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Flavivirus NS1 protein in infected host sera enhances viral acquisition by mosquitoes

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Summary

The arbovirus life cycle involves viral transfer between a vertebrate host and an arthropod vector, and acquisition of virus from an infected mammalian host by a vector is an essential step in this process. Here, we report that flavivirus nonstructural protein-1 (NS1), which is abundantly secreted into the serum of an infected host, plays a critical role in flavivirus acquisition by mosquitoes. The presence of dengue virus (DENV) and Japanese encephalitis virus (JEV) NS1s in the blood of infected interferon alpha and gamma receptor-deficient mice (AG6) facilitated virus acquisition by their native mosquito vectors because the protein enabled the virus to overcome the immune barrier of the mosquito midgut. Active immunization of AG6 mice with a modified DENV NS1 reduced DENV acquisition by mosquitoes and protected mice against a lethal DENV challenge, suggesting that immunization with NS1 could reduce the number of virus-carrying mosquitoes as well as the incidence of flaviviral diseases. Our study demonstrates that flaviviruses utilize NS1 proteins produced during their vertebrate phases to enhance their acquisition by vectors, which might be a result of flavivirus evolution to adapt to multiple host environments.

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The sequencing data of RNA-Seq analysis were deposited in the Short Read Archive (NCBI) with accession number GSE73967. The authors declare that they have no competing financial interests

Author Contributions

G.C. designed the experiments and wrote the manuscript. Y.L. and J.L. performed the majority of the experiments and analyzed data. K.N., S.D. and X.P. helped with RNA isolation and qPCR detection. J.Q. assisted in the statistical analysis. P.W. contributed experimental suggestions and improved the writing of the manuscript. All authors reviewed, critiqued and provided comments on the manuscript.

Introduction

Flavivirus is a genus of viruses belonging to the family *Flaviviridae*. Most flaviviruses are transmitted by an infected mosquito or tick vector to mammalian or avian hosts as the vector takes a blood meal. Mosquito-transmitted flaviviruses, including dengue virus (DENV), Japanese encephalitis virus (JEV), West Nile virus (WNV), Zika virus (ZIKV) and yellow fever virus (YFV), are etiologic agents of diseases with severe manifestations such as hemorrhage, shock, and encephalitis¹. DENV is transmitted between humans and its mosquito vectors, *Aedes aegypti* and *Aedes albopictus*². Worldwide, an estimated 390 million DENV infections occur each year, with 96 million manifesting with clinical symptoms³. JEV, which is transmitted by *Culex* mosquitoes, causes an estimated 67,900 annual cases of encephalitis diseases in 24 Asian and Oceanian countries⁴. Given the rapid increase in flavivirus spread and disease burden over the past decade, additional strategies are urgently needed to combat flavivirus infections worldwide.

Flaviviruses have a single-stranded, positive-sense RNA genome that encodes 3 structural proteins and 7 nonstructural proteins. Flavivirus nonstructural protein-1 (NS1) is expressed in multiple oligomeric forms and is present in different cellular locations, including on intracellular membranes, on the cell surface and extracellularly as a soluble, secreted lipoparticle^{5, 6}. Both secreted and cell-surface associated NS1 are highly immunogenic and might contribute to the pathogenesis of flavivirus infection in a host⁵. During acute DENV infection, secreted NS1 protein (sNS1) is present in patient sera at high levels², ranging from 70–15,000 ng/ml⁷; in exceptional cases, this level can reach up to 50,000 ng/ml⁸. Based on results from DENV studies in animals, sNS1 can contribute to the pathogenesis of severe DENV illness by increasing the permeability of capillaries^{9, 10} and might augment DENV infection by interfering with the immune system¹¹. As a virus-encoded extracellular component, NS1 is a potential vaccination candidate against flavivirus infection. Indeed, immunization of mice with DENV NS1 protects them from lethal DENV challenge^{9, 12}. Nonetheless, antibodies against DENV NS1 have been reported to cross-react with surface components on human platelets and endothelial cells, resulting in inhibition of platelet aggregation and apoptosis of endothelial cells^{13–16}. Although NS1 antibodies might contribute in the pathogenesis of DENV infection, the dynamics of NS1 antibody kinetics over the course of DENV infection have been found to be inconsistent with the course of illness⁵. Taken together, both NS1 and its antibodies have been implicated in the complicated roles of protection from and pathogenesis of DENV infection of humans.

The life cycles of many flaviviruses involve viral transfer between vertebrate hosts and mosquito vectors. Viral acquisition by vectors from an infected mammalian host is an essential step in the flavivirus life cycle^{17, 18}. During this process, sNS1 molecules that are in circulation in infected hosts can be simultaneously transferred with viruses to a mosquito. Here, we demonstrate that mosquito-borne flaviviruses utilize sNS1 proteins produced during their vertebrate phases to enhance their acquisition by vectors and provide a NS1-based immunization strategy to reduce the number of infected mosquitoes as well as infection in hosts.

Results

DENV sNS1 facilitates DENV acquisition via membrane blood feeding

The acquisition of a flavivirus by a mosquito from an infected host is an indispensable process in the flavivirus lifecycle. During the viremic stage in an infected host, abundant quantities of sNS1 can be found in blood circulation along with viruses, and together these components are transferred to a mosquito as it takes a blood meal. Therefore, we investigated the role of sNS1 in flavivirus acquisition by mosquitoes. To accomplish this, a recombinant DENV2 sNS1 protein was expressed and purified using a *Drosophila* S2 expression system (Supplementary Fig. 1a). Then, a mixture containing human blood (50% v/v), supernatant from DENV2-infected Vero cells (50% v/v) and the purified DENV2 sNS1 protein (final concentration up to 10 µg/ml) was used to feed *A. aegypti* via an *in vitro* blood feeding system (Fig. 1a). The ratios of infection in the mosquitoes were significantly enhanced by the presence of sNS1, regardless of whether a low (1×10^5 pfu/ml, Fig. 1b,c) or high (1×10^6 pfu/ml, Supplementary Fig. 1b,c) dose of DENV2 was used. It has been reported that sNS1 expression and secretion is associated with active viral replication in host cells¹⁹. Therefore, we measured the amount of sNS1 in the supernatant of DENV2-infected Vero cells using an ELISA. We found that sNS1 was continuously secreted into the supernatant of DENV2-infected Vero cells (Supplementary Fig. 1d). We next generated murine polyclonal antibodies against DENV2 NS1 (Supplementary Fig. 2a). Then, supernatant from DENV2-infected Vero cells (50% v/v) was mixed with fresh human blood (50% v/v) and serially diluted DENV2 NS1 antibodies for *in vitro* membrane feeding of *A. aegypti* (Fig. 1d). The presence of DENV2 NS1 antibodies significantly reduced mosquito infection ratios compared to those measures in the presence of pre-immune sera (Fig. 1e,f). Similar results were observed for mosquitoes fed antibodies against sNS1 proteins from DENV1 (GZ/XNC strain, *FJ176780*) or DENV3 (ThD3 strain, *AY676352*) during DENV1 or DENV3 infections, respectively (Supplementary Fig. 2a–c). In the above-mentioned experiments, DENV that was present in the supernatant of infected Vero cells was used for mosquito oral infection. To avoid any potential confounding effects caused by culture medium components on DENV infectivity, we purified native DENV2 sNS1 proteins (Supplementary Fig. 3a) and DENV2 virions from the supernatant of DENV2-infected Vero cells. The purified DENV2 particles were free of sNS1 (Supplementary Fig. 3b). Human whole blood (100% v/v) was incubated with the purified DENV2 infectious particles and native DENV2 sNS1 proteins, and then orally fed to mosquitoes (Fig. 1g). Native DENV2 sNS1 consistently facilitated DENV2 acquisition by *A. aegypti* (Fig. 1h,i).

