Microparticles in Dengue Infection Provide a Novel Biomarker to Predict Severe Clinical Outcomes

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Microparticles Provide a Novel Biomarker To Predict Severe Clinical Outcomes of Dengue Virus Infection

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ABSTRACT

Shedding of microparticles (MPs) is a consequence of apoptotic cell death and cellular activation. Low levels of circulating MPs in blood help maintain homeostasis, whereas increased MP generation is linked to many pathological conditions. Herein, we investigated the role of MPs in dengue virus (DENV) infection. Infection of various susceptible cells by DENV led to apoptotic death and MP release. These MPs harbored a viral envelope protein and a nonstructural protein 1 (NS1) on their surfaces. *Ex vivo* analysis of clinical specimens from patients with infections of different degrees of severity at multiple time points revealed that MPs generated from erythrocytes and platelets are two major MP populations in the circulation of DENV-infected patients. Elevated levels of red blood cell-derived MPs (RMPs) directly correlated with DENV disease severity, whereas a significant decrease in platelet-derived MPs was associated with a bleeding tendency. Removal by mononuclear cells of complement-opsonized NS1–anti-NS1 immune complexes bound to erythrocytes via complement receptor type 1 triggered MP shedding *in vitro*, a process that could explain the increased levels of RMPs in severe dengue. These findings point to the multiple roles of MPs in dengue pathogenesis. They offer a potential novel biomarker candidate capable of differentiating dengue fever from the more serious dengue hemorrhagic fever.

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- **DENV infection leads to apoptotic death and MP shedding**

