Anti-dengue virus nonstructural protein 1 antibodies contribute to platelet phagocytosis by macrophages

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Summary
Thrombocytopenia is an important clinical manifestation of dengue disease. The hypotheses concerning the pathogenesis of thrombocytopenia include decreased production and increased destruction or consumption of platelets. We previously suggested a mechanism of molecular mimicry in which antibodies (Abs) directed against dengue virus (DENV) nonstructural protein 1 (NS1) cross-react with platelets. Furthermore, several lines of evidence show activation of endothelial cells (ECs) and macrophages are related to dengue disease severity. Previous studies also suggested that Ab-opsonised platelets facilitate the engulfment of platelets by macrophages. Here we show that TNF-α-activated ECs upregulate adhesion molecule expression to enhance the binding of platelets and macrophages and lead to anti-DENV NS1 Ab-mediated platelet phagocytosis. We further demonstrate that the interaction between macrophages and TNF-α-activated ECs requires binding of FcγR with the Fc region of platelet-bound anti-DENV NS1 Abs. Importantly, the binding of anti-DENV NS1 Abs to platelets did not interfere with platelet adhesion to ECs. The adhesion molecules ICAM-1 and β3 integrin expressed on ECs as well as the FcγR expressed on macrophages were critical in anti-DENV NS1 Ab-mediated platelet phagocytosis on activated ECs. Moreover, anti-DENV NS1 Abs dramatically enhanced platelet engulfment by macrophages in a murine model of DENV infection. Our study provides evidence for a novel role for anti-DENV NS1 Abs in the pathogenesis of thrombocytopenia in dengue disease by enhancing platelet phagocytosis by macrophages.

Keywords
Dengue virus, nonstructural protein 1, platelet phagocytosis

Introduction
Dengue is an important arthropod-borne viral infection in many areas of the world. Nearly a third of the global population is at risk of exposure to dengue-carrier mosquitoes (1). Patients with dengue disease may have severe thrombocytopenia and/or plasma leakage, which can lead to death (2, 3). Despite considerable progress, the detailed mechanisms underlying the pathogenesis of thrombocytopenia in dengue infection remain to be completely defined.

Platelet-associated antibodies (Abs) likely contribute to enhanced destruction of platelets in dengue disease (3). Immune complexes containing DENV antigen are found on the surface of platelets which may contribute to their removal and destruction in the reticuloendothelial system, also called the mononuclear phagocyte system (4, 5). Further evidence indicates that platelet-associated Abs are associated with thrombocytopenia and disease severity (6). Moreover, a clinical trial with anti-D (Rh(D)-) Abs, which efficiently block Fcy receptor (FcγR)-mediated platelet phagocytosis, suggested the involvement of platelet-associated Abs in thrombocytopenia in dengue disease (7). Finally, sera elicited by DENV nonstructural protein 1 (NS1) either from patients or immunised mice cross-react with platelets (8–10), which may result in complement-mediated platelet lysis and accelerated clearance by phagocytes leading to the development of thrombocytopenia.

Other studies showed that DENV-infected endothelial cells (ECs) can lead to interaction between platelets and ECs. The adhesion of platelets to ECs is likely an alternative mechanism of thrombocytopenia in dengue disease (11, 12). Certain functions of ECs may be compromised by cytokines released from DENV-
infected cells. Tumour necrosis factor (TNF)-α released from DENV-infected monocytes is known to induce the expression of adhesion molecules including intercellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, and E-selectin (13). The initial interaction between platelets and ECs depends on P-selectin glycoprotein ligand 1 (PSGL-1)/P-selectin, glycoprotein (GP) Ib/von Willebrand factor (vWF), and GPIlb/\(\beta\)3. After initial interaction, the firm interaction between platelets and ECs requires GPLaβ1/collagen/\(\alpha\)β3, GPLlbβ3/fibronectin/\(\alpha\)β3, and GPLlbβ3/fibrinogen/ICAM-1 (14). Moreover, there is evidence that ECs bearing adhered platelets recruit phagocytes, promoting interaction between these three types of cells (15, 16). Taken together, the above studies suggest that DENV-infected or activated ECs might promote platelet adherence or clearance.