Immuno-blockade against sNS1 in AG6 mice prevents flavivirus acquisition by mosquitoes

Few animal models can accurately reproduce the dengue manifestations that are observed in infected humans. A type I and II interferon receptor-deficient mouse model (*ifnagr*^{-/-}) has been widely used as an animal model for studies of DENV infection²⁰. Therefore, we examined the role of sNS1 during DENV acquisition *in vivo* using an *ifnagr*^{-/-} model (C57BL/6 strain, AG6 strain)²¹. We first assessed whether DENV2 infection influences blood cell counts in AG6 mice. Compared to uninfected AG6 mice, DENV2-infected AG6 mice showed significantly reduced numbers of white blood cells and platelets; however, their numbers of erythrocytes were not altered (Supplementary Fig. 4a–c). These

observations are consistent with results from human studies²². Next, we intraperitoneally infected AG6 mice with the DENV2 43 strain (*AF204178*), a low-passage virus strain that was isolated from a dengue patient²³ (Fig. 2a). Similar to a human infection, DENV replication in AG6 mice resulted in the release of the sNS1 protein into blood circulation (Fig. 2b). Twelve hours after initiating the infection, we intraperitoneally inoculated the infected mice with DENV2 NS1 polyclonal antisera to neutralize any sNS1 protein present in the blood. As a control, a subset of infected mice was inoculated with the same amount of untreated sera. After an additional 12 hr incubation to allow for antibody dissemination, the infected mice were subjected to daily mosquito biting from day 1 to day 4 post mouse infection. The mouse blood-fed mosquitoes were reared for an additional 8 days to determine their DENV infection (Fig. 2a). Compared to the untreated sera, the anti-NS1 antisera completely neutralized the presence of sNS1 in the mouse sera until 4 days post infection (Fig. 2b). Furthermore, passive immunization with the anti-DENV2 NS1 sera did not influence DENV2 replication in AG6 mouse blood (Fig. 2c). The infection ratios of fed *A. aegypti* were reduced by the neutralization of DENV2 sNS1 (Fig. 2d,e). We observed the same results using a common DENV2 New Guinea C strain (*M29095*) (Supplementary Fig. 5a–d). In addition to *A. aegypti*, *A. albopictus* is another major vector for DENV transmission²⁴. The passive transfer of DENV2 NS1 antibodies into DENV2-infected AG6 mice also interrupted DENV acquisition by *A. albopictus* (Fig. 2f,g). Together, these data demonstrate a generalized and critical role for sNS1 in the acquisition of various DENV strains by different *Aedes* mosquito species.

NS1 secretion is a common property of flaviviruses. Similarly to DENV-infected cells, cells infected with JEV secrete NS1 into the extracellular milieu²⁵. Therefore, we applied similar procedures as above to infect AG6 mice with the JEV SA-14 strain (*U14163*), and subsequently allowed *Culex pipiens pallens*, a native mosquito vector for JEV transmission, to feed on the infected mice (Fig. 3a). JEV replication in AG6 mice was so rapid that all infected mice succumbed to their infections within 3–4 days, even with a low-dose JEV challenge. The passive immunization of the AG6 mice with JEV NS1 antibodies (Fig. 3b) had no impact on JEV viremia (Fig. 3c); however, it did significantly decrease the infection ratios of fed *Culex* mosquitoes (Fig. 3d,e). This result indicates a conserved role for sNS1 in flavivirus acquisition from infected hosts.

DENV sNS1 facilitates viral acquisition by suppressing the expression of immune genes in mosquito midguts

We next investigated the mechanism by which sNS1 facilitates flavivirus acquisition by mosquitoes. The midguts of mosquitoes that had acquired purified DENV2 sNS1 via blood meals were dissected for RNA-Seq analysis. Immune signaling components, enzymes and effectors were selected for analysis. The results showed that the expression levels of 26 genes at 4 hr, 36 genes at 8 hr, and 38 genes at 18 hr after NS1 treatment changed by more than 2-fold after the mosquitoes fed on blood containing DENV2 NS1 (Supplementary Table 1). Mosquitoes lack immunoglobulin-based humoral responses and instead rely heavily on efficient innate antiviral strategies to limit viral propagation, such as RNA interference (RNAi), reactive oxygen species (ROS) production and the propagation of several immune signals^{26–29}. The mRNA abundances of most genes in the RNAi, Toll and immune

deficiency (Imd) pathways, as well as those encoding antimicrobial peptides (AMPs), did not differ between the midguts of the DENV2 sNS1-fed mosquitoes and the control mosquitoes (gene regulation less than 2-fold). In contrast, the mRNA expression levels of genes related to ROS production and the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling pathway were suppressed in the midguts of the DENV2 sNS1-fed mosquitoes (Fig. 4a, Supplementary Fig. 6 and Supplementary Table 1). The changes in these immune-related genes at 18 hr post blood feeding were validated using qPCR (Fig. 4b). Furthermore, ROS activity in the mosquito midgut was consistently suppressed by DENV2 sNS1 (Fig. 4c,d). Inhibiting ROS activity with vitamin C³⁰ rendered the mosquitoes highly susceptible to DENV2 infection. In contrast, activating ROS with uracil³¹ reduced DENV acquisition by mosquitoes via blood meals (Fig. 4e,f). These results suggest that the ROS system acts as an essential player in restricting DENV infection in mosquitoes. In addition to the ROS system, the JAK-STAT pathway plays an important role in the control of viral infection in both *Drosophila* and mosquitoes^{28, 32}. Indeed, genetic interruption of the ROS system (via *Duox1* and *NoxM*) and JAK-STAT pathway (via *Dome*, *Hop* and *STAT*) enhanced DENV2 infection of mosquitoes via blood meals (Fig. 4g,h and Supplementary Fig. 7). To avoid any potential experimental noise introduced by the presence of sNS1 in supernatant from DENV2-infected Vero cells (Supplementary Fig. 1d), we used purified NS1-free DENV2 virions mixed with human blood for mosquito oral infection (Fig. 4e–h). These results indicated that sNS1 facilitates DENV infection by modulating mosquito antiviral mechanisms. Furthermore, the abundance of gut microbial flora can be used as an indicator of local immune activity: in the midguts of the DENV2 NS1-fed mosquitoes, the burden of commensal bacteria was enormously enhanced in comparison to the controls (Supplementary Fig. 8a–c), validating that NS1 mediates immune suppression in the mosquito gut.

Immunization with DENV NS1 prevents DENV acquisition and lethal infection

Each stage of the life cycle of a vector-borne pathogen can theoretically be modulated to reduce the incidence of the associated disease^{17, 33}. Immunization with DENV NS1 might disrupt the acquisition of DENV by a mosquito from an infected human, thereby reducing the number of infected mosquitoes and the subsequent spread of DENV. However, many studies have suggested that anti-DENV NS1 antibodies might cross-react with surface components on human platelets and endothelial cells. Such interactions may result in vascular leakage and other dengue-related symptoms^{14–16, 34}. Therefore, we constructed a DENV2 NS1 mutant (DENV2 Δ NS1) that harbored deletions of three potential antigenic regions that could elicit cross-reacting antibodies (Fig. 5a)^{14–16}. Unlike antibodies generated against full-length DENV2 NS1, the antibodies that were generated against the DENV2 Δ NS1 mutant (Supplementary Fig. 9a) showed no cross-reactivity with human umbilical vein endothelial cells (HUVECs) (Fig. 5b,c) or primary platelets (Supplementary Fig. 9b). Moreover, DENV2 Δ NS1 and full-length NS1 antibodies produced equivalent repressive effects on DENV acquisition (Fig. 5d,e).

We next immunized AG6 mice with either DENV2 Δ NS1 or full-length DENV2 NS1. As a negative control, mice were inoculated with phosphate-buffered saline (PBS) and the same adjuvant. NS1 antibody production was robustly elicited in immune-deficient AG6 mice.

