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

Jianying Liu1†, Yang Liu1,2,3†, Kaixiao Nie4, Senyan Du1, Jingjun Qiu4, Xiaojing Pang1, Penghua Wang5 and Gong Cheng1,2,*

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 NS1s in the blood of infected interferon-α and γ 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.

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 aetiological agents of diseases with severe manifestations such as haemorrhage, shock and encephalitis. DENV is transmitted between humans and its mosquito vectors, Aedes aegypti and Aedes albopictus. Worldwide, an estimated 390 million DENV infections occur each year, with 96 million manifesting with clinical symptoms. JEV, which is transmitted by Culicidae mosquitoes, causes an estimated 67,900 annual cases of encephalitis diseases in 24 Asian and Oceanian countries. 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 three structural proteins and seven non-structural 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. Both secreted and cell-surface-associated NS1 are highly immunogenic and might contribute to the pathogenesis of flavivirus infection in a host. During acute DENV infection, secreted NS1 protein (sNS1) is present in patient sera at high levels, ranging from 70 to 15,000 ng ml⁻¹ (ref. 7), but in exceptional cases this level can reach up to 50,000 ng ml⁻¹ (ref. 8). Based on results from DENV studies in animals, sNS1 can contribute to the pathogenesis of severe DENV illness by increasing the permeability of capillaries and might augment DENV infection by interfering with the immune system. 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. However, 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. 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 illness. 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. 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 an 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 life cycle. During the viremic stage in an infected host, abundant quantities of sNS1 can be found in blood circulation together with viruses, and together these components are transferred to a mosquito as it takes a blood meal. We therefore 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). A mixture containing human blood (50% vol/vol), supernatant from DENV2-infected Vero cells (50% vol/vol) and the purified DENV2 sNS1 protein (final concentration of up to 10 μg ml⁻¹) was then 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 × 10⁵ p.f.u. ml⁻¹, Fig. 1b,c) or high (1 × 10⁶ p.f.u. ml⁻¹, Supplementary Fig. 1b,c) dose of DENV was used. It has been reported that sNS1 expression and secretion is associated with active viral replication in host cells. We thus measured the amount of sNS1 in the supernatant of DENV2-infected Vero cells using an enzyme-linked immunosorbent assay (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). Supernatant from DENV2-infected Vero cells (50% vol/vol) was then mixed with fresh human blood (50% vol/vol) 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 with 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 feeding. 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% vol/vol) 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).

Immmuno-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 (ifnar−/−) has been widely used as an animal model for studies of DENV infection. We therefore examined the role of sNS1 during DENV acquisition in vivo using an ifnar−/− model (C57BL/6 strain, AG6 strain)²¹. We first assessed whether DENV2 infection influences blood cell counts in AG6 mice. Compared with 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 studies.²² 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 h 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 with 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 milieu.²⁵ We therefore applied procedures similar to those 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), but it significantly decreased 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 signalling components, enzymes and effectors were selected for analysis. The results showed that the expression levels of 26 genes at 4 h, 36 genes at 8 h and 38 genes at 18 h after NS1 treatment changed by more than twofold 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 twofold). In contrast, the mRNA expression levels of genes related to ROS production and the Janus kinase-signal transducer and activator of transcription (JAK-STAT) signalling 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 h post blood feeding were validated using quantitative PCR (qPCR; Fig. 4b). Furthermore, ROS activity in the mosquito midgut was consistently suppressed by DENV2 sNS1 (Fig. 4c,d). Inhibiting ROS activity with vitamin C rendered the mosquitoes highly susceptible to DENV2 infection. In contrast, activating ROS with uracil 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.²⁷,²³,²⁹,³² Indeed, genetic interruption of the ROS system (via Ddx1 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.

