemic disease. We first showed that recombinant NS1 as a vaccine could protect against lethal DENV–induced vascular leak syndrome and then followed this with studies of NS1 alone or NS1 combined with a sublethal DENV infection to characterize disease due to increased levels of NS1. Mice infected with DENV with and without NS1 were evaluated on a morbidity scale from 1 to 5 (*30*). Mice were monitored every 12 h after infection, followed every 6 h when displaying a score of >3, and euthanized immediately when they became moribund (score of 5). NS1 immunization experiments included negative controls consisting of OVA-immunized or unimmunized mice that were not protected and DENV2 PL046-immunized mice as positive controls that were protected against lethal challenge. In NS1 vascular leak experiments, OVA-injected mice were used as negative controls in comparison to NS1-injected mice, and sublethal DENV infection plus OVA was included as a negative control compared to sublethal DENV infection plus NS1. Mouse experiments testing mortality or protection from lethal DENV challenge had endpoints 10 days after infection, and no NS1-immunized, NS1-injected, or DENV-infected mice were excluded from the study. Mouse experiments were performed in duplicate, with numbers totaling four or greater in each group. We then hypothesized that DENV NS1 directly induces endothelial permeability and used in vitro TEER experiments with cultured human endothelial cell lines to evaluate this effect. Each TEER experiment was performed two to three times, with each condition including two replicate wells that were each read twice. Each experiment contained the following controls: (i) Transwell inserts containing only medium to determine transient resistance values of the permeable membrane, (ii) Transwell inserts with cultured human endothelial cells to obtain resistance values of the endothelial cell monolayer alone, and (iii) a positive control consisting of vasoactive molecules such as TNF-α, which is known to increase endothelial permeability. In addition, as an irrelevant protein control, OVA was included in each experiment. For the protection experiments using sera obtained from NS1-vaccinated mice or anti-NS1 mAbs, Transwells containing only sera or anti-NS1 mAbs were included to exclude any effect on endothelial permeability induced by these components. TEER values were collected every 2 h for 12 h and at 24 h, then the medium was replaced to reestablish the integrity of the monolayer, and the TEER values were read at 26 and 48 h. No blinding or randomization was performed for either the mouse studies or the TEER experiments.

Mice

Six- to eight-week old *Ifnar*$^{-/-}$ C57BL/6 mice were bred and maintained under specific pathogen–free conditions at the University of California, Berkeley, Animal Facility. Experiments were performed strictly following guidelines of the American Veterinary Medical Association and the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The Animal Care and Use Committee of the University of California, Berkeley, approved all experiments (protocol R252-1012B).

Cell cultures and viruses

All viruses were propagated in the *Aedes albopictus* C6/36 cell line [American Type Culture Collection (ATCC)] and titered by plaque assay on baby hamster kidney cells (BHK21, clone 15). DENV2 PL046 was obtained originally from H.-Y. Lei (National Cheng Kung University, Taiwan). DENV2 D220 was produced in our laboratory from the parental strain DENV2 PL046 (*42*). An HPMEC line (HPMEC.ST1.6R) was donated by J. C. Kirkpatrick (Institute of Pathology, Johannes Gutenberg University, Germany), propagated (passages 5 to 8), and maintained at 37°C in humidified air with 5% CO_2 in endothelial cell basal medium-2 supplemented with growth factors, antibiotics, and fetal bovine serum as per the manufacturer's specifications (Clonetics, Lonza). HUVECs (passage 3) were donated by M. Lodoen (University of California, Irvine) and maintained as described above.

Recombinant NS1 proteins and anti-NS1 mAbs

Recombinant NS1 proteins from DENV1, DENV2, DENV3, and DENV4 and WNV, greater than 95% purity and certified to be free of endotoxin

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contaminants, were produced by Native Antigen in HEK 293 cells; recombinant DENV2 NS1 produced in S2 cells was obtained from Merck. The recombinant DENV2 envelope (recE), expressed in a baculovirus expression vector system (GenScript) using High Five insect cells, was purified by an immune-affinity protein G column coupled to 4G2 (anti-E mAb). The NS1 and recE working stocks were also tested using the Endpoint Chromogenic Limulus Amebocyte Lysate (LAL) QCL-1000TM kit (Lonza) and confirmed to be bacterial endotoxin-free (<0.1 EU/ml per 25 μg of protein). Anti-NS1 mAbs were obtained as follows: 2B7 and 2E9.E2 were generated in the Harris laboratory (P.R.B. and E.H., unpublished), and 1H7.4 was a gift from P. Young (University of Queensland, Brisbane, Australia). mAb 3H5 (anti-DENV2 E) was obtained from ATCC. Hybridomas were propagated in CELLine culture flasks (Sigma); supernatants were collected, and the mAbs were affinity-purified using a Protein G Sepharose column. The mAbs were eluted from the column, concentrated, sterile-filtered, and titered before use.