Additionally, there was no significant difference in DENV2 NS1-specific antibody titers between the Δ NS1- and the full-length NS1-immunized AG6 mice (Supplementary Fig. 10). The immunized mice were intraperitoneally challenged with 1×10^6 pfu of the DENV2 43 strain two weeks after the final immunization (day 42) (Fig. 6a). No significant differences in viremia were found between the mice that were immunized with full-length DENV2 NS1 and the controls (Fig. 6b). Interestingly, immunization with DENV2 Δ NS1 significantly reduced DENV2 viremia (Fig. 6b). Immunization with both DENV2 Δ NS1 and full-length DENV2 NS1 effectively neutralized sNS1 (Fig. 6c). *A. aegypti* mosquitoes were allowed to feed on the infected, immunized mice from day 1 to day 4 after mouse infection and were then subsequently reared for an additional 8 days for DENV detection. The infection ratios of the mosquitoes that fed on the DENV2 Δ NS1-immunized mice were 3- to 8-fold lower than those of the mosquitoes that fed on the negative controls and 2- to 3-fold lower than those of the mosquitoes that fed on the full length NS1-immunized mice (Fig. 6d,e). Immunization with DENV NS1 has been shown to protect animals against lethal DENV challenge^{9, 35, 36}. To validate these results in our AG6 mouse model, we immunized AG6 mice with full-length NS1 or Δ NS1 three times and subsequently subjected them to intraperitoneal infection with the DENV2 43 strain (Fig. 6a). All control animals (immunized with PBS) succumbed to DENV2 infection by 28 days post infection, whereas 75% of the mice that were immunized with DENV2 Δ NS1 survived, and 33% of the mice immunized with full-length DENV2 NS1 were still showing protection by 40 days (Fig. 6f). In agreement with the survival results, the mice that underwent vaccination with DENV2 Δ NS1 showed reduced Evans blue dye intensity, an indicator of the vascular leakage caused by DENV infection, in various tissues (Fig. 6g,h). Altogether, these data demonstrate that immunization with DENV2 Δ NS1 reduces the number of infected mosquitoes that are produced after feeding on a DENV viremic host and protects animals against lethal DENV challenge.

Discussion

Viruses have evolved sophisticated strategies to efficiently infect their host organisms. Flaviviruses, which cycle between arthropods and vertebrates, must overcome several barriers to maintain their life cycles. Several flaviviral proteins, particularly their nonstructural (NS) proteins, help facilitate viral immune evasion in mammals. Most of these NS proteins interfere with cell-intrinsic antiviral mechanisms inside of host cells; however, NS1 is secreted in large quantities into the blood and also antagonizes extrinsic antiviral activities, such as the complement cascade, in mammals¹¹. Circulating NS1 may also be acquired by mosquitoes together with virions when they feed on a viremic mammalian host; as such, it may be prudent to determine what effects NS1 has on mosquitoes. The current study revealed that flaviviruses require NS1 to efficiently infect mosquitoes because it helps the viruses overcome the gut immune barrier. NS1 potently inhibits two important mosquito antiviral mechanisms: ROS production and the JAK-STAT pathway. In the harsh environment of the gut, viruses must rapidly overcome the gut immune barrier before activating the expression of their NS proteins. If a large amount of NS1 is taken up from a mammalian host, a flavivirus can more efficiently overcome this barrier; as such, the

abundant secretion of NS1 may be an evolutionary trait developed by flaviviruses to adapt to multiple host environments.

The mosquito gut is a pivotal natural entry site for arboviruses and the first barrier that can efficiently limit subsequent viral infection. Multiple inherent immune systems play essential roles in restricting flaviviral infection in the mosquito gut. Correspondingly, inhibition of the Toll and JAK-STAT pathways has been shown to increase DENV infection of the midgut in *A. aegypti*^{26, 28}. Toll pathway activation via *Wolbachia*-induced ROS production was found to stimulate AMP production and subsequently restrict DENV infection in *A. aegypti*³⁷. However, whether and how flaviviral proteins interfere with these antiviral mechanisms when infecting mosquitoes must still be elucidated. Interestingly, we found that flaviviruses exploit the secretion of NS1 in mammals to suppress the expression of immune-related genes in mosquitoes, particularly key components in the JAK-STAT pathway and the ROS system. In mammals, secreted NS1 proteins following DENV, WNV and YFV infections have also been shown to attenuate both the classical and lectin complement cascades by directly binding to multiple key complement components¹¹. In the current study, we showed that the secretion of NS1 following flavivirus infection plays a similar immune evasion role during viral acquisition by mosquitoes.

The immunization of mammalian hosts with NS1 is a potential strategy for flavivirus prevention. The immunization of mice with DENV NS1 has been shown to partially reduce the morbidity and mortality associated with DENV infection^{12, 35, 36}. Although the generation of antibodies against DENV NS1 can prevent DENV lethality in acute mouse infection models, such antibodies may cross-react with several coagulation factors and adhesion molecules on human platelets and endothelial cells^{16, 34}, resulting in inhibition of platelet aggregation or apoptosis of endothelial cells^{13–16}. To overcome this limitation, in the current study, we constructed a truncated form of DENV2 NS1 that harbors deletions of antigenic regions that could potentially elicit the production of cross-reactive antibodies. Antibodies produced against the DENV2 Δ NS1 construct did not cross-react with human platelets or endothelial cells. Furthermore, compared to mice immunized with full-length NS1 and to control mice, mice that were immunized with DENV2 Δ NS1 presented with much lower levels of viremia, less vascular leakage and higher survival rates after lethal DENV2 challenge, indicating that immunization with DENV Δ NS1 might be an effective and safe approach to prevent vascular leakage and lethal DENV infection in hosts.

For vector-borne diseases, immunization with key susceptibility factors that facilitate pathogen infection in vectors can disrupt microbial acquisition from an infected vertebrate host, thereby reducing the number of infected vectors and lowering the disease burden in nature^{17, 38, 39}. For example, a transmission-blocking vaccine targeting Pfs 48/45 and Pfs 25/28, two surface antigens on *Plasmodium* gametocytes, blocks parasite acquisition by mosquitoes and consequently impairs mosquito infectivity^{33, 40, 41}. The induction of an immuno-blockade against multiple mosquito C-type lectins that function as viral susceptibility factors significantly reduced vectorial capacity for DENV and WNV^{17, 38}. Given the critical role of NS1 in the DENV transmission cycle, the passive transfer of DENV NS1 antibodies may disrupt DENV acquisition from infected mice. Notably, the active immunization of mice with DENV Δ NS1 significantly and simultaneously reduced

DENV infection of both mice and mosquitoes, indicating the feasibility of reducing flaviviral disease burden as well as the number of virus-carrying mosquitoes, which might provide a novel avenue for controlling flavivirus circulation in nature.

Viral acquisition by mosquitoes from an infected mammalian host is an essential step in the flavivirus lifecycle. In this study, we demonstrate that the presence of flavivirus NS1 in the sera of infected hosts facilitates viral acquisition by mosquitoes, suggesting that the secretion of NS1 might be a survival strategy evolved by flaviviruses to enable them to adapt to multiple host environments. Overall, the current study offers unique insight into a previously unappreciated role of sNS1 in the flavivirus lifecycle.

Materials and Methods

Ethics statement

Human blood for mosquito feeding was taken from healthy donors who provided written informed consent. The collection of human blood samples was approved by the local ethics committee at Tsinghua University.

Mice, mosquitoes, cells and viruses

C57BL/6 mice deficient in type I and II interferon (IFN) receptors (AG6 mouse) were purchased from Institute Pasteur of Shanghai, Chinese Academy of Sciences. The mice were bred and maintained under a specific pathogen-free animal facility at Tsinghua University. Groups of age- and sex-matched AG6 mice, 6–8 weeks of age, were used for animal study. All experiments were approved by and performed under the guidelines of the Experimental Animal Welfare and Ethics Committee of Tsinghua University. *A. aegypti* (the Rockefeller strain), *A. albopictus* (the Beijing strain) and *C. pipiens pallens* (the Beijing strain) were reared in a low-temperature, illuminated incubator (Model 818, Thermo Electron Corporation, Waltham, MA) at 28 °C and 80% humidity according to standard rearing procedures³⁸. The DENV1 GZ/XNC strain (*FJ176780*), DENV2 New Guinea C strain (*M29095*), DENV2 43 strain (*AF204178*), DENV3 ThD3 strain (*AY676352*) and JEV SA-14 strain (*U14163*) were grown in Vero cells for blood meals¹⁷. The Vero cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% heat-inactivated fetal bovine serum (16000-044, Gibco). For DENV and JEV production, the cells were grown in VP-SFM medium (11681-020, Gibco). The *Drosophila melanogaster* S2 cell line was cultured in Schneider's Medium with 10% heat-inactivated fetal bovine serum and 1% antibiotic-antimycotic (15240-062, Invitrogen). The Vero cell line was purchased from ATCC (CCL-81). The *Drosophila* S2 cell line was sourced from a *Drosophila* expression system of Invitrogen (R690-07). The cell lines do not have mycoplasma contamination. DENVs and JEV were titrated by a plaque formation (PFU) assay as previously described⁴².

