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Supplemental Information

Flavivirus NS1 Triggers Tissue-Specific Vascular Endothelial Dysfunction Reflecting Disease Tropism

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Supplementary Figures

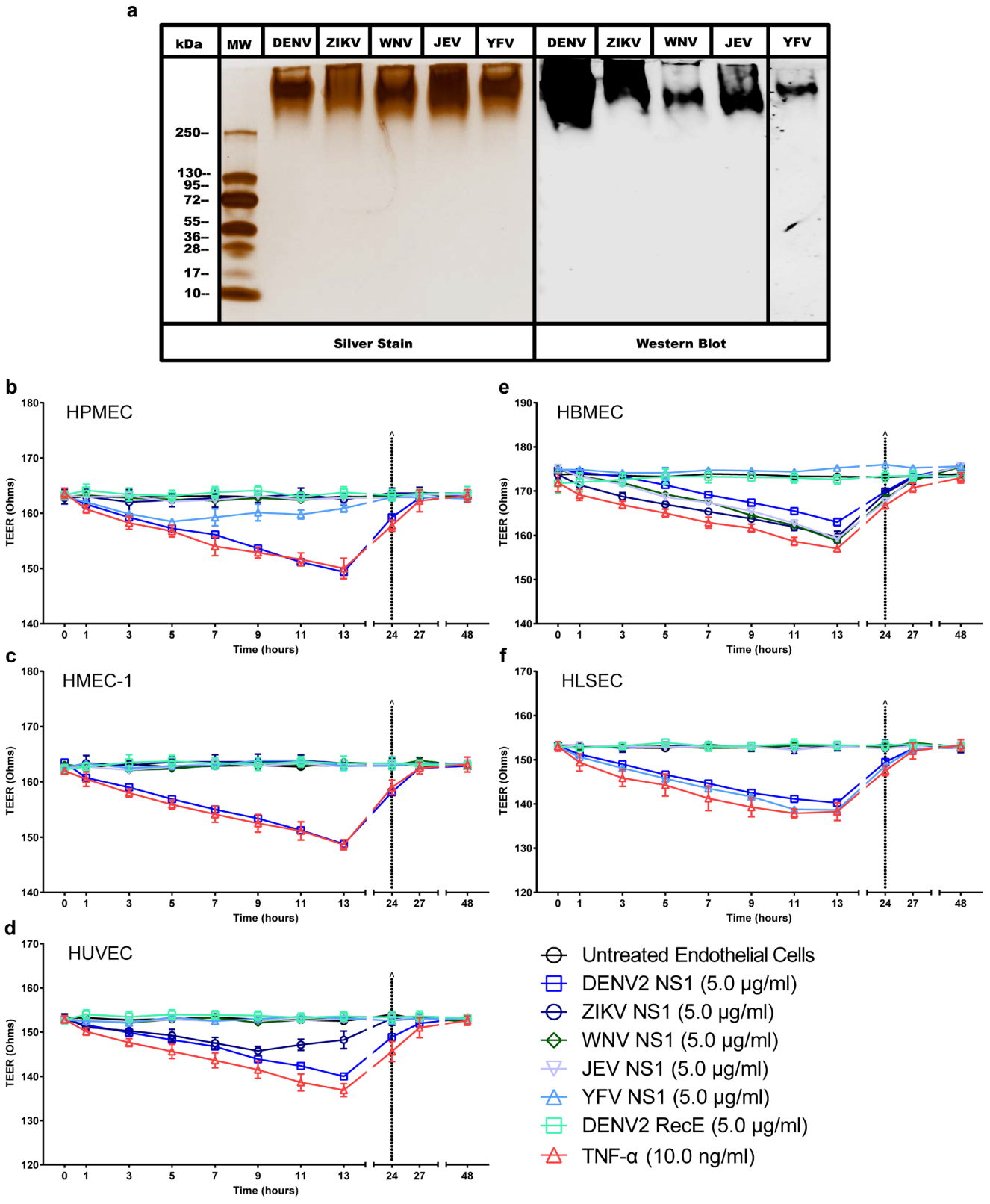


Figure S1. Recombinant flavivirus NS1 proteins from DENV2, ZIKV, WNV, JEV, and YFV are highly purified and oligomeric and increase the permeability of human endothelial cells in a tissue-specific manner.

Related to Figure 1. **(a)** All flavivirus NS1 proteins produced in HEK293 cells (The Native Antigen Company) were analyzed by native PAGE and silver staining (left panel) or Western blot (right panel) to visualize oligomeric NS1 proteins. For each sample, 2 µg of protein was diluted in non-reducing Laemmli sample buffer and separated on a 4-20% gradient polyacrylamide gel (Mini-Protean® TGX™ gels, Biorad) and analyzed by silver staining (left panel) or Western blot using an anti-6x-His-tag mAb. Molecular weight markers are shown on the left. **(b-f)** Transendothelial electrical resistance (TEER) was used to measure the effect of distinct flavivirus NS1 proteins on the barrier function of polarized monolayers of different human endothelial cells as previously described (Beatty et al., 2015; Puerta-Guardo, Glasner and Harris, 2016). TEER was collected using a voltmeter and expressed as electrical resistance in Ohms (Ω). Data shown here is non-normalized data from Figure 1.