intracellular NS1 staining

AnV labeling

HepG2, 5 MOI, 2 days postinfection

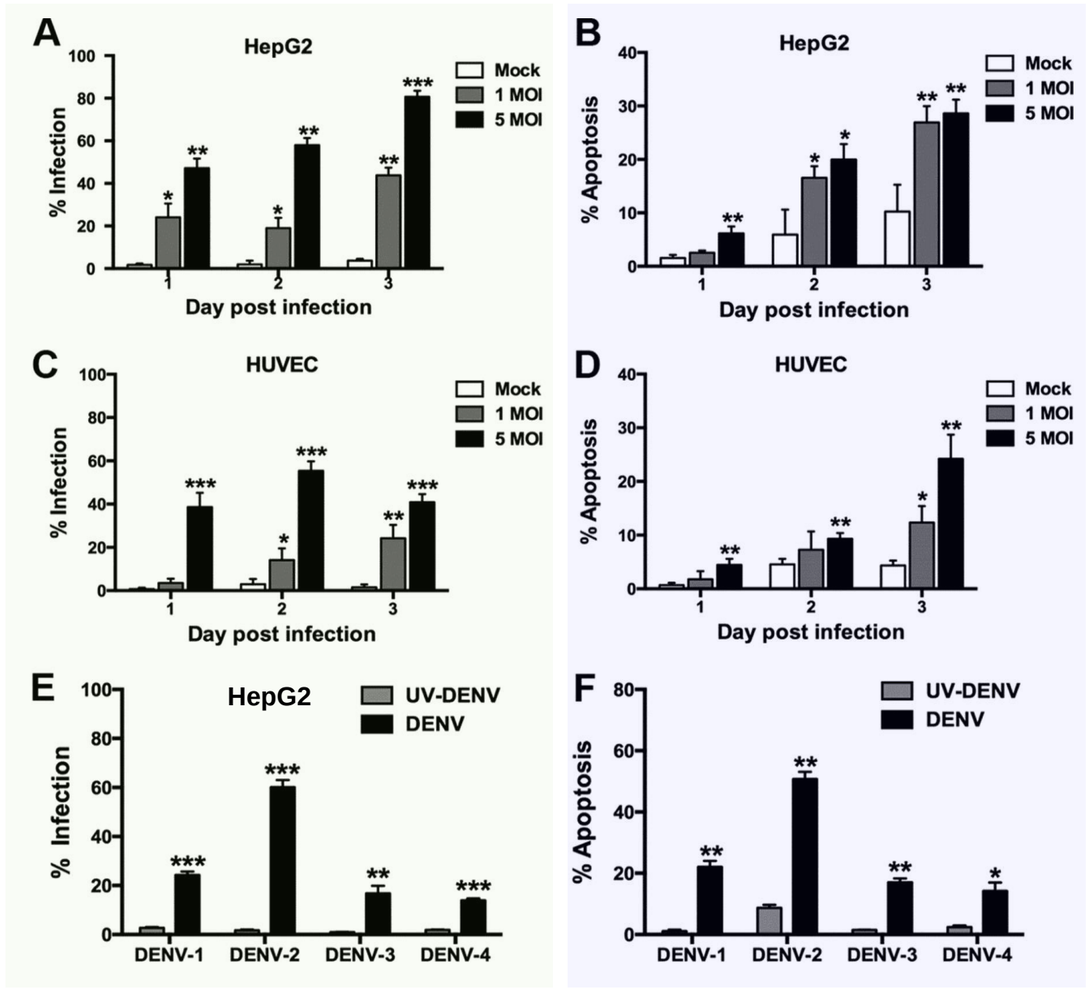

FIG 1. DENV infection causes apoptotic cell death.
Data are the mean ± SD from three to four independent experiments.
Asterisks denote statistically significantly differences between infected or apoptotic cells and mock-infected cells (*, P < 0.05; **, P < 0.001; ***, P < 0.0001).

