Immune-mediated thrombotic thrombocytopenic purpura plasma induces calcium- and IgG-dependent endothelial activation: correlations with disease severity

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SUPPLEMENTARY DATA

SUPPLEMENTARY METHODS

Patient characteristics

Twenty-five patients were diagnosed in Marseille with immune thrombotic thrombocytopenic purpura (iTTP), using the following criteria: ischemic and/or hemorrhagic clinical events associated with mechanical hemolytic anemia (hemoglobin<100g/L and presence of schistocytes>2% on blood smears), peripheral thrombocytopenia (<$150x10^9$/L), low plasmatic ADAMTS13 activity (<10%) (TECHNOZYM® ADAMTS-13 (Technoclone, Vienne, Autriche)) and detection of anti-ADAMTS13 auto-antibodies in serum (>20 IU/mL)[1] (Table 1). For each patient in the acute phase of the disease, platelet poor plasma was obtained from the first plasma exchange (PEX). For 10 patients, plasma on sodium citrate and serum were also obtained by peripheral vein puncture. Plasma from 14 of the 25 patients was also obtained during remission defined as normal hemoglobin and platelets, plasma ADAMTS13 activity >80%, and absence of anti-ADAMTS13 autoantibodies in serum 3-6 months after acute phase by vein puncture on sodium citrate. Plasma from patients with autoimmune neurologic diseases (myasthenia gravis or polyradiculoneuritis, n=13) treated by PEX constituted negative control. For 5 iTTP patients, von Willebrand Factor (VWF)-free plasma was prepared.

To study the correlation between endothelial activation and severity of the disease, in addition to the cohort from Marseille, we also included 37 iTTP patients from the French Reference center in Paris according to the same inclusion criteria for a total of 62 patients. (Table 1). Non-survivors patients were defined by the occurrence of an all-cause mortality in the acute phase of iTTP, i.e., occurring between the diagnosis and the remission phase.

To evaluate the role of anti-ADAMTS13 IgG, we also studied 5 serum samples from patients with acute episodes of hereditary TTP (hTTP) seen in the French Reference center, Paris. These patients were all tested negative for anti-ADAMTS13 autoantibodies.

Cells

Human microvascular dermal endothelial cells (HMVEC-d) and microvascular endothelial cell growth medium-2 bullet Kit (EGM2-MV) were from Lonza (Vivier, Switzerland) and were used between passages 3 and 7 in static conditions. Human umbilical vein endothelial cells (HUVEC) were grown in endothelial growth medium-2 bullet Kit (EGM2) from Lonza and used between passages 2 and 4.

Flow chamber

The formation of UL-VWF strings secreted by iTTP plasma-activated HMVEC-d was studied under flow conditions in a parallel-plate flow chamber device with HMVEC-d grown in endothelial cell growth basal medium-2 (EBM2) from Lonza (Vivier, Switzerland) containing 1% Fetal Bovine Serum (FBS). Briefly, a syringe pump connected to the outlet port injected thrombin, 1/100-diluted control or iTTP plasma samples through the chamber at 0.2ml/min in order to generate a shear rate of 4dyn/cm^2. After 1h of flow, the cells were washed, fixed in 1% paraformaldehyde for 10 minutes and labelled as described in immunofluorescence studies.

Intracellular Ca^{2+} flux measurement by microscopy

HMVEC-d were incubated for 1h with 5µM fluo-4 AM fluorescent dye in phosphate buffer saline (PBS) containing 1% of Bovine Serum Albumin (BSA), washed and incubated in PBS 1% BSA for 30min at 37°C. Cells were stimulated with 30µg of IgG from control or iTTP plasma samples, EBM2 or A23187 10µM. The fluorescence was captured every second during 120 seconds and total fluorescence quantified using a fluorescence microscope (Nikon ECLIPSE TE2000U) equipped with a CCD camera.

