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*J Immunol* published online 10 July 2009
http://www.jimmunol.org/content/early/2009/07/10/jimmunol.0800672

**Supplementary Material**
http://www.jimmunol.org/content/suppl/2009/07/13/jimmunol.0800672.DC1

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Deletion of the C-Terminal Region of Dengue Virus Nonstructural Protein 1 (NS1) Abolishes Anti-NS1-Mediated Platelet Dysfunction and Bleeding Tendency

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The mechanisms underlying dengue hemorrhagic disease are incompletely understood. We previously showed that anti-dengue virus (DV) nonstructural protein 1 (NS1) Abs cross-react with human platelets and inhibit platelet aggregation. Based on sequence homology alignment, the cross-reactive epitopes reside in the C-terminal region of DV NS1. In this study, we compared the effects of Abs against full-length DV NS1 and NS1 lacking the C-terminal aa 271 to 352 (designated ΔC NS1). Anti-ΔC NS1 Abs exhibited lower platelet binding activity than that of anti-full-length NS1. Anti-full-length NS1 but not anti-ΔC NS1 Abs inhibited platelet aggregation, which was shown to involve integrin αIIbβ3 inactivation. We found that the bleeding time in full-length NS1-hyperimmunized mice was longer than that in the normal control mice. By contrast, ΔC NS1-hyperimmunized mice showed a bleeding time similar to that of normal control mice. Passively administered anti-DV NS1, but not anti-ΔC NS1, Ab level decreased markedly in serum and this decrease was correlated with Ab binding to platelets. A transient platelet loss in the circulation was observed after anti-DV NS1, but not anti-ΔC NS1, Ab administration. In summary, platelet dysfunction and bleeding tendency are induced by anti-full-length DV NS1 but not by anti-ΔC NS1 Abs. These findings may be important not only for understanding dengue hemorrhagic disease pathogenesis but also for dengue vaccine development. The Journal of Immunology, 2009, 183: 1797–1803.

Infection with dengue viruses (DV) causes disease ranging from mild dengue fever to severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) (1). The clinical features of DHF/DSS include plasma leakage, bleeding tendency, and thrombocytopenia (2). The pathogenesis of DHF/DSS is complicated and is the subject of active investigation. In addition to a direct virus-mediated effect, dendritic cells, monocytes/macrophages, mast cells/basophils, T cells, Abs, cytokines, and complement may all contribute to the progression of dengue hemorrhagic disease (3–11). Abs derived from a first DV infection may enhance the secondary infection of different serotypes, by a phenomenon called Ab-dependent enhancement (12–18). In addition, Abs generated against DV nonstructural protein 1 (NS1) recognize common epitopes on coagulation-related proteins, platelets, and endothelial cells (19). We have also demonstrated the presence of Abs in patient sera which cross-react with platelets and endothelial cells (20, 21). Further investigation showed that anti-DV NS1 Abs cause endothelial cell apoptosis and immune activation (21–25).

Bleeding tendency is a marker of hematological abnormality in DHF/DSS patients (2). Both vascular endothelial cells and platelets play important roles in this phenomenon, although the pathogenic mechanisms are not fully understood. Platelet autoantibodies that cause thrombocytopenia have been reported in some virus infections, including hepatitis C virus, cytomegalovirus, and HIV (26–28). In DV infection, anti-platelet autoantibodies induce complement-mediated cell lysis, which may, at least in part, account for the pathogenic mechanisms of thrombocytopenia. In addition, these Abs also inhibit platelet aggregation (20). During blood vessel injury, activated platelets adhere to the injury site followed by changing shape, releasing granule contents, and eventually aggregating together through fibrin formation (29). Therefore, in this study, we aimed to clarify which step of platelet aggregation was influenced by anti-DV NS1 Abs.

Using sequence homology alignment, we found that the C-terminal region of DV NS1 protein contains cross-reactive epitopes with self-Ags (30, 31). To explore the pathological role of cross-reactive epitopes in the hemorrhagic syndrome, we deleted the C-terminal region of DV NS1 protein to generate ΔC NS1. Both full-length NS1 and ΔC NS1 proteins were used to produce Abs, the pathogenic effects of which were compared both in vitro and in vivo.

Materials and Methods

Mice

C3H/HeN mice were obtained from The Jackson Laboratory and maintained on standard laboratory food and water in the Laboratory Animal Center of National Cheng Kung University Medical College. Their 8-wk-old progeny were used for the experiments. Housing, breeding,
and experimental use of the animals were performed in strict accordance with the Experimental Animal Committee in National Cheng Kung University.