A number of case reports or studies suggested that haemophagocytic syndrome may play a role in dengue disease pathogenesis (17–19). Haemophagocytic syndrome mostly results from hyperproduction of cytokines such as interferon (IFN)-γ and TNF-α, and has been linked to viral infections (20, 21). Over-activated macrophages engulfing blood components including platelets have been proposed to cause transient haemophagocytic activity in dengue pathogenesis (22). In addition, platelets engulfed by monocytes have been detected in a DENV-infected nonhuman primate model (23). The current study aims to investigate the role of anti-DENV NS1 Ab-mediated platelet phagocytosis on an activated EC model (23).

The cDNAs of DENV NS1 and Japanese encephalitis virus (JEV) NS1 were cloned into the pRSETb vector containing a Histag. The preparations of recombinant proteins was induced with 1 M isopropyl β-D-1-thiogalactopyranoside (IPTG) (Calbiochem, San Diego, CA, USA) and proteins were solubilised in urea buffer (8 M urea, 0.5 M NaCl, 2 mM reduced glutathione, 0.2 mM oxidised glutathione, 1 mM EDTA, 0.1 mM PMSF) and concentrated by Amicon Ultra’ (Millipore, Billerica, MA, USA). Proteins were emulsified with complete Freund’s adjuvant (Sigma-Aldrich, St. Louis, MO, USA) and used to immunise mice (25 μg per mouse) followed by four further immunisations at the same dose in incomplete Freund’s adjuvant. The polyclonal Abs against DENV NS1 and JEV NS1 from the hyperimmunised sera were purified with protein G agarose columns (Millipore) and recovered with 0.1 M glycine-HCl (pH 2.7). The control IgG (clgG) was eluted from a protein G column loaded with normal mouse sera. The preparations were subjected to testing for endotoxin contamination using a Limulus amebocyte lysate assay (Pyrotell, Associates of Cape Cod, Falmouth, MA, USA); the endotoxin concentrations of anti-DENV NS1, anti-JEV NS1 and clgG were all <0.03 EU/ml.

Platelet preparation

Human whole blood (collected with ethical approval from the Institutional Review Board of National Cheng Kung University Hospital, No. A-ER-102–123, with written informed consent obtained from healthy volunteers) containing the anticoagulant ACD (29.9 mM sodium citrate, 113.8 mM glucose, 72.6 mM sodium chloride and 2.9 mM citric acid, pH 6.4) was centrifuged at 1000 rpm for 20 minutes (min) at room temperature to obtain platelet-rich plasma (PRP). PRP was centrifuged at 2000 rpm for 10 min in EDTA/PBS to obtain platelet pellets. The washed platelets were suspended in Tyrode’s solution (137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO\(_3\), 5 mM HEPES, 430 μM NaHPO\(_4\), 1 mM MgCl\(_2\)6H\(_2\)O, 2 mM CaCl\(_2\), 0.35 g/ml bovine serum albumin, pH 7.4) at a concentration of 10\(^8\) platelets/ml (24).

Cell and virus cultures

The human microvascular endothelial cell line-1 (HMEC-1) was obtained from the Centers for Disease Control and Prevention (Atlanta, GA, USA) (25), and passaged in culture flasks using endothelial cell growth medium M200 (Cascade Biologics, Portland, OR, USA) containing 2% fetal bovine serum (FBS), 1 μg/ml hydrocortisone, 10 ng/ml epidermal growth factor, 3 ng/ml basic fibroblast growth factor, 10 μg/ml heparin, and antibiotics. The human monocytic THP-1 cell line was cultured in RPMI-1640 (In-vitrogen, Carlsbad, CA, USA) containing 10% FBS. All cells were incubated at 37°C in a 5% CO\(_2\) atmosphere.

DENV serotype 2 (strain 16681) was propagated in C6/36 cells. Briefly, C6/36 monolayers were incubated with DENV at a multiplicity of infection (MOI) of 0.01 at 28°C in 5% CO\(_2\) for five days. The culture medium was harvested, and cell debris was removed by centrifugation at 900 × g for 10 min. After further centrifugation at 16,000 × g for 10 min, the virus supernatant was collected and stored at −70°C until use. Virus titre was determined by plaque assay using BHK-21 cells as described previously (26).