Histogram and density plots showing the gating protocol for MPs

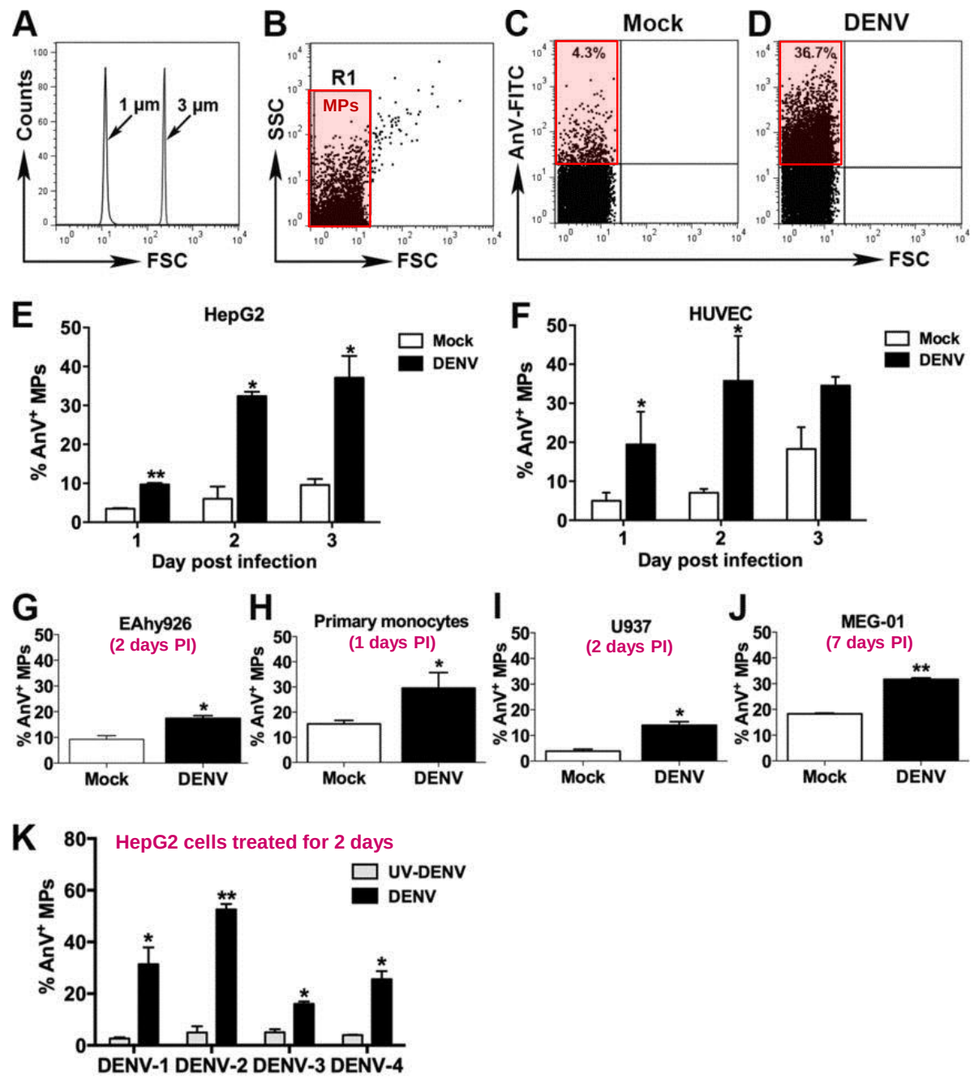

Fig 2. DENV infection induces MP production from various cell types.
All cell types were infected by DENV at an MOI of 5 Data are the mean ± SD from three to 4 independent experiments.
Asterisks denote statistically significantly differences between the percentage of AnV+ MPs produced by DENV-infected cells and the percentage produced by mock-infected cells (*, P < 0.05; **, P < 0.001).

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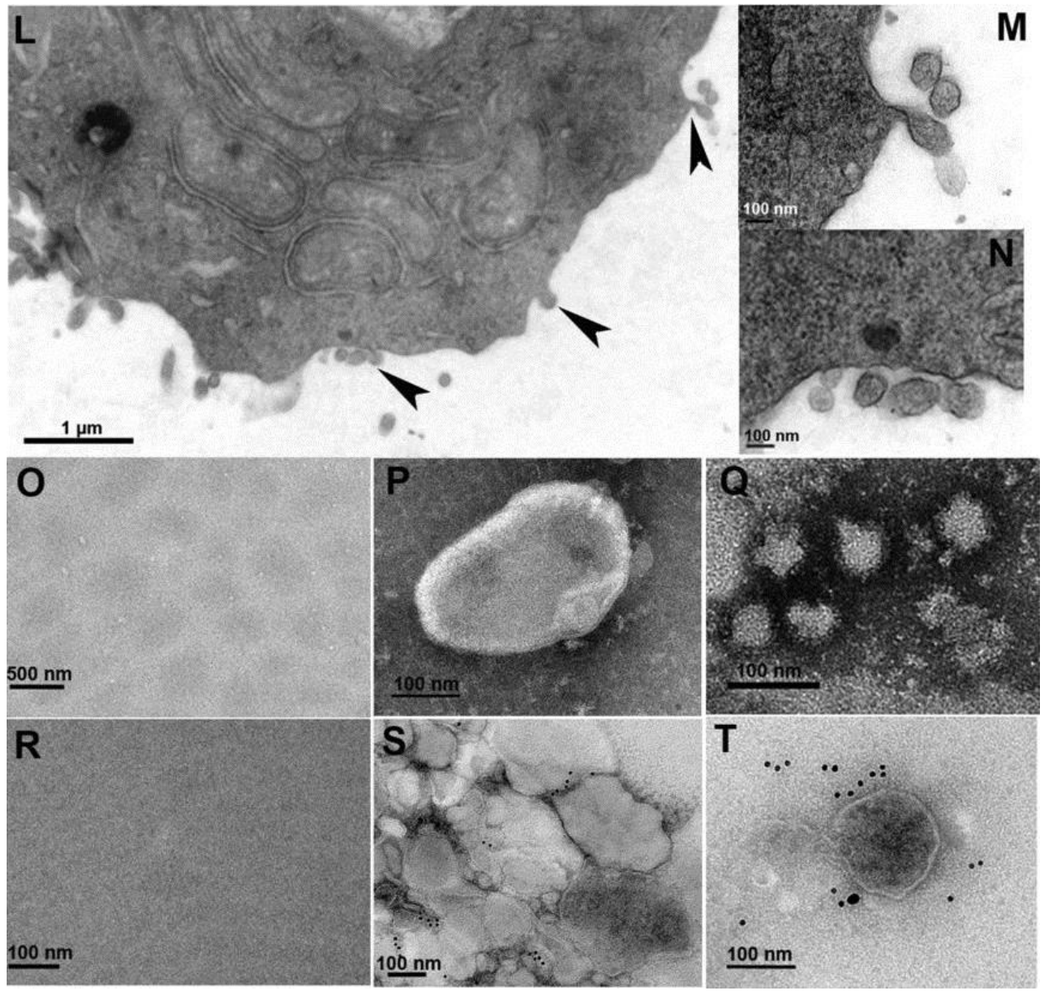
- **DENV infection leads to apoptotic death and MP shedding**



<u>Fig 2.</u> DENV infection induces MP production from various cell types.