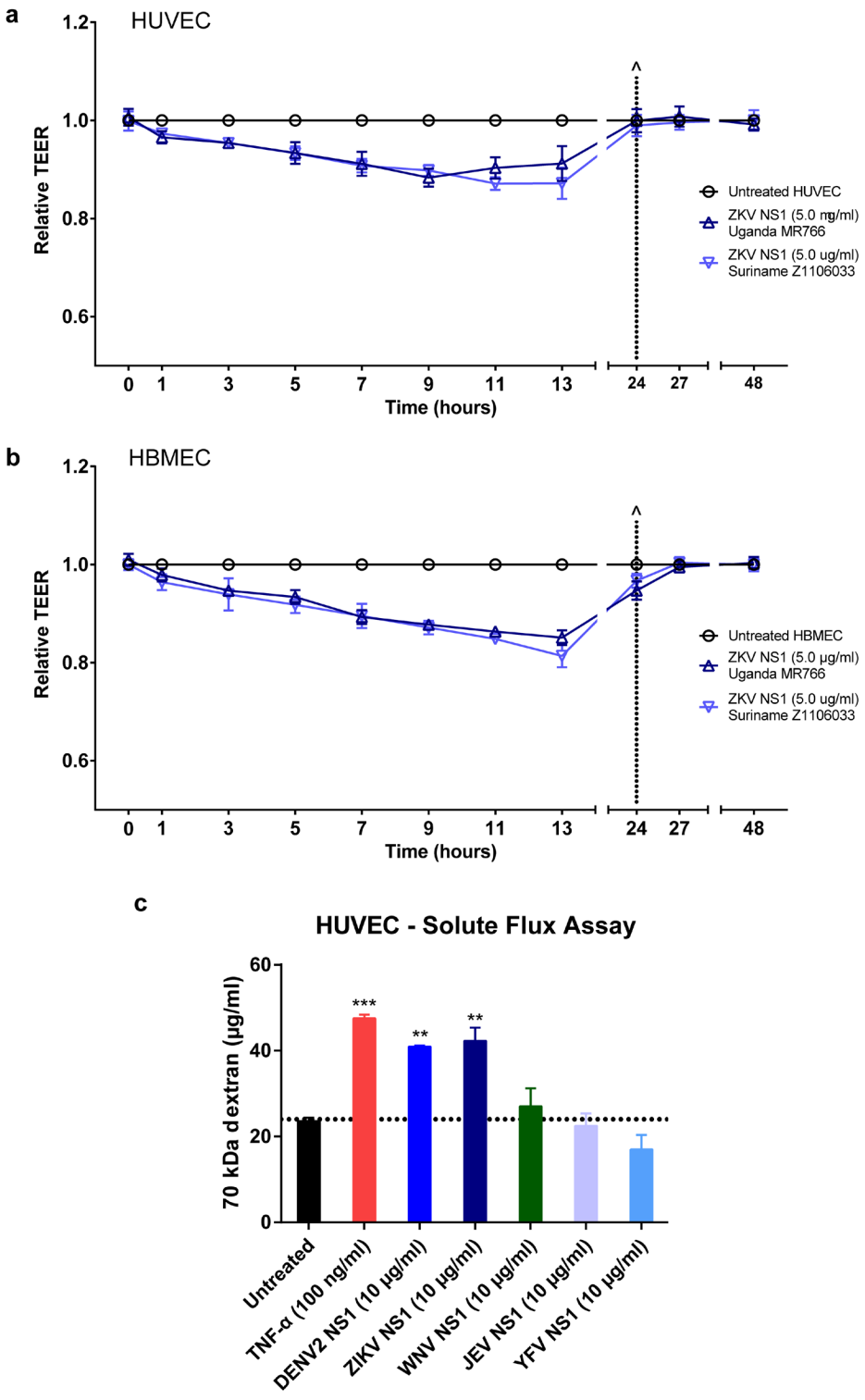
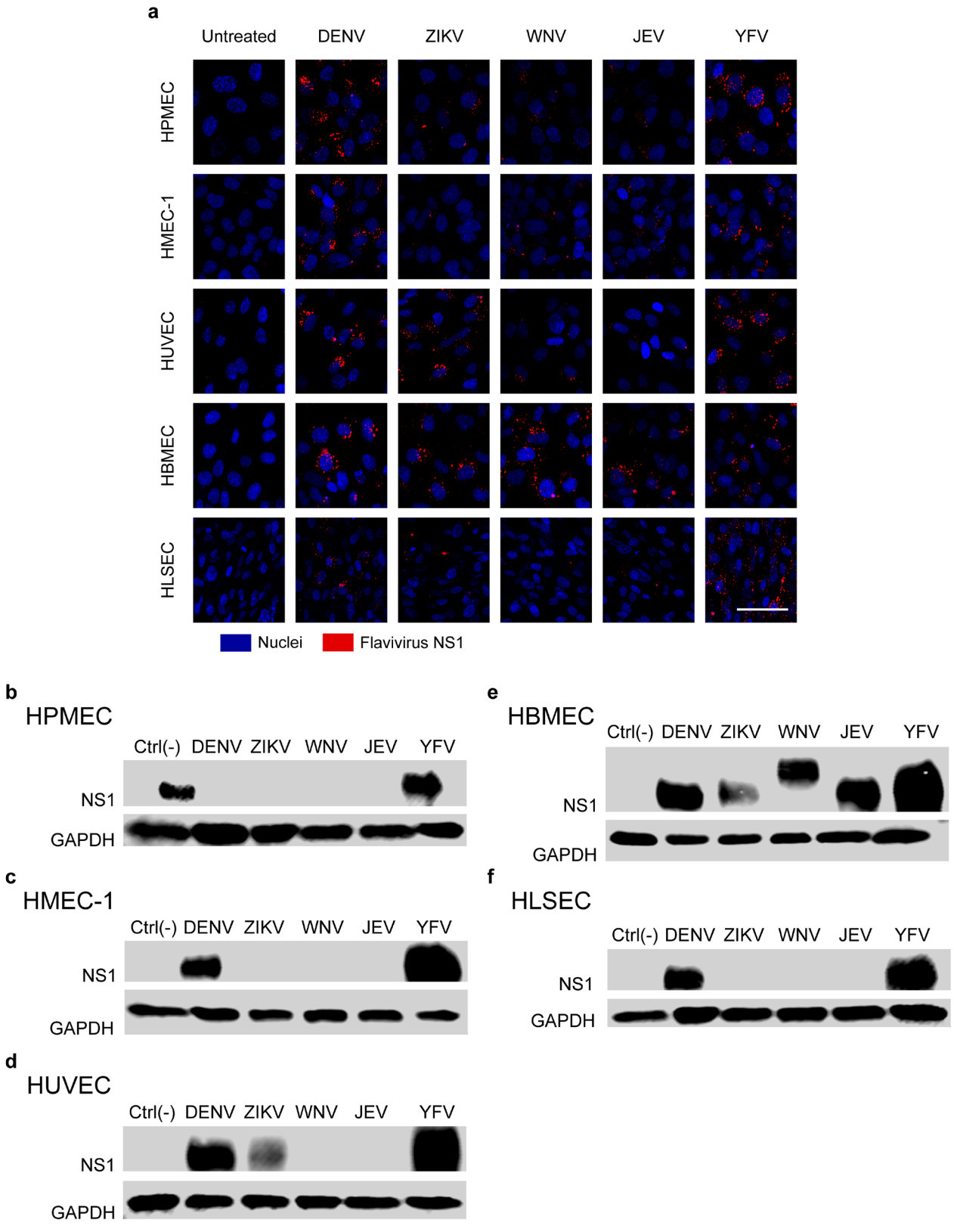