Platelet preparation

Human whole blood containing the anticoagulant (29.9 mM sodium citrate, 113.8 mM glucose, 72.6 mM NaCl, and 2.9 mM citric acid (pH 6.4)) was centrifuged at 1000 × g for 20 min at room temperature to obtain platelet-rich plasma. The platelet-rich plasma was centrifuged at 10,000 × g for 10 min at room temperature and washed in EDTA/PBS buffer twice. The washed platelets were suspended in Tyrodes’s solution (137 mM NaCl, 20 mM HEPES, 3.3 mM NaH2PO4, 2.7 mM KCl, 1 mg/ml BSA, and 5.6 mM glucose (pH 7.4)) at a concentration of 10^8 platelets/ml.

Recombinant protein and Ab preparation

Japanese encephalitis virus (JEV) NS1, DV2 NS1 (New Guinea C strain) (22), and C terminus (aa 271–352)-deleted DV2 NS1 (ΔC NS1) cDNA were cloned into the pET28a vector with His6 Tag. Plasmids were introduced into Escherichia coli BL21. The recombinant proteins were induced by 1 mM isopropyl B-D-1-thiogalactopyranoside (Calbiochem) and purified with Ni2⁺ columns. After purification, proteins were examined using SDS-PAGE. Proteins from SDS-PAGE were excised and homogenized in adjacent to immunize mice. Purified protein (25 μg) was emulsified in CFA for the first immunization, and 2 wk later in IFA for additional 1, 2, or 4 immunizations every week. Mouse sera were collected 3 days after the last immunization, and IgG was purified using protein G columns (Pharmacia Fine Chemicals).

Ab binding to platelet assay

Washed platelets were fixed with 1% formaldehyde in PBS at room temperature for 10 min and then washed with PBS. Various doses of anti-full-length DV NS1, anti-ΔC NS1, or anti-JEV NS1 were incubated with platelets for 30 min. After washing, platelets were incubated with FITC-conjugated anti-mouse IgG (Jackson ImmunoResearch Laboratories) for 30 min. Ab binding to platelets was analyzed using flow cytometry (BD Biosciences).

Platelet aggregation assay

Platelet-rich plasma depleted whole blood was centrifuged at 1000 × g for 10 min at room temperature and the supernatant was collected as platelet-poor plasma. The platelet number in platelet-rich plasma was determined and diluted to 10^8 in 450 μl of platelet-poor plasma. Platelets were preincubated with 25 μg of anti-full-length DV NS1, anti-ΔC NS1, or anti-JEV NS1 Abs at 37°C for 30 min, followed by addition of 20 μM ADP (Sigma-Aldrich). Platelet aggregation was detected using an automated aggreometer PACKS-4 (Helena Laboratories).

Granule secretion and integrin activation assay

Aliquots of 10^7 platelets were pretreated with 25 μg of anti-DV NS1, anti-ΔC NS1, or anti-JEV NS1 Abs for 30 min, followed by addition of 20 μM ADP for 10 min. For platelet factor-4 (PF-4) detection, samples were placed on ice for 5 min to stop the reaction and centrifuged at 12,000 × g for 1 min. The supernatant was assayed for PF-4 level using ELISA kit (American Diagnostica). For P-selectin and active-form glucose (pH 7.4)) at a concentration of 10^8 platelets/ml.

Bleeding time

Bleeding time was performed by a 3-mm tail-tip transection (32, 33). Blood droplets were collected on filter paper every 30 s for the first 3 min, and every 10 s thereafter. Bleeding time was recorded when the blood spot was smaller than 0.1 mm in diameter.

Ab titer determination

DV NS1, ΔC NS1, or JEV NS1 protein was coated on 96-well plates at 0.2 μg/well in coating buffer (NaCO3, 1.59 g, NaHCO3, 2.93 g (pH 9.6), in 1 liter H2O) at 4°C overnight, blocked with 1% BSA in PBS at 4°C overnight, and then washed three times with PBS. Mouse sera were pooled and diluted serially from 1/1000 to 1/2048000. The diluted mouse sera (100 μl) were added into the protein-coated wells, and incubated at 4°C overnight. After washing three times with PBS, peroxidase-conjugated anti-mouse IgG was added into each well (Calbiochem) and incubated for 2 h at room temperature. After the wells were washed three times with PBS, 200 μl ABTS (Sigma-Aldrich) was added into each well and the absorbance was measured at 405 nm.

