รายงานวิจัยฉบับสมบูรณ์

โครงการ การกระตุ้นและการสร้าง **NETs** ในเซลล์เม็ดเลือดขาว
ของผู้ป่วยที่ติดเชื้อไวรัสไข้เลือดออก

โดย ผศ.ดร.เฟเบี่ยน ลัวซอง และคณะ

มิถุนายน 2562

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Abstract (บทคัดย่อ)

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Project Title : Neutrophil activation and NETosis in patients infected with dengue virus

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(ระยะเวลาโครงการ)

Abstract: The involvement of the immune system in the protection and pathology of dengue virus (DENV) infection has been extensively explored. Several studies, mostly whole blood transcriptomic, have hinted at the activation of neutrophils during DENV infections. However, the exact contribution of neutrophils during infection remains elusive. In this work, we explored the phenotype of neutrophils as well as their function ex vivo using cells isolated from a cohort of Thai adult dengue patients. We have shown that during acute DENV infection, neutrophils were characterized with the upregulation of CD66b expression on the plasma membrane. These cells also released higher levels of reactive oxygen species compared to convalescent or healthy individuals. These observations confirmed neutrophil activation during DENV infection. Interestingly, we also observed that the CD66^{high} phenotype was positively correlated with the spontaneous decondensation of nuclei from DENV-infected patients compared to convalescent or healthy patients. The nuclei decondensation is an early event of the formation and release of neutrophil extracellular traps (NETs), a mesh of DNA release from PMN in order to trap and inactivate or kill microorganisms. Consequently, we tested the ability of NETs to inactivate DENV-2 and observed a marked and fast decrease of infectivity of the virus after incubation with NETs. Using ELISA, we also tested for the presence of marker of NETs in the serum of healthy volunteers, acute and convalescent dengue patients. Larger amounts of NET components were detected in the serum of for dengue hemorrhagic fever (DHF) compared to uncomplicated dengue fever. We also measured increased levels of the pro-inflammatory cytokines IL-8 and TNFα in DHF patients compared to healthy and uncomplicated Dengue Fever subjects. This suggested that NETsmay play dual roles during DENV infection. The increased ability for NET formation during acute DENV infection appeared to be independent of PAD4-mediated histone H3 hyper-citrullination. Our study suggests that neutrophils are involved in immunological responses to DENV infection.

บทคัดย่อ: บทบาทของระบบภูมิคุ้มกันในการป้องกันการติดเชื้อและพยาธิวิทยาของเชื้อไวรัสเด็งกี่ได้รับการศึกษาอย่างแพร่หลาย จากการศึกษาด้านทรานสคริปโตมบ่งชี้ว่าการติดเชื้อไวรัสเด็งกี่สามารถกระตุ้นการทำงานของเซลล์เม็ดเลือดขาวชนิดนิวโทรฟิลได้ อย่างไรก็ตามความรู้เกี่ยวกับบทบาทที่แท้จริงของนิวโทรฟิลต่อการติดเชื้อยังคงไม่ชัดเจน ในการศึกษานี้ได้ศึกษาคุณลักษณะและหน้าที่ของนิวโทรฟิล โดยนิวโทรฟิลถูกสกัดแยกจากเลือดผู้ป่วยติดเชื้อไวรัสเด็งกี่ชาวไทย พบว่าขณะที่เกิดการติดเชื้อ การแสดงออกของ CD66b ที่ผิวเซลล์เพิ่มสูงขึ้น ขณะเดียวกันพบว่าเซลล์เหล่านี้สร้างและปล่อยสารอนุพันธ์ออกซิเจนอิสระสูงกว่าเซลล์ผู้ป่วยจากระยะฟื้นไข้และกลุ่มควบคุมที่มีสุขภาพดี ซึ่งคุณลักษณะเหล่านี้สนับสนุนว่านิวโทรฟิลถูกกระตุ้นขณะเกิดการติดเชื้อไวรัสเด็งกี่ นอกจากนี้ เราพบว่าการลักษณะการเพิ่มขึ้นของ CD66 มีความสัมพันธ์เชิงบวกกับการคลายตัวของนิวเคลียสของนิวโทรฟิลจากผู้ป่วยติดเชื้อไวรัสเด็งกี่เมื่อเปรียบเทียบกับผู้ป่วยระยะฟื้นไข้และกลุ่มควบคุม เราพบว่านิวเคลียสของนิวโทรฟิลเกิดการคลายตัวในระยะเริ่มต้นของการสร้างและปล่อย NETs ซึ่งมีลักษณะเป็นเส้นใยดีเอนเอที่ใช้ในการยับยั้งหรือกำจัดเชื้อจุลชีพ คณะผู้วิจัยได้ศึกษาปริมาณของส่วนประกอบของ NETs ใน Serum ของผู้ป่วยที่ติดเชื้อไวรัสเด็งกี่ในระยะเฉียบพลัน เทียบกับระยะหายจากโรคและในกลุ่มควบคุมที่มีสุขภาพดี โดยวิธี ELISA ผลการทดลองแสดงให้เห็นว่ามีปริมาณของโมเลกุลที่เป็นส่วนประกอบสำคัญของ NETs ในผู้ป่วยที่มีภาวะเลือดออก (Dengue hemorrhagic fever, DHF) มากกว่าในกลุ่มผู้ป่วยที่ไม่มีอาการรุนแรง (Dengue fever, DF) นอกจากนั้นเรายังพบปริมาณของสารอักเสบชนิด IL-8 และ TNF-a สูงขึ้นในกลุ่มผู้ป่วยที่มีอาการรุนแรง เมื่อเทียบกับกลุ่มที่อาการไม่รุนแรงและกลุ่มควบคุม แสดงให้เห็นว่า NETs อาจมีบทบาทสองอย่าง ทั้งในการป้องกัน และทำให้เกิดอาการรุนแรงขึ้นในผู้ป่วยติดเชื้อไวรัสเด็งกี่ ในการศึกษาของเราการเพิ่มขึ้นของ NETs ในช่วงติดเชื้อไวรัสเด็งกี่เฉียบพลันนั้นไม่ขึ้นกับการเกิด histone H3 hypercitrullination โดยเอนไซม์ PAD4 การศึกษานี้แสดงให้เห็นว่านิวโทรฟิลมีบทบาทในการตอบสนองทางระบบภูมิคุ้มกันต่อการติดเชื้อไวรัสเด็งกี่

AIM i: To determine the activation state of neutrophils during dengue fever

AIM i.i.: To Measure the expression of surface adhesion molecules

Work by Hoang et. Al {Hoang, 2010 #597} previously suggested that PMN are activated during dengue infection. Whole blood microarray data showed that in severe dengue (24 DSS patients) genes related to neutrophil activation were upregulated compared to uncomplicated cases. These genes included Neutrophil Elastase (Ela2) and Cathepsin G (CTSG) and the adhesion molecules CEACAM 6 and CEACAM 8. CEACAM molecules promote cell-adhesion of neutrophils {Skubitz, 2008 #1814} and CEACAM activation prime neutrophils and induce ROS production {Stocks, 1995 #1817;Lund-Johansen, 1993 #1818}. Altogether, these data suggests that neutrophils are activated during dengue fever. As adhesion of neutrophils is one of the earliest events leading to the complete neutrophil activation, we will first measure the surface expression of adhesion molecules including CEACAM8 (CD66b), CD11b and CD16 using cohort samples and flow cytometry.

AIM i.ii.: Measure the level of Reactive Oxygen Species in vitro

CD66b primes neutrophil for the production of Reactive Oxygen Species (ROS). We will measure the background level of ROS produced by PMN using DHR and flow cytometry and compare the different group of subjects. Moreover, we will also measure the level of ROS released after exposure of the cells to a stimulus (PMA) in vitro, as increased expression of adhesion molecules can reflect neutrophil priming. An increased sensitivity of neutrophil toward PMA would indicate that PMN were primed *in vivo*.

AIM ii: Investigate the effect of dengue infection on the formation of Neutrophil Extracellular Traps (NETosis)

High levels of cell-free DNA have been measured in the plasma of Dengue patients {Ha, 2011 #496}. We hypothesize that part of this DNA originates from neutrophil extracellular traps. Therefore, we will measure the level of NETosis specific markers in the serum of subject and test the ability of PMN obtained from patients to release NETs in vitro.

AIM ii.i.: To test whether neutrophil isolated from dengue patients are more susceptible to NETosis

The exposure of neutrophil to stimuli can result in different outcomes, including priming, activation, respiratory burst, degranulation, and the release of NETs. To test whether neutrophil isolated from dengue patients release NETs, cells will be exposed to increasing concentration of PMA, a potent inducer of NETs formation.

AIM ii.ii.: To measure the level of NETs in the plasma of patients

Detection of DNA alone is not a proper and specific method to identify the presence of NETs in biological samples. DNA can be released by apoptotic or nectrotic as well. NETs are essentially a scaffold of cell-free DNA associated with proteins usually found in neutrophil granules, i.e. MPO or Neutrophil elastase. We will use a ELISA approach to quantify MPO-DNA complexes in the plasma of the cohort subjects and test whether a correlation exists between the different forms of the disease and the presence of NETs. Using Western Blotting, we will also measure the level of citrullination of Histones in patients cells, as it is a marker of NETosis in the NADPH-PAD4 pathway {Wang, 2009 #502}.

AIM iii: To investigate the role Neutrophil extracellular traps in dengue immune response and pathogenesis.

Increasing pieces of evidence suggest the capture and inactivation of enveloped viruses by NETs {Jenne, 2013 #964;Raftery, 2014 #1759;Saitoh, 2012 #976}. We hypothesize that the enveloped dengue virus can be trapped in NETs. Dengue virus (serotype 2) will be incubated with NETs generated *in vitro* and we will measure the virus titer after incubation in the presence or in the absence of NETs.

AIM iv: To determine the factors responsible for neutrophil activation and NETosis in dengue patient samples.

Neutrophils do not express the TLR required for the detection of RNA viruses {Hayashi, 2003 #1882; Berger, 2012 #1885}, but do express RIG-1 and MDA5 {Berger, 2012 #1885}. As neutrophils are activated during dengue infection, we would like to know if the virus can directly activates neutrophils, or if the PMN activation is only the consequence of the presence of high levels of pro-inflammatory mediators.

We hypothesize that neutrophils are indirectly activated by the pro-inflammatory cytokines released during the course of DENV infection, and will measure the level of pro-inflammatory and anti-inflammatory mediators in the plasma of uncomplicated and severe dengue patients at different phases of the diseases.

METHODS (from {Opasawatchai, 2018 #2908}):

Ethics statement

All participants were adults and provided written informed consents at the beginning of the study. Blood samples were collected from adult dengue patients and healthy individuals at the Vajira Hospital and the Tropical Medicine Hospital in Bangkok, and the Thasongyang Hospital in Tak province, Thailand. Ethics committees the Vajira Hospital and the Tropical Medicine Hospital (MUTM 2013-046-03) granted ethical approval.

Study cohort, blood sample collection

Nested-RT-PCR for dengue viral RNA (serotype 1, 2, 3, or 4) was used to include index cases. DENV severity was classified following WHO criteria into DF or DHF. Household members without signs of DENV infection, and with negative nested-RT-PCR were used as healthy controls. Blood samples from index cases were collected daily from admission until the fever decreased, and were classified using clinical symptoms. Functional ex-vivo experiments were performed on the day of blood collection.

Neutrophil isolation

Fresh heparinized blood samples were centrifuged (22°C, 800 g, 10 min) to separate cells and plasma. Cell suspensions were diluted (RPMI-1640 2% FBS) (Gibco, MA, USA) and separated using an Isoprep layer (Robbins Scientific Corporation, CA, USA). RBCs present in the pellet were lysed with a hypotonic NaCl solution (0.2%) and osmolarity restored with 1.6% NaCl after 30 sec. Recovered granulocytes were washed and resuspended in RPMI 1640-0.5% FBS. Neutrophil purity was routinely more than 95%. For western blot analysis, neutrophil pellets were dried and stored at ⁻80°C. Serum from the subject was also prepared and stored at ⁻80°C.

Flow cytometry

Leukocytes were separated from heparinized whole blood samples after RBC lysis (Biolegend, CA, USA), washed, and then incubated with fluorophore-conjugated antibodies against CD11b (PEcy7, #557743, BD Pharmigen), CD16 (data not shown), and CD66b (FITC, #555724, BD Pharmigen), or the corresponding isotype controls (BD Bioscience). Samples were run on a BD FACS Canto II, and data analyzed using the Flowjo v.8.7 software (Treestar, USA). Delta mean fluorescence intensity (MFI) was determined by subtracting the background fluorescence of the isotype control from specific MFIs of each pair of antibodies.

Detection of reactive oxygen species ex vivo

Leukocytes were isolated as described above. Following RBC lysis, leukocytes were incubated with DHR 123 for 15 min at 37°C, with or without PMA. Neutrophil (gated on FSC/SSC) ROS content was measured by the intensity of the FL1 channel.

Immunofluorescence

Neutrophils (2 × 105 cells, in RPMI-1640-0.5% FBS) were seeded onto a round glass coverslip in a 24-well plate. After 10 minutes, cells were incubated with various concentration of PMA for 2 hours (37°C, 5% CO2). Fixation was done using 4% paraformaldehyde, and permeabilization with a solution of PBS containing Triton-X 100 (0.5%). Nuclei and extracellular DNA fibers were stained either with Sytox Green (ThermoFisher, MA, USA), Hoechst 3342, or Hoechst and an anti-MPO antibody (Santa Cruz, sc-52707, 1:500) . Cells were mounted onto slides, and were observed using a Nikon Eclipse 80i microscope. At least three randomly pictured fields were used to count the percentage of cells with decondensed/rounded nuclei and extracellular Sytox green positive fibers.