Platelet adhesion assay

To assay platelet adhesion to HMEC-1 cells, 1.5 × 10\(^5\)/well HMEC-1 cells were seeded on microscope slides in 12-well plates.
The confluent cells were treated with or without 50 ng/ml of TNF-α (Peprotech) for 24 hours (h). After washing with PBS, HMEC-1 cells were cultured with 1 × 10^7 platelets per well, which were labelled with 20 µg/ml calcine-AM (Millipore) at 37°C for 50 min, in Tyrode’s buffer for 1 h. The cells were washed with PBS/2% FBS four times, and then fixed with 1% formaldehyde for 15 min. The number of adherent platelets (green) per HMEC-1 cell (blue in nucleus and the morphology in the bright field) was determined. At least 100–200 HMEC-1 cells were examined in each sample.

Ab binding to platelet assay

Purified platelets were incubated with various Abs (clgG, anti-JEV NS1 and anti-DENV NS1 Abs) at room temperature for 1 h on a shaker. After washing with PBS/EDTA, the platelets were labelled with Alexa-488-conjugated goat anti-mouse IgG (Invitrogen). The samples were fixed with 1% formaldehyde at room temperature for 15 min and analysed by flow cytometry (FACS Calibur; BD Biosciences, San Jose, CA, USA) with excitation set at 488 nm. The percentage of positive cells was determined by comparison with clgG.

Platelet phagocytic assay

For platelet phagocytic assay, HMEC-1 cells were activated with TNF-α for 24 h and cultured with calcine-AM labelled platelets in Tyrode’s buffer at 37°C for 1 h, which were pre-opsonised with Abs (anti-DENV NS1 Abs or clgG) at room temperature for 1 h. The plates were centrifuged at 1500 rpm for 5 min. After washing twice with HBSS/2% FBS, cells were cultured with IFN-γ (50 ng/ml for 48 h) (Peprotech) and PMA (100 nM for 30 min) (Sigma-Aldrich)-activated THP-1 cells for 4 h in HBSS (Invitrogen). The plates were centrifuged at 1500 rpm for 5 min, and cells were washed twice with HBSS/2% FBS, followed by fixation with 1% formaldehyde. The number of adhered THP-1 cells (red) per HMEC-1 cell (blue in nucleus and the morphology in the bright field) and the number of engulfed platelets (green) per THP-1 cell were analysed by confocal microscopy (Olympus BX-51, Richland, NC, USA) and quantified manually using ImageJ software (version 1.41o; W. Rasband, National Institutes of Health, Bethesda, MD, USA).

DENV-infection mouse model

Mice were intradermally inoculated with DENV (2 × 10^8 pfu/mouse) at four sites on the upper back as previously described (27). On day 1 post-infection, mice (n = 4) were further injected intravenously (i.v.) with Abs (clgG or anti-DENV NS1 Abs, 500 µg/mouse) and were sacrificed at day 4 after inoculation.

The liver sections were embedded in paraffin and sliced on slides. After deparaffining with xylene followed by washing in graded alcohol (100%, 95%, 85%, 70% and 50%), the sections were incubated in 2N HCl solution for 20 min and then with 20 µg/ml proteinase K in TE buffer (50 mM Tris Base, 1 mM EDTA, 0.5% Triton X-100, pH 8.0) for another 20 min at room temperature. The sections were then incubated with NH4Cl in PBS to quench reactive aldehyde groups and blocked with 10% bovine serum albumin in PBS for 30 min at room temperature. The primary and secondary Abs were adequately diluted in Ab diluents (Dako Corporation, Carpinteria, CA, USA). The infiltrating macrophages were stained with anti-mouse F4/80 Abs (Serotec, Raleigh, NC, USA) and the platelets were stained with anti-vWF Abs (Dako Corporation) overnight at 4°C, followed by Alexa-568-conjugated donkey anti-rabbit IgG or Alexa-647-conjugated donkey anti-rat IgG (Invitrogen) at room temperature for 2 h. After washing with PBS, the nuclei of cells were stained with DAPI (Calbiochem). Fluorescence images were detected using an Olympus BX-51 microscope and quantified manually using ImageJ software (version 1.41o).

Statistical analysis

Data are expressed as the mean ± SD of triplicate cultures. Multiple intergroup comparisons were assessed by one- or two-way ANOVA when appropriate, followed by post-hoc tests (mostly Tukey’s test and Dunnett’s test) with GraphPad Prism version 6.0. Two group comparisons were performed using Student t-test with GraphPad Prism version 6.0. Statistical significance was set at p < 0.05.