Results

Abs against DV NS1 lacking C terminus show lower binding activity to human platelets than that of anti-full-length NS1

We previously found that anti-DV NS1 Abs cross-reacted with platelets and endothelial cells (21, 22, 34), and the C-terminal region of DV NS1 protein contained cross-reactive epitopes (30, 31). We therefore deleted the C terminus of DV NS1 protein from aa 271 to 352 to generate C-terminus-truncated (ΔC) DV NS1. B. Target plasmids were digested with BamH1 and were identified using agarose gel electrophoresis. C. His6 Tag-containing fusion proteins, overexpressed in E. coli, were purified on Ni2⁺ columns and analyzed by SDS-PAGE.

Statistics

We used the paired t test for statistical analysis. Statistical significance was set at p < 0.05.

Anti-full-length NS1 but not anti-ΔC NS1 Abs inhibit ADP-induced platelet aggregation

We next examined the effect of anti-ΔC NS1 Abs on ADP-induced platelet aggregation. Results showed that only anti-full-length NS1 Abs inhibited platelet aggregation, while anti-ΔC NS1 Abs did not (Fig. 3). Anti-JEV NS1 Abs showed no effect on ADP-induced platelet aggregation as a negative control.

Anti-DV NS1 Abs inhibit ADP-induced platelet aggregation through integrin αIIbβ3 inactivation

The mechanism of anti-NS1 Ab-mediated platelet aggregation inhibition was investigated. We first checked whether anti-DV NS1
NS1 Abs inhibited ADP-induced activation of integrin/IIb/IIIa. We found that anti-full-length NS1 but not anti-plasma fibrinogen receptor, is essential for platelet aggregation. Moreover, following granule secretion, P-selectin undergoes re-expression (Fig. 4A). These results indicated that anti-NS1 Abs inhibited ADP-induced platelet aggregation via blocking integrin/IIb/IIIa activation.

Active immunization with full-length NS1, JEV NS1, or anti-JEV NS1 were generated in mice and purified on protein G columns. A, Platelets were incubated with various Abs for 30 min, followed by FITC-conjugated anti-mouse IgG staining and analyzed by flow cytometry. One representative histogram with 5 μg Ab treatment is shown. B, The percentages of platelet binding were quantified. Data are presented as the mean ± SD of triplicate cultures. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

FIGURE 2. Anti-ΔC NS1 Abs show lower binding to human platelets than that of anti-full-length DV NS1 Abs. Polyclonal Abs against full-length DV NS1, ΔC NS1, or JEV NS1 were generated in mice and purified on protein G columns. A, Platelets were incubated with various Abs for 30 min, followed by FITC-conjugated anti-mouse IgG staining and analyzed by flow cytometry. One representative histogram with 5 μg Ab treatment is shown. B, The percentages of platelet binding were quantified. Data are presented as the mean ± SD of triplicate cultures. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

FIGURE 3. Anti-DV NS1 but not anti-ΔC NS1 Abs inhibit ADP-induced platelet aggregation. Human platelet-rich plasma was preincubated with 25 μg of anti-full-length DV NS1, anti-ΔC NS1, anti-JEV NS1, or control IgG at 37°C for 30 min followed by stimulation with 20 μM ADP, and platelet aggregation was recorded for 6 min. A, Percentage light transmission was monitored using an aggregometer. B, Platelet aggregation was quantified and shown as the mean ± SD of triplicate cultures. The normal control, which was not pretreated with Abs, was normalized to 100% of platelet aggregation. **, p < 0.01.

platelet counts in DV NS1- , ΔC NS1- , and JEV NS1-immunized mice were all increased (Fig. 5B), ruling out the possible mechanism that the prolonged bleeding time in DV NS1-immunized mice might be due to reduced platelet counts. In this study, mice were actively immunized for a total of five times. We also immunized mice for two or three doses of DV NS1, and the results showed prolonged bleeding time in these mice, yet statistically nonsignificant, as compared with PBS-immunized controls (Fig. S2A). The platelet numbers were increased after mice were immunized with PBS in adjuvant for three times or with DV NS1 in adjuvant for two or three times (Fig. S2B).