MPO-DNA ELISA

Flat bottom 96-well plates (Thermofisher Scientific, MA, USA) were coated with an anti-MPO polyclonal antibody (1:500, #2329755, Millipore, CA, USA) overnight in the cold. Plates were washed (3 times, 0.05% Tween in PBS), and 20 μL of patient serum pre-mixed with anti-DNA peroxidase antibody (1:80, Cell Death ELISA Plus kit, Roche, Germany) was added to each well. Plates were incubated (RT, in the dark, three hours, with agitation) and washed five times. The substrate ReserveTM TMB (KPL, PA, USA) was added and the reaction stopped by adding an equal amount of 1 N HCl. The O.D. (450 nm) was measured using a microplate reader (EZ400, Biochrom, UK).

Detection of plasma cytokines

EDTA plasma (25 μL) from healthy subjects, DF, and DHF patients with acute infection were analyzed for a panel of 38 cytokines including IL-8 and TNFα by Luminex bead-based multiplex assay using the Milliplex$^{\circledR}$ MAP human cytokine/chemokine magnetic bead panel kit (Millipore, USA). The FlexMAP 3D (Luminex$^{\circledR}$) platform was used according to manufacturer's instructions.

Neutrophil Elastase ELISA

The level of neutrophil elastase in the plasma of patients was measured with a Human PMN Elastase ELISA Kit (Abcam) following manufacturer's instruction. The average reading between duplicate wells subtracted by blank was used to calculate the plasma NE concentration (ng/ml).

Cell lysis and histone extraction

Frozen cell pellets were lysed in a low salt buffer (20 mM Hepes, 1 mM Sodium EDTA, 1 mM Sodium EGTA, 500 mM KCl, 50 mM PMSF, and 75 mM MgCl2) complemented with a protease inhibitor cocktail (cOmplete ULTRA, Roche, USA). After centrifugation (16,000 g, 4°C, 15 min), histones were extracted from the nuclear and membrane fractions overnight with 0.5 N HCl at 4°C. Histones in the HCl soluble fraction were precipitated with TCA acid (33.33%) and washed with cold acetone. The HCl-insoluble fractions were also collected for the detection of PAD4. Protein concentration was determined by the Bradford protein assay (Biorad, CA, USA).

Detection of citrullinated histone H3 and PAD4 by western blot

Acid-soluble histone extracts were separated on a 14% acid-urea gel. Proteins were then transferred onto a PVDF membrane in 0.7% acetic acid buffer. Acid-insoluble nuclear fractions were separated by 10% SDS-PAGE before transferring onto a PVDF membrane using a semi-dry blotting system (Biorad, CA, USA). Membranes were blocked with 5% skimmed milk in TBS-0.2% Tween for 1 hour, and were incubated with rabbit anti-citrullinated histone3 antibodies (1:2000, #ab5103, Abcam, Cambridge, UK) for histone extracts or mouse anti-PAD4 antibodies (1:2000, #ab57167, Abcam, Cambridge, UK) for acid-insoluble nuclear fractions. Membranes were stripped and probed with rabbit anti-total histone3 (Abcam, #1791) for loading control. Chemiluminescence was captured using a GBox (Syngene, Cambridge, UK), and band intensity was quantified by densitometry using the ImageJ software (NIH).

Coincubation of neutrophils with DENV in vitro

Neutrophils were plated for 10 min before PMA (100 nM) was added for 3 hours to induce NET formation in a coverslip containing well. The supernatant was carefully removed prior to the addition of the virus (DENV2, MOI 1:1). The virus was incubated for 10, 30, or 180 min with neutrophils or NETs. Following the incubation period, supernatants were collected and viral titers were determined by FFU. Coverslips were recovered, fixed (4% paraformaldehyde), and stained with Hoechst 33342 (Thermo-Fisher Scientific).

Foci Forming Unit (FFU) assay

Ten-fold serial dilutions of C6/36 cell supernatants used for the propagation of DENV-2 were incubated with BHK cells (3×10^5) in 96-well plates at 37°C for 90 min. This was followed by addition of 1.5% carboxymethyl cellulose (CMC), and cells were incubated at 37°C for 3 days. After fixation with 3.7% paraformaldehyde, cells were permeabilized with 0.1% TritonX and washed with PBS. DENV-specific anti-E antibody (4G2) was added for three hours at 37°C before washing with PBS. Cells were stained with anti-mouse Ig secondary antibody tagged with HRP for 45 min at 37°C, and washed three more times with PBS. Diammonium phosphate (DAP) was added at room temperature for color visualization; FFU were counted under a light microscope.

Statistical analysis

Statistical analyses were performed using the Prism version 5.0 software. The Mann-Whitney U-test was a used to compare the median between two different groups of donors (e.g., healthy control vs DENV infected patients). The Wilcoxon-signed rank test was used to compare the median obtained from the same patient at two different time points (e.g. febrile vs convalescent phase). Differences between more than two groups of samples were determined by Kruskal-Wallis tests, followed by Dunn's post-hoc tests. Correlation between the two sets of data obtained from the same donor (e.g. level of CD66b expression versus percent spontaneous delobulation) was evaluated by the Spearman-rank correlation test.

RESULTS

Neutrophils were stimulated during acute DENV infection

To identify and assess the phenotype of neutrophils in whole blood, we measured expression levels of the activation markers CD11b and CD66b by flow cytometry. No significant change was observed in CD11b expression (Fig. 1A&B). However, CD66b expression was markedly increased in febrile patients (Feb DENV) as compared with that of healthy controls (p-value < 0.001) (Fig. 1C); this difference was maintained until defervescence (Def DENV, Fig. 1D). At convalescence, CD66b level was significantly decreased (p-value < 0.001), and was comparable to that of healthy donors (Fig. 1C). The DENV infection-associated CD66b upregulation was found in both mild (DF) and severe (DHF) patients (Fig. 1C& 1D). We did not observe any correlation between CD66b expression and viral titer or virus serotype (data not shown). In line with CD66b upregulation, PMA induced a stronger respiratory burst in neutrophils from febrile patients as compared with that in convalescent and healthy individuals (Fig. 1C), respiratory bursts are characteristic of primed neutrophils. Altogether, these results suggested that neutrophils are activated during acute DENV infections.
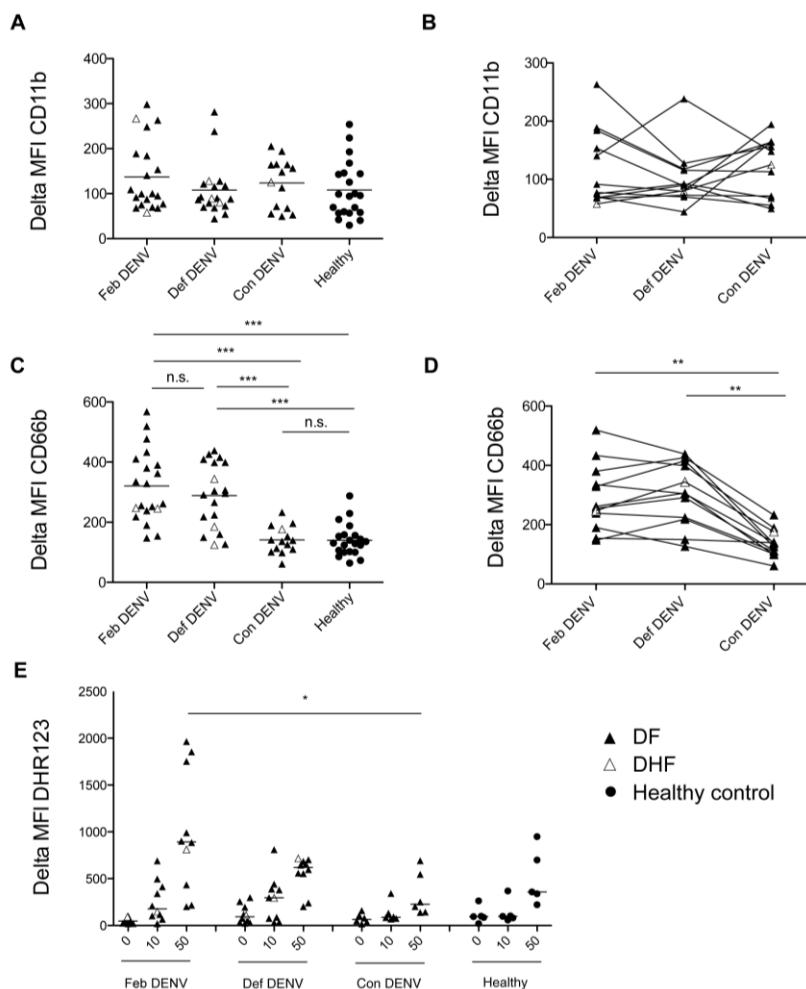

Fig. 1

Fig. 1. Neutrophil activation during acute DENV infection

Delta MFI of CD11b (A) and CD66b (C) in healthy donors (n = 21), during febrile illness (Feb DENV) (n = 21), deferevescence (Def DENV) (n=20), and convalescence (Con DENV) (n = 14). Bars represent the mean of each population. ***p < 0.001, as assessed by the Kruskal-Wallis test, followed by Dunn's post-hoc test. Neutrophil CD11b (B) and CD66b (D) surface expression at the different phase of the disease in individual patients. **p < 0.01, assessed by the Wilcoxon-signed rank test. (E) Granulocytes were gated by size and granularity prior to ROS production analysis by flow cytometry. Dot plots show level of ROS production by the mean fluorescent intensity of DHR-123 in Feb DENV (n = 10), Def DENV (n = 10), Con DENV (n = 6), and healthy controls (n = 5). *p < 0.05, assessed by the Kruskal-Wallis test, followed by Dunn's post-hoc test. In all panels, DF patients are represented by a dark arrowhead, DHF patients by a white arrowhead, and healthy control by a dark circle.

Neutrophil activation during DENV infection resulted in increased nuclei delobulation

ROS are required for NET formation in response to various stimuli. As high levels of cell-free DNA are associated with severe DENV infection, and elevated levels of NET components, myeloperoxidase and neutrophil elastase, have been found during the acute phase of the infection, we hypothesized that neutrophils form NETs during DENV infections. We found that nuclei of unstimulated healthy neutrophils remained lobulated after 2 hours, as did the cells for all conditions before incubation (Fig. S1). In contrast, nuclei of unstimulated neutrophils from acute, but not convalescent, patients were delobulated after 2 hours (p < 0.01) (Fig. 2A&C). Moreover, the percentage of delobulated cells was greater in acute DENV neutrophils as compared with that of healthy and convalescent cells for each PMA concentration tested (Fig. 2B). The presence of PMN with delobulated nuclei was not due to contaminating cells as shown by FACS and Giemsa staining prior incubation (Fig. S1). In healthy human neutrophils, the increased number of cells with delobulated nuclei was associated with the release of NETs in unstimulated cells and in response to LPS and PMA (Fig. S2). The loss of lobes has been shown to be an early mark of NET formation. This observation suggested a possible increased propensity for acute DENV neutrophils to form NETs. Interestingly, cell expression of CD66b and percentage of nuclei lobule loss were positively correlated in matched donors (p < 0.01) which suggested that CD66b may be an early marker of NETs during DENV infection (Fig. 2D). No association was observed between the expression of CD11b or CD16 and spontaneous delobulation (data not shown). To assess if PMN delobulation and activation were observable in vivo, the SSC values of neutrophils for healthy controls and DENV patients obtained at different time were analyzed. The SSC mean fluorescence intensity for febrile DENV patient neutrophils (442.7 +/- 72.62) was lower compared to healthy controls (481.7 +/- 89.74), but the difference was not statistically different (Fig. S3).
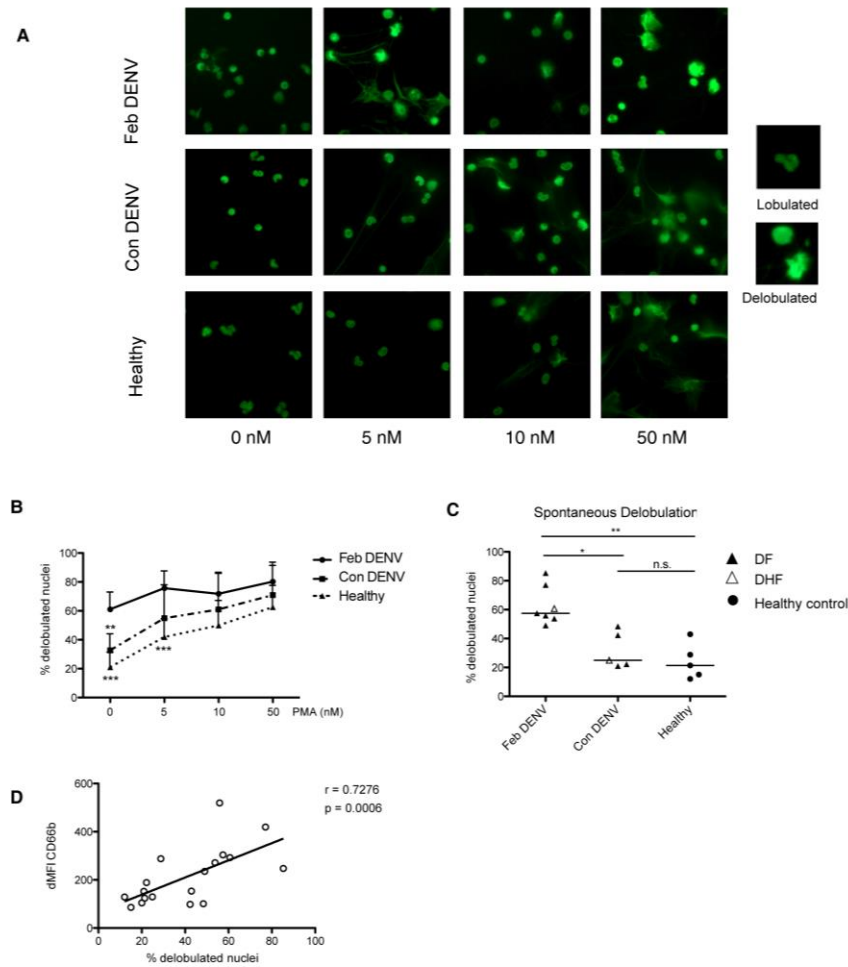
Fig. 2. Neutrophils from acute DENV infected patients are susceptible to NET formation