Figure S2. NS1 from two different ZIKV lineages induces similar levels of hyperpermeability of human brain and umbilical vein endothelial cells. Related to Figure 1. **(a-b)** Human endothelial cells from **(a)** umbilical vein (HUVEC) and **(b)** brain (HBMEC) were grown on Transwell semi-permeable membrane inserts (0.4 µm pore size), and NS1 (5 µg/ml, 1.5 µg of total protein) from two different ZIKV strains, Uganda (MR766) and Suriname (Z1106033), was added to the apical chamber. A TEER assay was used to evaluate the effect of these proteins on endothelial permeability at indicated time-points over 48 hours. (^) represents media change. Relative TEER values from two independent experiments performed in duplicate are plotted. Error bars indicate standard error of the mean

(SEM). **(c)** HUVEC were grown on collagen-treated Transwell semi-permeable membrane inserts (0.4 μm pore size), and distinct flavivirus NS1 proteins (10 μg/ml, 3 μg of total protein) were added to the apical chamber. At 6 hpt, 70-kDa dextran conjugated to FITC (1 mg/ml) was added to the apical chamber and allowed to circulate for one hour, after which 100 μl of supernatant from the basolateral chamber was collected and analyzed on a plate reader in duplicate wells. Data from two independent experiments performed in duplicate are plotted. Error bars indicate SEM. Statistically significant differences were determined by an ordinary one-way ANOVA with multiple comparisons to the untreated group between distinct experimental groups. **, $P<0.01$; ***, $P<0.001$.


a
Untreated
DENV
ZIKV
WNV
JEV
YFV
HPMEC
HMEC-1
HUVEC
HBMEC
HLSEC
Nuclei
Flavivirus NS1
b
HPMEC
Ctrl(-) DENV ZIKV WNV JEV YFV
NS1
GAPDH
c
HMEC-1
Ctrl(-) DENV ZIKV WNV JEV YFV
NS1
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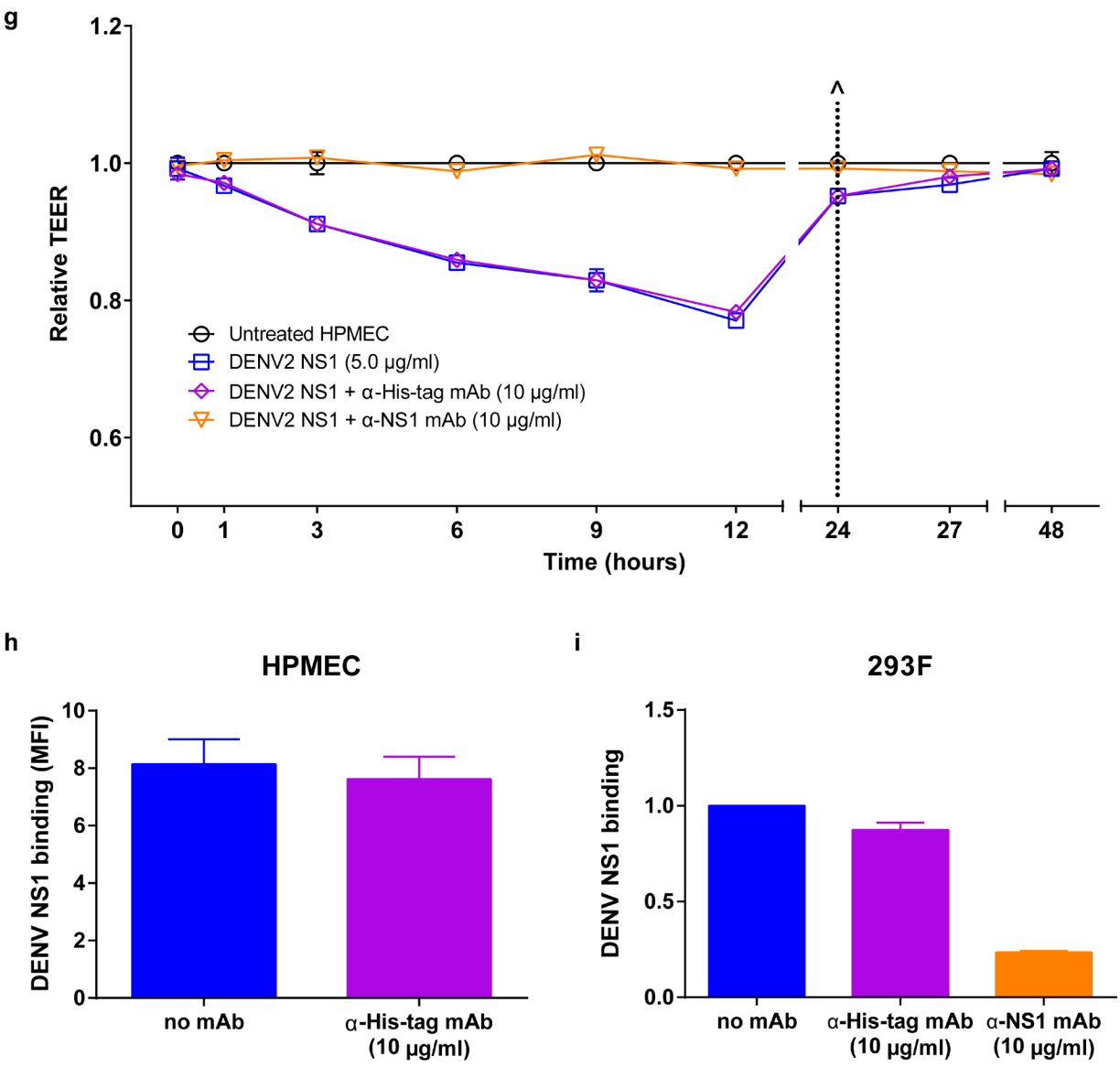