Antibodies

To produce murine polyclonal antibodies, DENV and JEV *NS1* genes were amplified from the cDNA of infected cells and cloned into a pET-28a (+) expression vector. The cloning primers are presented in Supplementary Table 2. Recombinant NS1 proteins were expressed

in the *Escherichia coli* BL21 DE3 strain in an insoluble form in inclusion bodies. The proteins were then solubilized using 8 M urea and purified with a TALON Purification Kit (635515, Clontech). Murine antisera were generated following 3 boosters of recombinant NS1. Polyclonal antibodies were purified from the immunized antisera using protein A/G agarose (20423, Thermo). All antibodies for tag detection were purchased from Medical & Biological Laboratory (MBL, Japan).

DENV2 NS1 protein generation in a *Drosophila* expression system

The DENV2 NS1 gene was cloned into a pMT/BiP/myc-His A vector (modified from pMT/BiP/V5-His A, V4130-20, Invitrogen) for expression in *Drosophila* S2 cells. The cloning primers are shown in Supplementary Table 2. The procedure used to generate stable cells is described in the manual of the *Drosophila* expression system (K5130-01, Invitrogen). NS1-expressing, stable S2 cells were then amplified in regular Schneider's Medium in a 175- cm² flask and transferred into spinner flasks containing Express Five serum-free medium (10486-025, Gibco) for protein expression. The cells were cultured for 3 days and induced with 500 μ M copper sulfate for another 4 days. The supernatant was centrifuged, filtered, and then concentrated for purification using a TALON Purification Kit (635515, Clontech). Protein purity was verified by SDS-PAGE and immunostaining with an anti-myc mouse monoclonal antibody (M047-3, MBL, Japan).

Purification of native DENV sNS1

Native DENV2 sNS1 was purified on an immunoaffinity column with DENV2 NS1 antibodies⁴³. Polyclonal antibodies isolated from the DENV2 NS1 immunized antisera were coupled to cyanogen bromide-activated Sepharose 4B beads (C9142, Sigma-Aldrich). Native DENV2 sNS1 protein was isolated from the supernatant of DENV2-infected Vero cells at 5 days post infection. The eluted native sNS1 was concentrated using Amicon filter units (UFC801096, Millipore). The purified protein was diluted in PBS, aliquoted and stored at -80°C .

Purification of infectious DENV virions

Infectious DENV2 virions were purified by high-speed centrifugation⁴⁴. Briefly, supernatant from DENV2-infected Vero cells was harvested 5 days after inoculation. Cell fragments were removed by centrifugation at 25,000 g and 4 $^{\circ}\text{C}$ for 20 min. Following this, the supernatant was carefully transferred into a clean centrifuge tube and centrifuged at 25,000 g and 4 $^{\circ}\text{C}$ for an additional 6 hr to pellet the virions. The precipitated virions were washed twice and then solubilized in VP-SFM medium (11681-020, Gibco). Insoluble material was removed by an extra centrifugation step at 12,000 g and 4 $^{\circ}\text{C}$ for 2 min. The virions in the VP-SFM medium were aliquoted and stored at -80°C .

DENV NS1 detection by ELISA

DENV2 NS1 concentrations in Vero cell supernatant and mouse serum were measured using a Dengue NS1 Antigen ELISA Kit (8404-25, Diagnostic Automation). The experiment was performed according to the kit manual. The optical density (OD) was measured at 450 nm

with an ELISA reader (Varioskan Flash Multimode Reader, Thermo Scientific). NS1 concentration was calculated using the NS1 standard provided in the kit.

Gene silencing in mosquitoes

Detailed procedures for gene silencing in mosquitoes have been described elsewhere^{17, 38}. Briefly, female mosquitoes were anesthetized on a cold tray, and 1 µg/300 nl of double-stranded RNA (dsRNA) was microinjected into their thoraxes. The injected mosquitoes were allowed to recover for 3 days under standard rearing conditions. They were subsequently used for oral infection. Gene silencing efficiency was assessed by qPCR. The primers used for gene detection are shown in Supplementary Table 2.

Membrane blood feeding

We detected DENV IgG antibody levels in donor sera using a commercial ELISA kit (01PE10, Panbio). All blood samples were negative for DENV antibodies. Fresh human blood was placed in heparin-coated tubes (367884, BD Vacutainer) and centrifuged at 1,000 g and 4 °C for 10 min to separate plasma from blood cells. The plasma was collected and heat-inactivated at 55 °C for 60 min. The separated blood cells were washed 3 times with PBS to remove the anticoagulant. The cells were then resuspended in the heat-inactivated plasma. Purified proteins, antibodies, and vitamin C or uracil were mixed with viruses and human blood for mosquito oral feeding via a Hemotek system (6W1, Hemotek Limited, England). Only a short time interval elapsed (usually less than 10 min) between mixing the materials and allowing the mosquitoes to take a blood meal. Engorged female mosquitoes were transferred into new containers and maintained under standard conditions for an additional 8 days. The mosquitoes were subsequently sacrificed for further analysis.

Complete blood count

Blood from DENV2 (43 strain)-infected and uninfected AG6 mice was collected for blood cell counting using a hemocytometer (Z359629, Sigma). The blood was diluted with specific counting fluids for red blood cells (RBC), white blood cells (WBC) and platelets. The RBC dilution fluid contained normal saline to prevent hemolysis. The WBC dilution fluid contained 3% acetic acid and 1% gelatin violet to lyse RBCs without harming WBCs. The platelet dilution fluid included 1% ammonium oxalate buffer to lyse all blood cells except platelets. After diluting the blood in the different fluids, cells were manually counted using a hemocytometer and a microscope⁴⁵.

Mosquito feeding on infected mice

Female mosquitoes were separated into a netting-covered container for blood feeding. The mosquitoes were starved for 24 hr before engorgement. DENV or JEV-infected AG6 mice were anesthetized and placed on top of the container. The mosquitoes were allowed to feed on the mice for 30 min in darkness. After anesthetization using ice, the engorged mosquitoes were transferred to new containers and maintained under standard conditions for an additional 8 days. The mosquitoes were subsequently sacrificed for further analysis.

Viral genome quantitation by TaqMan qPCR

Total RNA was isolated from homogenized mosquitoes using an RNeasy Mini Kit (74106, Qiagen) and reverse-transcribed into cDNA using an iScript cDNA synthesis kit (170-8890, Bio-Rad). Viral genomes were quantified via TaqMan qPCR amplification of DENV and JEV genes. The primers and probes used for this analysis are shown in Supplementary Table 2. Gene quantities were normalized against *A. aegypti actin* (AAEL011197).

Determination of virus titer in infected mice by plaque assay

Blood samples were collected from the tail veins of infected mice in 0.4% sodium citrate and were centrifuged for 5 min at 6,000 g and 4 °C for plasma isolation. The presence of infectious viral particles in plasma was determined using a plaque assay as previously described⁴².