Results

FcyR is required for anti-DENV NS1 Ab-mediated macrophage adhesion to activated ECs

Previous studies indicated that TNF-α-activated ECs trigger the expression of adhesion molecules to increase platelet binding and promote phagocyte recruitment (13–16, 28). Moreover, phagocytosis of immune complexes relies on the interaction between immune complexes and FcγRs (29). Previous studies demonstrated that anti-DENV NS1 Abs cross-react with platelets (8–10), which prompts speculation that anti-DENV NS1 Ab-bound platelets may be phagocytosed by macrophages. In order to establish a model in vitro to analyse the role of anti-DENV NS1 Abs and activated ECs in haemophagocytosis, we co-cultured anti-DENV NS1-opsinised platelets adhering on TNF-α-activated HMEC-1 cells with activated THP-1 cells to explore the role of anti-DENV NS1 Abs in platelet phagocytosis by macrophages.

First, we found an increase in the number of adherent THP-1 cells, which were pre-activated with IFN-γ, after opsonising platelets with anti-DENV NS1 Abs as compared with clgG (Figure 1A). Based on the quantification of the number of adherent THP-1 cells per HMEC-1, platelets opsonised with 10 µg anti-DENV NS1 Abs significantly increased the THP-1 cell binding to TNF-α-activated HMEC-1 cells (Figure 1B). Moreover, platelet-anti-DENV NS1 Ab complexes as compared with anti-DENV NS1 Ab alone caused more macrophage adherence to TNF-α-activated HMEC-1 cells (Suppl. Figure 1, available online at www.thrombosis-online.com), indicating that platelet-anti-DENV NS1 Ab complexes...
Wan, Yang, et al. Anti-DENV NS1 Ab-mediated platelet phagocytosis. We next determined the surface molecule expression and activation of THP-1 cells after IFN-\(\gamma\) pretreatment, which is well known to induce the expression of certain Fc\(\gamma\)Rs on monocytes (30). Results showed that the activation markers CD40 and HLA-DR on THP-1 cells were elevated after stimulation with 50 ng/ml IFN-\(\gamma\) for 48 h. We also confirmed that the expression of Fc\(\gamma\)RII (CD32) and Fc\(\gamma\)RI (CD64) were higher with IFN-\(\gamma\) treatment (Suppl. Figure 2, available online at www.thrombosis-online.com). Using blocking Abs, we demonstrated that the adherence of THP-1 cells to TNF-\(\alpha\)-activated HMEC-1 cells was mediated through the interaction between Fc\(\gamma\)RI/II and anti-DENV NS1 Abs (Figure 1 C and D). Therefore, the observed increased interaction between THP-1 and HMEC-1 cells requires binding of Fc\(\gamma\)R with the Fc region of anti-DENV NS1 Abs bound on platelets.

**Adhesion molecule expression on activated ECs mediates the adherence of anti-DENV NS1 Ab-bound platelets to activated ECs**

One of the consequences of adhesion molecule expression on the EC surface is an increased binding of platelets to ECs (14). Furthermore, TNF-\(\alpha\) and EC activation are associated with DENV infection and severity of dengue disease (13, 27, 31). In the present study, we first demonstrated that TNF-\(\alpha\) treatment of HMEC-1 cells enhances expression of a larger panel of adhesion molecules than that previously examined, i.e. ICAM-1, VCAM-1, platelet endothelial cell adhesion molecule-1 (PECAM-1/CD31) and \(\beta_3\) integrin (Suppl. Figure 3, available online at www.thrombosis-online.com). The level of ICAM-1 was most significantly increased, followed by \(\beta_3\) integrin. We then detected the functional change after activating the HMEC-1 cells with TNF-\(\alpha\). Results showed that the number of adherent platelets per HMEC-1 cell was significantly elevated after TNF-\(\alpha\) treatment (Figure 2 A). Furthermore, ICAM-1 and \(\beta_3\) integrin play important roles in platelet adhesion to TNF-\(\alpha\)-activated HMEC-1 cells. Results showed that the increased level of platelet adhesion to TNF-\(\alpha\)-activated HMEC-1 cells was inhibited by treatment with anti-ICAM-1 and anti-\(\beta_3\) integrin blocking Abs, but not with anti-\(\beta_1\) integrin blocking Abs (Suppl. Figure 4A, available online at www.thrombosis-online.com). Moreover, transfecting HMEC-1 cells with ICAM-1 and \(\beta_3\) integrin siRNA also yielded similar results as using anti-ICAM-1 and anti-\(\beta_3\) integrin blocking Abs (Suppl. Figure 4B, available online at www.thrombosis-online.com).