- **(L)** Transmission electron micrograph of a DENV-infected HepG2 cell displaying small vesicles of 80 to 200 nm in size (arrowheads) near the cell periphery.
- **(M and N**) Budding MPs at higher magnification.
- **(O to Q)** Negative staining on the grids absorbed by buffer (O), isolated MPs (P), and sucrose density-purified virus particles (Q) released from DENV-infected HepG2 cells are depicted.
- **(R to T**) Immunogold labeling of isolated MPs.
- **(S and T)** The clusters of 10-nm gold particles (black dots) at the periphery of bilamellar vesicular structures of MPs indicate the externalization of AnV-bound PS at the outer leaflet of the MP membrane.
- **(R)** Grids adsorbed with buffer instead of MPs that then underwent the same immunogold labeling procedure used for the isolated MPs in the images shown in panels S and T.

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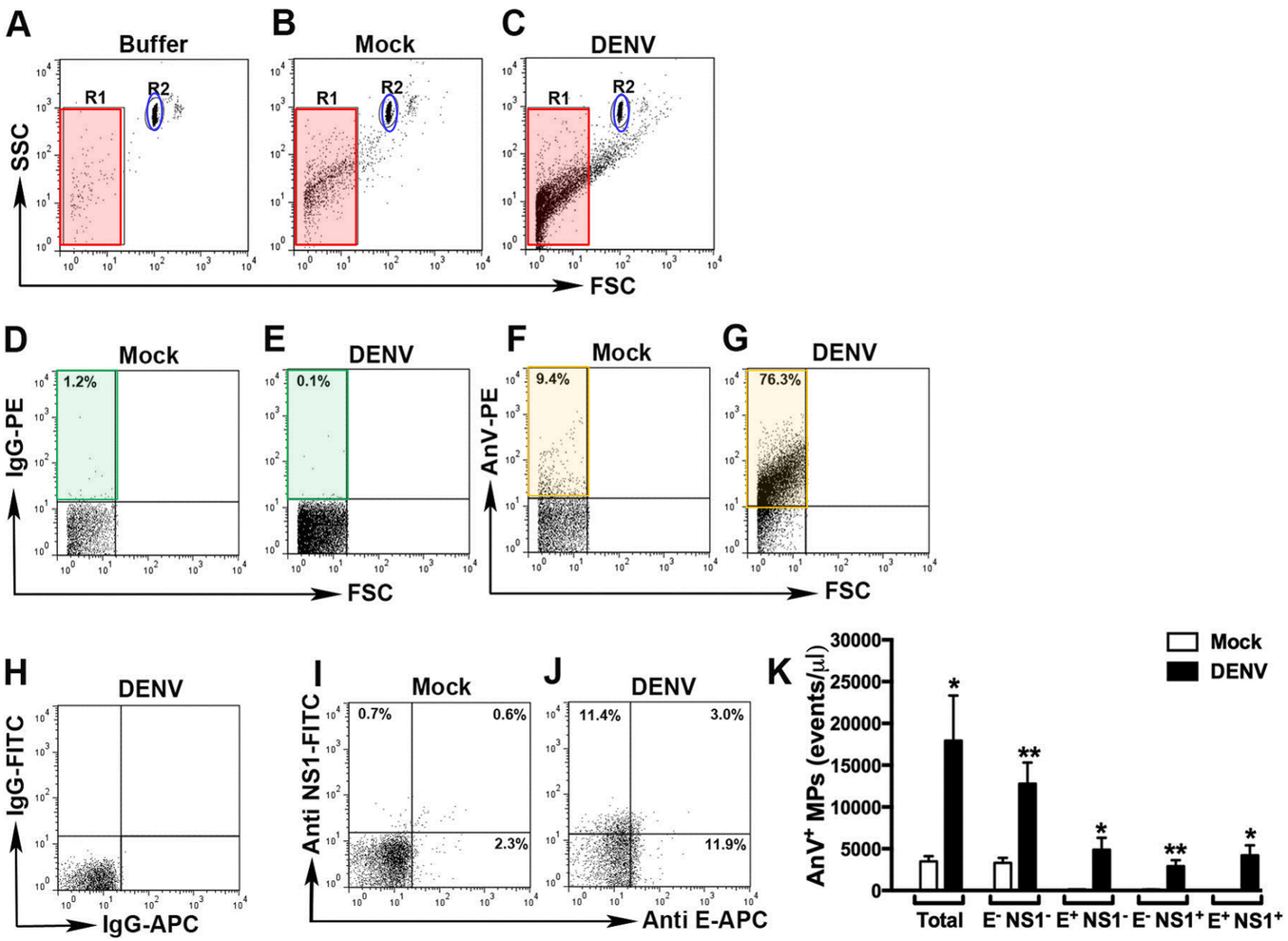
- **MPs released from DENV-infected cells express viral antigens on their surfaces**
  - **Region R1: MPs FSC/SSC light scatter gate (size, <1 μm)**
  - **Region R2: TruCount beads known density**



