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IgA-driven neutrophil activation underlies severe dengue disease after primary Zika virus infection in humans

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Supplemental Materials for

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Supplementary Methods

Description of studies

The **Pediatric Dengue Cohort Study (PDCS)** is an ongoing prospective cohort study initiated in 2004 and based at the Sócrates Flores Vivas Health Center (HCSFV), the primary health care facility for District II of Managua, and part of the Municipal Health System of Managua. Located adjacent to Lake Xoxitlan, District II encompasses neighborhoods spanning a spectrum of low to middle socioeconomic status. The HCSFV serves ~62,000 residents across 18 neighborhoods within District II[48]. The illiteracy rate in this area is approximately 7%, and over 90% of the households have access to running water and a sewage system. All residents have access to electricity. The PDCS tracks approximately 4,000 active participants yearly and has followed ~12,000 children since its initiation. Initial enrollment in 2004 was children 2-9 years old; adjustments to the age limit were implemented in 2008, 2013, 2018, 2019, and 2022, extending the eligibility to 11, 14, 15, 16, and 17 years, respectively. To preserve the original age distribution of the cohort, approximately 250 2-year-old children, along with children aged 3-11 years as needed, are enrolled annually. Participants benefit from free, round-the-clock 24/7 medical care provided by study physicians at the HCSFV. In cases requiring hospitalization, study staff facilitate patient transfer to the study hospital, the National Pediatric Reference Hospital (Hospital Infantil Manuel de Jesús Rivera). Children presenting with fever and, since July 2016, rash are screened for signs and symptoms of dengue, Zika, and chikungunya. Symptomatic infections are captured through "enhanced" passive surveillance, complemented with periodic home visits. Each suspected case is characterized with more than 250 demographic, clinical, laboratory, and imaging variables and, both acute (1-6 days post-symptom onset) and early convalescent samples (14-21 days) are collected to confirm infection. A standardized questionnaire is used to record the subject's demographic and epidemiologic characteristics and data digitized by double-data entry, with quality control checks performed daily and weekly. Approximately 97% of cases present to the HCSFV within the first three days of illness, and convalescent samples are collected from 94% of cases; similar statistics have been maintained to date. Additionally, the study collects annual healthy blood samples from all participants in March-April (previously in July from 2004 to 2010), which enables the detection of inapparent arboviral infections[11].

The **Pediatric Dengue Hospital-Based Study (PDHS)** is based at the Hospital Infantil Manuel de Jesús Rivera (HIMJR), Managua, Nicaragua (2005-present)[22,49]. Children are invited to participate if they: 1) present to the hospital 1-7 days post-onset of symptoms and 2) meet case definitions for dengue, Zika, and/or chikungunya, which consists of either i) ≥1 signs or symptoms, including headache, arthralgia, myalgia, retro-orbital pain, positive tourniquet test, petechiae, or signs of bleeding; or ii) rash with ≥2 signs/symptoms including fever, conjunctivitis, arthralgia, myalgia, or peri-articular edema[22]. Blood samples are collected at presentation (acute), daily for days 1-3, and at early convalescence (14-28 days). Clinical information (>250 variables) and fluid intake/output is collected every 12 hours, and, after systematic monitoring by a clinical supervisor, is digitized by double-data entry, with quality control checks performed daily and weekly. A standardized questionnaire is used to record the subject's demographic and epidemiologic characteristics. As part of a longitudinal arm of the study, consenting participants return to provide healthy blood samples 3, 6, 12, and 18 months post-illness.

Arboviral infections in both studies are identified using real-time RT-PCR, virus isolation, and DENV, ZIKV, and CHIKV serological assays (detailed in the main text).