Immunofluorescence experiments and quantification

After 1h stimulation, confluent HMVEC-d were washed twice in PBS, fixed in 1% paraformaldehyde for 10 minutes and labelled with anti-VWF (Dako, Denmark), anti-P-selectin antibodies (1/400 dilution) (BD Biosciences, Europe) or rabbit non-immune serum (1/100 dilution) (ThermoFisher Scientific, USA) for 1h at RT. To visualize VWF and P-selectin, the secondary antibodies Alexa Fluor 594-conjugated or Alexa Fluor 488-conjugated donkey anti-rabbit IgG, respectively, were used (1/1000, ThermoFisher Scientific, USA) for 1h. Nuclei was then stained with DAPI (4'6-di-amidino-2-phenylindole)

(ThermoFisher Scientific, USA). To study Weibel-Palade Bodies degranulation, the same experiment was repeated in 1% Triton-X permeabilized HMVEC-d. Quantification of IF was performed using a previously reported method.[2] Images of VWF secretion and membrane tethering were obtained with a fluorescence microscope (Nikon ECLIPSE TE2000U) equipped with a CCD camera. Images of VWF degranulation on 0.02 % saponin permeabilized cells and of P-selectin were realized with a confocal microscope (Leica TCS SP5). For quantification of VWF immunofluorescence, a trained operator opened with a drawing program (Microsoft, USA) the TIFF-formatted 8-bit RGB fluorescence images, and surrounded in color each cell and its exocytosed VWF.[2] A program written in Java (Oracle, USA) incorporated in ImageJ (National Institute for Health, USA) detected the border and counted the number of pixels n_i inside for each VWF-antibody fluorescence intensity i (from 0 to 255). Total signal for an image was $\boldsymbol{S} = (\sum \boldsymbol{i} \times \boldsymbol{n_i}) / \boldsymbol{N}$ where N was the number of cells in the image. Images contained 6 or 7 cells and five images at least were analyzed per condition. For quantification of the P-selectin, the total fluorescence intensity (FI) was measured with ImageJ on each image and this FI was normalized to cell number. Images contained 3 to 5 cells and five images at least were analyzed per condition.

Some experiments were made in the presence of Ca^{2+} ionophore A23187 (10 µM), of calcium chelator MAPTAM (1,2-bis-5-methylaminophenoxylethane-NNN'-tetraacetoxymethyl acetate) (1µM) from Calbiochem (Merck Chemicals Ltd, Nottingham, UK), thrombin (≥2000NIH units/mg, 4IU/mL or 0.5µM), hirudin (40U), hemopexin (170µM), Annexin V (10ng/mL), PPACK (10µM) from Sigma Aldrich (Saint Louis, USA), decomplemented-iTTP-plasma (1/100) (heating for 30 minutes at 56°C) or anti-C5 monoclonal antibody 200µg/mL (Eculizumab, Alexion, France).

To visualize ADAMTS13, HMVEC-d were stimulated, washed in PBS and labeled with anti-ADAMTS13 (1/500 dilution) (ThermoFisher Scientific, USA) for 1h at 4°C, then fixed in 1% paraformaldehyde for 10 minutes, before labeling with secondary Alexa Fluor 488-coupled anti-mouse IgG (ThermoFisher Scientific, USA) and total fluorescence quantification. DAPI labelling (1/5000 dilution) was performed for nucleus visualization.

Preparation of VWF-free plasma

VWF-free plasma was obtained using a purification column (Microlink protein coupling kit, Thermo Fisher Scientific,) coupled with rabbit anti-human VWF antibodies. VWF concentrations were measured by enzyme linked-immunoSorbent assay (ELISA) (Thermo Fisher Scientific, Rockford, USA) in control and iTTP plasma samples before and after passage on column and 99% of the VWF concentration was lost.

Quantification of IgG and purification of F(ab)'2 fragments

IgG quantification in native plasma, depleted plasma or eluted IgG were realized with Fast ELISA kit dosage (RD-Biotech, Besançon). F(ab)'2 fragments were isolated with PIERCE F(ab)'2 fragment preparation kit (Thermo Fisher Scientific, Rockford, USA).