**Figure 1:** Platelet-anti-DENV NS1 Ab complexes increase macrophage adherence to TNF-\(\alpha\)-activated endothelial cells through Fc\(\gamma\)R. A) Platelet and Ab complexes were incubated with 50 ng/ml TNF-\(\alpha\)-treated HMEC-1 cells for 1 h. After washing, platelets, Abs and HMEC-1 cells were co-cultured with Alexa-594-conjugated cholera toxin B-labelled THP-1 cells (red) which were pretreated with 50 ng/ml IFN-\(\gamma\) and PMA for 4 h. Cells were washed twice, fixed with 1% formaldehyde, and examined by confocal microscopy. B) The quantification of adherent THP-1 cells per HMEC-1 cell is shown. C) Platelet and Ab complexes were incubated with 50 ng/ml TNF-\(\alpha\)-treated HMEC-1 cells for 1 h. After washing, platelets, Abs and HMEC-1 cells were co-cultured with cholera toxin B-labelled IFN-\(\gamma\) and PMA-activated THP-1 cells (red) which were pretreated with blocking Abs, including anti-Fc\(\gamma\)RII (CD32), anti-Fc\(\gamma\)RI (CD64) and isotype control Abs, for 4 h. Cells were washed twice, fixed with 1% formaldehyde, and examined by confocal microscopy. D) The quantification of adherent THP-1 cells per HMEC-1 cell is shown. Statistical significance was based on repeated measures one-way ANOVA followed by Tukey’s multiple comparison test. *P < 0.05, **P < 0.01.
We previously showed that anti-DENV NS1 Abs cross-reacted with human platelets and inhibited ADP-induced platelet aggregation (9). However, anti-DENV NS1 Abs could not inhibit the expression of P-selectin which might influence the platelets adhesion (24). To investigate whether the anti-DENV NS1 Abs cause an effect on platelet adhesion to TNF-α-activated HMEC-1 cells, we first confirmed the binding of anti-DENV NS1 Abs to platelets (Figure 2B). We then pre-incubated Abs with platelets and detected the adherent number of platelets per HMEC-1 cell. Anti-DENV NS1 Abs may slightly but not significantly reduce platelet binding to TNF-α-activated HMEC-1 cells as compared with cIgG. However, there was still a significant increase in the adherent platelets, after opsonisation with anti-DENV NS1 Abs, on TNF-α-treated HMEC-1 cells (Figure 2C).

Anti-DENV NS1 Abs increase platelet phagocytosis by macrophages on the activated ECs

In this co-culture experiment, we prelabelled platelets with calcein-AM (green) and THP-1 cells with Alexa-594-conjugated CT-B (red). The labelled Ab-opsonised adherent platelets (green) were then co-cultured with THP-1 (red) for phagocytic assay. From the confocal 3D images, we identified the yellow spots as engulfed platelets and green spots as adherent platelets (Figure 3A). Under confocal microscopic observation, we found that anti-DENV NS1 Abs increased the number of engulfed platelets (Figure 3B). Furthermore, we counted the number of engulfed platelets per THP-1 and found there was significantly higher platelet engulfment in the anti-DENV NS1-treated group as compared with the clgG group (Figure 3C). The average number of engulfed platelets per THP-1 cell was also increased after opsonisation with anti-DENV NS1 Abs on TNF-α-activated ECs (Figure 3D). Similar to clgG, anti-JEV NS1 Abs did not increase the percentage of platelet-engulfed THP-1 cells (Suppl. Figure 5A, available online at www.thrombosis-online.com) or the average number of engulfed platelets per THP-1 cell (Suppl. Figure 5B, available online at www.thrombosis-online.com). In addition, anti-DENV NS1 but not anti-JEV NS1 Abs increased platelet adhesion to THP-1 cells (Suppl. Figure 5C and D, available online at www.thrombosis-online.com). Furthermore, anti-DENV NS1-induced platelet engulfment was lower in the absence of HMEC-1 cells (see Suppl. Figure 5A and B), suggesting a possible role of ECs as a platform to help platelet binding and engulfment by macrophages.