RNA-Seq analysis of mosquito midguts

For this experiment, we fed *A. aegypti* mosquitoes with 10 µg/ml purified DENV2 sNS1. An equal amount of BSA was used as a negative control. Total RNA was extracted with TRIzol (15596018, Ambion) from pools of 10 midguts at 4 hr, 8 hr, and 18 hr after blood feeding. The samples were delivered to the Beijing Genomics Institute (Shenzhen, China) for commercial RNA-Seq services and data analysis. Clean reads were mapped to the *A. aegypti* transcript database using SOAPaligner/SOAP2 mismatches. The number of clean reads for each gene was calculated and then normalized to reads per kb per million reads (RPKM), which associates read numbers with gene expression levels. The log₂ ratio (read number in NS1-fed midgut/read number in BSA-fed midgut) was exploited to determine gene regulation. Immune genes with log₂ ratio ≤ -1 or log₂ ratio ≥ 1 were selected for further analysis. The sequencing data were deposited in the Short Read Archive (NCBI) with accession number GSE73967.

H₂O₂ assay

Either 10 µg/ml DENV2 sNS1 or 10 µg/ml BSA was orally introduced into female *A. aegypti* mosquitoes by blood feeding. The midguts of the fed mosquitoes were dissected in PBS with 2 mg/ml of the catalase inhibitor 3-amino-1,2,4-triazole (A8056-10G, Sigma) at different time points. After homogenization, the samples were filtered through a spin filter with a 10K molecular weight cutoff (431486, Corning Spin-XUF, Corning). The eluate from each experimental group was then collected and tested using a hydrogen peroxide assay kit (K265-200, BioVision). Fluorescence intensity was measured at an excitation wavelength of 550 nm and an emission wavelength of 590 nm using a fluorescence microplate reader according to the manufacturer's instructions.

DHE staining

The midguts of mosquitoes fed 10 µg/ml DENV2 NS1 or BSA were dissected in PBS containing the catalase inhibitor 3-amino-1,2,4-triazole (A8056, Sigma) at 30 hr after feeding. Immediately after dissection, the midguts were incubated with 2 µM dihydroethidium (DHE) (D7008, Sigma) in PBS at room temperature for 30 min in darkness. Then, the midguts were fixed with 4% paraformaldehyde (PFA) for 30 min and

incubated for an additional 30 min with Triton X-100. Nuclei were stained blue with To-Pro-3 iodide (T3605, Thermo-Fisher Scientific). Slides were imaged using a 10×objective lens on a Zeiss LSM 780 meta confocal microscope (Carl Zeiss, Germany) in a multi-track mode.

Antibody attachment to human endothelial cells

HUVECs were cultured in endothelial cell medium (1001, Sciencell) in 96-well plates until they reached 100% confluence. For ELISA, the cells were fixed in 2% PFA and blocked with 1% bovine serum albumin (BSA). Then, they were incubated with 1 µg of murine antibody at 37 °C for 30 min. The plates were then washed 6 times with PBS containing 0.05% Tween 20 (PBST), and they were stained with anti-mouse horseradish peroxidase (HRP)-conjugated IgG (JM-6402-05, MBL, Japan). After an additional 1 hr of incubation, the signal was detected using a commercial peroxidase substrate system (52-00-01 and 50-85-04, Kirkegaard & Perry Laboratories), and the OD at 450 nm was measured with an ELISA reader (Varioskan Flash Multimode Reader, Thermo Scientific). For flow cytometry, HUVECs were detached with 2 mM EDTA in PBS and washed with FACS buffer (PBS containing 2% FBS). The cells were incubated with 5 µg murine antibody at 4 °C for 1 hr. After washing, the cells were subsequently incubated with Alexa 488-conjugated anti-mouse IgG (S-11223, Thermo) at 4 °C for 30 min. The treated cells were then suspended in FACS buffer and analyzed on a flow cytometer (Accuri C6, BD Biosciences).

Antibody attachment to human platelet cells

Platelets were isolated from fresh human blood⁴⁶. The isolated platelets were resuspended, plated onto a 96-well plate, and fixed in 2% PFA in PBS for 15 min. After washing with PBS, the cells were blocked with 1% BSA for 1 hr. Following this, 1 µg of murine antibody was incubated with the platelets at 37 °C for 30 min. After additional washing, the cells were stained with anti-mouse HRP-conjugated IgG (JM-6402-05, MBL, Japan). A commercial peroxidase substrate system (52-00-01 and 50-85-04, Kirkegaard & Perry Laboratories) was employed for signal detection. The OD was measured at 450 nm with an ELISA reader (Varioskan Flash Multimode Reader, Thermo Scientific).

DENV NS1 immunization and viral challenge in AG6 mice

AG6 mice were immunized three times with 40 µg of either full-length DENV2 NS1 or DENV2 ΔNS1 (on the 0th, 2nd and 4th week post initial immunization). Mice inoculated with PBS plus adjuvant served as mock controls. After two additional weeks (day 42), the mice were challenged with 1x10⁶ pfu of the DENV2 43 strain via intraperitoneal injection. All of the challenged mice were monitored daily for 40 days. Mouse sera were collected at different time points and stored at -80 °C for further investigation.

Measurement of vascular leakage using Evans blue dye

Vascular leakage was determined following intravascular administration of Evans blue dye (E2129, Sigma)⁹. Briefly, 150 µl Evans blue dye (0.5% in PBS) was intravenously injected per mouse 18 days post infection. After 2 hr of dissemination, the mice were anesthetized and perfused with PBS. The following tissues were harvested: kidney, liver, spleen, small

intestine, large intestine and stomach. The dye was extracted from the tissues using formamide (F7503, Sigma). The dye concentrations in the formamide extracts were then quantified by measuring the absorbance at 610 nm.

Titration of DENV NS1 antibodies

A total of 1 µg purified DENV2 NS1 was coated per well in 96-well plates at 4 °C overnight. The coated wells were then blocked using 1% BSA for 1 hr at room temperature. After washing with PBST, antisera from the immunized AG6 mice were serially diluted from 1/250 to 1/10,000,000. Subsequently, the diluted antisera were added to the coated wells and incubated for an additional 2 hr. After washing with PBST, anti-mouse HRP-conjugated IgG (JM-6402-05, MBL, Japan) was added to the wells for 1 hr. A commercial peroxidase substrate system (52-00-01 and 50-85-04, Kirkegaard & Perry Laboratories) was used for signal detection. The OD at 450 nm was measured with an ELISA reader (Varioskan Flash Multimode Reader, Thermo Scientific).

Statistics

Animals were randomly allocated into different groups. Mosquitoes that died before measurement were excluded from analysis. The investigators were not blinded to the allocation during the experiments or to the outcome assessment. All experiments were performed independently at least three times. For DENV and JEV infections, at least five AG6 mice were included in each group. No statistical methods were used to predetermine sample size.

Descriptive statistics have been provided in the figure legends. A Kruskal-Wallis analysis of variance was conducted to detect any significant variation among replicates. If no significant variation was detected, the results were pooled for further comparison. Given the nature of the experiments and the type of samples, differences in continuous variables were assessed with the non-parametric Mann-Whitney test. Differences in mosquito infective rates were analyzed using Fisher's exact test. *P* values were adjusted using Bonferroni correction to account for multiple comparisons. The survival rates of the infected mice were statistically analyzed using the Log-rank (Mantel-Cox) test. All analyses were performed using GraphPad Prism statistical software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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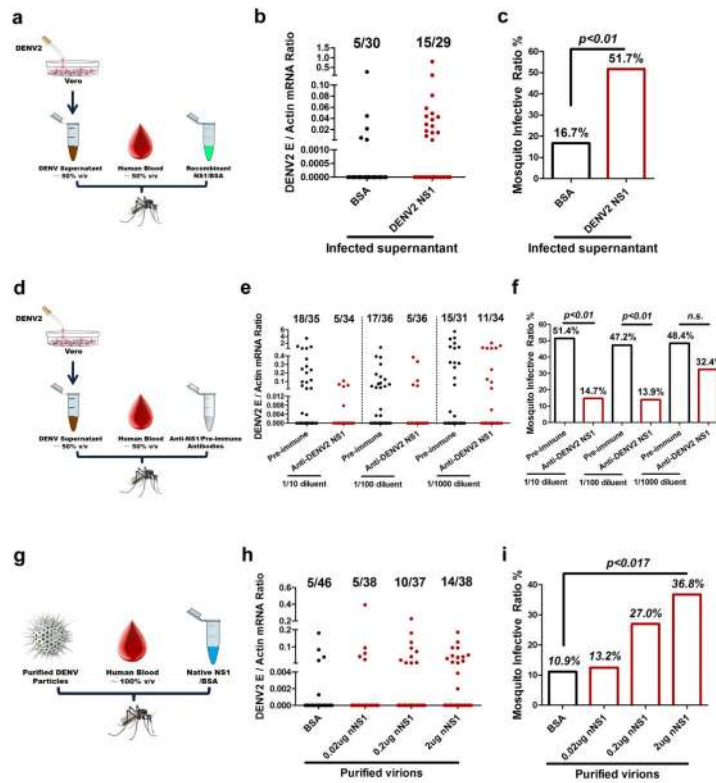