Isolated neutrophils from healthy donors and DENV infected patients during febrile illness (Feb DENV) and convalescent phase of the disease (Con DENV) were cultured for two hours without stimulation or with 5 nM, 10 nM, or 50 nM PMA. The cells were fixed, permeabilized and stained with the DNA dye Sytox green, and were visualized with a fluorescent microscope. (A) Representative images of nuclear morphologies of isolated neutrophils from Feb DENV, Con DENV, and healthy donors in response to varying concentration of PMA stimulation.Representative high magnifications of lobulated and delobulated nuclei are shown on the right.. (B) Average percentage of cells with delobulated nuclei from Feb DENV (n = 7), Con DENV (n = 5) and Healthy donors (n = 5) in response to varying PMA concentration. Error bars represent mean ± SEM, analyzed by two-way ANOVA and Bonferrini posttest, **p < 0.01, ***p < 0.001. (C) Percentage of cells undergoing nuclei decondensation without PMA stimulation from Feb DENV (n = 7), Con DENV (n = 5), and healthy donors (n = 5). Bars represent mean of each group. *p < 0.05, **p < 0.01, analyzed by Kruskal-Wallis test followed by Dunn's post-hoc test. (D) Correlation between cells with decondensed nuclei with CD66b expression in matched donors, as determined by Spearman-ranked correlation (r = 0.620, p = 0.0010, n = 17).
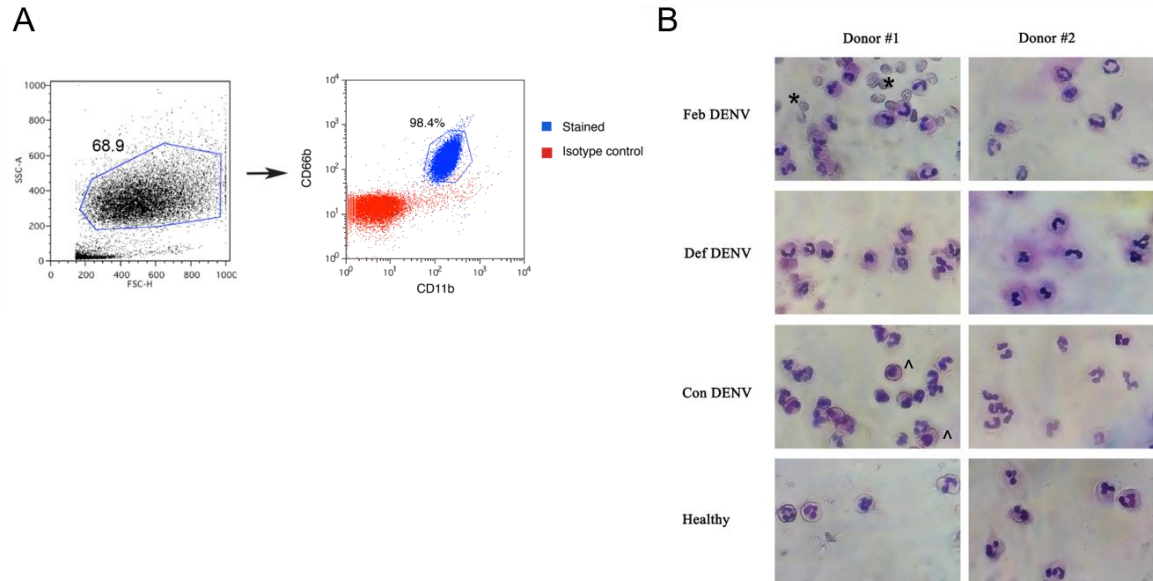
Figure S1. Flow cytometry and Wright Giemsa staining of cells isolated from DENV patients.

Representative flow cytometry dot plots (A) and Giemsa staining (B) of neutrophils isolated from dengue patients. (A) After isolation, the cells were stained using CD11b, CD66b, or the corresponding isotype controls. Granulocytes were gated first on FSChigh/SSChigh, and the expression of CD11b and CD66b measured, showing that more than 95% of the cells were double positive. (B) Giemsa staining of isolated cells from 2 donors at different time points. Note the typical multilobed nuclei of neutrophils. Some red blood cells (*) can be seen for Donor #1 during the Febrile phase and monocytes (^) can be observed for the same donor at convalescence.
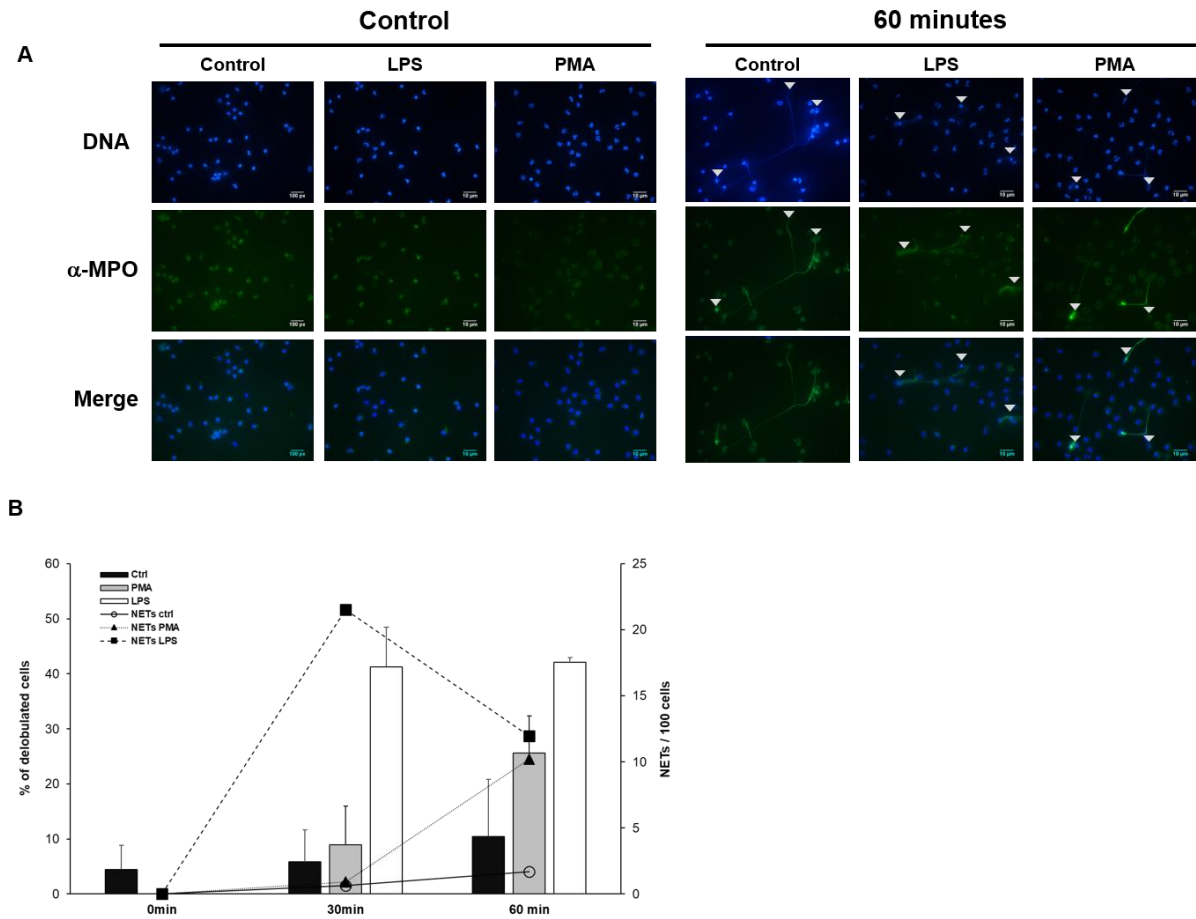
Figure S2. Nuclei delobulation is associated with the increased release of NET over time.

(**A**) After isolation, cells were stimulated with LPS (100 ng/ml) or PMA (50 nM) for 30 or 60 minutes, and stained using Hoechst (DNA) and an anti-MPO antibody (NETs). Arrowheads indicate delobulated nuclei. (**B**) Quantification of (A), a minimum of 70 cells in 3 different fields were counted for each condition. The percentage of cells with delobulated nuclei (histograms, +/- SD) and the number of NETs per 100 cells (lines) are reported (right y-axis).
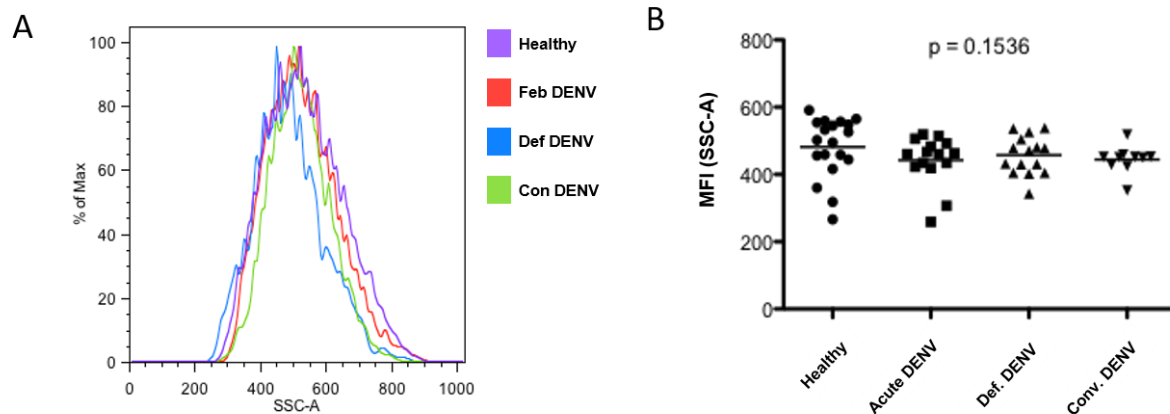
Figure S3. Analysis of neutrophil SSC MFI during dengue infection.

(A) Representative histograms of SSC MFI for DENV neutrophils isolated from dengue patients at different time points. (**B**) Cells isolated from DENV patients showed a decrease in SSC MFI (442.7 +/- 72.62) compared to Heathy individual (481.7 +/- 89.74). p=0.1536, Anova.

NETs impaired DENV infectivity in vitro

NETs have been shown to trap and inhibit viruses in vitro, and can prevent viral dissemination in vivo (10, 32). As DENV neutrophils were prone to spontaneous formation of NETs in vitro, we investigated whether PMA-induced NETs from healthy neutrophils could inactivate DENV (Fig. 3A). After 30 min of incubation, DENV titer was dramatically decreased in the presence of NETs as compared with that when incubated with the virus alone or in the presence of unstimulated healthy neutrophils (Fig. 3B&C). This suggested that NETs could trap or inactivate the virus in vitro.
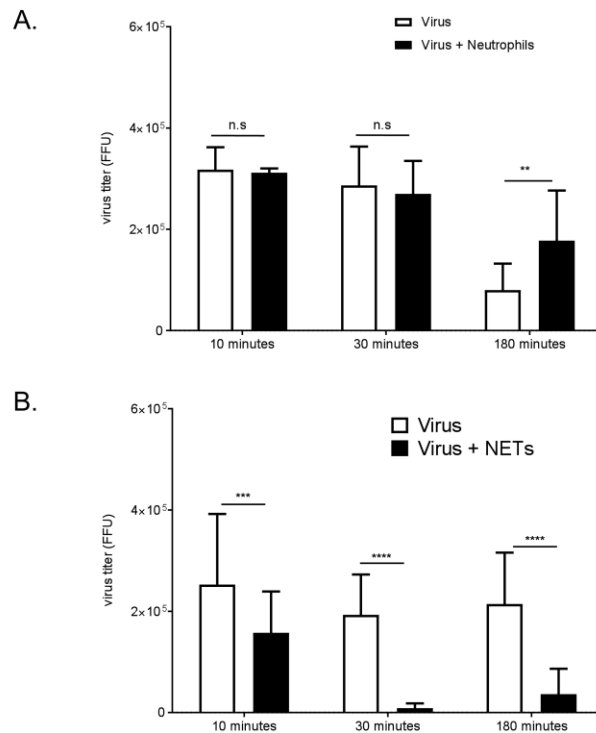
Fig. 3

Fig. 3. NETs impair DENV infectivity in vitro

Neutrophils from healthy donors were cultured in the presence (A) or absence of PMA (100 nM) (B) for three hours to induce NETs formation. After removal of the supernatant, the virus was added for 10, 30, or 180 min to neutrophils, NETs, or empty well controls. The virus titer recovered after incubation with neutrophils (B) or with NETs (A) was determined by the FFU assay. Data are shown means ± SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001, analyzed by two-way ANOVA.