Figure S3. Flavivirus NS1 proteins bind differentially to the surface of human endothelial cells, and binding and NS1-induced hyperpermeability are not prevented by an anti-His-tag mAb. Related to Figure 2. Binding of flavivirus NS1 proteins to the surface of different human endothelial cells grown on coverslips was examined by confocal microscopy **(a)** and Western blot analyses **(b-f)**. The amount of NS1 bound to different human endothelial cell monolayers (red) was quantified 1 hpt at 37°C using an anti-His Tag mAb conjugated to Alexa 647 and expressed as MFI (Figure 2a-e). Nuclei were stained with *Hoechst* (blue). Images (20X) are representative of two individual experiments run in duplicate. Scale bar, 10 µm. MFI values are quantified in Figure 2a-e. **(b-f)** Cell lysates from monolayers of **(b)** HPMEC, **(c)** HMEC-1, **(d)** HUVEC, **(e)** HBMEC, and **(f)** HLSEC treated with different flavivirus NS1 proteins (10 µg/ml, 3 µg of total protein) were collected 1 hpt and were analyzed by Western blot. Representative images of Western blots from two independent experiments. Densitometry values are quantified in Figure 2f-j. **(g)** HPMEC were cultured on Transwell semi-permeable membrane inserts (0.4 µm pore size) until confluent and treated with DENV2 NS1 (5 µg/ml, blue squares), DENV2 NS1 and an anti-His-tag mAb (10 µg/ml, purple diamonds), or DENV2 NS1 and an anti-NS1 mAb (10 µg/ml, orange triangles). A TEER assay was used to evaluate the effect of these treatments on endothelial permeability at indicated time-points over 48 hours. (^) represents media change. Relative TEER values from one experiment performed in duplicate are plotted. Error bars indicate standard error of the mean (SEM). NS1 binding assay on HPMEC **(h)** and 293F cells **(i)** evaluated by confocal microscopy and flow cytometry, respectively. **(h)** Quantification of MFI from confocal microscopy images

of DENV2 NS1 (5 μg/ml) binding to HPMEC in the presence or absence of an anti-His-tag mAb (10 μg/ml). **(i)** Relative NS1 binding to the surface of 293F cells in the presence or absence of an anti-His-tag mAb (10 μg/ml) and an anti-NS1 mAb. The relative binding of NS1 to 293F cells was determined using the percentage of NS1-positive cells (treated with NS1 in the absence of mAbs) and the negative control (cells not treated with NS1 but stained using an anti-NS1 mAb). Error bars indicate SEM.