To further confirm the role of adhesion molecules in anti-DENV NS1-mediated platelet phagocytosis, we analysed the percentages of different engulfed platelet numbers and the average number of engulfed platelets per THP-1 cell on TNF-α-activated HMEC-1 cells which were pretreated with blocking Abs or siRNA. The higher platelet engulfment and the increased average number of engulfed platelets per THP-1 cell were significantly inhibited after treatment with anti-β3 integrin and anti-ICAM-1 Abs (Figure 4A and B) as well as β3 integrin siRNA (Figure 4D and E). The knockdown efficiency was confirmed by determining the expression of adhesion molecules (Figure 4C). Although not statistically significant, there was also a trend of inhibition by...
ICAM-1 siRNA. The results indicate that β3 integrin and ICAM-1 are involved in anti-DENV NS1-mediated platelet phagocytosis. Moreover, the blockade of β3 integrin by blocking Abs inhibited the platelet engulfment both in the absence or presence of unbound platelets (Suppl. Figure 6, available online at www.thrombosis-online.com). These results indicated that the reduced platelet engulfment after blocking of adhesion molecules was due to the decreased numbers of platelet adhesion rather than availability of platelets to be engulfed.

Blocking with anti-FcγR Abs on THP-1 cells showed that the anti-DENV NS1 Ab-mediated increase in the number of engulfed platelets per THP-1 cell was inhibited, especially by anti-FcγRI Abs (Figure 5A). The higher platelet engulfment per THP-1 cell (Figure 5B) and the average number of engulfed platelets per THP-1 cell (Figure 5C) were significantly reduced by anti-FcγRI Ab treatment. The results indicate that anti-DENV NS1 Abs interact with FcγRI on THP-1 cells and trigger phagocytosis of platelets.

From the in vitro studies, we found that ICAM-1 and β3 integrin are important in platelet adherence to TNF-α-activated ECs. Furthermore, platelets opsonised with anti-DENV NS1 Abs can adhere to TNF-α-activated ECs. Importantly, the ICAM-1 and β3 integrin expressed on ECs as well as FcγRI expressed on macrophages play crucial roles in anti-DENV NS1 Ab-mediated platelet phagocytosis on activated ECs (Figure 5D).

**Anti-DENV NS1 Abs increase platelet phagocytosis by macrophages in vivo**

To further extrapolate our in vitro findings to an in vivo setting, we used a DENV-induced haemorrhagic mouse model in which both virus and TNF-α were considered important for haemorrhagic manifestations (27). Furthermore, we previously demonstrated that i.v. injection with anti-DENV NS1 Abs reduced the platelet number within hours which is related to the binding of anti-DENV NS1 Abs to platelets (24). We therefore verified the role of anti-DENV NS1 Abs in platelet phagocytosis in vivo by i.v. injection with anti-DENV NS1 Abs after infection with DENV. We observed platelet phagocytosis in the mouse liver tissues by immunofluorescence assay. The sections were then co-immunostained using anti-F4/80 (macrophage marker) and anti-vWF (platelet marker) Abs. Under confocal microscopic observation, we found that anti-DENV NS1 Abs could increase the number of engulfed platelets especially in DENV-infected mice (Figure 6A). Furthermore, we counted the number of engulfed platelets per F4/80+ macrophage and found that the percentage of platelet-co-localised macrophages was significantly increased in the anti-DENV NS1-treated group as compared with the clgG group after DENV infection (Figure 6B). The average number of engulfed platelets per F4/80+ macrophage was also increased after treatment with anti-DENV NS1 Abs in DENV-infected mice (Figure 6C). Anti-JEV NS1 Abs, as compared with anti-DENV NS1 Abs, did not
To correlate enhanced platelet phagocytosis with thrombocytopenia in dengue disease, we determined the platelet numbers at different time points after Ab administration. Anti-DENV NS1 Abs caused a transient platelet loss in the circulation at 6 h after administration with Abs in the mock mice (Suppl. Figure 8A, available online at www.thrombosis-online.com). After DENV infection, anti-DENV NS1 Abs not only caused sustained platelet loss at least up to 12 h after Ab administration (see Suppl. Figure 8A), but also induced a higher percentage of platelet reduction than the cIgG group (Suppl. Figure 8B, available online at www.thrombosis-online.com). These results indicate that anti-DENV NS1 Abs can dramatically enhance the co-localisation of platelets and macrophages as well as cause platelet loss after DENV infection.
Figure 5: FcγR expressed on macrophages mediates platelet phagocytosis on TNF-α-activated endothelial cells. A) Platelet and Ab complexes were incubated with 50 ng/ml TNF-α-treated HMEC-1 cells for 1 h. After washing, platelets, Abs and HMEC-1 cells were co-cultured with cholera toxin B-labelled IFN-γ and PMA-activated THP-1 cells (red), pretreated with blocking Abs (i.e. anti-FcγRII, anti-FcγRI, and isotype control Abs), for 4 h. Cells were washed twice, fixed with 1% formaldehyde, and examined by confocal microscopy. B) The percentages of different numbers of engulfed platelets (yellow) per THP-1 cell were determined by counting more than 100 THP-1 cells per sample. ***P < 0.001 vs isotype control Ab based on two-way ANOVA followed by Dunnett’s multiple comparison test. C) The quantification of engulfed platelets (yellow) per THP-1 cell is shown. *P < 0.05 vs isotype control Abs based on one-way ANOVA followed by Dunnett’s multiple comparison test. D) A schematic model of in vitro study. Opsonisation of platelets with anti-DENV NS1 Abs mediates platelet phagocytosis by macrophages on a TNF-α-activated endothelial cell layer. The ICAM-1 and β3 integrin expressed on endothelial cells as well as FcγR expressed on macrophages play important roles in anti-DENV NS1 Ab-mediated platelet phagocytosis on activated endothelial cells.