Figure 1. DENV sNS1 facilitates DENV acquisition via membrane blood feeding
(a–c) The presence of recombinant DENV2 sNS1 in blood increased DENV2 acquisition by *A. aegypti*. **(a)** Recombinant DENV2 sNS1 protein was expressed and purified from *Drosophila S2* cells. A total of 10 μ g of purified sNS1 was incubated with fresh human blood (500 μ l) and supernatant from DENV2-infected Vero cells (500 μ l) to feed *A. aegypti* via an *in vitro* membrane blood meal. **(b,c)** A concentration of 1×10^5 pfu/ml DENV2 was used for mosquito oral infection. Mosquitoes fed the same amount of BSA served as negative controls.
(d–f) Immunoblockade of DENV sNS1 in supernatant from infected Vero cells reduced DENV acquisition by *A. aegypti*. **(d)** Serially diluted anti-DENV2 NS1 antisera were mixed with supernatant from DENV2-infected Vero cells (500 μ l) and fresh human blood (500 μ l) for *in vitro* membrane feeding of *A. aegypti*. **(e,f)** A concentration of 1×10^6 pfu/ml DENV2 was used for oral infection. As a mock control, mosquitoes were fed the same dilution of pre-immune sera.
(g–i) The presence of purified native sNS1 (nNS1) in viremic human blood increased DENV2 acquisition by *A. aegypti*. Infectious DENV2 particles and native sNS1 protein were purified from the supernatant of DENV2-infected Vero cells. Following this, native sNS1 and 1×10^6 pfu purified DENV2 virions were incubated with human blood (1 ml) for mosquito oral feeding. The equivalent amount of BSA was used as a negative control.
(a–i) The DENV2 NGC strain was used for mosquito oral infection. Mosquito infectivity was determined by TaqMan qPCR at 8 days post blood meal. **(b,e,h)** The number of infected mosquitoes relative to total mosquitoes is shown at the top of each column. Each dot represents a mosquito. **(c,f,i)** The data are represented as the percentage of mosquito

infection. Differences in mosquito infective ratio were compared using Fisher's exact test. **(i)** The p values were adjusted using Bonferroni correction to account for multiple comparisons. Differences were considered significant if $p < 0.017$.

(a-i) The experiments were biologically repeated at least 3 times with similar result.

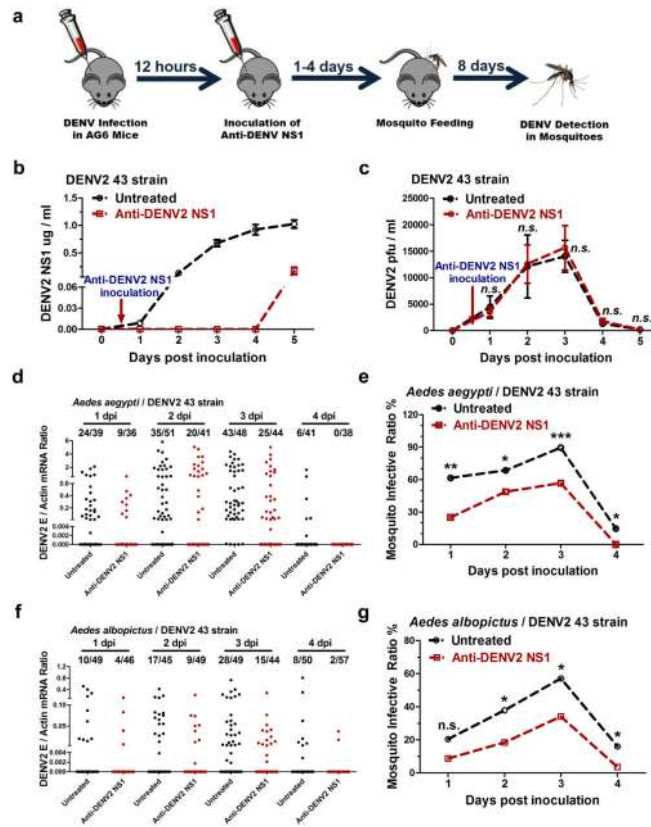


Figure 2. Passive transfer of DENV2 sNS1 antibodies into infected AG6 mice prevents DENV acquisition by mosquitoes

(a) Schematic representation of the study design. AG6 mice were intraperitoneally infected with 1×10^6 pfu of the DENV2 43 strain. Subsequently, 100 μ l of a DENV2 NS1 antibody was intraperitoneally inoculated into the mice at 12 hr post infection. An equivalent quantity of untreated serum was inoculated as a mock control. After allowing 12 hr for antibody dissemination, the infected mice were subjected to daily mosquito biting from day 1 to day 4 post mouse infection. The mouse blood-fed mosquitoes were reared for an additional 8 days for DENV detection.

(b,c) DENV2 infection of AG6 mice. (b) Measurement of DENV2 sNS1 concentration. Mouse sera were used to determine the amounts of DENV2 sNS1 present from 0 to 5 days post infection by ELISA. (c) Detection of DENV2 viremia in the blood of infected AG6 mice. The presence of infectious viral particles in the blood plasma was assessed using a plaque assay. (b,c) The data are representative of at least five infected AG6 mice. The values in the graph represent the mean \pm SEM. A non-parametric Mann-Whitney test was used for statistical analysis.

(d–g) Immunoblockade of DENV2 sNS1 in the infected AG6 mice reduced the infection of fed *A. aegypti* (d,e) and *A. albopictus* (f,g). (d,f) The number of infected mosquitoes relative to total mosquitoes is shown at the top of each column. Each dot represents a mosquito.

(b–g) The DENV2 43 strain was used for animal infections. (e,g) The data are represented as the percentage of mosquito infection. Differences in mosquito infective ratios were

assessed using Fisher's exact test. "*", "**" and "***" represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

(b–g) The experiments were biologically reproduced at least 3 times.

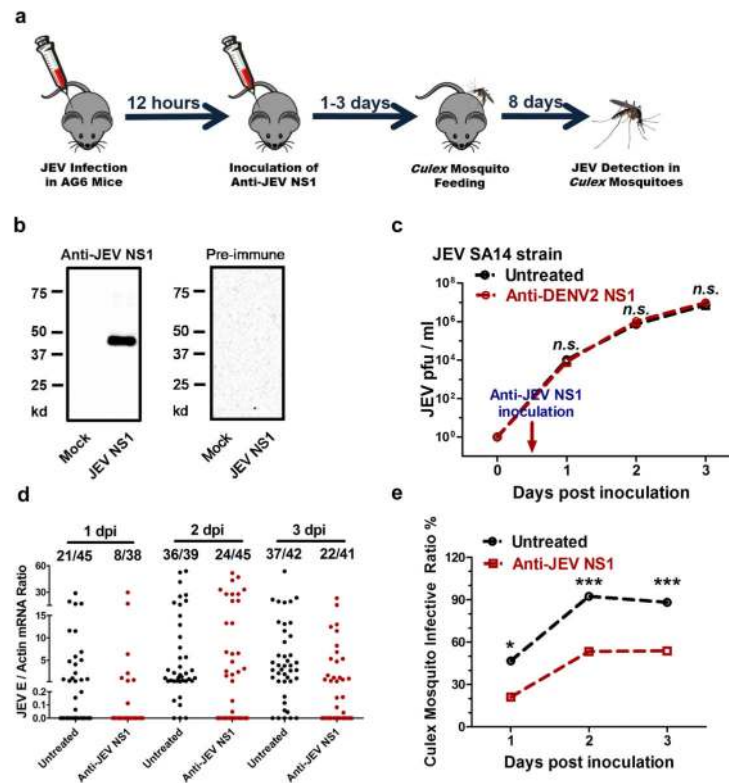


Figure 3. Passive transfer of antibodies against JEV NS1 in infected AG6 mice prevents JEV acquisition by *Culex pipiens pallens*

(a) Schematic representation of the study design. AG6 mice were infected with the JEV SA-14 strain. Subsequently, a 100 μ l aliquot of a JEV NS1 antibody stock was intraperitoneally injected per mouse 12 hr post infection. Infected mice were inoculated with an equivalent quantity of untreated serum as a mock control. After waiting an additional 12 hr for antibody dissemination, the infected mice were subjected to daily mosquito biting from day 1 to day 3 post mouse infection. The mouse blood-fed mosquitoes were reared for an additional 8 days for JEV detection.