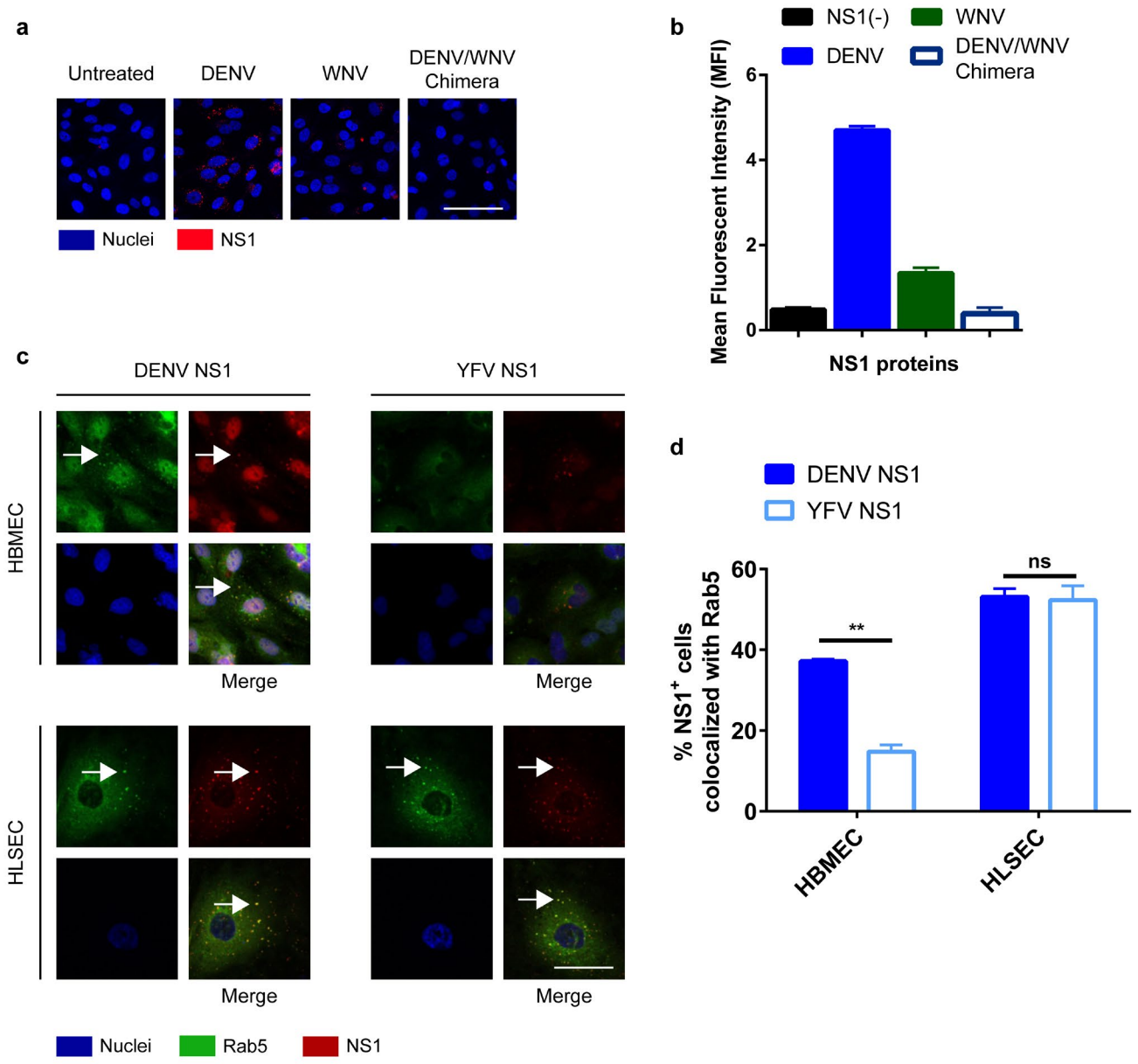


Figure S4. Chimeric DENV/WNV NS1 recombinant protein does not bind to the surface of HPMEC, and DENV NS1 is efficiently internalized into both HLSEC and HBMEC while YFV NS1 is only efficiently internalized into HLSEC. Related to Figure 2. Binding of wild type DENV and WNV NS1 proteins to the surface of HPMEC cells grown on coverslips was examined by confocal microscopy. **(a)** Representative images of NS1 interaction with the surface of HPMEC detected by immunofluorescence assay using an anti-His Tag mAb conjugated to Alexa 647 (in red). Nuclei are stained in blue. Scale bar, 10 µm. **(b)** Mean fluorescence intensities (MFI) values obtained by image J analyses of two different experiment run in duplicate. Error bars indicate SEM. **(c)** Internalization of DENV and YFV NS1 proteins into HBMEC (*top panels*) and HLSEC (*bottom panels*) monolayers grown on coverslips. Co-staining of NS1 protein (red) and Rab5, an early endosome marker (green) after 90 minutes of NS1 interaction with endothelial cell monolayers at 37°C. Images were processed and acquired using confocal microscopy as described in the STAR Methods (Fluorescence microscopy section). Scale bar, 10 µm. **(d)** NS1-Rab5 colocalization analyses. Quantification of colocalization between NS1 and Rab5 was achieved by manually counting NS1-positive cells and evaluating them for colocalization with Rab5 puncta in ImageJ. At least 50 NS1-positive events were quantified for two individual experiments. Error bars indicate SEM. Unpaired T-tests was used for statistical analyses; **, $p<0.01$, and ns (not significant), $p>0.05$.