Elevated IL-8 and TNFα were associated with severe DENV infection

To investigate whether neutrophil activation was correlated with a proinflammatory environment, we measured plasma levels of IL-8 and TNFα, cytokines known to activate neutrophils, during the febrile phase of DENV infection. We found significantly elevated IL-8 in DHF patients as compared with that of healthy controls (p < 0.001) and DF patients (p < 0.001) (Fig.4 A-B). TNFα was significantly increased in DF and DHF patients as compared to healthy controls (p < 0.05, p < 0.001); however, no significant difference was found between DF and DHF patients. We also measured the level of Neutrophil Elastase, a maker of neutrophil activation, degranulation and NETosis, in the serum of patients (Fig. 4 C-D). We observed a slight increase of NE in the serum of DHF patients during the febrile phase as compared to convalescence, while the difference at the two time points was less obvious for DF patients (Fig. 4C). The level of NE was significantly increased in Acute Dengue (DF and DHF) as compared to convalescence (p < 0.0411) (Fig.4 D).
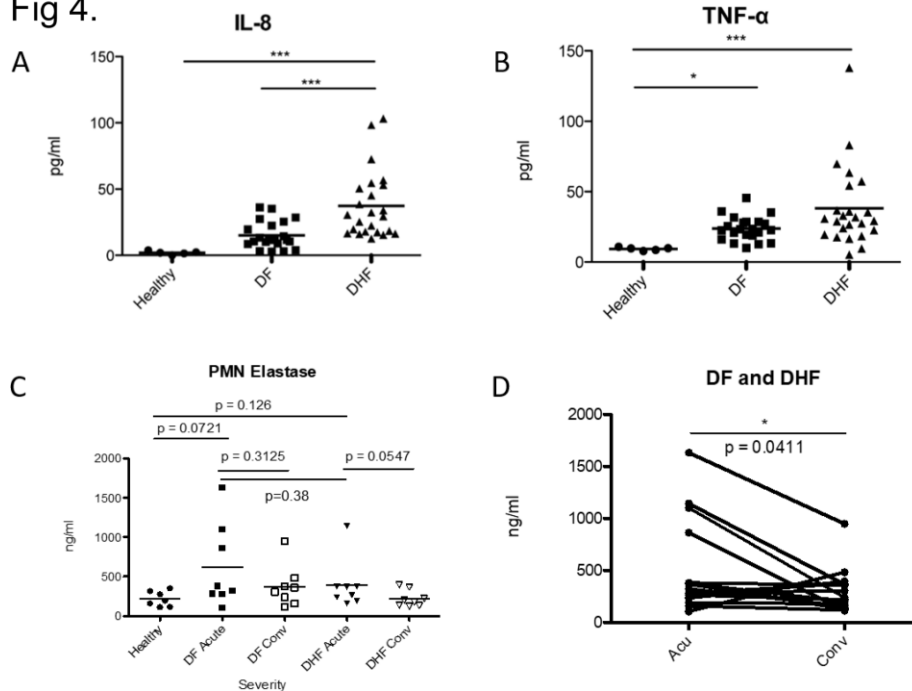
Fig. 4. Elevated levels of IL-8, TNFα and Neutrophil Elastase during acute DENV infection

Plasma samples from healthy donors (healthy), mild (DF) and severe (DHF) DENV infected patients during febrile illness (Feb DENV) and convalescent phase (Con DENV) were used. (A) Plasma level of IL-8 and (B) TNFα during febrile DENV infection. *p < 0.05 and ***p < 0.001, Kruskal-Wallis test followed by Dunn's post-hoc test. (C) Level of NE (ng/ml) in the serum of healthy, DF and DHF patients during the febrile and convalescence phase. Mann-Whitney U test. (D) Level of NE in the plasma of acute DENV patients (DF and DHF) versus convalescent patients. NE concentration from the same patients are connected with lines. Wilcoxon-signed rank test, *p < 0.05.

Elevated NET-derived products were associated with severe DENV infection

Nuclei delobulation (Fig. 2) and change in SSC (Fig. S3) were indirectly suggesting DENV neutrophils increased susceptible to NETosis. To determine whether NETs were produced during Dengue virus infection, and the association of NETs with clinical outcomes of DENV infection, we measured the level of MPO-DNA complexes in the serum of DENV-infected patients by ELISA. Ex vivo experiments indicated that febrile DENV infections increased susceptibility to spontaneous delobulation regardless of disease severity (Fig. 2). However, serum levels of MPO-DNA complexes were elevated during febrile DENV infection in DHF patients, but not in DF patients, when compared with those in convalescent individuals (Fig. 5A-C). Therefore, excessive NETs may be an aggravating factor that enhances disease severity during an acute DENV infection.
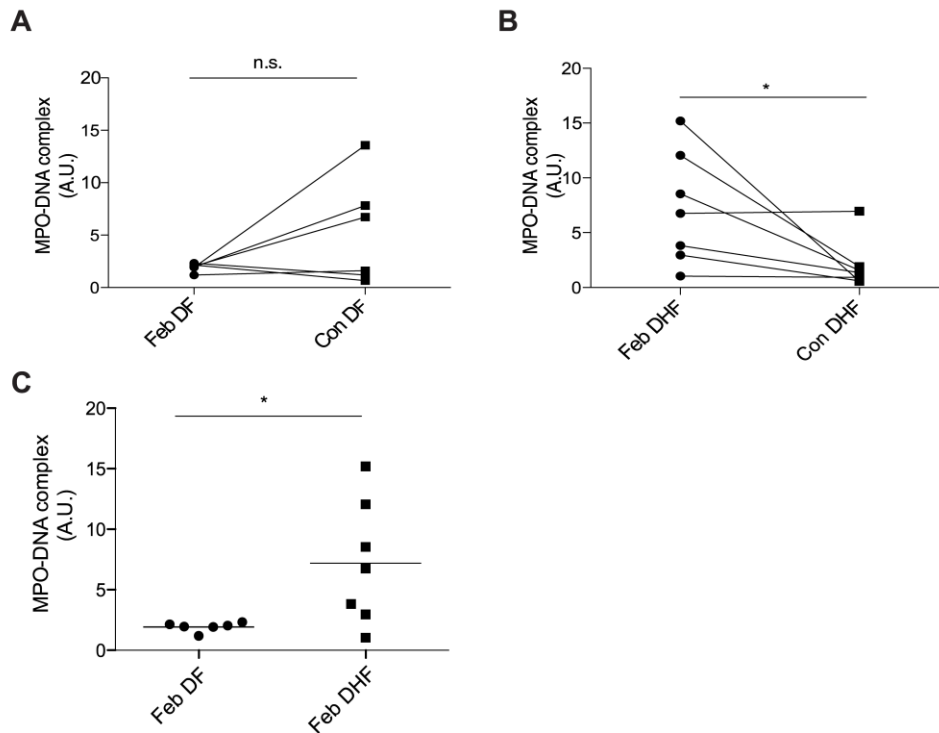
Fig. 5

Fig. 5. NETs are associated with severe DENV infection

Serum samples from healthy donors as well as mild (DF) and severe (DHF) DENV infected patients during febrile illness (Feb DENV) and convalescent phase (Con DENV) were used for the MPO-DNA ELISA. (A-B) O.D. ratios from the same DF and DHF patients are connected with lines. Analysis was performed with the Wilcoxon-signed rank test, *p < 0.05. (C) Each symbol represents O.D. at 450 nm normalized to inter-experimental control. Mann-Whitney U test, *p < 0.05.

Histone H3 citrullination was suppressed during acute DENV infection

Protein arginine deiminase 4 (PAD4)-dependent histone citrullination is one of the pathways responsible for NET formation in microbial infections, systemic inflammations, and autoimmune diseases. We measured levels of Histone H3 citrullination (Fig. S4) and PAD4 (Fig. S5) in nuclear extracts of frozen neutrophils from DENV patients by western blot. Surprisingly, histone H3 citrullination was markedly decreased during the acute phase of DENV infection as compared with that during the convalescent stage and in healthy controls (Fig. S4). PAD4 level was also slightly decreased during the acute phase of the infection compared with that during convalescence, albeit non-significant (Fig. S5).
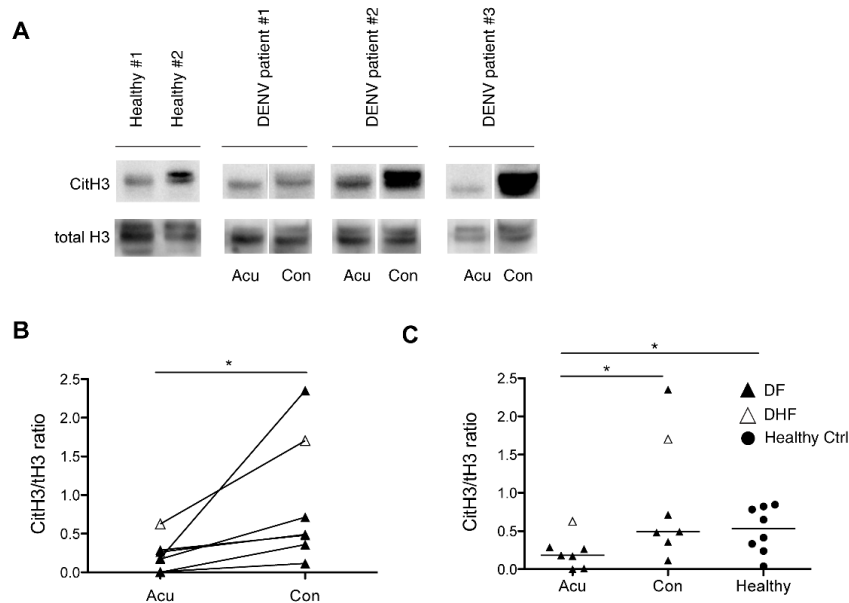
Fig. S4. Citrullination of Histone H3 is suppressed in neutrophils from acute dengue patients

Histones were extracted from neutrophils obtained from acute (Acu) or convalescent (Con) dengue patients. The relative amount of acid-extracted histones was measured with anti-citrullinated H3 (CitH3) or total histone 3 (tH3) antibodies. (A) Western blot showing levels of CitH3 and tH3 in neutrophils from three DENV patients and two healthy donors. (B) Paired analysis of the normalized citrullinated histone 3 (CitH3) levels in acute and convalescent DENV patients. Wilcoxon signed-rank test, *p < 0.05. (C) CitH3 levels in acute (DF = 6, DHF = 1) or convalescent dengue patients and healthy controls. Mann-Whitney U, *p < 0.05.
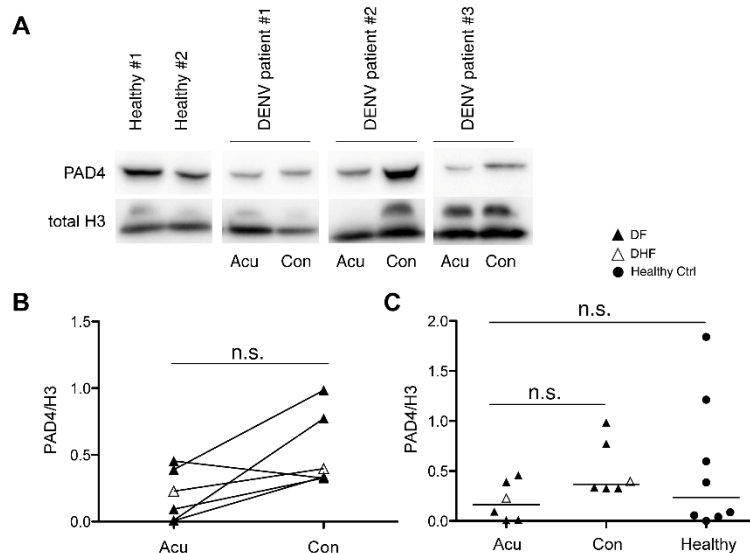

Fig. S5. PAD4 expression is reduced in neutrophils from acute dengue patients

(A) Western blot analysis on acid insoluble nuclear extracts in neutrophils obtained from the same patients as in Fig. 5. (B) Paired analysis of normalized PAD4 expression in acute and convalescent DENV patients; histone 3 was used as loading control. (C) Relative expression of PAD4 in neutrophils obtained from acute (Acu) (DF = 6, DHF = 1), convalescent (Con), and healthy donors.

Summary – Discussion (for full references, see attached manuscript):

Evidences of excessive inflammation during DENV infection suggested that activation of neutrophils and formation of NETs may be involved in disease pathogenesis (34). Due to access to neutrophil samples from DENV-infected patients, we assessed both the phenotype and function of neutrophils in naturally- infected DENV patients. We showed that neutrophils were primed in vivo (Fig. 6, iii), and were prone spontaneous delobulation. We also highlighted the dual roles of NETs in inhibiting DENV, as well as its possible contribution to disease severity.

Neutrophils overexpressed CD66b during acute DENV infection. CD66b is a marker of granulocyte activation involved in adhesion to endothelial cells (51), degranulation (52), and increased reactive oxygen species (ROS) production (51). Similar CD66b upregulation has been reported in patients during bacterial sepsis, a condition involving systemic inflammation (53). We did not observe CD11b overexpression associated with DENV infection. Interestingly, an increase in the CD66b:CD11b ratio has been reported for neutrophils in the presence of S. aureus, and this was associated with their reduced ability to phagocytosed (54). Our findings also confirm, at the protein level, the CD66b overexpression reported at the transcriptional level during DENV infection (18). In line with the elevated CD66b expression, we found robust ROS production by granulocytes in response to ex vivo PMA stimulation during acute dengue infection. This could be the result of priming by the pro-inflammatory environment found during acute dengue virus infection, as TNFα and IL-8, cytokines known to prime neutrophils for ROS production, were also found elevated in dengue patients as previously reported (55) and in our own study cohort (Figure 4 A-B). ROS are essential both for neutrophil antimicrobial activity and for ROS-dependent NET formation (28, 56).

Emerging evidence has supported the presence and role of NETs in viral infections. Direct activation of neutrophils to form NETs by viral particles has been demonstrated in HIVs (32), RSVs (35), and hantaviruses (15). We provide evidences, in the present study, that during DENV infection, neutrophils are more susceptible to spontaneous delobulation ex vivo. Delobulation is an early feature of NET formation, but is not limited to the release of NETs and is found in other form of cell death. However, we confirmed that the limited spontaneous NETosis of healthy neutrophils, detected with an anti-PMO antibody, was also associated with the increased percentage of spontaneous delobulation (Fig. S2). How DENV infection could result in such susceptibility remains to be elucidated. The ability of DENV particles to directly induce formation of NETs varies from one study to another. While Yost et al. showed that DENV could induce production of NETs in healthy neutrophils (20), Moreno-Altamirano et al. demonstrated that DENV-2 inhibited NET formation in response to PMA stimulation by altering the glycolysis pathway (36). In the present study, we could not characterize the mechanisms responsible for the elevated delobulation or the increase MPO-DNA complexes detected in vivo in natural DENV infection. However, we proposed that elevated levels of pro-inflammatory cytokines IL-8 and TNF☐, which were reported to be inducers of NETs (7, 28, 37, 38) during febrile DENV infection, could contribute to such susceptibility through the priming or the activation of neutrophils (Figure 4A-B and (4)). Neutrophil-platelet interactions may also promote NET formation during

DENV infections (39); studies have shown that neutrophil-platelet interactions promoted production of NETs in the bacterial sepsis model (40) and murine pox virus infections (10). Furthermore, DENV has been shown to activate platelets (41, 42). Of note, we could not amplify viral genome from neutrophils isolated from dengue patients (data not shown).