(b) Production of murine polyclonal antibodies against JEV NS1. The JEV NS1 gene was cloned into a pET-28a (+) expression vector and expressed in *E. coli* BL21 DE3. Recombinant JEV NS1 in inclusion bodies was dissolved in 8 M urea and purified for antibody generation. The antibodies were validated by immunostaining with S2-expressed JEV NS1 protein.

(c) JEV infection in AG6 mice. Blood was collected from mouse tail veins from 0 to 3 days post JEV infection. The presence of infectious viral particles in the blood plasma was assessed using a plaque assay. The data are representative of at least five infected AG6 mice. Differences between untreated and JEV NS1 antibody-treated groups were assessed using a non-parametric Mann-Whitney test.

(d,e) Immunoblockade of JEV sNS1 in JEV-infected AG6 mice reduced JEV acquisition by *C. pipiens pallens*. The fed mosquitoes were reared for an additional 8 days for JEV detection by TaqMan qPCR. (d) The number of infected mosquitoes relative to total mosquitoes is shown at the top of each column. Each dot represents a mosquito. (e) The data

are represented as the percentage of mosquito infection. Differences in mosquito infective ratios were assessed using Fisher's exact test. "*", "**" and "***" represent $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

(b–e) The experiments were biologically reproduced at least 3 times.

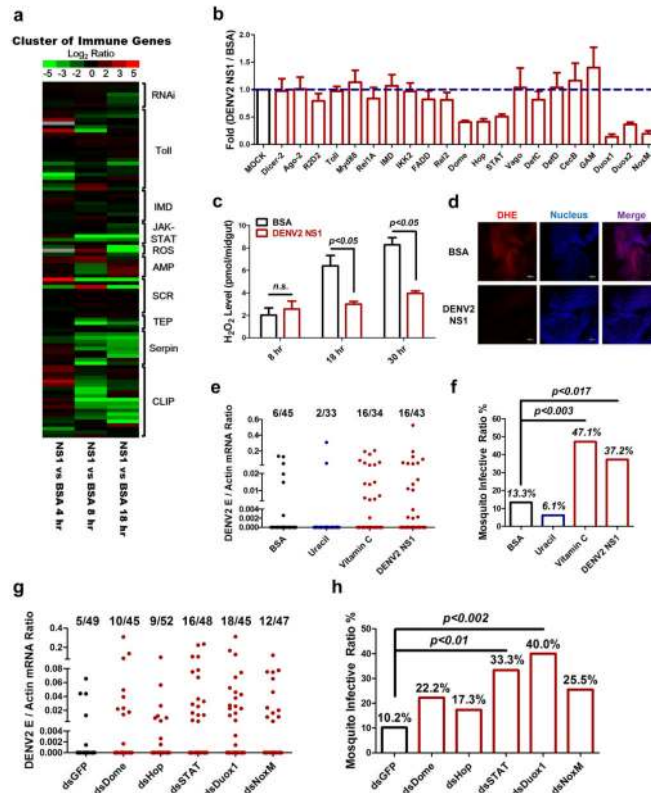


Figure 4. DENV2 sNS1 suppresses the expression of immune-related genes in the mosquito midgut

(a) Regulation of immune-related genes in the midguts of *A. aegypti*. The midguts of mosquitoes that acquired 10 μ g purified DENV2 sNS1 or BSA via blood meals were dissected for RNA-Seq. Immune-related genes were clustered according to immune pathways and factors.

(b) Validation of immune-related gene regulation. The expression levels of the immune-related genes listed in Supplementary Fig. 6 were assessed in the mosquito midguts by qPCR at 18 hr post DENV2 sNS1 feeding. Gene regulation is represented as the mRNA ratio between sNS1-fed and BSA-fed midguts. The primers are described in Supplementary Table 2.

(c,d) ROS activities in the midguts of mosquitoes fed DENV2 sNS1. The midguts of mosquitoes fed either DENV2 sNS1 or BSA were dissected for H₂O₂ detection (c) and dihydroethidium (DHE) staining (d). (d) Nuclei were stained blue with To-Pro-3 iodide. Images were examined using a 10 \times objective lens on a Zeiss LSM 780 meta confocal microscope. The scale bars represent 100 μ m.

(b,c) The values in the graph represent the mean \pm SEM. A non-parametric Mann-Whitney test was used to determine significant differences.

(e,f) The role of the ROS system in DENV infection of mosquitoes. Either 3.3 mM vitamin C or 1 mM uracil mixed with 1×10^6 pfu purified DENV2 virions and human blood was used for a mosquito blood meal. Mosquito infectivity was determined by TaqMan qPCR at 8 days post blood meal.

(g,h) Genetic suppression of the JAK-STAT pathway and the ROS system enhanced DENV2 infection in mosquitoes. *GFP* dsRNA served as a negative control. Mosquito infectivity was determined by TaqMan qPCR at 8 days post blood meal.

(e,g) The number of infected mosquitoes relative to total mosquitoes is shown at the top of each column. Each dot represents a mosquito.

(f,h) Differences in mosquito infective ratios were compared using Fisher's exact test. The p values were adjusted using Bonferroni correction to account for multiple comparisons. Differences were considered significant if $p < 0.017$ (**f**) or $p < 0.01$ (**h**).

(a-h) The experiments were biologically repeated at least 3 times with similar result.