Quantification of nucleosomes

Nucleosomes were quantified by ELISA (Cell Death Detection ELISA Plus), (Sigma Aldrich, Saint Louis, USA) according to the manufacturer's instructions. One unit of nucleosomes refers to the average amount of nucleosomes quantified in plasma from negative controls.

Determination of plasma heme concentrations

Heme concentrations were measured in diluted plasma (1/10 in PBS) as described by Belcher et al.[3] In diluted plasma (1/10 in PBS), the free heme concentration was determined using oxidization of the 3,3',5,5'-tetramethylbenzidine (Turbo-TMB, Thermo Fisher scientific, Waltham, MA) by the pseudo-peroxidase activity of heme. The reaction was stopped by adding sulfuric acid (2M) and the absorbance was measured at 450 nm.

Statistical analysis

Values are presented as median with interquartile range (25% and 75% percentile) for the indicated number of experiments. Data are compared using non-parametric Mann-Whitney two-tailed test with Prism software in all data, except to compare the effects of the plasma from the same patients obtained in acute phase or remission, with or without treatment with hemopexin, Annexin V, eculizumab, hirudin, plasma heating or depleted in IgG in which Wilcoxon matched-pairs signed rank

test was used. A p value less than 0.05 was considered statistically significant.

SUPPLEMENTARY REFERENCES

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SUPPLEMENTARY FIGURE LEGENDS

Figure S1: iTTP plasmas induced-VWF release in endothelial cells in different conditions

(A) VWF string formation detected by fluorescence microscopy on HMVEC-d incubated with either control (a), or iTTP plasma samples (b) in dynamic conditions using a flow chamber (n=3) (original magnification X40, scale bar=10µm). (B) VWF string formation detected by fluorescence microscopy in HUVECs incubated with either control (a), or iTTP plasma samples (b-c) (original magnification X40, scale bar=10µm). (C) VWF string formation detected by immunofluorescence microscopy in HMVEC-d incubated with (a) iTTP plasma obtained by PEX, (b) iTTP serum or (c) iTTP citrated plasma from the same patient during the acute phase of the disease (original magnification X40, scale bar=10µm). (D) VWF concentrations in the supernatants of HMVEC-d stimulated with VWF-free plasma samples (n=5, * $P<.05$). (E) Endothelin-1 concentrations in the supernatants of HUVECs incubated with control or iTTP plasma samples (n=7, ** $P<.01$). (F) Endothelin-1 concentrations (pg/mL) in supernatants of HMVEC-d incubated with iTTP plasma obtained by PEX (n=8), iTTP serum (n=8, *$P<.05$) or iTTP citrated plasma (n=8, *P*: ns). (G) Fluorescence intensity (AU) of the Ca^{2+} flux in HUVECs after 20 second-incubation with either control (n=5) or iTTP plasma samples (n=17, ** $P<.01$). (H) Fluorescence intensity (AU) of the Ca^{2+} flux at 20s in HMVEC-d treated with iTTP plasma obtained by PEX (n=10), iTTP serum (n=10) or iTTP citrated plasma (n=10).

Figure S2: IgG-induced calcium flux on microscope observations

(A) Ca^{2+} flux filmed on living cells during 120s after stimulation with medium alone (EBM2), iTTP IgG or control IgG. (B) Quantification of the Ca^{2+} flux filmed on living cells treated as in (A). Time 0 correspond to cell stimulation with A23187 (orange line), iTTP IgG (red line) or control IgG (blue line). The quantification of each condition is normalized with the EBM2 stimulation (green line).

Figure S3: ADAMTS13 expression on endothelial cells is not modified by incubation with IgG from iTTP plasma patients

(A) ADAMTS13 detection by fluorescence microscopy on HMVEC-d incubated with medium alone (EBM2) (a), control IgG (b) or iTTP IgG (c) (original magnification X40, scale bar=50µm) (B) ADAMTS13

FI quantification in HMVEC-d incubated with either EBM2 (representing the 100%), control IgG or iTTP IgG (n=7, *P =* ns).