Quantification of markers of neutrophil degranulation

Neutrophil degranulation and NETosis were assessed in plasma immediately after thawing, using commercially available kits following the manufacturers' protocols. Myeloperoxidase (MPO) and Matrix Metalloproteinase-9 (MMP9) concentrations in plasma were quantified using DuoSet™ ELISA kits (R&D Systems, DY3174 and DY911, respectively). Briefly, 96-well plates were coated with specific capture antibodies diluted in PBS (pH 7.2–7.4) and incubated overnight at room temperature. Plates were blocked with 1% BSA in PBS for 1 hour at room temperature. Samples and standards, diluted in PBS with 1% BSA (with 2% normal goat serum for MMP9), were added (100 µL/well) and incubated for 2 hours at room temperature. Detection antibodies conjugated to biotin were applied, followed by a 20-minute incubation with streptavidin-horse radish peroxidase (HRP). Signal development was achieved using tetramethylbenzidine (TMB), and the reaction was stopped with 2N sulfuric acid. Optical density (OD) was measured at 450 nm with background correction at 570 nm. Standard curves were generated using serial dilutions of recombinant MPO and MMP9, and sample concentrations were interpolated using linear regression.

Human neutrophil elastase (NE) concentrations in plasma were quantified using the Hycult Biotech commercial kit (HK319). Plasma samples were diluted and added to microtiter wells pre-coated with a human elastase-specific capture antibody. Standards and samples (100 µL/well) were incubated for 1 hour at room temperature. After washing four times with wash buffer, 100 µL of biotinylated tracer antibody was added, and the plate was incubated for another hour at room temperature. Following additional washes, 100 µL of streptavidin-peroxidase conjugate was applied, and the plate was incubated for 1 hour. Signal development was performed by adding 100 µL of TMB substrate and incubating for 30 minutes in the dark. The reaction was terminated with 2% oxalic acid, and absorbance was measured at 450 nm using a microplate reader.

Human lactoferrin levels in plasma were measured using the CatchPoint® SimpleStep ELISA® kit (ab229392, Abcam), which employs fluorescence detection. Plasma samples were processed following the manufacturer's recommendations. Standards were prepared by serial dilutions from a reconstituted lactoferrin stock solution. Standards and samples (50 µL each) were added to pre-coated wells, followed by 50 µL of an antibody cocktail containing capture and detector antibodies. Plates were incubated for 1 hour at room temperature on a plate shaker set to 400 rpm and subsequently washed three times with Wash Buffer. Detection was performed by adding 100 µL of CatchPoint HRP Development Solution, containing Stoplight Red Substrate and hydrogen peroxide, followed by a 10-minute incubation in the dark. Fluorescence intensity was measured at 530 nm (excitation), 570 nm (cutoff), and 590 nm (emission) using a fluorescence plate reader.

Neutrophil extracellular traps (NETs) in plasma samples were quantified using the GENLISA™ Human NETs ELISA Kit (KBH4548, Krishgen BioSystems). Plasma samples were diluted in the Standard Diluent provided. A standard curve was generated using serial dilutions of the NETs standard stock solution. Samples and standards (100 µL each) were

added to wells of a microtiter plate pre-coated with anti-NETs antibodies, followed by a 60-minute incubation at 37°C. After aspiration, 100 µL of Biotinylated NETs Antibody Working Solution was added, and the plate was incubated for another 60 minutes at 37°C. Wells were then washed four times with 1X Wash Buffer to remove unbound components. Next, 100 µL of Streptavidin-HRP Working Solution was added to each well and incubated for 30 minutes at 37°C. Following additional washes, 100 µL of TMB substrate was added to develop the signal during a 10-minute incubation in the dark at 37°C. The reaction was terminated by adding 100 µL of Stop Solution. Absorbance was measured at 450 nm using a microplate reader within 15 minutes of adding the Stop Solution.

A standard curve was generated by plotting absorbance or fluorescence values against known analyte concentrations, and sample concentrations were interpolated and adjusted for dilution factors. Background correction was performed by subtracting the blank (zero standard) signal from all optical density (OD) or fluorescence values. Samples exceeding the upper limit of quantification were reanalyzed at higher serum dilutions when possible; otherwise, data was analyzed as background-corrected OD or fluorescence values. All assays were conducted in duplicate, and results were reported as the mean of the technical replicates. Outliers were identified and removed using the Interquartile Range (IQR) method. Boundaries for identifying outliers were defined as follows: Lower Bound: Q1 – (1.5 × IQR); Upper Bound: Q3 + (1.5 × IQR), where Q1 and Q3 corresponded to the first and third quartiles, respectively. Observations with values outside these thresholds were considered outliers and excluded from further analysis.