**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. Recombinant DENV2 sNS1 protein was expressed and purified from Drosophila S2 cells (a). A total of 10 µg of purified sNS1 was incubated with fresh human blood (500 µl) and supernatant from DENV2-infected Vero cells (500 µl) to feed A. aegypti via an in vitro membrane blood meal. In b and c, 1 x 10⁶ p.f.u. ml⁻¹ 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. Serially diluted anti-DENV2 NS1 antisera were mixed with supernatant from DENV2-infected Vero cells (500 µl) and fresh human blood (500 µl) for in vitro membrane blood feeding of A. aegypti (d). In e and f, 1 x 10⁶ p.f.u. ml⁻¹ 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 x 10⁶ p.f.u. 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. In 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. In b, e and h, the number of infected mosquitoes relative to total mosquitoes is shown at the top of each column. Each dot represents a mosquito. In c, f and i, data are represented as the percentage of mosquito infection. Differences in mosquito infective ratio were compared using Fisher’s exact test. In i, P values were adjusted using the Bonferroni correction to account for multiple comparisons. Differences were considered significant if P < 0.017. In a–i, experiments were biologically repeated at least three times with similar results. NS, not significant.
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 p.f.u. of the DENV2 43 strain. Subsequently, 100 µl of a DENV2 NS1 antibody was intraperitoneally inoculated into the mice at 12 h post-infection. An equivalent quantity of untreated serum was inoculated as a mock control. After allowing 12 h 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. For measurement of DENV2 sNS1 concentration (b), mouse sera were used to determine the amounts of DENV2 sNS1 present from 0 to 5 days post-infection by ELISA. For detection of DENV2 viremia in the blood of infected AG6 mice (c), the presence of infectious viral particles in the blood plasma was assessed using a plaque assay. Data in b and c are representative of at least five infected AG6 mice. Values in the graph represent the mean ± s.e.m. A non-parametric Mann–Whitney test was used for statistical analysis. d–g, Immunoblockade of DENV2 sNS1 in the infected AG6 mice (d, f, g) reduced the infection of fed A. aegypti (d, e) and A. albopictus (f, g). In d and f, the number of infected mosquitoes relative to total mosquitoes is shown at the top of each column. Each dot represents a mosquito. In e and g, data are represented as the percentage of mosquito infection. Differences in mosquito infective ratios were assessed using Fisher’s exact test. *P < 0.05, **P < 0.01, ***P < 0.001. Experiments reported in b–g were biologically reproduced at least three times.
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 disease14,15,33. 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 symptoms14,15,34. We therefore constructed a DENV2 NS1 mutant (DENV2 ΔNS1) that harboured deletions of three potential antigenic regions that could elicit cross-reacting antibodies (Fig. 5a)14,15. Unlike antibodies generated against full-length DENV2 NS1, the antibodies that were generated against the DENV2 ΔNS1 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 ΔNS1 and full-length NS1 antibodies produced equivalent repressive effects on DENV acquisition (Fig. 5d,e).

We next immunized AG6 mice with either DENV2 ΔNS1 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...
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 µg 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. Expression levels of the immune-related genes listed in Supplementary Fig. 6 were assessed in the mosquito midguts by qPCR at 18 h 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**). In **d**, nuclei were stained blue with To-Pro-3 iodide. Images were examined using a ×10 objective lens on a Zeiss LSM 780 meta confocal microscope. Scale bars, 100 µm. In **b** and **c**, values in the graph represent the mean ± s.e.m. 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 p.f.u. 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. In **e** and **g**, the number of infected mosquitoes relative to total mosquitoes is shown at the top of each column. Each dot represents a mosquito. In **f** and **h**, differences in mosquito infective ratios were compared using Fisher’s exact test. P values were adjusted using the Bonferroni correction to account for multiple comparisons. Differences were considered significant if P < 0.017 (**f**) or P < 0.01 (**h**). Experiments reported in all panels were biologically repeated at least three times with similar results. NS, not significant.
AG6 mice. Additionally, there was no significant difference in DENV NS1-specific antibody titres between the ΔNS1- and the full-length NS1-immunized AG6 mice (Supplementary Fig. 10). The immunized mice were intraperitoneally challenged with 1 × 10^6 p.f.u. of the DENV2 43 strain two weeks after final immunization. The infection ratios of the mosquitoes that fed on the DENV2 ΔNS1-immunized mice were three- to eightfold lower than those of the mosquitoes that fed on the negative controls and two- to threefold 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. To validate these results in our AG6 mouse model, we immunized AG6 mice with full-length NS1 or ΔNS1 three times, then subjected them to intraperitoneal infection with the DENV2 43 strain (Fig. 6a). All control animals (immunized with PBS) succumbed to DENV infection by 28 days post-infection, whereas 75% of the mice that were immunized with DENV2 ΔNS1 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 ΔNS1 showed reduced Evans blue dye intensity—an indicator of the vascular leakage caused by DENV infection—in various tissues (Fig. 6g,h).