Figure S4: Heme and nucleosomes circulating concentrations are not correlated with iTTP severity

(A) Heme concentrations (µM) in survivors (n=41) and non-survivors (n=21) patient's plasmas (*P*= ns). (B) Nucleosomes quantification in survivors (n=41) and non-survivors (n=21) patient's plasmas (*P*= ns).

Figure S5: Complement and thrombin are not involved in iTTP plasma induced endothelial activation

(A) VWF secretion detected by immunofluorescence on HMVEC-d incubated with either iTTP plasma (a), heated-iTTP plasma (b) or iTTP plasma samples in the presence of eculizumab (c) (original magnification X40, scale bar=30µm). (B) Endothelin-1 concentrations (pg/mL) in the supernatants of HMVEC-d incubated with either iTTP plasma (n=17), heated-iTTP plasma (n=9) or iTTP plasma samples in the presence of eculizumab (n=8). (C) Fluorescence intensity of the Ca^{2+} flux at 20s in HMVEC-d incubated with iTTP plasma (n=25), heated iTTP plasma (n=14) or iTPP plasma samples with eculizumab (n=12). (D) Fluorescence intensity of the Ca^{2+} flux at 20s in HMVEC-d incubated with iTTP from PEX in presence or absence of hirudin (n=18). Horizontal dotted line represents median Ca^{2+} mobilization induced in HMVEC-d by control plasma. (E) VWF concentrations (ng/mL) in the supernatants of HMVEC-d incubated with medium (EBM2), thrombin (THR), iTTP plasma (n=10) in presence or not of PPACK 10µM. (F) Fluorescence intensity of the Ca^{2+} flux at 20s in HMVEC-d treated with medium (EBM2), A23187 or iTTP plasma (n=10) in presence or not of PPACK 10µM.

SUPPLEMENTARY VIDEOS LEGENDS

Supplementary Figure S2A medium (EBM2): Ca^{2+} flux filmed on living cells during 120s after stimulation with medium alone (EBM2) (original magnification X10, scale bar=50µm).

Supplementary Figure S2A iTTP IgG: Ca^{2+} flux filmed on living cells during 120s after stimulation with 30 µg of IgG from iTTP patient's plasma (original magnification X10, scale bar=50µm).

Supplementary Figure S2A Control IgG: Ca^{2+} flux filmed on living cells during 120s after stimulation with 30 µg of IgG from control patient's plasma (original magnification X10, scale bar=50µm).