We next investigated the effects of anti-full-length NS1 or anti-ΔC NS1 Abs after passive immunization in mice. Results showed a marked decrease of anti-full-length NS1 Ab titers in mouse sera by 24 h (Fig. 6A) while the titers of anti-ΔC NS1 (Fig. 6B) and anti-JEV NS1 (Fig. 6C) showed only a slight decrease or no change up to 48 h after Ab administration. The decrease of serum Ab titers was correlated with their platelet binding activity as evidenced by the presence of Abs on the platelets isolated from mice passively immunized with anti-full-length NS1 Abs (Fig. 6D). In addition to using Abs obtained from mice immunized five times with DV NS1 as shown in Fig. 6, we also tested Abs from mice immunized two or three times with DV NS1. Results showed that anti-DV NS1 Abs derived

Abs might interfere with the steps of ADP-induced platelet granule secretion. Platelet α-granules contain many proteins, such as PF-4, platelet-derived growth factor, β-thromboglobulin, fibrinogen, von Willebrand factor, and fibronectin (35–38). Moreover, following granule secretion, P-selectin undergoes redistribution from the platelet α-granule membrane to the plasma membrane (39, 40). We used P-selectin and PF-4 as granule secretion markers (41, 42). Results showed that neither anti-full-length NS1 nor anti-ΔC NS1 Abs inhibited P-selectin expression (Fig. 4A) and PF-4 secretion (Fig. 4B).

The activation of platelet membrane glycoprotein αmβ3, plasma fibrinogen receptor, is essential for platelet aggregation (43–45). We found that anti-full-length NS1 but not anti-ΔC NS1 Abs inhibited ADP-induced activation of integrin αmβ3 (Fig. 4C). These results indicated that anti-NS1 Abs inhibited ADP-induced platelet aggregation via blocking integrin αmβ3 activation.
from sera of mice immunized two or three times with NS1 also showed the decrease of serum Abs and the increase of platelet binding activity (Fig. S3). Furthermore, anti-full-length NS1 Abs, but not anti-ΔC NS1 or anti-JEV NS1, caused a transient platelet loss in the circulation at 6 h after administration with anti-full-length NS1 Abs (Fig. 6E).

**Discussion**

We previously showed that anti-DV NS1 Abs cross-reacted with human platelets and caused platelet dysfunction (20, 34). In the present study, we further showed that anti-NS1 inhibited ADP-induced platelet aggregation via blocking integrin \( \alpha_{IIb}\beta_3 \) activation. In addition, the major cross-reactive epitopes are located in the C-terminal region of NS1 protein. Compared with Abs against full-length NS1, Abs against NS1 lacking the C terminus showed lower platelet binding ability and did not inhibit ADP-induced platelet aggregation. These results correlated with the bleeding tendency in mice. We found prolonged bleeding times after mice were immunized with NS1 as compared with that of mice immunized with ΔC NS1 or JEV NS1.

Anti-dengue Abs play various crucial roles in the development of DV-caused disease, ranging from amplifying the number of DV-infected target cells at the beginning of infection to the later stages of immune-mediated cell or tissue damage. The generation and titer of the anti-dengue Abs as well as the status of primary infection or secondary infection are very important to explain their roles in the dengue pathogenesis. In this study, we address the role of anti-NS1 in platelet dysfunction and bleeding tendency. Because NS1 is a nonstructural protein, anti-NS1 Abs cannot enhance DV infection. However, anti-NS1 Abs can influence dengue immunopathogenesis due to the ability of anti-NS1 Abs to bind platelets via cross-reactive epitopes (34). This is also a problem with using NS1 as a candidate vaccine. In this study, we demonstrate that carboxy-truncated NS1 is largely depleted of platelet cross-reactivity, thereby providing a novel vaccine candidate with improved safety characteristics.

Thrombocytopenia is a common feature in patients after DV infection. One of the possible mechanisms of DV-induced

**FIGURE 4.** Anti-NS1 Abs inhibit ADP-induced integrin \( \alpha_{IIb}\beta_3 \) activation but not α-granule secretion in platelets. Human platelet-rich plasma was preincubated with 25 μg of anti-full-length NS1, anti-ΔC NS1, anti-JEV NS1, or control IgG at 37°C for 30 min followed by stimulation with 10 or 20 μM of ADP for 10 min. A, The platelets were stained with PE-conjugated anti-CD62 Ab and analyzed by flow cytometry. The percentages of P-selectin-expressing cells were quantified and shown as the mean ± SD of triplicate cultures. Bacitracin (BCT; 5 mM) was used as the control showing an inhibition of ADP-induced platelet activation. B, ADP-stimulated platelet supernatants were collected to determine the concentrations of PF-4 by ELISA. C, ADP-stimulated platelets were stained with FITC-conjugated PAC-1 Ab and analyzed by flow cytometry. The percentages of active-form integrin \( \alpha_{IIb}\beta_3 \)-expressing cells were quantified and shown as the mean ± SD of triplicate cultures. *, \( p < 0.05 \); **, \( p < 0.01 \).