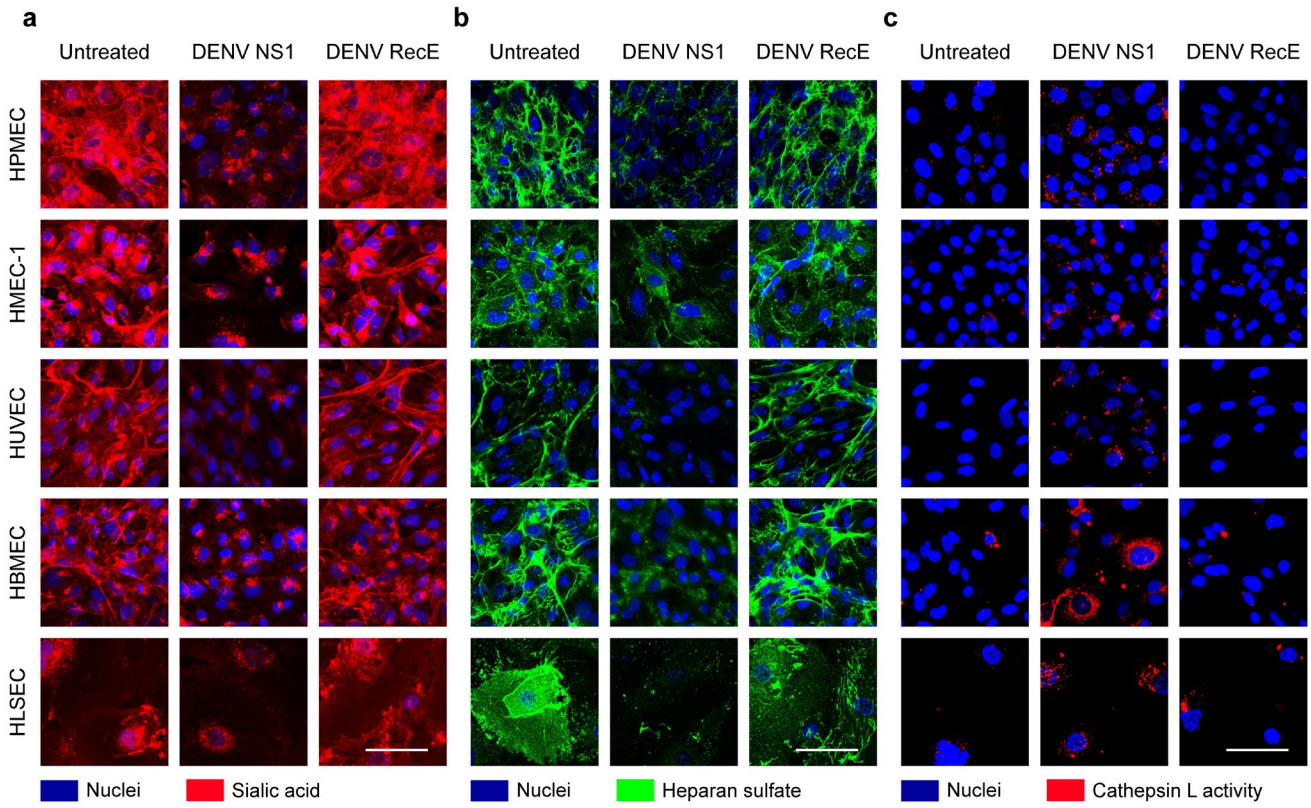
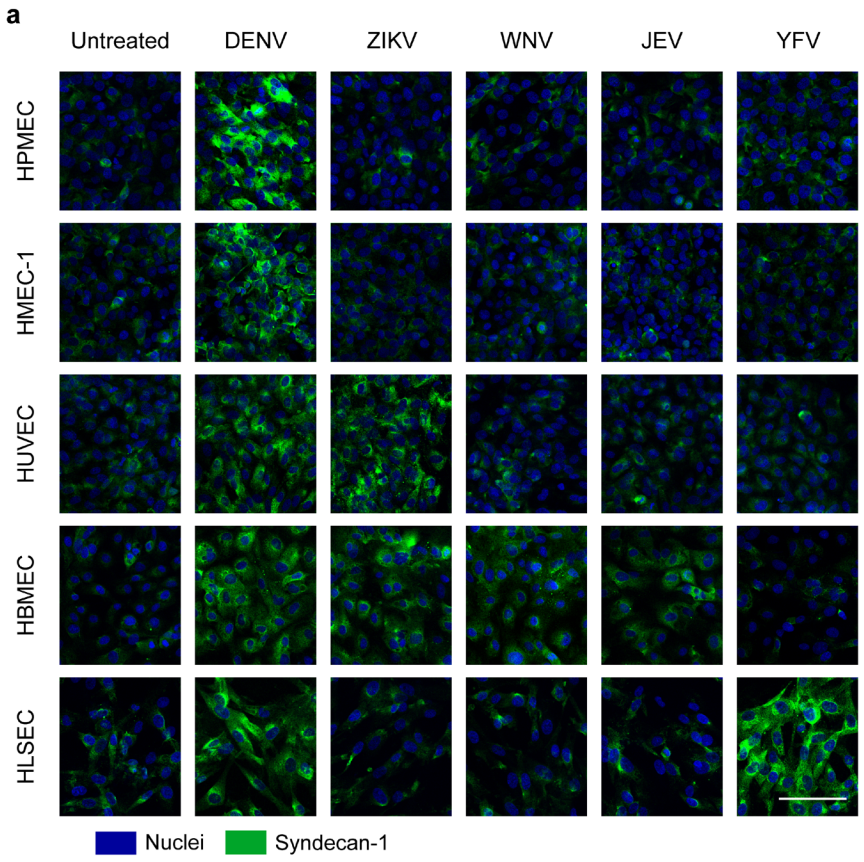


Figure S5. Recombinant envelope protein of DENV does not alter the expression of glycocalyx components of distinct human endothelial cells. Related to Figures 3, 4, and 5. **(a)** Sialic acid and **(b)** heparan sulfate on the cell surface and **(c)** cathepsin L activity in different human endothelial cells 6 hpt with DENV NS1 and DENV recombinant E protein (5 µg/ml). Sialic acid, heparan sulfate and cathepsin L activity were visualized as previously described (Figures 3, 4, and 5). Nuclei are stained in blue (*Hoechst*). Scale bars, 10 µm. Untreated cells were used as control for sialic acid, heparan sulfate expression and basal cathepsin L activity. Images were acquired using confocal microscopy and analyzed by ImageJ software. Images are representative of two independent experiments.