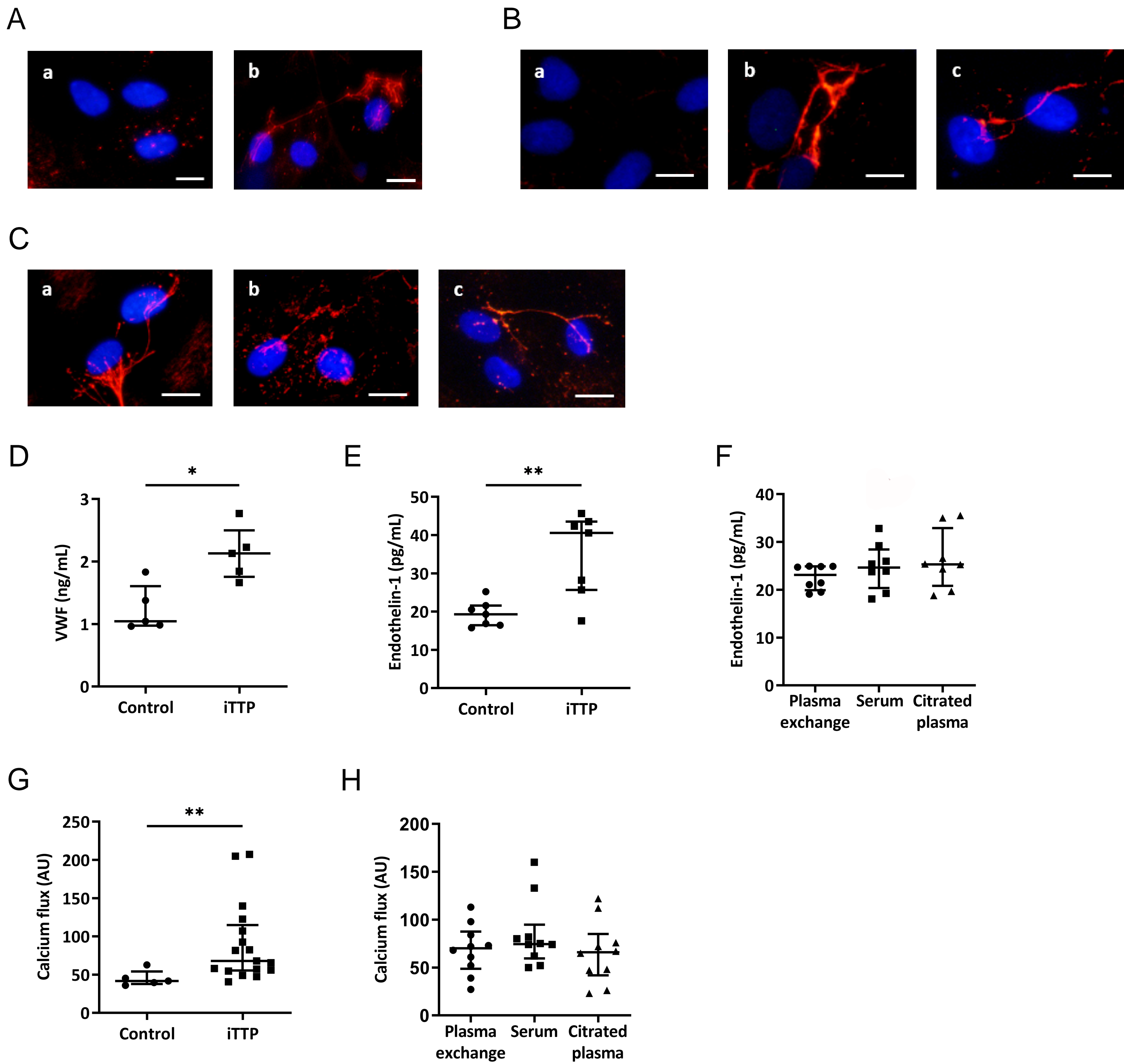
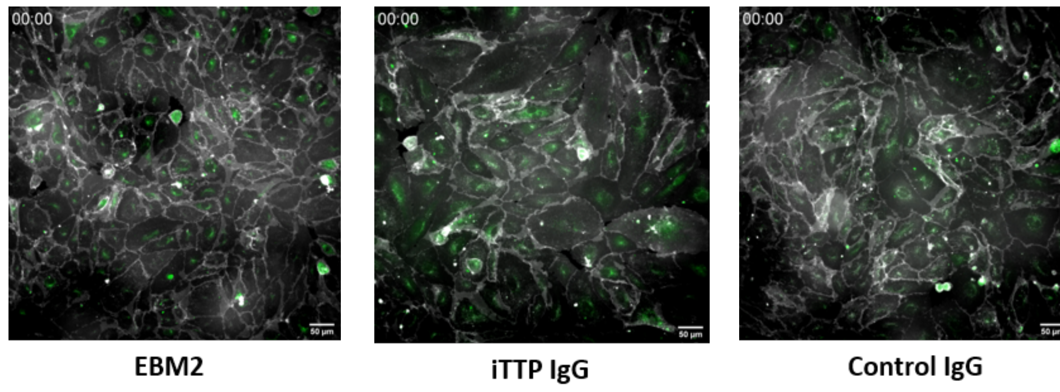


FIGURE S1

A



(see supplementary videos)