NETs have been described as powerful antimicrobial weapons as well as threats to the host (43). In this study, we found higher susceptibility for NET formation in acute DENV infections regardless of disease severity. In addition, NETs could potentially inhibit DENV in vitro. Possible antiviral immunity elicited by NETs include immobilization of viral particles, inhibition of virus by antimicrobial proteins (32), and possibly through NET-induced type I interferon production by plasmacytoid dendritic cells (44). The precise mechanisms that underlie NET-mediated DENV inhibition need to be further investigated.

We detected elevated levels of Neutrophil Elastase in the plasma of Acute infected patients (Fig. 4 C-D). While not significant, the differences seemed more marked for DHF patients as compared to DF patients. NE elastase is a marker of neutrophil degranulation, and also found on NETs. Interestingly, higher levels of NET-derived components, such as the MPO-DNA complex, were also found in the serum of DHF patients as compared with that in DF patients (Fig. 5A-C). Previously, two independent studies suggested that cell-free DNA (30) and plasma histone H2A (42), two other known components of NETs, were markedly elevated in severe form of DENV infection. These findings were in agreement with our observation, and showed that DENV infection may induce NET formation, and that elevated NET components are associated with severe disease.

The mechanisms underlying neutrophil activation and NET formation in dengue pathogenesis are currently unknown. Several studies have shown that canonical NETosis starts with the loss of neutrophil nucleus lobules (28, 29, 31). This is however not a proper marker of NETosis as other forms of cell death, such as leukotoxic hypercitrullination, can display similar features (48). However, the detection of NET products by ELISA in the serum of DENV patients, together with the low level of Histone citrullination, support an increased propensity of DENV neutrophils to release NETs. As we did not observe any difference in spontaneous formation of NETs between DF and DHF patients ex vivo (Fig. 2), we propose that NET release may be inhibited, or that NETs were degraded in DF, but not DHF patients in vivo (Fig. 6, (iv) and (v)). It was proposed that metalloproteinases and histones in NETs may participate in vascular leakage in dengue infection by disrupting the vascular endothelial cell layer (45, 46). This suggest that the NET formation and degradation may affect the final outcomes of DENV infections. This phenomenon was also observed in SLE patients who exhibited excessive neutrophil-derived immune complex formation due to impairments in NET degradation (47).

Recent studies have suggested that PAD4 and histone citrullination-mediated NET formation is stimuli-dependent (48). Histone citrullination was found to be decreased and increased by PMA and ionomycin, respectively; however, both molecules stimulated NET formation (49). Little is known regarding

NET formation in viral infections. However, PAD4-dependent NETs were not required for host protection during influenza infection in vivo (12). Similar to PMA-induced NET formation, we suggest that DENV-induced susceptibility to NET formation may be independent of PAD4 activity and histone H3 citrullination.

Targeting NETs using DNase have shown promising results in improving NET-mediated inflammation in the RV infection model (14) and in intestinal ischemic perfusion injury (50). Understanding the precise pathway responsible for NET formation in DENV infection, its regulation, and its impact on disease outcomes may shed light on potential therapeutic interventions for DENV infections in the future.

Future direction:

It is still unclear whether the ability of neutrophils to release NETs during DENV infection is just the consequence of specific activation of neutrophils, or if we are looking at specific polarization/subset of PMN, for several reasons. First, it is believed that DENV can infect progenitor cells in the bone marrow. Also, DENV infection is often, in some reference up to 80% (2), associated with neutropenia.

Therefore, we hypothesize that DENV perturbs PMN homeostasis and would like to explore the mechanisms of this disturbance of the homeostasis. We would like to know if we have a different type of PMNs during the infection, i.e. if we have differently matured PMN, or differently activated cells in response to the virus. The presence of Low Density Neutrophil or Myeloid Derive Suppressive Cells is unlikely, as we were isolating cells using a Ficoll-hypaque gradient, therefore using normal density PMN.

To address these questions in the future, we have initiated discussion and collaboration with Professor Hongbo Luo (Children's Hospital Boston, Harvard Medical School) to perform a study at the single neutrophil level, as they do have experience working with neutrophil transcriptomics. We think that pseudo time and single cell transcriptome will highlight the events perturbing myelopoiesis during DENV infection, and potentially uncover the differences between asymptomatic dengue patients (infected but without symptoms, we have a few samples), dengue fever and severe dengue fever patients (dengue hemorrhagic fever).

As we do only have frozen samples, we would need to work on isolated nuclei. One of my PhD students have been testing several protocols for the nuclei isolation and I think have found a suitable one (see the attached pictures/protocol). I'll have him try to amplify some candidate genes to see if we can extract any RNA from the recovered nuclei.

Output จากโครงการวิจัยที่ได้รับทุนจาก สกว.

1. ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ (ระบุชื่อผู้แต่ง ชื่อเรื่อง ชื่อวารสาร ปี เล่มที่ เลขที่ และหน้า) หรือผลงานตามที่คาดไว้ในสัญญาโครงการ

Our manuscript has been accepted and published in Frontiers in Immunology, a Q1 publication in the field of Immunology (IF=5.51), and amongst the top 10 most cited journal in Immunology. Also, this TRF grant has enabled the development of two other projects related to neutrophil biology, with two manuscripts expected to be published in the next 6 months in APJAI and in Acta Parasitology, respectively.

2. การนำผลงานวิจัยไปใช้ประโยชน์
    -   เชิงพาณิชย์ (มีการนำไปผลิต/ขาย/ก่อให้เกิดรายได้ หรือมีการนำไปประยุกต์ใช้โดยภาคธุรกิจ/บุคคลทั่วไป)
    -   เชิงนโยบาย (มีการกำหนดนโยบายอิงงานวิจัย/เกิดมาตรการใหม่/เปลี่ยนแปลงระเบียบข้อบังคับหรือวิธีทำงาน)
    -   เชิงสาธารณะ (มีเครือข่ายความร่วมมือ/สร้างกระแสความสนใจในวงกว้าง)
    -   เชิงวิชาการ (มีการพัฒนาการเรียนการสอน/สร้างนักวิจัยใหม่) อื่นๆ (เช่น ผลงานตีพิมพ์ในวารสารวิชาการในประเทศ การเสนอผลงานในที่ประชุมวิชาการ หนังสือ การจดสิทธิบัตร)

The TRF grant also supported two master projects, one that is part of the publication in Frontiers in Immunology, the other that will be published in the next 6 months. This second project was presented at the TBS meeting 2017:

"Effect of Terrein (a secondary metabolite produced from Aspergillus terreus) on neutrophil phagocytosis and ROS production, MP37-MP46, TSB 2017"

Neutrophil Activation and Early Features of NET Formation Are Associated With Dengue Virus Infection in Human

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The involvement of the immune system in the protection and pathology of natural dengue virus (DENV) has been extensively studied. However, despite studies that have referred to activation of neutrophils in DENV infections, the exact roles of neutrophils remain elusive. Here, we explored the phenotypic and functional responses of neutrophils in a cohort of adult dengue patients. Results indicated that during an acute DENV infection, neutrophils up-regulate CD66b expression, and produce a more robust respiratory response as compared with that in convalescent or healthy individuals; this confirmed *in vivo* neutrophil activation during DENV infection. Spontaneous decondensation of nuclei, an early event of neutrophil extracellular trap (NET) formation, was also markedly increased in cells isolated from DENV-infected patients during the acute phase of the infection. *In vitro* incubation of NETs with DENV-2 virus significantly decreased DENV infectivity. Interestingly, increased levels of NET components were found in the serum of patients with more severe disease form—dengue hemorrhagic fever (DHF), but not uncomplicated dengue fever, during the acute phase of the infection. Levels of pro-inflammatory cytokines IL-8 and TNFα were also increased in DHF patients as compared with those in healthy and DF subjects. This suggested that NETs may play dual roles during DENV infection. The increased ability for NET formation during acute DENV infection appeared to be independent of PAD4-mediated histone H3 hyper-citrullination. Our study suggests that neutrophils are involved in immunological responses to DENV infection.

Keywords: innate immunity, neutrophil, viral infection, dengue, NETs

INTRODUCTION

The dengue virus (DENV) is a growing public health concern worldwide. There are four DENV serotypes, which cause a multitude of clinical outcomes that range from self-limiting febrile illness, dengue fever (DF), and dengue hemorrhagic fever (DHF) to life-threatening dengue shock syndrome (DSS) (1). The mechanisms underlying the pathogenesis of the disease and immune responses to DENV infection are not completely understood; however, these are likely the result of complex interplays between viral factors such as viral load, serotypes, and viral proteins (2, 3) and host factors, such as pre-existing immune responses and changes in endothelial cell composition (4–6).

The roles of neutrophils during viral infections have been re-evaluated following the discovery of neutrophil extracellular traps (NETs). NET formation (NETosis) consists of a series processes characterized by nuclear decondensation and delobulation, rupture of the plasma membrane, and release of DNA fibers with antimicrobial peptides (7). While NETosis is a potent anti-microbial mechanism, excessive formation of NETs, or the inability to clear NETs from the circulation, contributes to pathogenesis of autoimmune diseases (8). The roles of NETs in viral infections vary greatly from virus to virus. Beneficial roles of NETs in trapping (HIV) (9) and inhibiting viral dissemination (murine pox virus) (10) have been reported. NETs and neutrophil-derived products have been found in lungs of influenza virus-infected mice (11). However, inhibition of NET formation did not affect infection outcomes (12). On the contrary, excessive NET formation resulted in airway obstruction during respiratory syncytial virus (RSV) infection (13) and exacerbated allergic airway inflammation during rhinovirus (RV) infection (14). Moreover, components of NETs were shown to serve as autoantigens in patients infected with the hemorrhagic hantavirus (15). Interestingly, the mechanisms underlying NET formation and the factors that dictate the role of NETs during viral infections remain largely unknown.

Converging data suggest that neutrophils are not simple bystanders during DENV infection. While it is known that DENV-infected patients exhibit sustained neutropenia, the clinical relevance of this phenomenon is still poorly understood (16). Increased levels of pro-inflammatory mediators such as IL-8 and TNFα can activate neutrophils (4). In addition, anaphylatoxins (17) were found in acute and severe dengue patients. Importantly, expression of genes associated with neutrophil function (DEF4A, CEACAM8, BPI, and ELA2) was found to be increased in whole blood transcriptomes of severe DENV infections (18). A recent study showed that neutrophil elastase levels were higher in DENV-infected patients as compared with those in healthy controls (19). DHF patients also had significantly higher level of elastase activity as compared with that of DF patients, which suggested that neutrophil activation is associated with severe form of the disease (19).

Intriguingly, another recent study showed that DENV could induce formation of NETs *in vitro* (20). However, due to their short half-lives, the study of neutrophil functions, NETs, and their association with disease severity in naturally-infected dengue patients is challenging, and to our knowledge, have never been reported.

Here, neutrophils obtained from DENV-infected patients were collected, processed, and analyzed in a timely manner. We performed a longitudinal study that examined neutrophil phenotypes and functional responses across different severity levels of DENV infection. Our results were the first to show neutrophil activation and their susceptibility to NET formation; our study highlighted possible roles of neutrophils during human DENV infections. Our findings provide new understanding of host immune responses during DENV infections by targeting potential roles of neutrophils.

MATERIALS AND METHODS

Ethics Statement

All participants included in the present study were adults; written informed consents were provided by all subjects prior to study onset. Blood samples were collected from adult dengue patients and healthy individuals at the Vajira Hospital and the Tropical Medicine Hospital in Bangkok, as well as the Thasongyang Hospital in Tak province, Thailand. Ethical approval was obtained from ethics committees at the Vajira Hospital and the Tropical Medicine Hospital, Mahidol University (2013-046-03).

Study Cohort, Blood Sample Collection, and Neutrophil Isolation

Index cases were included based on positive nested-RT-PCR for dengue viral RNA (serotype 1, 2, 3, or 4). Severity of DENV infection was classified into DF or DHF according to WHO criteria (1). Household members who did not show signs of DENV infection, and for which DENV could not be detected by nested-RT-PCR in the serum, served as healthy controls. The number of patients and control subjects used at least once for each assay are presented in **Table S1**. Blood samples from index cases were collected daily from admission until day of defervescence, and were further classified according to the phase of infection using clinical symptoms. Febrile samples (Feb DENV) refer to specimen collected on days of high fever until the fever subsided (Defervescence, Def DENV). Convalescent samples (Con DENV) were collected 2 weeks following the first admission, when patients were fully recovered. All functional *ex vivo* experiments were performed on the day of blood collection. For western blot, neutrophil pellets were dried and stored at −80°C. In addition, serum was prepared and stored at −80°C. To isolate neutrophils, fresh heparinized blood samples were centrifuged (22°C, 800 g, 10 min) to separate cells from plasma. Cell suspensions were diluted in RPMI-1640 medium supplemented with 2% fetal bovine serum (FBS) (Gibco, MA, USA) before isolation on an Isoprep layer (Robbins Scientific Corporation, CA, USA). The pellet containing red blood cells (RBCs) and granulocytes was subjected to RBC lysis via the addition of a hypotonic NaCl solution (0.2%), cells were

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Abbreviations: Feb DENV, Febrile phase of Dengue virus infection; Def DENV, Defervescent phase of Dengue virus infection; Con DENV, Convalescent phase of Dengue virus infection; Acu DENV, Acute phase of Dengue virus infection; tH3, Total histone H3; CitH3, Citrullinated histone H3.

incubated for 30 s before adding an equal volume of 1.6% NaCl. Recovered cell pellets were washed and resuspended in RPMI 1640 completed with 0.5% FBS. Giemsa staining and FACS staining were performed and showed that the pellets contained routinely more than 95% neutrophils.