- (H to J) Representative density plots of AnV+ MPs from DENV-infected cells stained with FITC- and APC-conjugated isotype control Abs (IgG-FITC and IgG-APC, respectively) (H) and AnV+ MPs from mock-infected (I) and DENV-infected (J) cells double stained with FITC-conjugated anti-NS1 MAb clone 2G6 (anti-NS1-FITC) and APC-conjugated anti-E MAb clone 4G2 (anti-E-APC). The percentages of AnV+ MPs positive for NS1 alone (left upper quadrants), E alone (right lower quadrants), and both E and NS1 (right upper quadrants) generated by mock-infected (I) and DENV-infected cells (J) are depicted.
- (K) The absolute numbers of total AnV+ MPs and AnV+ MPs negative for both E and NS1 (E− NS1−), positive for E alone (E+ NS1−), positive for NS1 alone (E− NS1+), and positive for both E and NS1 (E+ NS1+) were determined by using TruCount beads of known density (region R2 in panel A).
- Data are the mean ± SD from four independent experiments.
- Asterisks note statistically significantly differences between the percentage of AnV+ MPs produced by DENV-infected cells and the percentage produced by mock-infected cells (*, $P < 0.05$; **, $P < 0.001$).

FIG 3. MPs generated from DENV-infected cells express E and NS1 antigens on their surfaces.

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- **Circulating MPs in dengue patients**

TABLE 1 Demographic, clinical, and key laboratory characteristics of patients enrolled in the study[a]

Characteristic	Result for the following patient groups:			
	OFI patients ($n = 10$)	DF patients ($n = 19$)	DHF patients ($n = 24$)	P value
No. (%) male patients	5 (50)	10 (53)	19 (79)	0.1012
Age (yr)	9.5 (6, 10.8)	10 (8, 11)	11 (8.3, 13.8)	0.1824
Body wt (kg) at enrollment	22.5 (18.5, 35.7)	33.0 (23.2, 49.0)	35.5 (25.5, 46.0)	0.0683
Platelet nadir (10^9/liter)	184.5 (161.0, 198.0)	96.0 (52.5, 140.0)	27.5 (21.2, 48.8)	<0.0001
Day of acute illness of platelet nadir	−0.5 (−1.8, 0.0)	0 (0, 1)	0 (0, 1)	0.0454
Maximum RBC count (10^{12}/liter)	4.7 (4.5, 4.9)	5.4 (4.9, 5.8)	5.5 (5.2, 6.0)	0.0040
Day of acute illness of maximum RBC count	0 (−2, 1)	0.0 (−0.5, 0.5)	0 (−1, 1)	0.5826
No. (%) of patients with mucosal bleeding	0 (0)	5 (29)	12 (71)	0.0078
Day of acute illness of first episode of mucosal bleeding	—	−1 (−1.5, −0.5)	0 (−1, 0)	0.2007

[a] Data are presented as the number (percentage) for categorical variables and median (25th, 75th percentiles) for continuous variables. OFI, other febrile illness; DF, dengue fever; DHF, dengue hemorrhagic fever; RBC, red blood cell; —, OFI patients did not have mucosal bleeding.