B

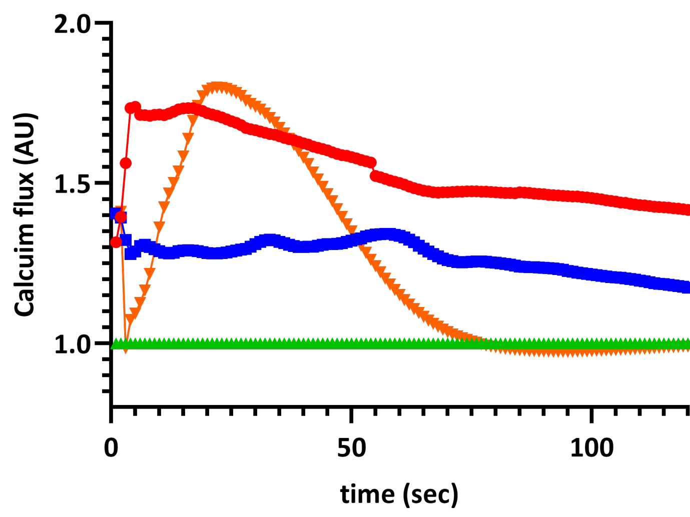


FIGURE S2

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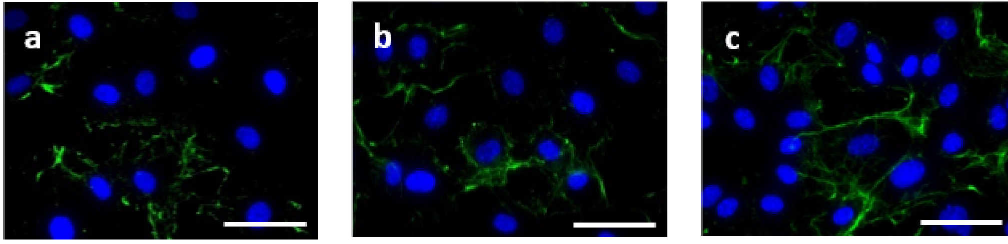

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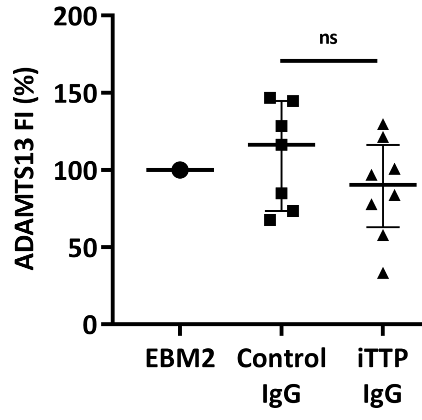
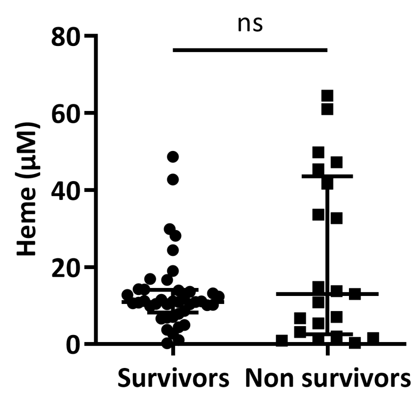


FIGURE S3

A



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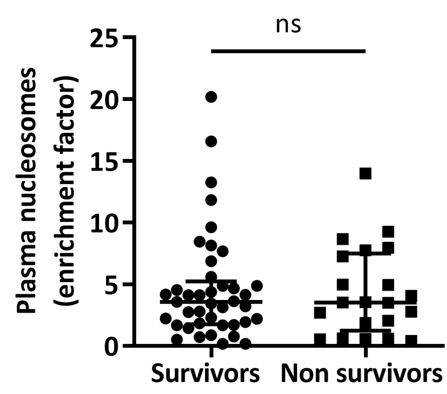
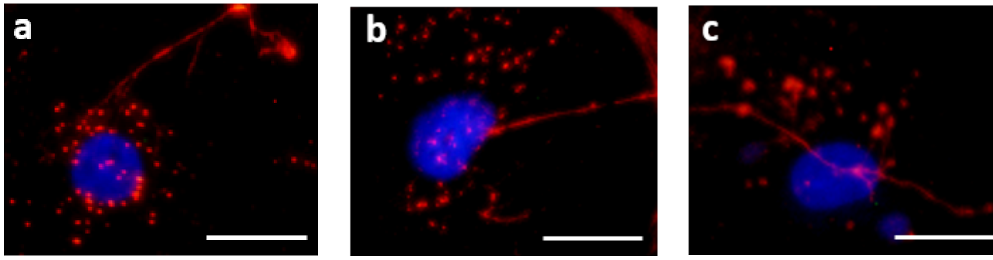