Definition of severe dengue disease

Severe disease in our study was defined as a composite outcome of children meeting the WHO 1997 criteria for Dengue Hemorrhagic Fever or Dengue Shock Syndrome[47]. **Dengue Hemorrhagic Fever** was defined as experiencing at least one of each of the following criteria: fever plus 1) hemorrhagic manifestations defined as the presence of at least one of the following manifestations: i) positive tourniquet test, ii) ecchymosis, petechia, hematoma, purpura, iii) bleeding at injection site, hypermenorrhea or vaginal bleeding, subconjunctival hemorrhage, hematuria, hemoptysis, bleeding gums or nose, iv) hematemesis or melena; 2) thrombocytopenia defined as platelet count $\leq$100,000 platelets per mm^3; 3) evidence of plasma leakage as a result of increased vascular permeability defined as i) $\geq$20% hemoconcentration, ii) age- and sex-specific elevated hematocrit, iii) pleural effusion, iv) ascites, v) hypoproteinemia or hypoalbuminemia, or vi) edema. **Dengue Shock Syndrome** (1997 WHO criteria) was defined as cases meeting criteria for DHF and at least one of each of the following criteria: 1) i) age-specific hypotension or ii) narrow pulse pressure <20 mmHg), and 2) i) cold, clammy skin and restlessness or ii) rapid and weak pulse. Symptomatic participants infected with DENV2 who did not meet the criteria for severe disease were categorized as having **Dengue Fever (DF)**.

Outcome	n	% Male	Mean Days	Mean Age	Leukocyte count		Platelet count	
					Mean	SD	Mean	SD
Pediatric Dengue Cohort study								
DF	14	42.86	1.5	10.44	3.31	0.88	129.57	40.59
DHF-DSS	10	40.00	1.9	10.67	2.94	0.83	43.20	21.34
Inapparent	17	53.00	-	9.11	-	-	-	-
Pediatric Dengue Hospital study								
DF	36	47.22	2.61	9.41	3.02	0.96	127.14	55.73
DHF-DSS	18	44.44	2.39	10.49	2.41	0.62	49.16	28.95

Table S1. Clinical and laboratory characteristics of pediatric dengue cases in the cohort and hospital-based studies. Summary of demographic, clinical, and laboratory features of children with dengue fever (DF) or dengue hemorrhagic fever-dengue shock syndrome (DHF-DSS) in the Pediatric Dengue Cohort Study and Pediatric Dengue Hospital Study. For each outcome group, the table presents the total number of cases (n), percentage of male participants, mean time to sample collection post-fever onset (days), mean age (in years), mean leukocyte count ($10^3/\mu L$) with standard deviation (SD), and mean platelet count ($10^3/\mu L$) with standard deviation (SD).

Supplementary Video 1. NETosis ex-vivo assay for E antigen, mock-depleted condition
Representative video of neutrophil activation from an *ex-vivo* experiment using polyclonal serum of a participant who subsequently developed DHF-DSS. Shown under the mock-depleted condition in the context of E antigen.

Supplementary Video 2. NETosis ex-vivo assay for E antigen, IgA-depleted condition
Representative video of the same participant who developed DHF-DSS, showing neutrophil activation after IgA depletion in the context of E antigen.

Supplementary Video 3. NETosis ex-vivo assay for NS1 antigen, mock-depleted condition
Representative video of neutrophil activation from the same participant (DHF-DSS case) under the mock-depleted condition in the context of NS1 antigen.

Supplementary Video 4. NETosis ex-vivo assay for NS1 antigen, IgA-depleted condition
Representative video of the same participant who developed DHF-DSS, showing neutrophil activation after IgA depletion in the context of NS1 antigen.