Flow Cytometry

White blood cells were separated from heparinized whole blood samples using RBC lysis buffer (Biolegend, CA, USA). After washing with PBS, recovered white blood cells were incubated with fluorophore-conjugated antibodies against CD11b (PEcy7, #557743, BD Pharmigen) and CD66b (FITC, #555724, BD Pharmigen), or the corresponding isotype controls (BD Bioscience). Samples were acquired on a BD FACS Canto II. Granulocytes were gated using FSC/SSC, as well as double expression of CD11b and CD66b. Data were analyzed using the Flowjo v.8.7 software (Treestar, USA). Delta mean fluorescence intensity (MFI) was determined by subtracting the background fluorescence of the isotype control from specific MFIs of each pair of antibodies.

Detection of Reactive Oxygen Species *ex vivo*

The level of neutrophil intracellular reactive oxygen species (ROS) was measured *ex vivo* by flow cytometry using dihydrorhodamine (DHR) 123 (Invitrogen, MA, USA). DHR 123 is converted into green fluorescent rhodamine 123 by hydrogen peroxide. Following RBC lysis, fresh heparinized blood samples were incubated in the presence of DHR 123 for 15 min at 37°C, with or without PMA. ROS level was determined based on green fluorescence intensity of neutrophils gated on FSC/SSC using flow cytometry.

Immunofluorescence

The protocol for NET visualization by immunofluorescence was as previously described (15, 21). Briefly, 2×10^5 cells in RPMI-1640-0.5% FBS were seeded onto a round glass coverslip in a 24-well plate. Cells were allowed to adhere for 10 min before addition of PMA at various concentrations for 2 h (37°C, 5% CO_2). Cells were then fixed with 4% paraformaldehyde, and were permeabilized with PBS containing Triton-X 100 (0.5%). Nuclear morphology and NETs were stained using Sytox Green (1 μM, ThermoFisher, MA, USA), Hoechst 3342, or Hoechst and an anti-MPO antibody (Santa Cruz, sc-52707, 1:500). Cells were mounted onto slides, and were observed using a Nikon Eclipse 80i microscope. The percentage of NET formation was quantified by counting cells with decondensed/rounded nuclei and extracellular Sytox green positive fibers in at least three randomly pictured fields.

MPO-DNA ELISA

MPO-DNA ELISA was adapted from Caudrillier et al. (22). Briefly, flat bottom 96-well plates (Thermofisher Scientific, MA, USA) were coated with an anti-MPO polyclonal antibody (1:500, #2329755, Millipore, CA, USA) at 4°C overnight. Plates were washed with 0.05% Tween in PBS three times before addition of 20 μL patient serum pre-mixed with anti-DNA peroxidase antibody (1:80, Cell Death ELISA Plus kit, Roche, Germany).

Cells were allowed to incubate at room temperature in the dark for 3 h under agitation. Plates were then washed five times prior to addition of the substrate Reserve™ TMB (KPL, PA, USA). The reaction was stopped by adding an equal amount of 1 N HCl; O.D. (450 nm) was measured using a microplate reader (EZ400, Biochrom, UK).

Detection of Plasma Cytokines

EDTA plasma (25 μL) from healthy controls, DF, and DHF patients with acute infection were analyzed for a panel of 38 cytokines including IL-8 and TNFα by Luminex bead-based multiplex assay using the Milliplex® MAP human cytokine/chemokine magnetic bead panel kit (Millipore, USA). The FlexMAP 3D (Luminex®) platform was used according to manufacturer's instructions.

Neutrophil Elastase ELISA

Plasma level of Neutrophil Elastase was measured with a Human PMN Elastase ELISA Kit (Abcam) following manufacturer's instruction. Briefly, standard human NE and 1:100 plasma samples were incubated on 96-well plate pre-coated with polyclonal antibody against human NE in duplicates. The NE was then probed by the addition of HRP-conjugated anti-Human alpha1-P1 polyclonal antibody. After the addition of TMB for 20 min, followed by stop solution, the O.D. was read at 450 nm using a microplate reader (EZ400, Biochrom, UK). The average reading between duplicate wells subtracted by blank was used to calculate the plasma NE concentration (ng/ml).

Cell Lysis and Histone Extraction

Neutrophils were frozen immediately after isolation, and were kept at −80°C until use. Frozen cell pellets were lysed in low salt buffer (20 mM Hepes, 1 mM Sodium EDTA, 1 mM Sodium EGTA, 500 mM KCl, 50 mM PMSF, and 75 mM $MgCl_2$) in a protease inhibitor cocktail (cOmplete ULTRA, Roche, USA). After centrifugation (16,000 g, 4°C, 15 min), pellets containing the nuclear and membrane fractions were treated overnight with 0.5 N HCl at 4°C to extract histones. Histones present in the HCl soluble fraction were then precipitated with TCA acid (33.33%), and were washed with cold acetone. The acid-insoluble fractions were also collected for detection of PAD4. Protein concentration was determined by the Bradford protein assay (Biorad, CA, USA).

Detection of Citrullinated Histone H3 and PAD4 by Western Blot

Histone extracts were separated on a 14% acid-urea gel, as previously described (23). Proteins were then transferred onto a PVDF membrane in 0.7% acetic acid buffer. Alternatively, acid-insoluble nuclear fractions were separated by 10% SDS-PAGE before transferring onto a PVDF membrane using a semi-dry blotting system (Biorad, CA, USA). Membranes were blocked with 5% skimmed milk in TBS-0.2% Tween for 1 h, and were incubated with rabbit anti-citrullinated histone3 antibodies (1:2,000, #ab5103, Abcam, Cambridge, UK) for histone extracts or mouse anti-PAD4 antibodies (1:2,000, #ab57167, Abcam, Cambridge, UK) for acid-insoluble nuclear fractions. Membranes were stripped and probed with rabbit anti-total histone3 (Abcam, #1791) for loading control. Chemiluminescence was captured

using a GBox (Syngene, Cambridge, UK), and band intensity was quantified by densitometry using the ImageJ software (NIH).

Cell Culture and DENV Propagation

C6/36 cells were maintained at 25°C in L-15 medium supplemented with tryptose phosphate broth and 10% FBS. At 80–90% confluency, DENV-2 diluted in 2% FBS L-15 (MOI:0.01) was added to the C6/36 monolayer at room temperature under gentle agitation. The cell monolayer was washed to remove unbound viruses prior to addition of fresh 2% FBS L-15. Cell supernatant was harvested 3 and 5 days post-infection, and the viral titer was determined by FFU assay.

Coincubation of Neutrophils With DENV *in vitro*

Neutrophils isolated from healthy volunteers (2×10^5) were placed onto a glass coverslip in a 24-well plate, and were incubated with 500 μL RPMI-2% autologous serum for 10 min. The supernatant was removed prior to addition of the virus (DENV2, MOI 1:1). Alternatively, neutrophils were plated for 10 min before PMA (100 nM) was added for 3 h to induce NET formation. The supernatant was then carefully removed, and the virus was added. The virus was incubated for 10, 30, or 180 min with neutrophils or NETs. At the end of the incubation period, supernatants were collected, and viral titers were determined by FFU. Coverslips were recovered, fixed (4% paraformaldehyde), and stained with Hoechst 33342 (Thermo-Fisher Scientific).

Foci Forming Unit (FFU) Assay

BHK-21 cells were maintained at 37°C in DMEM with 10% FBS. Ten-fold serial dilutions of C6/36 cell supernatants were incubated with 3×10^5 BHK cells in 96-well plates at 37°C for 90 min. This was followed by addition of 1.5% carboxymethyl cellulose (CMC), and cells were incubated at 37°C for 3 days. After fixation with 3.7% paraformaldehyde, cells were permeabilized with 0.1% TritonX and washed with PBS. DENV-specific anti-E antibody (4G2) was added for 3 h at 37°C before washing with PBS. Cells were stained with anti-mouse Ig secondary antibody tagged with HRP for 45 min at 37°C, and washed three more times with PBS. Diammonium phosphate (DAP) was added at room temperature for color visualization; FFU were counted under a light microscope.

Statistical Analysis

Statistical analyses were performed using the Prism version 5.0 software. The Mann-Whitney U-test was a used to compare the median between two different groups of donors (e.g., healthy control vs. DENV infected patients). The Wilcoxon-signed rank test was used to compare the median obtained from the same patient at two different time points (e.g., febrile vs. convalescent phase). Differences between more than two groups of samples were determined by Kruskal–Wallis tests, followed by Dunn's *post-hoc* tests. Correlation between the two sets of data obtained from the same donor (e.g., level of CD66b expression vs. percent spontaneous delobulation) was evaluated by the Spearman-rank correlation test.

RESULTS

Neutrophils Were Stimulated During Acute DENV Infection

To identify and assess the phenotype of neutrophils in whole blood, we measured expression levels of the activation markers CD11b and CD66b by flow cytometry (24, 25). No significant change was observed in CD11b expression (**Figures 1A,B**). However, CD66b expression was markedly increased in febrile patients (Feb DENV) as compared with that of healthy controls ($p < 0.001$) (**Figure 1C**); this difference was maintained until defervescence (Def DENV, **Figure 1D**). At convalescence, CD66b level was significantly decreased ($p < 0.001$), and was comparable to that of healthy donors (**Figure 1C**). The DENV infection-associated CD66b upregulation was found in both mild (DF) and severe (DHF) patients (**Figures 1C,D**). We did not observe any correlation between CD66b expression and viral titer or virus serotype (data not shown). In line with CD66b upregulation, PMA induced a stronger respiratory burst in neutrophils from febrile patients as compared with that in convalescent and healthy individuals (**Figure 1E**), respiratory bursts are characteristic of primed neutrophils (26). Altogether, these results suggested that neutrophils are activated during acute DENV infections.

Neutrophil Activation During DENV Infection Resulted in Increased Nuclei Delobulation

ROS are required for NET formation in response to various stimuli (27, 28). As high levels of cell-free DNA are associated with severe DENV infection (29), and elevated levels of NET components, myeloperoxidase, and neutrophil elastase, have been found during the acute phase of the infection (18, 19), we hypothesized that neutrophils form NETs during DENV infections. We found that nuclei of unstimulated healthy neutrophils remained lobulated after 2 h, as did the cells for all conditions before incubation (**Figure S1**). In contrast, nuclei of unstimulated neutrophils from acute, but not convalescent, patients were delobulated after 2 h ($p < 0.01$) (**Figures 2A,C**). Moreover, the percentage of delobulated cells was greater in acute DENV neutrophils as compared with that of healthy and convalescent cells for each PMA concentration tested (**Figure 2B**). The presence of PMN with delobulated nuclei was not due to contaminating cells as shown by FACS and Giemsa staining prior incubation (**Figure S1**). In healthy human neutrophils, the increased number of cells with delobulated nuclei was associated with the release of NETs in unstimulated cells and in response to LPS and PMA (**Figure S2**). The loss of lobes has been shown to be an early mark of NET formation (27, 28, 30). This observation suggested a possible increased propensity for acute DENV neutrophils to form NETs. Interestingly, cell expression of CD66b and percentage of nuclei lobule loss were positively correlated in matched donors ($p < 0.01$) which suggested that CD66b may be an early marker of NETs during DENV infection (**Figure 2D**). No association was observed between the expression of CD11b
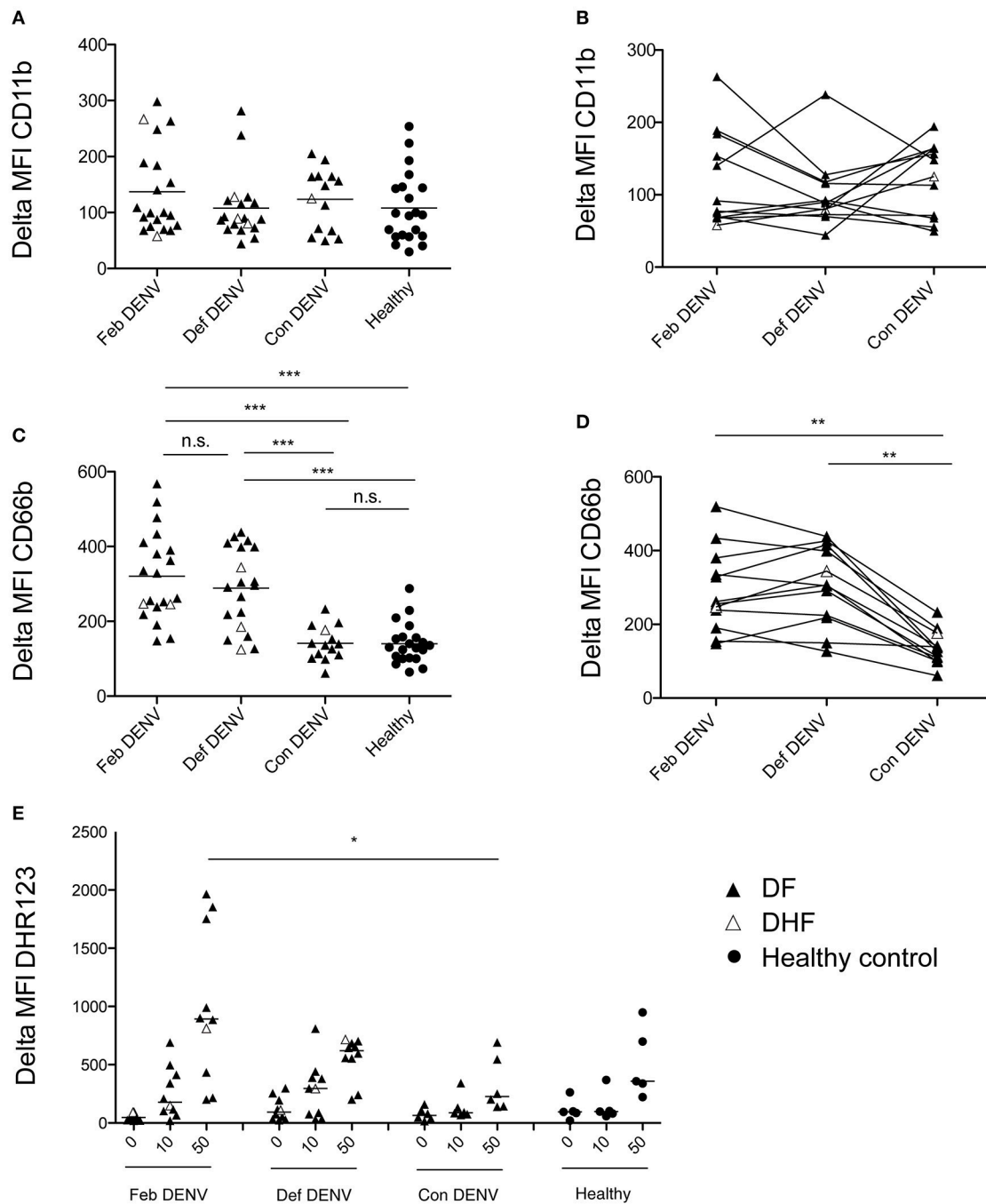
FIGURE 1 | Neutrophil activation during acute DENV infection. Delta MFI of CD11b **(A)** and CD66b **(C)** in healthy donors ($n = 21$), during febrile illness (Feb DENV) ($n = 21$), defervescence (Def DENV) ($n = 20$), and convalescence (Con DENV) ($n = 14$). Bars represent the mean of each population. ***$p < 0.001$, as assessed by the Kruskal-Wallis test, followed by Dunn's *post-hoc* test. Neutrophil CD11b **(B)** and CD66b **(D)** surface expression at the different phase of the disease in individual patients. **$p < 0.01$, assessed by the Wilcoxon-signed rank test. **(E)** Granulocytes were gated by size and granularity prior to ROS production analysis by flow cytometry. Dot plots show level of ROS production by the mean fluorescent intensity of DHR-123 in Feb DENV ($n = 10$), Def DENV ($n = 10$), Con DENV ($n = 6$), and healthy controls ($n = 5$). *$p < 0.05$, assessed by the Kruskal–Wallis test, followed by Dunn's *post-hoc* test. In all panels, DF patients are represented by a dark arrowhead, DHF patients by a white arrowhead, and healthy control by a dark circle.