**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 non-structural (NS) proteins, help facilitate viral immune evasion in mammals. Most of these NS proteins interfere with cell-intrinsic antiviral mechanisms inside host cells. However, NS1 is secreted over time, and hence, 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. Accordingly, the abundant secretion of NS1...
Figure 6 | NS1 vaccination prevents DENV infections in mice and mosquitoes. a–e, Active immunization with DENV2 ΔNS1 prevented DENV2 acquisition by mosquitoes. a, Schematic representation of the study design. b, 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. In d, the number of infected mosquitoes relative to total mosquitoes is shown at the top of each column. Each dot represents a mosquito. In e, 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 ΔNS1 protected mice from lethal DENV-induced vascular leakage. In f, AG6 mice were immunized and infected following the same procedure as shown in a. n, number of mice in each group. Survival rates of the infected mice were statistically analysed using the log-rank (Mantel-Cox) test. In g and 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 OD610 was measured (g). Infected mice were used to visualize DENV-induced vascular leakage in various tissues (h). In b–g, the red P values represent a comparison between full-length DENV2 NS1-immunized and control mice, and the blue P values represent a comparison between DENV ΔNS1-immunized and control mice. P values were adjusted using the Bonferroni correction to account for multiple comparisons. Differences were considered significant if P < 0.025. *P < 0.025, **P < 0.005, ***P < 0.0005. In b, c, and g, data are representative of at least five infected AG6 mice. Values in the graph represent the mean ± s.e.m. A non-parametric Mann-Whitney test was used to determine significant differences. In b–h, experiments were biologically reproduced at least three times. d.p.i., days post inoculation; NS, not significant.
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. aegypti26,28. Toll pathway activation via Wolbachia-induced ROS production was found to stimulate AMP production and subsequently restrict DENV infection in A. aegypti27. 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 components41. 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 infection12,25,36. 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 cells16,34, resulting in inhibition of platelet aggregation or apoptosis of endothelial cells15–16. To overcome this limitation, in the current study we constructed a truncated form of DENV2 NS1 that harbours deletions of antigenic regions that could potentially elicit the production of cross-reactive antibodies. Antibodies produced against the DENV2 ΔNS1 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 ΔNS1 presented with much lower levels of viremia, less vascular leakage and higher survival rates after lethal DENV2 challenge, indicating that immunization with DENV ΔNS1 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 nature17,38,39. 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 impedes mosquito infectivity33,40,41. 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 WNV17,38. Given the critical role of NS1 in the DENV transmission cycle, the passive transfer of DENV NS1 antibodies may disrupt DENV infection from infected mice. Notably, the active immunization of mice with DENV ANS1 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 may 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 life cycle. 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 life cycle.