Mouse experiments of NS1-induced vascular leak and NS1 vaccination

For studies of NS1-induced vascular leak, *Ifnar*$^{-/-}$ mice were injected intravenously with NS1 (5 to 20 μg/kg) or OVA protein (10 μg/kg) alone or NS1 (5 to 10 μg/kg) or OVA (10 μg/kg) in combination with a sublethal dose of 10^6 PFU of DENV2 D220. Separate groups of mice were administered 10^6 PFU of DENV2 D220 alone as a sublethal control or 10^7 PFU of DENV2 D220 as a lethal control. Mice were observed every 12 h for morbidity using a scoring system on a scale of 1 to 5 as follows: 1 = healthy; 2 = ruffled fur and mild signs of lethargy; 3 = hunched posture, ruffled fur, and intermediate level of lethargy and failure to groom; 4 = very lethargic, limited mobility, ruffled fur, and hunched posture; and 5 = moribund with limited to no mobility and inability to reach food or water. Mice were euthanized immediately when they became moribund. For vascular leak studies and measurement of cytokines and levels of sNS1 and viral RNA, mice were euthanized 72 h after injection, blood was collected via cardiac puncture, and tissues were harvested. For mortality studies, mice were monitored for 10 days after injection. For vaccination studies, *Ifnar*$^{-/-}$ mice were injected intraperitoneally three times (days 0, 14, and 42) with 20 μg of NS1 or 20 μg of OVA with 1 μg of MPLA (InvivoGen) and AddaVax (0.5% sorbitan trioleate, 5% squalene, and 0.5% Tween 80 in 10 mM sodium citrate buffer; InvivoGen). Two weeks after the third immunization (day 56), mice were challenged with 10^7 PFU of DENV2 D220 intravenously or 5 μg of 4G2 (anti-E mAb) 24 h before 10^5 PFU of D220 (ADE). To test the effects of the anti–NS1-immune serum on NS1-induced vascular leak, *Ifnar*$^{-/-}$ mice were injected with 10 μg of NS1 or 10 μg of OVA in combination with 10^6 PFU of DENV2 D220, immediately administered 300 μl of polyclonal anti-NS1, anti-OVA, or anti–PL046-immune serum intraperitoneally, and then followed for morbidity and mortality for 10 days. The immune serum used for passive transfer was collected from NS1- or OVA-immunized mice or DENV PL046–infected mice 6 weeks after initial immunization or infection. To test the effects of anti-NS1 mAb on NS1-induced vascular leak, *Ifnar*$^{-/-}$ mice were injected with 10 μg of NS1 or 10 μg of OVA in combination with 10^6 PFU of DENV2 D220, immediately given 200 μg of anti-NS1 mAb (1H7.4) or isotype control antibody intraperitoneally, and then followed for morbidity and mortality for 10 days. Separate groups of mice were given 10^6 PFU of DENV2 D220 alone as a sublethal control or 10^7 PFU of DENV2 D220 as a lethal control.

Enzyme-linked immunosorbent assays

Serum was collected 3 days after the intravenous injection of NS1 with or without DENV infection. Serum was analyzed by NS1-specific capture ELISA using mAb 7E11 (5 μg/ml; a gift from R. Putnak, Walter Reed Army Institute of Research) as a coating antibody, 50 μl of a 1:100 dilution of each mouse serum sample, followed by detection using 100 μl of a biotinylated NS1-specific mAb, 2B7 (4 μg/ml) (P.R.B. and E.H., unpublished). A standard curve of NS1 (7 to 2000 ng/ml) was run in parallel to quantify NS1 concentration in all samples. Absorbance [optical density at 405 nm (OD_{405})] was measured using an Applied Biosystems 7200 microplate reader. To determine levels of NS1-specific antibodies in immunized mice before challenge, serum was obtained 1 week after the third immunization via submandibular bleed. For the ELISA, 50 μl of NS1 (0.5 μg/ml) was coated onto a Nunc Immulon polystyrene plate; after blocking with 5% nonfat dry milk in phosphate-buffered saline (PBS) + 0.05% Tween 20 (PBS-T), 50 μl of a 1:100 dilution of each test serum was added and incubated for 24 h. After washing with PBS-T, biotinylated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch) was added, followed by washing and addition of streptavidin–alkaline phosphatase (Life Technologies) and para-Nitrophenylphosphate (Sigma-Aldrich) substrate, which was read at OD_{405} as above. Capture ELISAs were performed to quantitate the levels of TNF-α or IL-6 in citrated plasma. Mouse TNF-α and IL-6 ELISA Ready-SET-Go! kits (eBioscience) were used according to the manufacturer's instructions. The OD_{405} of each sample was measured, and cytokine levels in serum were expressed in picograms per milliliter.