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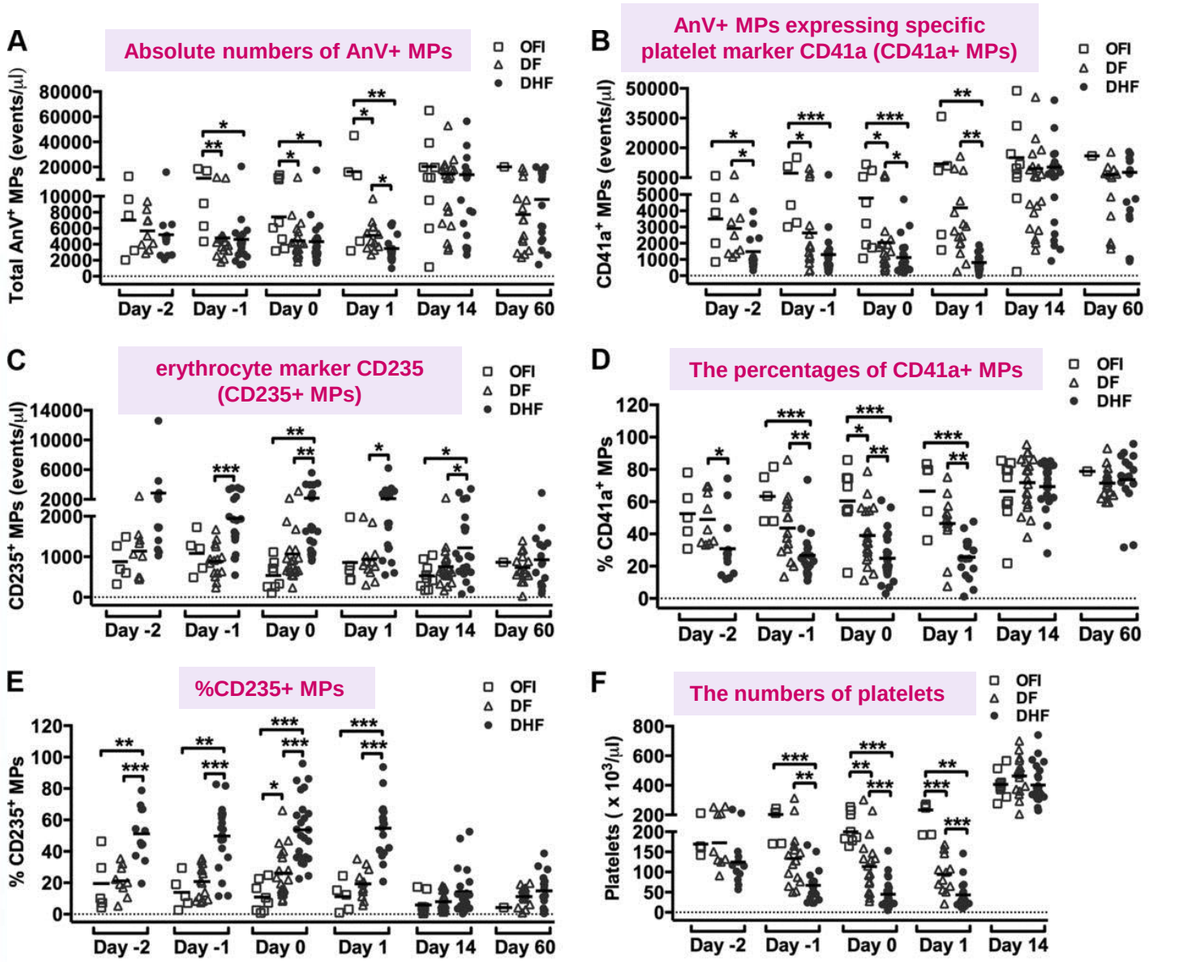
- **Circulating MPs in dengue patients**