Discussion

In this study we have modeled various cellular, viral and Ab components to elucidate the roles of anti-DENV NS1 Abs, macrophages and activated ECs in dengue-associated thrombocytopenia involving a mechanism of platelet phagocytosis. The results indicate that both ICAM-1 and β3 integrin are important in platelet adhesion as well as anti-DENV NS1 Ab-mediated platelet phagocytosis on TNF-α-activated ECs. On the other hand, anti-FcγRI significantly and anti-FcγRII slightly inhibit anti-DENV NS1 Ab-mediated platelet phagocytosis. The superiority of FcγRI over FcγRII in facilitating anti-DENV NS1 Ab-mediated platelet phagocytosis may reflect functional differences as well as different affinities for Abs between FcγRI (higher affinity) and FcγRII (lower affinity) receptors (32). We also found higher expression levels of FcγRI than FcγRII on THP-1 cells after IFN-γ treatment. In contrast to FcγRI and FcγRII, the level of FcγRIII was very low even after IFN-γ treatment in THP-1 cells (data not shown), which is consistent with the findings of others (33). Therefore, we did not determine the role of FcγRIII either on THP-1 cell adhesion to ECs or in the platelet phagocytosis assay. It is conceivable that FcγRIII may play a role in one or more of these processes under conditions in which FcγRIII is upregulated, for example by treatment of THP-1 cells with TGF-β plus retinoic acid (34).

The results of the present study indicate a complex interplay between ECs, platelets, Abs and monocytes in order to achieve efficient platelet phagocytosis which may contribute to the thrombocytopenia often associated with dengue disease. Our in vitro results showed that platelets firmly adhered to activated ECs, followed by platelet phagocytosis by macrophages. We first focused...
on the adhesion molecules on ECs, however, many other molecules are involved in platelet adhesion and aggregation on activated ECs (35). In addition to adhesion molecules, collagen, fibronectin, fibrinogen and vWF are important bridging factors between adhesion molecules on ECs and platelets (14). Tissue factor, vWF and coagulation factors have been reported at higher levels in dengue haemorrhagic fever than in dengue fever patients (36, 37). We only analysed the expression of adhesion molecules on ECs but not platelets and monocytes. The interactions between these three cells are complicated and remain to be investigated in this co-culture model.

In addition to TNF-α used in this study, IL-1 is another prototypic activator for ECs. Although these two cytokines use different signalling molecules, they trigger a similar effect on ECs, which include upregulation of ICAM-1, VCAM-1 and E-selectin. IL-1 has potential to change the morphology of ECs and to induce plasma leakage (38). However, little information is available on the correlation between IL-1 and disease severity. Another cytokine, IFN-γ, is able to trigger the expression of ICAM-1 and induce leukocyte adhesion to ECs (39). However, IFN-γ is much weaker than TNF-α in stimulating the surface expression of EC ICAM-1. Although we used the most potent mediator, i.e. TNF-α, to activate the ECs, possible contributions and cross-talk from these and other mediators cannot be excluded in the authentic disease setting.