a
Untreated
DENV
ZIKV
WNV
JEV
YFV
HPMEC
HMEC-1
HUVEC
HBMEC
HLSEC
Nuclei
Syndecan-1
b
DENV
ZIKV
WNV
JEV
YFV
Syndecan-1 expression
(Fold change/control cells)
0
1
2
3
4
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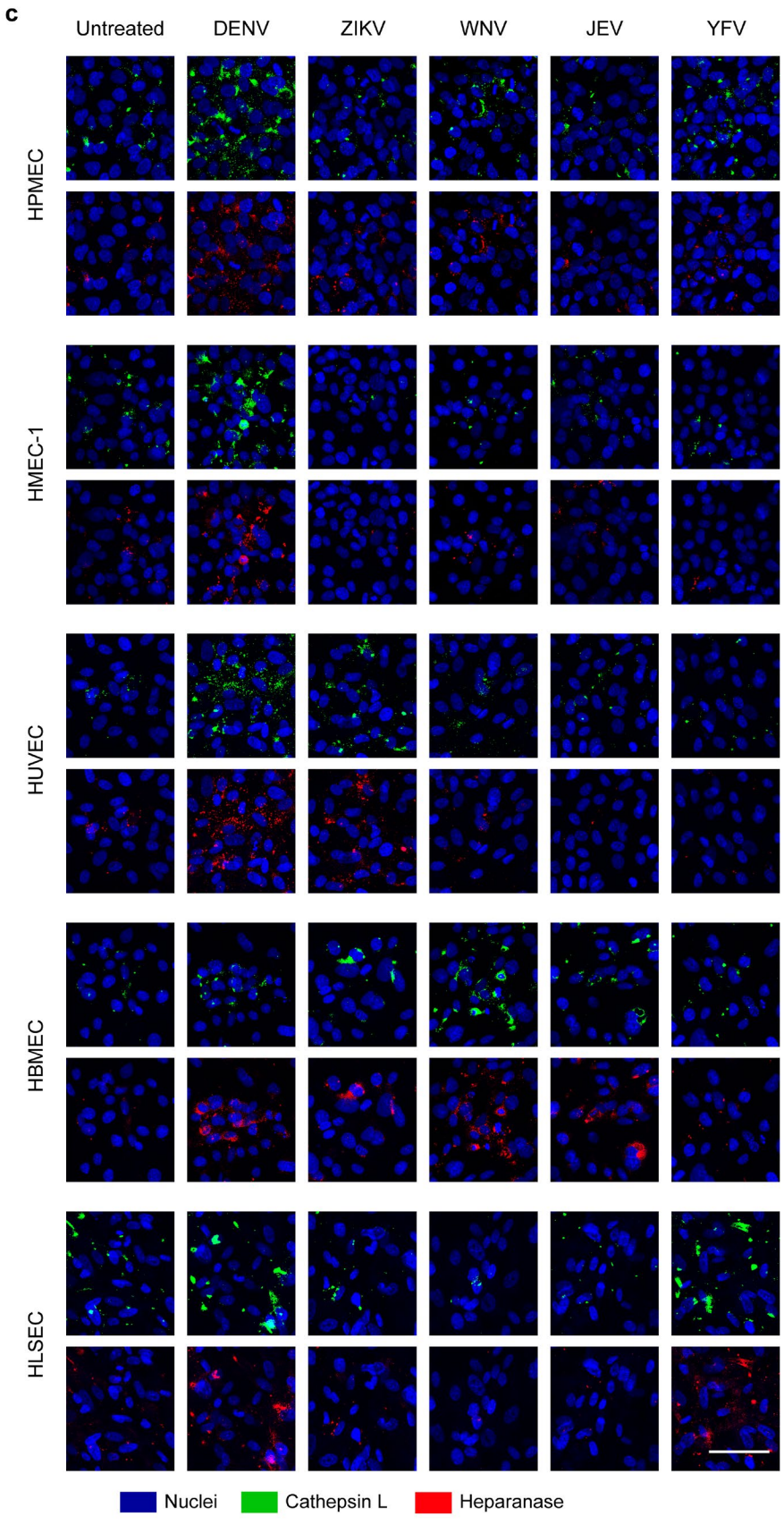
HPMEC
HMEC-1
HUVEC
HBMEC
HLSEC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Untreated
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ZIKV
WNV
JEV
YFV
HPMEC
HMEC-1
HUVEC
HBMEC
HLSEC
Nuclei
Cathepsin L
Heparanase

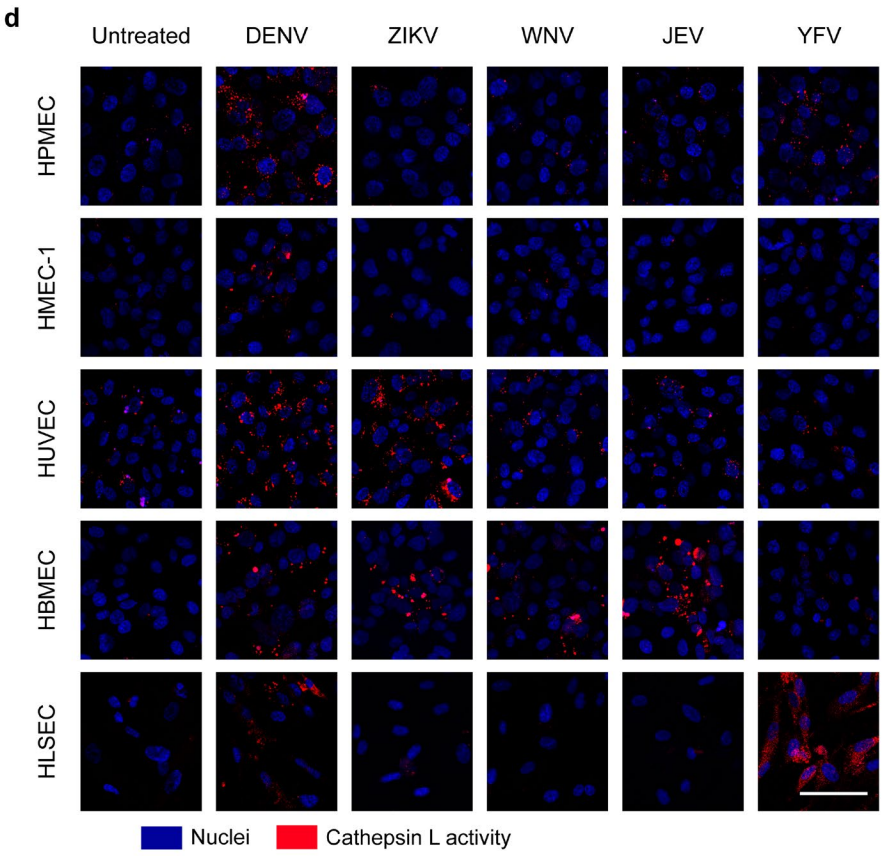

Figure S6. Flavivirus NS1 proteins alter the expression of syndecan-1 in a cell type-dependent manner and induce differential expression of cathepsin L and heparanase and activity of cathepsin L in human endothelial cells. (a) Related to Figure 4. Syndecan-1 expression on human endothelial cell monolayers grown on coverslips 6 hpt with different flavivirus NS1 proteins (5 µg/ml, 1.5 µg of total protein) and examined by confocal microscopy. Syndecan-1 was stained with anti-Syndecan-1 mAb (green). Nuclei were stained with *Hoechst* (blue). Images (20X) and MFI values are representative of three independent experiments. Scale bar, 10 µm. **(b)** Quantification of MFI in **(a)** from three independent experiments. Error bars indicate SEM. Fold-change in MFI for Syndecan-1 expression in NS1-treated monolayers was normalized to untreated controls. Statistically significant differences between distinct treatment groups were determined by ANOVA and unpaired t-tests as indicated, with *, $p<0.05$. **(c)** Related to Figure 5a and f. Co-staining analyses of cathepsin L (green) and heparanase (red) in human endothelial cell monolayers grown on coverslips 6 hpt with different flavivirus NS1 proteins (5 µg/ml), examined by confocal microscopy. Images (20X) are representative of three independent experiments. Scale bar, 10 µm. MFI values are quantified in Figure 5a and f. **(d)** Flavivirus NS1 proteins differentially activate cathepsin L in human endothelial cells *in vitro*. Related to Figure 5b. Cathepsin L proteolytic activity (Magic Red assay, red) in human endothelial cell monolayers grown on coverslips 6 hpt with different flavivirus NS1 proteins (5 µg/ml), examined by confocal microscopy. Images (20X) are representative of three independent experiments. Scale bar, 10 µm. MFI values are quantified in Figure 5b.