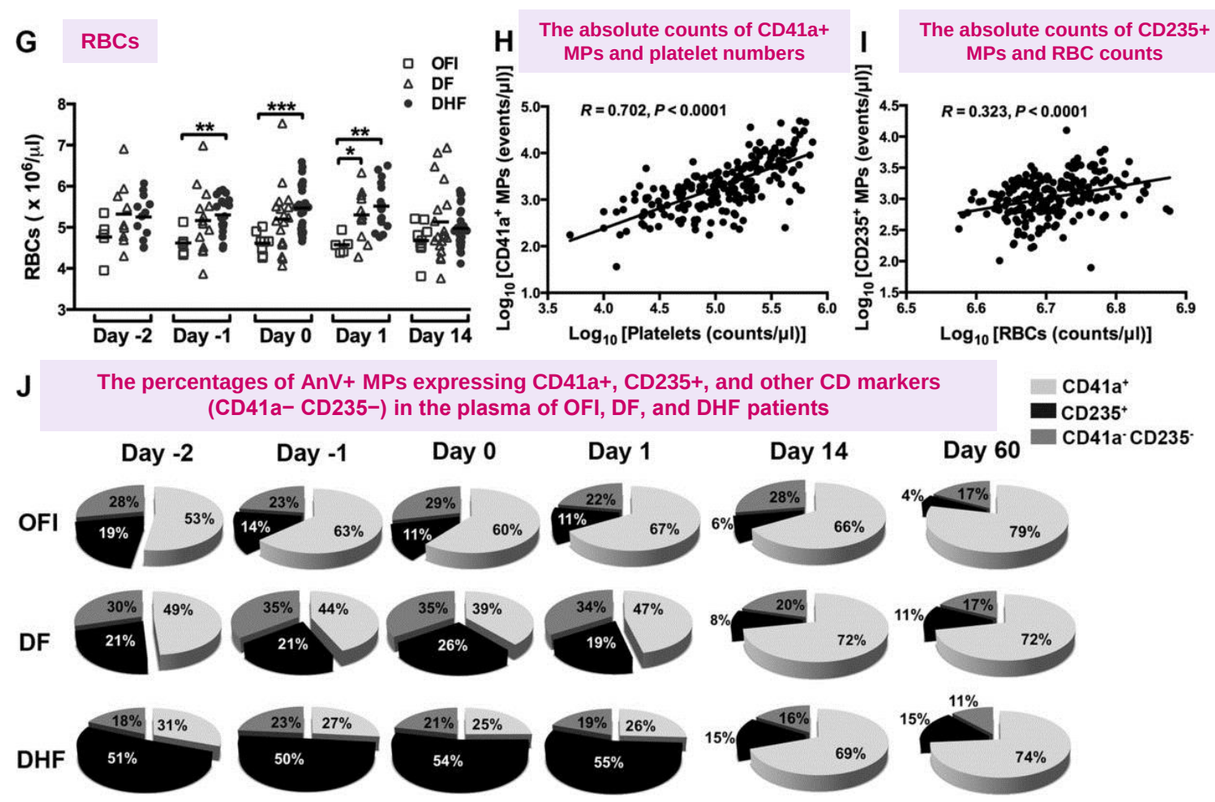


Fig 4. Circulating MP levels in DENV-infected patients.
The linear regression, correlation coefficient, and P value are presented in the graphs.

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- **DENV infection leads to apoptotic death and MP shedding**

TABLE 2 Absolute number of AnV^+ MPs in three groups of patients[a]

	Total AnV^+ MPs (mean ± SD no. of events/μl)			*P* value		
Day	OFI patients	DF patients	DHF patients	OFI vs DF patients	OFI vs DHF patients	DF vs DHF patients
−2	7,027 ± 1,973	5,202 ± 1,068	5,668 ± 774	0.393	0.458	0.744
−1	11,060 ± 2,863	4,800 ± 761	4,606 ± 947	0.007	0.011	0.879
0	7,429 ± 1,385	4,434 ± 546	4,324 ± 648	0.021	0.031	0.901
1	16,370 ± 7,583	5,110 ± 560	3,468 ± 413	0.031	0.004	0.023
14	20,410 ± 6,650	14,380 ± 2,629	13,940 ± 2,701	0.317	0.285	0.906
60	ND	7,713 ± 1,326	9,604 ± 1,580	ND	ND	0.300

[a] MPs, microparticles; OFI, other febrile illness; DF, dengue fever; DHF, dengue hemorrhagic fever; ND, not done.