We utilised a dengue haemorrhagic mouse model in this study to validate our *in vitro* findings *in vivo*. In this model, infected mice develop haemorrhage and show prolonged bleeding time due to DENV and TNF-α (27). Langerhans cells (dermal dendritic cells) are generally proposed to be the initial target for DENV...
infection at the site of the mosquito bite (40). However, subsequent cell targets involve elements of the reticuloendothelial system (in liver, bone marrow and spleen) including monocytes/macrophages, lymphocytes and Kupffer cells (41). Moreover, DENV-infected immune cells secrete abundant cytokines, chemokines and other soluble factors which may affect the functions and integrity of ECs (42). Here, we used i.v. injection with anti-DENV NS1 Abs after infection with DENV to produce enhanced platelet phagocytosis in the mouse liver tissues. A “two-hit” mouse model has recently been proposed in which DENV (first hit) and anti-platelet Ig (second hit) were injected at the same skin location in the mouse. DENV and anti-platelet Ig (anti-CDA1 and anti-NS1 Abs) synergistically induced haemorrhage, platelet loss, and cytokine surge through the Nlrp3-inflammasome and FcγRIII pathways (43). Although our in vitro results showed that FcγRI expressed on macrophages play a more important role in anti-DENV NS1 Ab-mediated platelet phagocytosis, we did not determine the role of individual FcγRs on anti-DENV NS1 Ab-mediated platelet phagocytosis in vivo. In addition, besides TNF-α and IFN-γ, other factors which may be involved in vivo require further investigation.

We have demonstrated a mechanism for anti-DENV NS1 Abs in platelet phagocytosis by macrophages associated with thrombocytopenia. A clinical study showed the involvement of platelet-associated Abs in thrombocytopenia in dengue disease (7). However, another study showed that treatment with high-dose i.v. immunoglobulin had no effect on thrombocytopenia in dengue patients (44). It should be noted that thrombocytopenia in dengue is likely multifactorial, involving more than one type of Abs. For example, dengue antigens and Abs are present on the surface of platelets from dengue patients with haemorrhagic fever (45). Anti-DENV envelope (E) protein Abs mediate the binding between platelets and DENV, which suggests a role for immune-mediated clearance of platelets in thrombocytopenia (6). These lines of evidence suggest that in severe dengue infection, not only anti-DENV NS1 Abs but also anti-DENV E protein Abs may have a role in thrombocytopenia.

In conclusion, the ICAM-1 and β3 integrin expressed on ECs as well as FcγR expressed on macrophages play crucial roles in anti-DENV NS1 Ab-mediated platelet phagocytosis on activated ECs. Our findings address not only the phagocytic mechanisms associated with dengue pathogenesis but also the possible impact of immunopathogenic complications induced by anti-DENV NS1 Abs on the safety of dengue vaccination and therapy.

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Author contributions
Conceived and designed the experiments: SWW, YWY, CFL, CPC, TMY, RA and YSL. Performed the experiments: SWW, YWY and YTC. Analysed the data: SWW, YWY, YTC, CFL and YSL. Wrote the paper: SWW, YWY, RA and YSL.

What is known about this topic?
- Dengue virus (DENV) infections may result in thrombocytopenia and plasma leakage, which may lead to severe disease or even death. The mechanisms underlying thrombocytopenia in dengue infection are incompletely understood.
- Platelet-associated antibodies (Abs) may contribute to enhanced destruction of platelets and result in thrombocytopenia. DENV-infected or cytokine-activated endothelial cells (ECs) might promote platelet adherence or clearance. In addition, activated macrophages might remove platelets by a haemophagocytic mechanism.
- Abs against DENV nonstructural protein 1 (NS1) either from patients or immunised mice cross-react with platelets. However, the role of anti-DENV NS1 Ab-mediated platelet phagocytosis on activated ECs in the pathogenesis of thrombocytopenia is not clear.

What does this paper add?
- We demonstrate platelet phagocytosis by macrophages in a reconstituted model system comprising monocytic THP-1 cells, cytokine-activated ECs and anti-DENV NS1 Ab-treated platelets. We show further that this process requires ICAM-1 and β3 integrin expression on ECs as well as the FcγR expressed on macrophages.
- We extrapolate the above in vitro results to an in vivo situation by showing that anti-DENV NS1 Abs dramatically enhances platelet engulfment by macrophages in a DENV-infected murine model.
- This study validates a novel mechanism for dengue-associated thrombocytopenia and further illustrates the multifaceted roles of anti-DENV NS1 Abs in dengue pathogenesis.

Conflicts of interest
None declared.

References