or CD16 and spontaneous delobulation (data not shown). To assess if PMN delobulation and activation were observable *in vivo*, the SSC values of neutrophils for healthy controls and DENV patients obtained at different time were analyzed. The SSC mean fluorescence intensity for febrile DENV patient neutrophils (442.7 ± 72.62) was lower compared to healthy
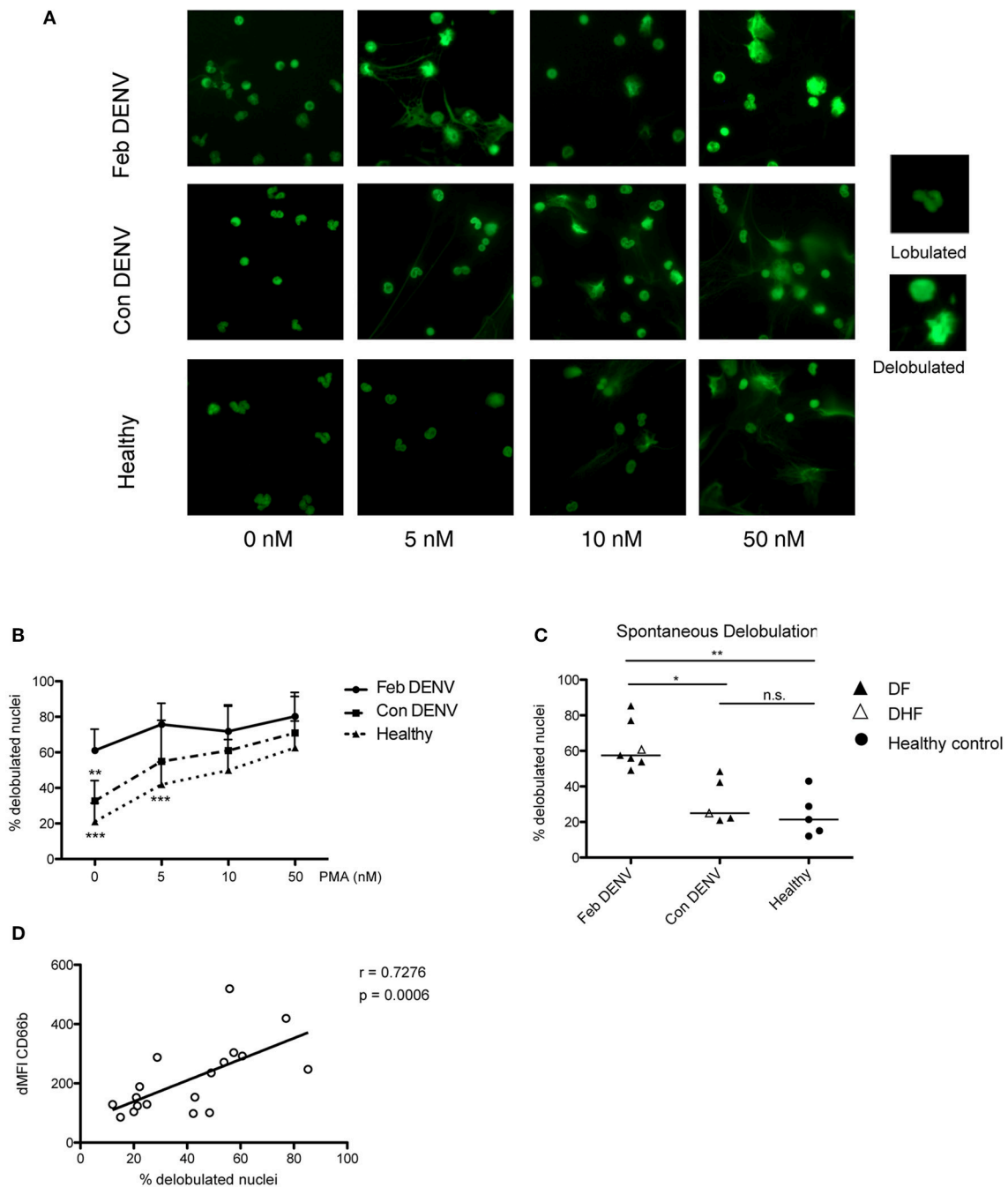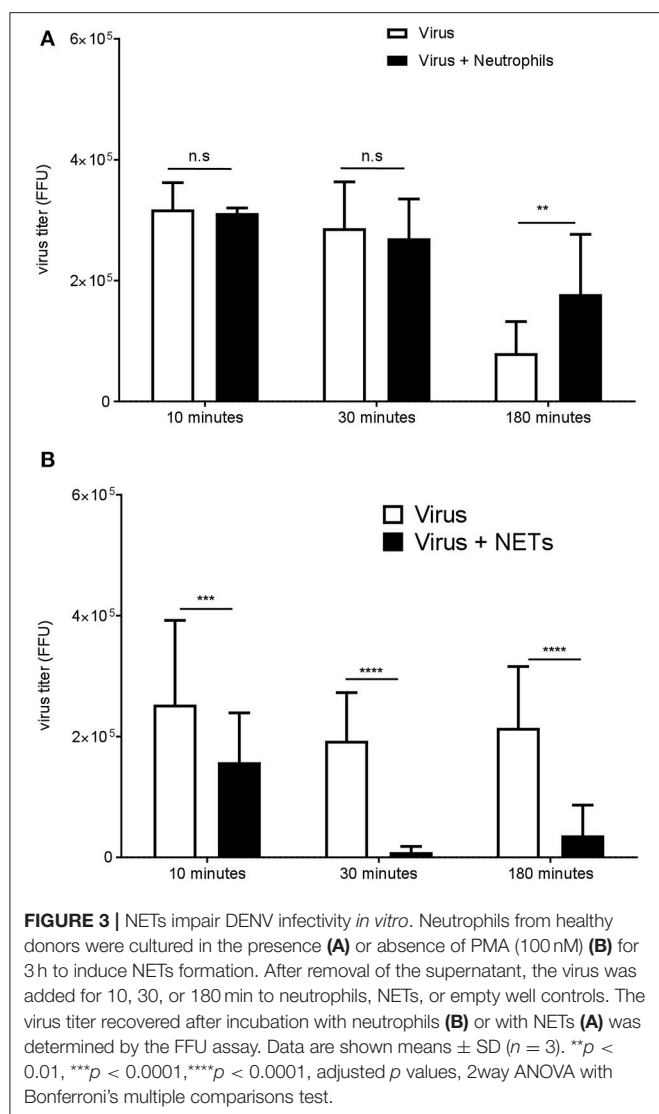
FIGURE 2 | Neutrophils from acute DENV infected patients are susceptible to NET formation. Isolated neutrophils from healthy donors and DENV infected patients during febrile illness (Feb DENV) and convalescent phase of the disease (Con DENV) were cultured for 2 h without stimulation or with 5, 10, or 50 PMA. The cells were fixed, permeabilized, and stained with the DNA dye Sytox green, and were visualized with a fluorescent microscope. **(A)** Representative images of nuclear morphologies of isolated neutrophils from Feb DENV, Con DENV, and healthy donors in response to varying concentration of PMA stimulation. Representative high magnifications of lobulated and delobulated nuclei are shown on the right. **(B)** Average percentage of cells with delobulated nuclei from Feb DENV ($n = 7$), Con DENV ($n = 5$), and Healthy donors ($n = 5$) in response to varying PMA concentration. Error bars represent mean ± SEM, $**p < 0.01$, $***p < 0.001$, 2way ANOVA with Bonferroni's multiple comparisons test. **(C)** Percentage of cells undergoing nuclei decondensation without PMA stimulation from Feb DENV ($n = 7$), Con DENV ($n = 5$), and healthy donors ($n = 5$). Bars represent mean of each group. $*p < 0.05$, $**p < 0.01$, analyzed by Kruskal–Wallis test followed by Dunn's *post-hoc* test. **(D)** Correlation between cells with decondensed nuclei with CD66b expression in matched donors, as determined by Spearman-ranked correlation ($r = 0.620$, $p = 0.0010$, $n = 17$).

FIGURE 3 | NETs impair DENV infectivity *in vitro*. Neutrophils from healthy donors were cultured in the presence **(A)** or absence of PMA (100 nM) **(B)** for 3 h to induce NETs formation. After removal of the supernatant, the virus was added for 10, 30, or 180 min to neutrophils, NETs, or empty well controls. The virus titer recovered after incubation with neutrophils **(B)** or with NETs **(A)** was determined by the FFU assay. Data are shown means ± SD ($n = 3$). **$p < 0.01$, ***$p < 0.0001$,****$p < 0.0001$, adjusted p values, 2way ANOVA with Bonferroni's multiple comparisons test.

controls (481.7 ± 89.74), but the difference was not statistically different (**Figure S3**).

NETs Impaired DENV Infectivity *in vitro*

NETs have been shown to trap and inhibit viruses *in vitro*, and can prevent viral dissemination *in vivo* (10, 31). As DENV neutrophils were prone to spontaneous formation of NETs *in vitro*, we investigated whether PMA-induced NETs from healthy neutrophils could inactivate DENV (**Figure 3A**). After 30 min of incubation, DENV titer was dramatically decreased in the presence of NETs as compared with that when incubated with the virus alone or in the presence of unstimulated healthy neutrophils (**Figure 3B**). This suggested that NETs could trap or inactivate the virus *in vitro*.

Elevated IL-8 and TNFα Were Associated With Severe DENV Infection

To investigate whether neutrophil activation was correlated with a proinflammatory environment, we measured plasma levels of

IL-8 and TNFα, cytokines known to activate neutrophils, during the febrile phase of DENV infection. We found significantly elevated IL-8 in DHF patients as compared with that of healthy controls ($p < 0.001$) and DF patients ($p < 0.001$) (**Figures 4A,B**). TNFα was significantly increased in DF and DHF patients as compared to healthy controls ($p < 0.05$, $p < 0.001$); however, no significant difference was found between DF and DHF patients. We also measured the level of Neutrophil Elastase, a maker of neutrophil activation, degranulation and NETosis, in the serum of patients (**Figures 4C,D**). We observed a slight increase of NE in the serum of DHF patients during the febrile phase as compared to convalescence, while the difference at the two time points was less obvious for DF patients (**Figure 4C**). The level of NE was significantly increased in Acute Dengue (DF and DHF) as compared to convalescence ($p < 0.0411$) (**Figure 4D**).

Elevated NET-Derived Products Were Associated With Severe DENV Infection

Nuclei delobulation (**Figure 2**) and change in SSC (**Figure S3**) were indirectly suggesting DENV neutrophils increased susceptible to NETosis. To determine whether NETs were produced during Dengue virus infection, and the association of NETs with clinical outcomes of DENV infection, we measured the level of MPO-DNA complexes in the serum of DENV-infected patients by ELISA (22). *Ex vivo* experiments indicated that febrile DENV infections increased susceptibility to spontaneous delobulation regardless of disease severity (**Figure 2**). However, serum levels of MPO-DNA complexes were elevated during febrile DENV infection in DHF patients, but not in DF patients, when compared with those in convalescent individuals (**Figures 5A–C**). Therefore, excessive NETs may be an aggravating factor that enhances disease severity during an acute DENV infection.