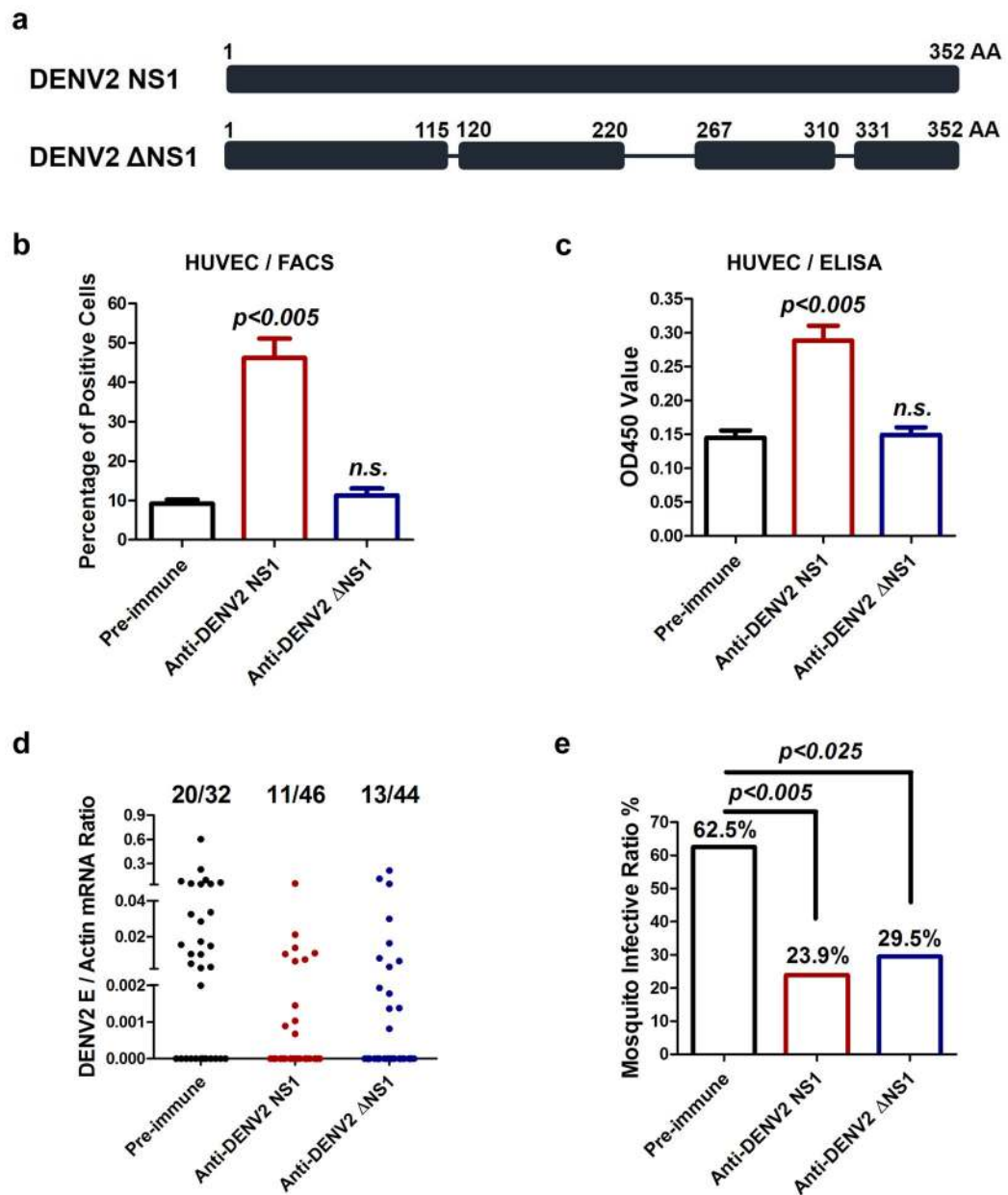


Figure 5. An antibody generated against DENV2 Δ NS1 does not cross-react with human cells and prevents DENV acquisition by mosquitoes

(a) Schematic representation of DENV2 Δ NS1. DENV2 Δ NS1 lacks the following antigenic regions from full-length DENV2 NS1 that potentially elicit the production of cross-reactive antibodies: amino acids 116–119¹⁴, 221–266¹⁵ and 311–330¹⁶.

(b,c) DENV NS1 antibody cross-reactivity with HUVEC cells. Purified antibodies against DENV2 full-length NS1 or DENV2 Δ NS1 were incubated with HUVECs. A pre-immune antibody served as a mock control. Antibody attachment to the HUVECs was quantified by flow cytometry (b) and ELISA (c). DENV2 NS1 antibodies were stained using anti-mouse Alexa 488-conjugated IgG for FACS assay and anti-mouse HRP-conjugated IgG for ELISA. The values in the graph represent the mean \pm SEM. A non-parametric Mann-Whitney test

was used to determine significant differences. The p values were adjusted using Bonferroni correction to account for multiple comparisons. Differences were considered significant if $p < 0.025$.

(d,e) An antibody generated against DENV2 Δ NS1 prevents DENV acquisition by mosquitoes. Murine antisera (1:100 dilution) against DENV2 full-length NS1 or DENV2 Δ NS1 were incubated with supernatant from DENV-infected Vero cells (50% v/v) and fresh human blood (50% v/v). The mixture was used for *in vitro* membrane feeding of *A. aegypti*. The same dilution of pre-immune serum served as a mock control. Mosquito infectivity was determined by TaqMan qPCR at 8 days post blood meal. **(d)** The number of infected mosquitoes relative to total mosquitoes is shown at the top of each column. Each dot represents a mosquito. **(e)** The data are represented as the percentage of mosquito infection. Differences in mosquito infective ratios were compared using Fisher's exact test. The p values were adjusted using Bonferroni correction to account for multiple comparisons. Differences were considered significant if $p < 0.025$.

(b–e) The experiments were biologically repeated at least 3 times with similar result.

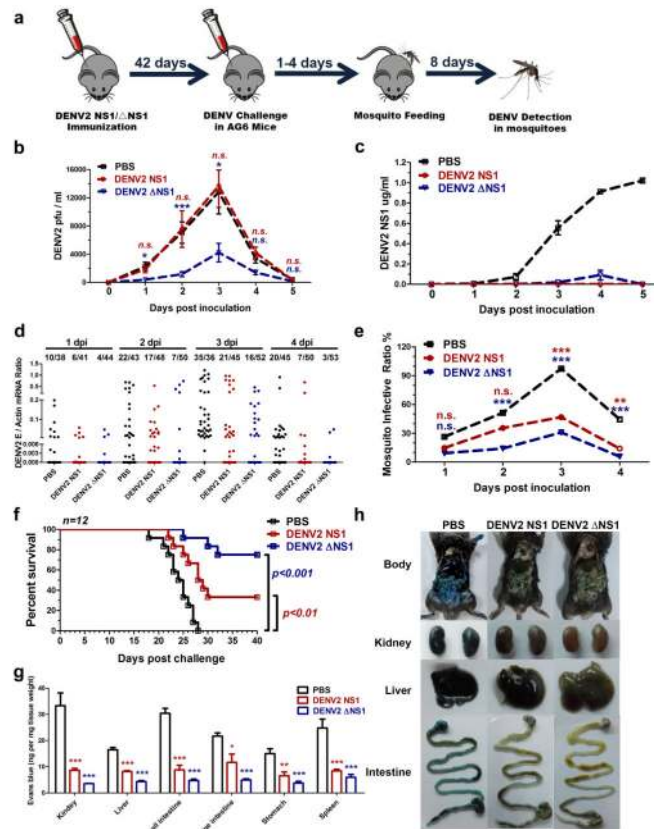


Figure 6. NS1 vaccination prevents DENV infections in mice and mosquitoes (a–e) Active immunization with DENV2 Δ NS1 prevented DENV2 acquisition by mosquitoes. (a) Schematic representation of the study design. (b,c) DENV2 infection in immunized AG6 mice. (b) Detection of DENV2 viremia. Blood was collected from the tail veins of infected mice from 0 to 5 days post infection. The presence of infectious viral particles in the blood plasma was assessed using a plaque assay. (c) Detection of DENV2 sNS1 concentration. Mouse sera were used to determine DENV2 sNS1 quantities by ELISA. (d,e) NS1 immunization reduced DENV acquisition by *A. aegypti*. (d) The number of infected mosquitoes relative to total mosquitoes is shown at the top of each column. Each dot represents a mosquito. (e) The data are represented as the percentage of mosquito infection. Differences in mosquito infective ratios were compared using Fisher's exact test. (f–h) Vaccination with DENV2 Δ NS1 protected mice from lethal DENV-induced vascular leakage. (f) AG6 mice were immunized and infected following the same procedure as shown in Fig. 6a. “n” represents the number of mice in each group. The survival rates of the infected mice were statistically analyzed using the Log-rank (Mantel-Cox) test. (g,h) Evans blue dye was intravenously injected into the mice at 18 days post infection. The dye was extracted from various tissues using formamide, and the OD₆₁₀ was measured (g). The infected mice were used to visualize DENV-induced vascular leakage in various tissues (h). (b–g) The red *p* value represents a comparison between full-length DENV2 NS1-immunized and control mice. The blue *p* value represents a comparison between DENV Δ NS1-immunized and control mice. The *p* values were adjusted using Bonferroni correction to

account for multiple comparisons. Differences were considered significant if $p < 0.025$. “*”, “**”, and “***” represent $p < 0.025$, $p < 0.005$, and $p < 0.0005$, respectively.

(b,c,g) The data are representative of at least five infected AG6 mice. The values in the graph represent the mean \pm SEM. A non-parametric Mann-Whitney test was used to determine significant differences.

(b-h) The experiments were biologically reproduced at least 3 times.