FIGURE S4

A



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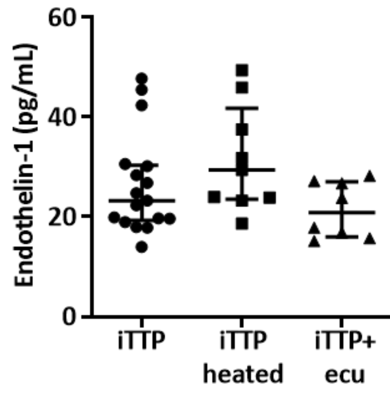

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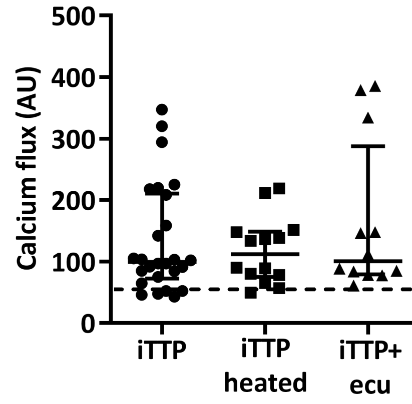

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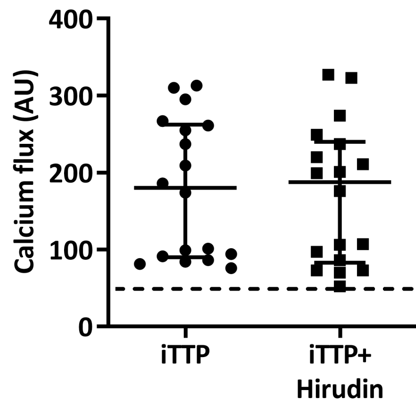

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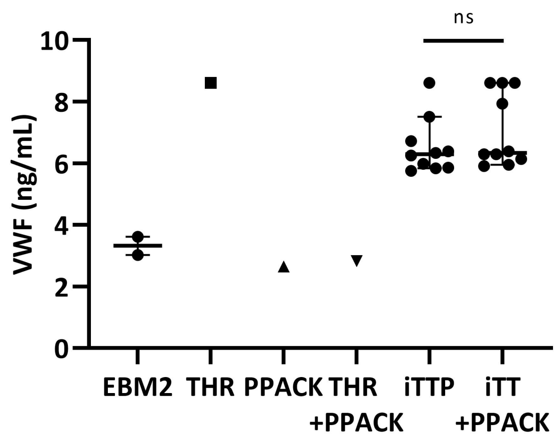

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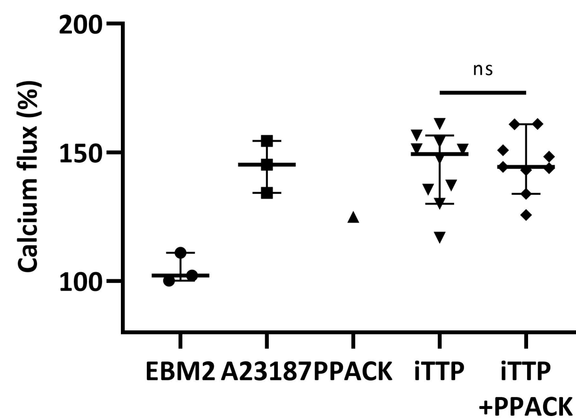

FIGURE S5