Histone H3 Citrullination Was Suppressed During Acute DENV Infection

Protein arginine deiminase 4 (PAD4)-dependent histone citrullination is one of the pathway responsible for NET formation in microbial infections, systemic inflammations, and autoimmune diseases (32). We measured levels of Histone H3 citrullination (**Figure S4**) and PAD4 (**Figure S5**) in nuclear extracts of frozen neutrophils from DENV patients by western blot. Surprisingly, histone H3 citrullination was markedly decreased during the acute phase of DENV infection as compared with that during the convalescent stage and in healthy controls (**Figure S4**). PAD4 level was also slightly decreased during the acute phase of the infection compared with that during convalescence, albeit non-significant (**Figure S5**).

DISCUSSION

Evidences of excessive inflammation during DENV infection suggested that activation of neutrophils and formation of NETs may be involved in disease pathogenesis (33). Due to access to neutrophil samples from DENV-infected patients, we assessed
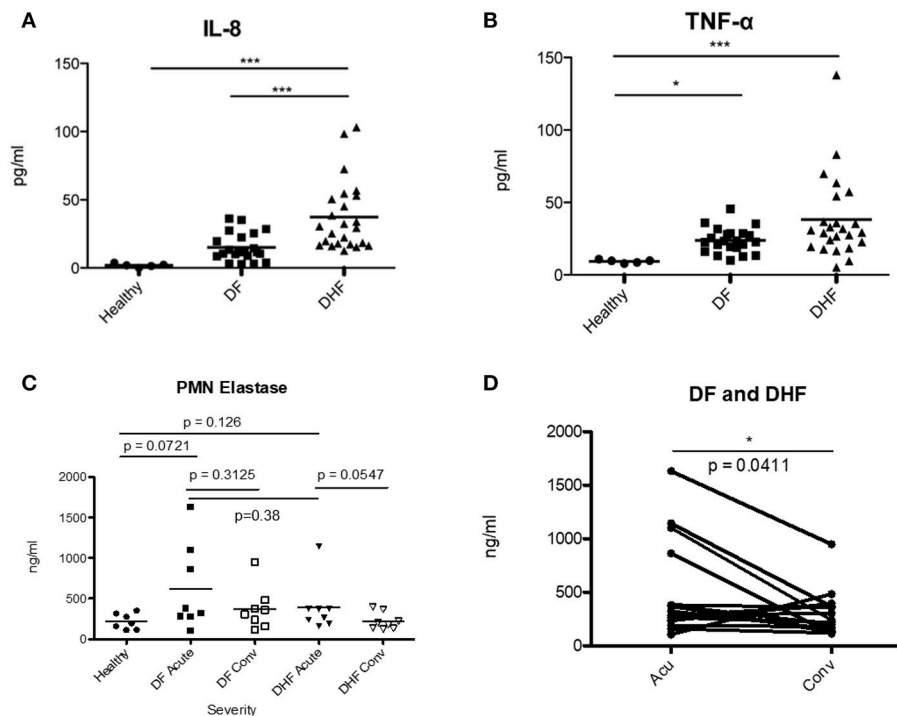
FIGURE 4 | Elevated levels of IL-8, TNFα and Neutrophil Elastase during acute DENV infection. Plasma samples from healthy donors (healthy), mild (DF) and severe (DHF) DENV infected patients during febrile illness (Feb DENV) and convalescent phase (Con DENV) were used. **(A)** Plasma level of IL-8 and **(B)** TNFα during febrile DENV infection. *$p < 0.05$ and ***$p < 0.001$, Kruskal–Wallis test followed by Dunn's *post-hoc* test. **(C)** Level of NE (ng/ml) in the serum of healthy, DF and DHF patients during the febrile and convalescence phase. Mann–Whitney *U*-test. **(D)** Level of NE in the plasma of acute DENV patients (DF and DHF) vs. convalescent patients. NE concentration from the same patients are connected with lines. Wilcoxon-signed rank test, *$p < 0.05$.

both the phenotype and function of neutrophils in naturally-infected DENV patients. We showed that neutrophils were primed *in vivo* (**Figures 6i–iii**), and were prone spontaneous delobulation. We also highlighted the dual roles of NETs in inhibiting DENV, as well as its possible contribution to disease severity.

Neutrophils overexpressed CD66b during acute DENV infection. CD66b is a marker of granulocyte activation involved in adhesion to endothelial cells (34), degranulation (35), and increased reactive oxygen species (ROS) production (34). Similar CD66b upregulation has been reported in patients during bacterial sepsis, a condition involving systemic inflammation (36). We did not observe CD11b overexpression associated with DENV infection. Interestingly, an increase in the CD66b:CD11b ratio has been reported for neutrophils in the presence of *S. aureus*, and this was associated with their reduced ability to phagocytosed (37). Our findings also confirm, at the protein level, the CD66b overexpression reported at the transcriptional level during DENV infection (18). In line with the elevated CD66b expression, we found robust ROS production by granulocytes in response to *ex vivo* PMA stimulation during acute dengue infection. This could be the result of priming by the pro-inflammatory environment found during acute dengue virus infection, as TNFα and IL-8, cytokines known to prime neutrophils for ROS production, were also found elevated in

dengue patients as previously reported (38) and in our own study cohort (**Figures 4A,B**). ROS are essential both for neutrophil antimicrobial activity and for ROS-dependent NET formation (27, 39).

Emerging evidence has supported the presence and role of NETs in viral infections. Direct activation of neutrophils to form NETs by viral particles has been demonstrated in HIVs (31), RSVs (40), and hantaviruses (15). We provide evidences, in the present study, that during DENV infection, neutrophils are more susceptible to spontaneous delobulation *ex vivo*. Delobulation is an early feature of NET formation, but is not limited to the release of NETs and is found in other form of cell death. However, we confirmed that the limited spontaneous NETosis of healthy neutrophils, detected with an anti-PMO antibody, was also associated with the increased percentage of spontaneous delobulation (**Figure S2**). How DENV infection could result in such susceptibility remains to be elucidated. The ability of DENV particles to directly induce formation of NETs varies from one study to another. While Yost et al. showed that DENV could induce production of NETs in healthy neutrophils (20), Moreno-Altamirano et al. demonstrated that DENV-2 inhibited NET formation in response to PMA stimulation by altering the glycolysis pathway (41). In the present study, we could not characterize the mechanisms responsible for the elevated delobulation or the
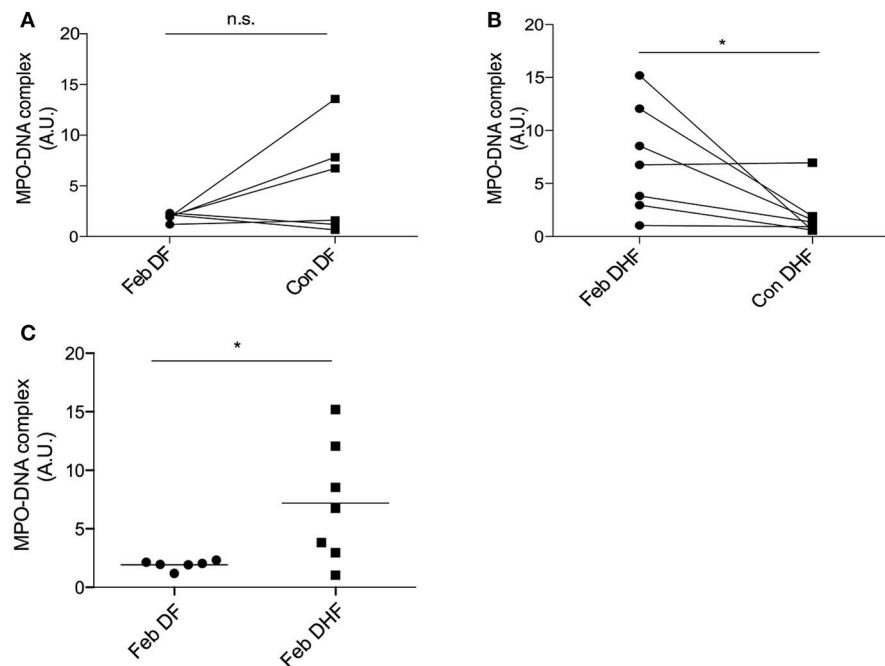
FIGURE 5 | NETs are associated with severe DENV infection. Serum samples from healthy donors as well as mild (DF) and severe (DHF) DENV infected patients during febrile illness (Feb DENV) and convalescent phase (Con DENV) were used for the MPO-DNA ELISA. **(A,B)** O.D. ratios from the same DF and DHF patients are connected with lines. Analysis was performed with the Wilcoxon-signed rank test, *$p < 0.05$. **(C)** Each symbol represents O.D. at 450 nm normalized to inter-experimental control. Mann–Whitney U-test, *$p < 0.05$.

increase MPO-DNA complexes detected *in vivo* in natural DENV infection. However, we proposed that elevated levels of pro-inflammatory cytokines IL-8 and TNFα, which were reported to be inducers of NETs (7, 27, 42, 43) during febrile DENV infection, could contribute to such susceptibility through the priming or the activation of neutrophils [**Figures 4A,B** and (4)]. Neutrophil-platelet interactions may also promote NET formation during DENV infections (44); studies have shown that neutrophil-platelet interactions promoted production of NETs in the bacterial sepsis model (45) and murine pox virus infections (10). Furthermore, DENV has been shown to activate platelets (46, 47). Of note, we could not amplify viral genome from neutrophils isolated from dengue patients (data not shown).

NETs have been described as powerful antimicrobial weapons as well as threats to the host (48). In this study, we found higher susceptibility for NET formation in acute DENV infections regardless of disease severity. In addition, NETs could potentially inhibit DENV *in vitro*. Possible antiviral immunity elicited by NETs include immobilization of viral particles, inhibition of virus by antimicrobial proteins (31), and possibly through NET-induced type I interferon production by plasmacytoid dendritic cells (49). The precise mechanisms that underlie NET-mediated DENV inhibition need to be further investigated.

We detected elevated levels of Neutrophil Elastase in the plasma of Acute infected patients (**Figures 4C,D**). While not significant, the differences seemed more marked for DHF patients as compared to DF patients. NE elastase is a marker of
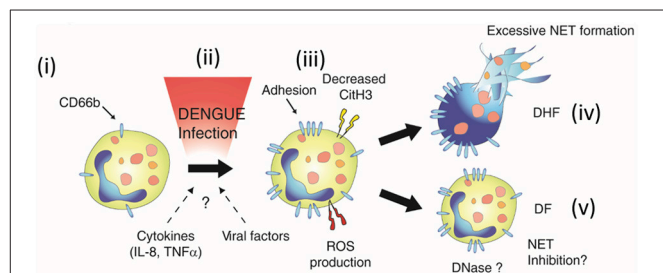

FIGURE 6 | Neutrophil activation during DENV infection. During dengue infection, resting neutrophils **(i)** are activated **(ii)**. This activation is characterized by the upregulation of CD66b, increased production of ROS **(iii)**, and might be due to the proinflammatory environment (IL-8, TNFa), or unidentified viral or host factors **(ii)**. Neutrophil activation was also associated *ex vivo* with increased spontaneous delobulation for both DF and DHF patients (**Figure 2**). However, markers of NETs were found elevated only in the serum of the severe form of dengue (DHF, **iv**). This suggested that either NETosis is inhibited by a unknown mechanism or NETs degraded in the serum of DF patients (NET Inhibition and DNASe, respectively) **(v)**, and that these regulatory mechanisms are absent in DHF patients, leading to excessive NET formation **(iv)**.

neutrophil degranulation, and also found on NETs. Interestingly, higher levels of NET-derived components, such as the MPO-DNA complex, were also found in the serum of DHF patients as compared with that in DF patients (**Figures 5A–C**). Previously, two independent studies suggested that cell-free DNA (29) and

plasma histone H2A (47), two other known components of NETs, were markedly elevated in severe form of DENV infection. These findings were in agreement with our observation, and showed that DENV infection may induce NET formation, and that elevated NET components are associated with severe disease.

The mechanisms underlying neutrophil activation and NET formation in dengue pathogenesis are currently unknown. Several studies have shown that canonical NETosis starts with the loss of neutrophil nucleus lobules (27, 28, 30). This is however not a proper marker of NETosis as other forms of cell death, such as leukotoxic hypercitrullination, can display similar features (50). However, the detection of NET products by ELISA in the serum of DENV patients, together with the low level of Histone citrullination, support an increased propensity of DENV neutrophils to release NETs. As we did not observe any difference in spontaneous formation of NETs between DF and DHF patients *ex vivo* (**Figure 2**), we propose that NET release may be inhibited, or that NETs were degraded in DF, but not DHF patients *in vivo* (**Figures 6iv,v**). It was proposed that metalloproteinases and histones in NETs may participate in vascular leakage in dengue infection by disrupting the vascular endothelial cell layer (51, 52). This suggest that the NET formation and degradation may affect the final outcomes of DENV infections. This phenomenon was also observed in SLE patients who exhibited excessive neutrophil-derived immune complex formation due to impairments in NET degradation (53).

Recent studies have suggested that PAD4 and histone citrullination-mediated NET formation is stimuli-dependent (50). Histone citrullination was found to be decreased and increased by PMA and ionomycin, respectively, however, both molecules stimulated NET formation (54). Little is known regarding NET formation in viral infections. However, PAD4-dependent NETs were not required for host protection during influenza infection *in vivo* (12). Similar to PMA-induced NET formation, we suggest that DENV-induced susceptibility to NET formation may be independent of PAD4 activity and histone H3 citrullination.

Targeting NETs using DNase have shown promising results in improving NET-mediated inflammation in the RV infection model (14) and in intestinal ischemic perfusion injury (55). Understanding the precise pathway responsible for NET formation in DENV infection, its regulation, and its impact on disease outcomes may shed light on potential therapeutic interventions for DENV infections in the future.

AUTHOR CONTRIBUTIONS

AO, PM, JM, AS, and FL designed the experiments. AO, PA, NS, NJ, and WC did the experiments. AO, PA, NS, and FL analyzed data. PM, PS, TY, SS, KM, and AS helped with sample collection and AO and FL wrote the manuscript.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fimmu.2018.03007/full#supplementary-material

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