TABLE 3 Absolute number of AnV^+ MPs expressing the specific platelet marker CD41a in three groups of patients[a]

	$CD41a^+$ MPs (mean ± SD no. of events/μl)			*P* value		
Day	OFI patients	DF patients	DHF patients	OFI vs DF patients	OFI vs DHF patients	DF vs DHF patients
−2	3,503 ± 926	2,911 ± 611	1,485 ± 337	0.589	0.021	0.042
−1	7,291 ± 2,414	2,637 ± 695	1,299 ± 329	0.018	0.0002	0.072
0	4,784 ± 1,320	2,041 ± 429	1,117 ± 221	0.049	0.0001	0.049
1	11,920 ± 6,238	4,182 ± 1,067	811.2 ± 126	0.061	0.002	0.002
14	15,060 ± 5,178	9,595 ± 2,476	10,280 ± 2,155	0.288	0.317	0.834
60	ND	6,399 ± 1,318	7,696 ± 1,465	ND	ND	0.531

[a] MPs, microparticles; OFI, other febrile illness; DF, dengue fever; DHF, dengue hemorrhagic fever; ND, not done.

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TABLE 4 Absolute number of AnV^+ MPs expressing the specific erythrocyte marker CD235 in three groups of patients[a]

	$CD235^+$ MPs (mean ± SD no. of events/μl)			*P* value		
Day	OFI patients	DF patients	DHF patients	OFI vs DF patients	OFI vs DHF patients	DF vs DHF patients
−2	876.3 ± 217	1,138 ± 216	2,852 ± 928	0.449	0.199	0.134
−1	1,084 ± 218	1,946 ± 208	1,946 ± 208	0.372	0.056	0.0002
0	534.8 ± 123	1,065 ± 159	2,167 ± 260	0.053	0.001	0.002
1	857.3 ± 288	934.7 ± 147	2,092 ± 343	0.795	0.076	0.012
14	529.9 ± 104	752.9 ± 104	1,221 ± 185	0.029	0.029	0.042
60	ND	731.5 ± 92	926.4 ± 181	ND	ND	0.355

[a] MPs, microparticles; OFI, other febrile illness; DF, dengue fever; DHF, dengue hemorrhagic fever; ND, not done.

TABLE 5 Number of circulating platelets in three groups of patients[a]

	Mean ± SD platelet count (10^3/μl)			*P* value		
Day	OFI patients	DF patients	DHF patients	OFI vs DF patients	OFI vs DHF patients	DF vs DHF patients
−2	169 ± 14	172 ± 20	124 ± 15	0.917	0.146	0.072
−1	202 ± 14	133 ± 18	66 ± 9	0.053	<0.0001	0.001
0	199 ± 10	113 ± 16	44 ± 7	0.003	<0.0001	0.0002
1	233 ± 16	94 ± 13	43 ± 8	<0.0001	<0.0001	0.002
14	406 ± 29	463 ± 30	401 ± 26	0.248	0.912	0.138
60	ND	ND	ND	ND	ND	ND

[a] OFI, other febrile illness; DF, dengue fever; DHF, dengue hemorrhagic fever; ND, not done.

TABLE 6 Number of circulating RBCs in three groups of patients[a]

	Mean ± SD RBC counts (10^6/μl)			*P* value		
Day	OFI patients	DF patients	DHF patients	OFI vs DF patients	OFI vs DHF patients	DF vs DHF patients
−2	4.8 ± 0.2	5.3 ± 0.3	5.2 ± 0.1	0.185	0.103	0.817
−1	4.6 ± 0.1	5.2 ± 0.2	5.3 ± 0.1	0.158	0.004	0.524
0	4.6 ± 0.1	5.2 ± 0.2	5.5 ± 0.1	0.059	0.0005	0.221
1	4.5 ± 0.1	5.3 ± 0.2	5.5 ± 0.1	0.017	0.002	0.326
14	4.7 ± 0.1	5.1 ± 0.2	4.9 ± 0.1	0.152	0.105	0.492
60	ND	ND	ND	ND	ND	ND

[a] RBC, red blood cell; OFI, other febrile illness; DF, dengue fever; DHF, dengue hemorrhagic fever; ND, not done.