Quantitation of virus by qRT-PCR

Samples of all tissues were stored in RNA later (Ambion), and RNA was extracted using an RNeasy Mini kit (Qiagen). Serum was generated from whole blood by centrifugation, and RNA was extracted using a QIAamp Viral Recovery RNA kit (Qiagen). Serum viremia levels and tissue viral load were measured by qRT-PCR as described previously (*29*). Viral load was expressed as either glyceraldehyde phosphate dehydrogenase in genome equivalents per microgram (tissue) or genome equivalents per millilter (serum).

Quantitation of Evans blue vascular leakage

Vascular leakage was quantified by Evans blue dye as previously described (*42*). Briefly, 200 μl of 0.5% Evans blue dye was injected intravenously 3 days after treatment and allowed to circulate for 1 h before mice were euthanized and cardiac puncture was performed. Tissues were collected in preweighed tubes containing 1 ml of formamide and incubated at 37°C and 5% CO_2 for 24 h. Evans blue concentration in extracts was quantified by measuring OD_{610} in samples and comparing to a standard curve. Data were expressed as nanograms of Evans blue dye per milligram of tissue weight.

Transendothelial electrical resistance

To evaluate the effect of sNS1 on the integrity of the endothelium, HPMEC or HUVEC monolayers grown on a 24-well Transwell polycarbonate membrane system (Transwell permeable support, 0.4 μM, 6.5-mm insert; Corning Inc.) were incubated with NS1 (0.2 to 20 μg/ml) or OVA (1 to 20 μg/ml) as a negative control. Endothelial permeability was evaluated by measuring TEER in ohms at sequential 2-h time points after the addition of test proteins. TEER was measured using an epithelial voltohmmeter with "chopstick" electrodes (World Precision Instruments). Transwell inserts containing untreated HPMEC cells were

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used as a negative control, and inserts with medium alone were used for blank resistance measurements. Relative TEER was expressed as the ratio of resistance value as follows: [ohm (experimental condition) − ohm (medium alone)]/[ohm (nontreated endothelial cells) − ohm (medium alone)]. After 24 h of treatment, 50% of upper and lower chamber media was replaced by fresh endothelial cell medium.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software, and all graphs were generated using Prism 6. Comparison between ELISA and qRT-PCR titers was conducted using a nonparametric Mann-Whitney test. Comparison of survival rates was conducted using a nonparametric log-rank (Mantel-Cox) test and graphed as Kaplan-Meier survival curves. For TEER experiments, statistical significance was determined using a two-way analysis of variance (ANOVA), and differences in treatment were considered significant for P values <0.05.

SUPPLEMENTARY MATERIALS

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Fig. S1. Recombinant NS1 from DENV1, DENV2, DENV3, DENV4, and WNV is highly purified and oligomeric.

Fig. S2. Morbidity and vascular leakage are induced by NS1.

Fig. S3. NS1 increases endothelial cell permeability in two different endothelial cell lines, HUVEC and HPMEC.

Fig. S4. Viremia after lethal DENV challenge is reduced by immunization with DENV2 NS1.

Fig. S5. Immunization with DENV NS1 and DENV infection induces serotype cross-reactive anti-NS1 antibodies.

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Dengue virus NS1 triggers endothelial permeability and vascular leak that is prevented by NS1 vaccination

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A leak in the dike

Everyone knows how mosquitos can wreck an end-of-summer picnic. But in some climates, these pesky intruders persist and carry a variety of detrimental diseases—some with no preventative vaccines or targeted therapies. One such passenger is dengue virus (DENV), which infects up to 400 million people each year and comes in several serotypes (1 to 4) and disease presentations—from mild infection to severe disease and sometimes death. But to treat or prevent dengue requires that we have a more complete picture of the disease pathology. Now, Modhiran *et al.* and Beatty *et al.* describe the results of in vitro and in vivo experiments that point to circulating dengue virus non-structural protein 1 (NS1) and the innate immune Toll-like receptor 4 (TLR4) as a focus for basic scientists as well as vaccine and drug developers.

DENV infection protects a patient from future reinfection with the same DENV serotype as well as producing temporary immune protection from severe dengue disease caused by a different DENV serotype. But unlike diamonds, this immune protection doesn't last forever, and when the protected period passes, the patient becomes at increased risk of enhanced infection and progression to severe disease if he or she is infected with a second DENV serotype. This severe form of dengue infection is believed to result from immunopathogenic processes that induce cytokine storm and cause vascular leakage that leads to shock. Until now, no dengue viral proteins have been linked to vascular endothelium permeability (that is, vascular leakage).

Beatty *et al.* show that inoculation of mice with DENV NS1 protein alone induces both vascular leak and secretion of inflammatory cytokines and that administration of NS1 with a sublethal dose of DENV2 leads to lethal vascular leak syndrome. In human endothelial cell monolayers in culture, NS1 from any of the four DENV serotypes triggered endothelial barrier permeability. NS1's pathogenic effects were blocked by NS1-immune polyclonal mouse serum or monoclonal antibodies to NS1 (in vivo and in vitro), and immunization of mice with NS1 protected against lethal DENV2 challenge.

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