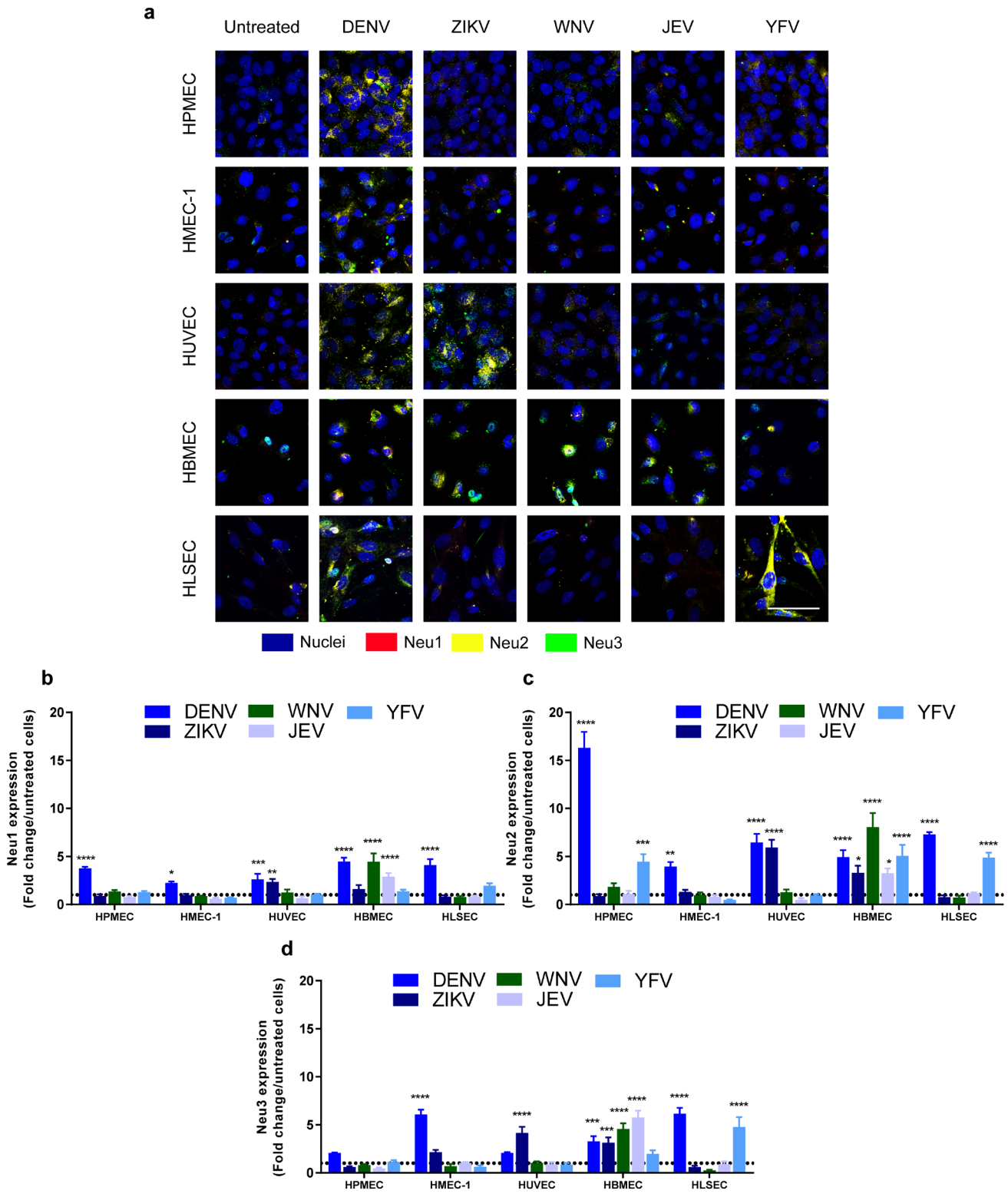

Figure S7. Flavivirus NS1 proteins increase the expression of human endothelial sialidases in a cell type-dependent manner. Related to Figure 5. **(a)** Neu1, Neu2, and Neu3 expression in human endothelial cell monolayers grown on coverslips 6 hpt with different flavivirus NS1 proteins (5 µg/ml, 1.5 µg of total protein) and examined by confocal microscopy. Neu1, Neu2, and Neu3 were stained with Neu1 antibody H-300 (Santa Cruz Biotech, red), Neu2 polyclonal antibody (PA5-35114, Thermo Scientific, yellow) and Ganglioside sialidase antibody N-18 (Santa Cruz Biotech, green), respectively. The merged image is shown for each condition. Nuclei were stained

with *Hoechst* (blue). Images (20X) are representative of two independent experiments. Scale bar, 10 μm. **(b-d)** Quantification of MFI in **(a)** from two independent experiments. Error bars indicate SEM. Fold-change in MFI for **(b)** Neu1, **(c)** Neu2, and **(d)** Neu3 expression in NS1-treated monolayers was normalized to untreated controls. Statistically significant differences between distinct treatment groups were determined by unpaired t-tests as indicated. *, $p<0.05$.