**FIGURE 5.** Prolonged bleeding time in NS1-hyperimmunized mice. A, C3H/HeN mice were i.p. immunized with recombinant full-length NS1 (\( n = 10 \)), ΔC NS1 (\( n = 9 \)), or JEV NS1 (\( n = 9 \)) proteins or nonimmunized as the normal control (\( n = 10 \)), and the bleeding time was determined 3 days after the last injection as described in Materials and Methods. B, After the bleeding time experiment, mouse blood samples were collected and the platelet numbers were counted using an automatic blood-cell counter. **, \( p < 0.01 \); ***, \( p < 0.001 \).
thrombocytopenia is that DV impairs hematopoietic progenitor cell growth resulting in a decrease in thrombopoiesis (46). Also, anti-envelope protein Abs enhanced binding of DV to platelets, supporting a role for platelet clearance in the pathogenesis of thrombocytopenia (47). We previously demonstrated that cross-reactive Abs in dengue patient sera and mouse anti-NS1 Abs caused platelet lysis (20, 34), illustrating a further potential mechanism for platelet loss in dengue disease. A recent study demonstrated that anti-platelet autoantibodies elicited by DV NS1 caused thrombocytopenia and mortality in mice (48). We also showed that passive immunization with anti-full-length NS1 Abs caused transient platelet loss, a common feature of dengue disease. Most interestingly, anti-ΔC NS1 Abs did not cause platelet loss. At present, our data do not elucidate the mechanism of platelet loss. Whether it is due to sequestration from the circulation or platelet destruction or by other mechanisms remains for further investigation. We determined the NS1 titer, complement C5a, and LDH level in mouse sera after passive immunization with anti-NS1 Abs. At 6 h, anti-DV NS1 titer in mouse sera showed a decrease as compared with that at 1 h. In addition, both LDH and C5a levels were increased in the anti-NS1-treated group as compared with the control group at 6 h (our unpublished data). Furthermore, anti-NS1 Abs can also bind to endothelial cells and stimulate the expression of adhesion molecules (24). These adhesion molecules trap platelets on endothelial cell surface (49), which may provide another reason for the low platelet numbers in circulation at 6 h. By 24 h, platelet replenishment allows for platelets to reach normal levels.

Nevertheless, we found that DV NS1-, ΔC DV NS1-, and JEV NS1-immunized mice all had elevated platelet counts, suggesting that the differences in bleeding times were not due to different platelet counts. The mechanism of blood coagulation is complicated in that, in addition to platelet number, platelet function, blood vessel function, and coagulation factors all contribute to stop bleeding. The increased bleeding time in NS1-immunized mice is very likely, at least in part, due to the dysfunction of platelet aggregation, which was demonstrated in our in vitro studies. The increased platelet numbers observed in all the active-immunization groups may be explained by the phenomenon of adjuvant-induced inflammation, which involves induction of proinflammatory cytokines, leading to inflammatory thrombopoiesis (50, 51). The effect of adjuvant-induced thrombopoiesis was confirmed by immunizing mice with adjuvant plus PBS only to give a platelet number of 1264 ± 276 × 10³/μl (n = 14) as compared with normal control of 825 ± 83 × 10³/μl (n = 10).

Mice passively administered anti-NS1 Abs showed decreased Ab titers in the circulation as compared with those mice given anti-ΔC NS1 or anti-JEV NS1 Abs. Our finding that anti-NS1 (but not anti-ΔC NS1 or anti-JEV NS1) shows good binding to platelets in vivo may provide a possible explanation for the observed decreases in serum Ab titers. Other than binding to platelets, the decrease of anti-DV NS1 Abs in the circulation might also be caused by their binding to endothelial cells as we have previously demonstrated (21).