**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 (IFN−/−) 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 the animal studies. All experiments were approved by and performed under the guidelines of the Experimental Animal Welfare and Ethics Committee of Tsinghua University. Aedes 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) at 28 °C and 80% humidity according to standard rearing procedures42. The DENV1 GZ/XNC strain (FJ176780), DENV2 New Guinea C strain (M20909), DENV2-43 strain (AF204178), DENV3 THD3 strain (AY767352) and JEV SA-14 strain (U41683) were grown in Vero cells for blood meals47. 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 VPE-SFM medium (11668-023, 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 (CCL81). The Drosophila S2 cell line was sourced from a Drosophila expression system of Invitrogen (R690-07). These cell lines do not have mycoplasma contamination. DENVs and JEV were titrated by a plaque formation (p.f.u.) 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 antiseras were generated following three boosters of recombinant NS1. Polyclonal antibodies were purified from the immunized antiseras using protein A/G agarose (25423, Thermo). All antibodies for tag detection were purchased from the Medical & Biological Laboratory (MBL, Japan).

**DENV2 NS1 protein generation in a Drosophila expression system.** The DENV2 NS1 gene was cloned into a pMT/BP/myc-His A vector (modified from pM/cBP/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 (K310-01, 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 (10486-035, Gibco) for protein expression. The cells were cultured for 3 days and induced with 500 µM 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 antiseras 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 collected 5 days after inoculation. Cell fragments were removed by centrifugation at 25,000g and 4 °C for 20 min. Following this, the supernatant was carefully transferred into a clean centrifuge tube and centrifuged at 25,000g and 4 °C for an additional 6 h 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 12,000g and 4 °C for 2 min. The viroms 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 elsewhere36,37. Briefly, female mosquitoes were anaesthetized on a cold tray and 1 µg per 300 ml of double-stranded RNA (dsRNA) was microinjected into their thoraxae. 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 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 three 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). 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 killed for further analysis.

Complete blood count. Blood from DENV2 (43 strain)-infected and uninfected AG66 mice was collected for blood cell counting using a haematocytometer (Z359629, Sigma). The blood was diluted with specific counting fluids for red blood cells (RBCs), white blood cells (WBCs) and platelets. The RBC dilution fluid contained normal saline to prevent haemolysis. 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 haematocytometer and a microscope35.

Mosquito feeding on infected mice. Female mosquitoes were separated into a netting-covered container for blood feeding. The mosquitoes were starved for 24 h before engorgement. DENV or JEV-infected AG66 mice were anaesthetized and placed on top of the container. The mosquitoes were allowed to feed on the mice for 30 min in darkness. After anaesthetization using ice, the engorged mosquitoes were transferred to new containers and maintained under standard conditions for an additional 8 days. The mosquitoes were subsequently killed for further analysis.

Virulence gene quantification by TaqMan qPCR. Total RNA was isolated from homogenized mosquitoes using an RNeasy Mini Kit (74106, Qiagen) and reverse-transcribed 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 (AAE011197).

Determination of virus titre 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 6,000g and 4 °C for plasma isolation. The presence of infectious viral particles in plasma was determined using a plaque assay as previously described40.

RNA-Seq analysis of mosquito midguts. For this experiment, we fed A. aegypti mosquitoes with 10 µg ml⁻¹ 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, 8 and 18 h after blood feeding. The samples were delivered to the Beijing Genomics Institute for commercial RNA-Seq services and iScript cDNA synthesis kit (170-8890, Bio-Rad). Viral genomes were quantified via 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 (AAE011197).

Antibody attachment to human platelet cells. Antibody attachment to human platelet cells was determined following intravascular administration of Evans blue dye (E2129, Sigma) 9. The midgut of mosquitoes fed 10 µg ml⁻¹ DENV2 NS1 or BSA were dissected in PBS containing the catalse inhibitor 3-amino-1,2,4-triazole (A8056, Sigma) at 30 h after feeding. Immediately after dissection, the midguts were immobilized with 2 µM dimethylsulfoxide (DMSO; D7008, Sigma) on ice for at least 30 min in darkness. The midguts were then 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 x10 objective lens on a Zeiss LSM 780 meta confocal microscope (Carl Zeiss) in a multi-track mode.

Antibody attachment to human endothelial cells. HUVECs were cultured in endothelial cell medium (1001, Science) in 96-well plates until they reached 100% confluence for ELISA, the cells were fixed in 2% PFA and blocked with 1% BSA. These were incubated with 1 µg of murine antibody at 37 °C for 30 min. The plates were then washed six times with PBS containing 0.05% Tween 20 (PBST) and then stained with anti-mouse horseshard peroxidase (HRP)-conjugated IgG (JM-6402-05, MBL). After an additional 1 h 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 h. 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 analysed on a flow cytometer (Ascru C6, BD Biosciences).

Antibody attachment to human platelet cells. Platelets were isolated from fresh human blood26. 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 h. 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). A commercial peroxidase substrate system (52-00-01 and 50-85-04, Kirkegaard & Perry Laboratories) was used 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 AG66 mice. AG66 mice were immunized three times with 40 µg of either full-length DENV2 NS1 or DENV2 ANS1 (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 1 × 10⁴ p.f.u. of the DENV2 43 strain via intraperitoneal injection. All the challenged mice were monitored daily for 40 days. Mouse sera were collected at different time points and stored at −80 °C for further evaluation.

Measurement of vascular leakage using Evans blue dye. Vascular leakage was determined following intravascular administration of Evans blue dye (E2129, Sigma). Briefly, 150 µl Evans blue dye (0.3% in PBS) was intravenously injected per mouse 18 days post-infection. After 2 h dissemination, the mice were anaesthetized and perfused with PBS. The following tissues were collected: 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 blocked using 1% BSA for 1 h at room temperature. After washing with PBST, anti-sera from the immunized AG66 mice were serially diluted from 1/250 to 1/100,000. Subsequently, the diluted antisera were added to the coated wells and incubated for an additional 2 h. After washing with PBST, anti-mouse HRP-conjugated IgG (JM-6402-05, MBL) was added to the wells for 1 h. 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 are provided in the figure legends. A Kruskal–Wallis analysis of variance was conducted to detect any significant variation among groups. For ELISA and qPCR data, a Student’s t-test was used 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 analysed using Fisher’s exact test. P values were adjusted using the Bonferroni correction to account for multiple comparisons. The survival rates of the infected mice were statistically analysed using the log-rank (Mantel–Cox) test. All analyses were performed using GraphPad Prism statistical software.

Accession codes. The sequencing data of RNA-Seq analysis were deposited in the Short Read Archive (NCBI) with accession number GSE73987.