In the present study, NS1 and ΔC NS1 derived from DV2 were used. We previously reported that the levels of anti-endothelial cell Abs were similar in patients infected by different DV serotypes (52). These findings suggested that there is no serotype-specificity for anti-NS1 autoantibody production. We also showed that the cross-reactivity of DV3-infected patient sera to endothelial cells, which led to induction of endothelial cell apoptosis, could be inhibited by DV2 NS1 preabsorption (21). We recently showed that anti-DV2 NS1 Abs cross-reacted with liver vessel endothelium (53). Furthermore, IgG purified from DV3-infected patient sera caused liver injury in mice, and liver injury induced by these IgG was inhibited by preabsorption with DV2 NS1. Therefore, anti-NS1-mediated cross-reactivity shows no dengue serotype-specificity. The sequence alignment by ClustalW2 showed that the identity between DV2 and DV3 is 81.71%, and between DV2 and DV4 is 76.83%.

Using two-dimensional gel electrophoresis and Western blot analysis, we have previously identified anti-DV NS1 cross-reactive proteins from endothelial cell membrane extract (31). Among them, protein disulfide isomerase (PDI) can be expressed on the platelet surface and participate in platelet aggregation (54–56). Our unpublished results show that anti-DV NS1 can also cross-react with PDI on the platelet surface. PDI may regulate the activation of integrin αIIbβ3, the fibrinogen receptor, which is required for the late stage of platelet aggregation (55, 57). Therefore, the inhibitory effect of anti-DV NS1 on the activation of integrin αIIbβ3 may be through the recognition of platelet surface PDI to block its active sites and interfere with PDI function. This hypothesis is currently under investigation.

Numerous strategies of dengue vaccine design are based on the protective efficacy of Abs against viral E or NS1 protein
(58–61). Although the E protein is responsible for eliciting major neutralizing Abs during DV infection, it is also associated with the induction of infection-enhancing Abs. A limitation to the vaccine regimen containing NS1, however, is that anti-NS1 Abs may cause cross-reaction with platelets. The resultant consequences of platelet lysis and impaired platelet aggregation may lead to thrombocytopenia and bleeding tendency. The findings in this study suggest that C-terminal deletion of NS1 protein may provide a possible strategy for dengue vaccine development.

Acknowledgments
We thank the Proteomic Research Core facility, Academia Sinica, Taiwan, for preparing C terminus (aa 271–352) deleted DV2 NS1 (AC NS1) plasmid. We thank Dr. Shu-Ying Sherry Wang for helping with sequence alignment of NS1 proteins from different dengue serotypes. We also thank Dr. S. L. Hsieh (National Yang-Ming University, Taipei, Taiwan) and Dr. Y. L. Lin (Institute of Biomedical Science, Academia Sinica, Taipei, Taiwan) for providing JEV NS1 plasmid.

Disclosures
The authors have no financial conflict of interest.

References
Supplemental Figure legends

FIGURE S1. Platelet binding activity and anti-NS1 C-terminus titer of anti-NS1 Abs from mice immunized with 2, 3 or 5 doses of NS1. Polyclonal Abs were generated in mice after immunization with various doses of NS1 and IgG was purified on protein G columns. A, Platelets were incubated with Abs for 30 min, followed by FITC-conjugated anti-mouse IgG staining and analyzed by flow cytometry. The percentages of platelet binding were quantified. B, ELISA plate was coated with 0.5 mg/well of a mixture of NS1 C-terminal peptides (aa 271-280, 281-290, 291-309, 310-330 and 331-350) and various dilutions of Abs were added, followed by peroxidase-conjugated anti-mouse IgG. Data are presented as the mean ± SD of triplicate cultures. *p < 0.05, **p < 0.01.

FIGURE S2. The bleeding times and platelet numbers in mice immunized with 2 or 3 doses of NS1. C3H/HeN mice were i.p. immunized with PBS or DV NS1 in adjuvant for 2 or 3 times, and the bleeding times (A) and platelet numbers (B) were determined 3 days after the last injection.

FIGURE S3. Passively administered anti-NS1 Abs are diminished in mouse sera and this decrease is correlated to the binding of Abs to platelets. Mice were i.v.
administered with 500 μg of purified IgG obtained from mice after immunized with 2, 3 or 5 doses of DV NS1 (n = 3). A-C, After 1, 24, 48, and 72 h, blood samples were collected. The serum Ab titers were analyzed by ELISA. D, Freshly isolated mouse platelets after Ab administration for 24 h were washed and fixed with 1% formaldehyde in PBS, and then stained with FITC-conjugated anti-mouse IgG. Ab binding to platelets was detected and quantified by flow cytometry. **p < 0.01, ***p < 0.001.
Fig. S3