Received 24 December 2015; accepted 5 May 2016; published 20 June 2016

References

1. Gould, E. A. & Solomon, T. Pathogenic flaviviruses. Lancet 371, 500–509 (2008).
2. Rigau-Perez, J. G. et al. Dengue and dengue haemorrhagic fever. Lancet 352, 971–977 (1998).
3. Bhatt, S. et al. The global distribution and burden of dengue. Nature 496, 504–507 (2013).
4. Campbell, G. L. et al. Estimated global incidence of Japanese encephalitis: a systematic review. Bull. World Health Organ. 89, 766–774 (2011).
5. Müller, D. A. & Young, P. R. The flavivirus NS1 protein: molecular and structural biology, immunology, role in pathogenesis and application as a diagnostic biomarker. Antiviral Res. 99, 192–208 (2013).
6. Akey, D. L. et al. Flavivirus NS1 structures reveal surfaces for associations with membranes and the immune system. Science 343, 881–884 (2014).
7. Young, P. R., Hilditch, P. A., Bletchaty, C. & Halloran, W. An antigen capture enzyme-linked immunosorbent assay reveals high levels of the dengue virus protein NS1 in the sera of infected patients. J. Clin. Microbiol. 38, 1053–1057 (2000).
8. Alcon, S. et al. Enzyme-linked immunosorbent assay specific to dengue virus type 1 nonstructural protein NS1 reveals circulation of the antigen in the blood during the acute phase of disease in patients experiencing primary or secondary infections. J. Clin. Microbiol. 40, 376–381 (2002).
9. Beaty, P. R. et al. Dengue virus NS1 triggers endothelial permeability and vascular leak that is prevented by NS1 vaccination. Sci. Transl. Med. 7, 304ra141 (2015).
10. Modhiran, N. et al. Dengue virus NS1 protein activates cells via Toll-like receptor 4 and disrupts endothelial cell monolayer integrity. Sci. Transl. Med. 7, 304ra142 (2015).
11. Avirutnan, P. et al. Antagonism of the complement component C4 by flavivirus nonstructural protein NS1. J. Exp. Med. 207, 793–806 (2010).
12. Schlesinger, J. J., Brandris, M. W. & Walsh, E. E. Protection of mice against dengue 2 virus encapsidation by immunization with the dengue 2 virus nonstructural glycoprotein NS1. J. Gen. Virol. 68, 853–857 (1987).
13. Lin, C. F. et al. Expression of cytokine, chemokine, and adhesion molecules during endothelial cell activation induced by antibodies against dengue virus nonstructural protein 1. J. Immunol. 174, 395–403 (2005).
14. Liu, I. J., Chiu, Y. C., Chen, Y. C. & Wu, H. C. Molecular mimicry of human endothelial cell antigen by autoantibodies to nonstructural protein 1 of dengue virus. Viral Immunol. 26, 972–9736 (2013).
15. Omokodo, M. D. et al. A highly conserved region between amino acids 221 and 266 of dengue virus nonstructural protein 1 is a major epitope region in infected patients. Am. J. Trop. Med. Hyg. 91, 146–155 (2014).
16. Cheng, H. J. et al. Anti-dengue virus nonstructural protein 1 antibodies recognize dengue capsid protein dimer and isomer on platelets and inhibit platelet aggregation. Mol. Immunol. 47, 398–406 (2009).
17. Liu, Y. et al. Transmission-blocking antibodies against mosquito C-type lectins for dengue prevention. PLoS Pathogens 10, e1003931 (2014).
18. Cheng, G., Liu, Y., Wang, P. & Xiao, X. Mosquito defense strategies against viral infection. Trends Parasitol. 32, 177–186 (2016).
19. Chung, K. M. & Diamond, M. S. Defining the levels of secreted non-structural protein NS1 after West Nile virus infection in cell culture and mice. J. Med. Virol. 80, 547–556 (2008).
20. Tan, G. K. et al. A non mouse-adapted dengue virus strain as a new model of severe dengue infection in AG129 mice. PLoS Negl. Trop. Dis. 4, e672 (2010).
21. Orozco, S. et al. Characterization of a model of lethal dengue virus infection in C57BL/6 mice deficient in the alpha/beta interferon receptor. J. Gen. Virol. 93, 2152–2157 (2012).
22. Halsead, S. B. Dengue. Lancet 370, 1644–1652 (2007).
23. Guo, X. et al. Vector competence of Aedes albopictus and Aedes aegypti (Diptera: Culicidae) for DEN2-43 and New Guinea C virus strains of dengue 2 virus. Acta Trop. 128, 566–570 (2013).
24. Sang, S. et al. Predicting unprecedented dengue outbreak using imported cases and climatic factors in Guangzhou, 2014. PLoS Negl. Trop. Dis. 9, e0003808 (2015).
25. Takamatu, Y. et al. NS1 protein expression facilitates production of Japanese encephalitis virus in avian cells and embryonated chicken eggs. J. Gen. Virol. 95, 373–383 (2014).
26. Xi, Z., Ramirez, J. L. & Dimopoulos, G. The Aedes aegypti toll pathway controls dengue virus infection. PLoS Pathogens 4, e1000098 (2008).