Supplemental Material

Empagliflozin and Dapagliflozin Reduce ROS Generation and Restore NO Bioavailability in Tumor Necrosis Factor α-Stimulated Human Coronary Arterial Endothelial Cells

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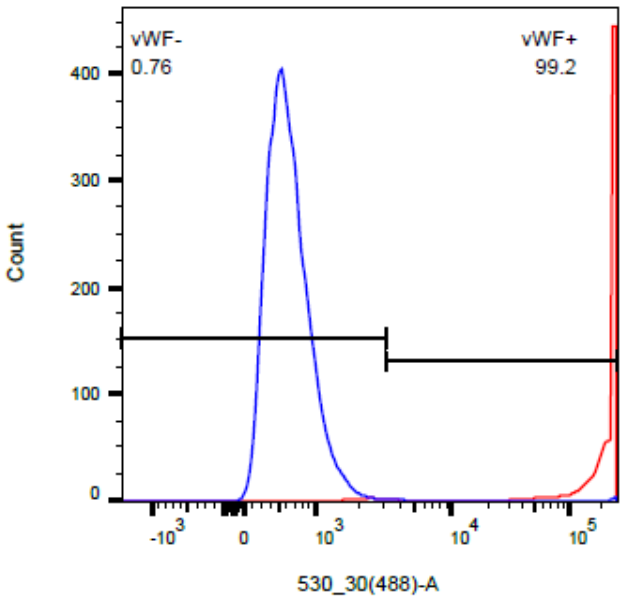
Supplementary Fig. 1

vWF FACS analysis

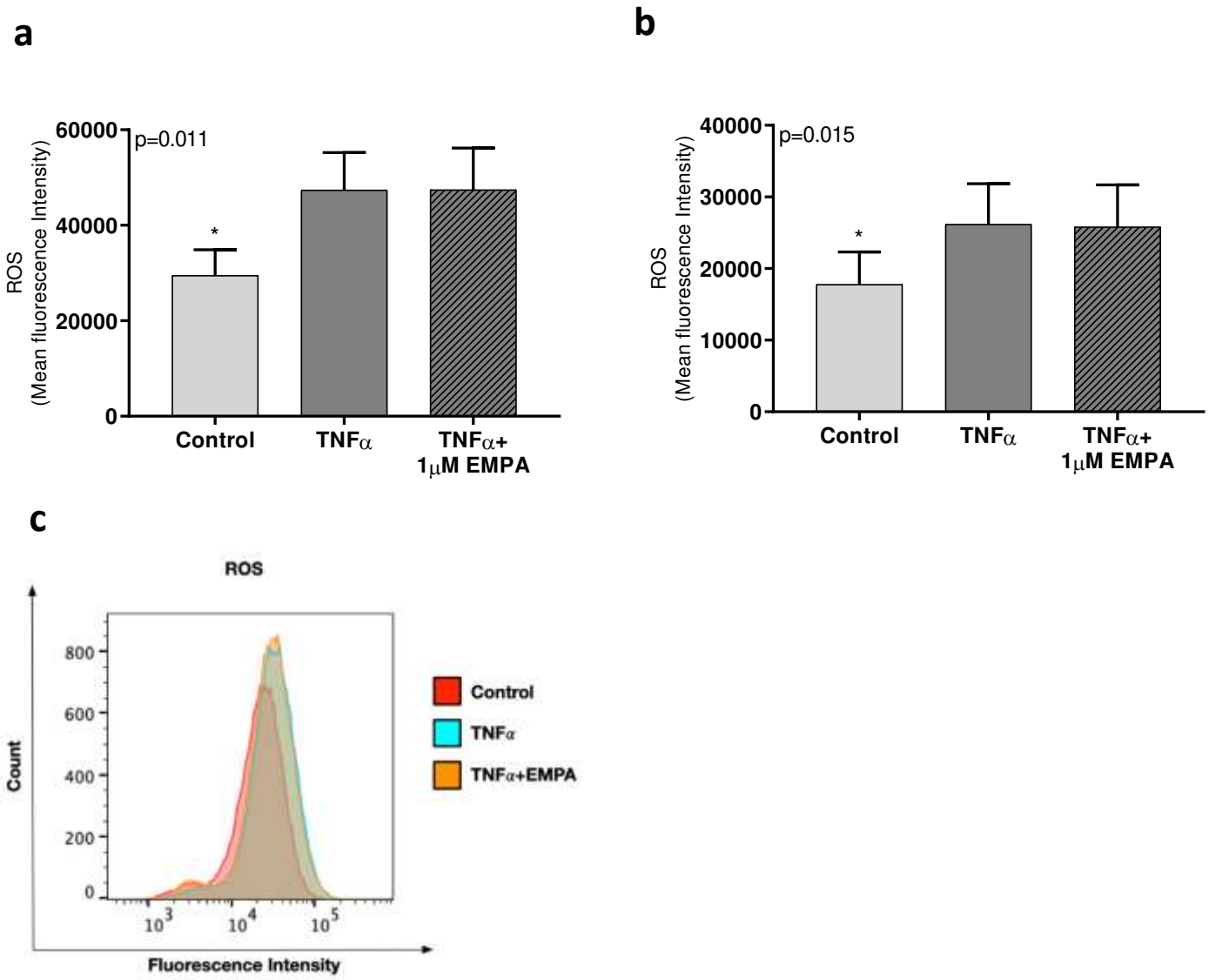
After culturing and harvesting of HUVECs, the cell number was adjusted using Z2 coulter counter (Beckman Coulter, Brea, CA, USA) to one million HUVEC. Cells were permeabilized for 15 min using 2% Tween-20/PBS at 37 °C. Cells were washed and centrifuged twice (241 rcf, 5 min, 4 °C) and subsequently blocked for 30 min with 20% normal sheep-serum (Jackson Immuno Research #013-000-121) in PBS at room temperature. Next, 2µl FITC-conjugated anti-vWF antibody (Bio-Rad, Wiesbaden, Germany) was added and cells were incubated on ice for 30 min in the dark. Again, cells were washed twice and then resuspended in 300 µl cold PBS. Cells were kept in the dark and on ice until the analysis.



→99.2% Purity of HUVECs

Supplementary Fig. 1 Characterization of HUVEC culture. Cells purity was determined by measurement of von Willebrand Factor (vWF) expression level in HUVECs culture using FACS analysis. 99.2% of the cells were positive for vWF, indicating a robust endothelial cell culture.

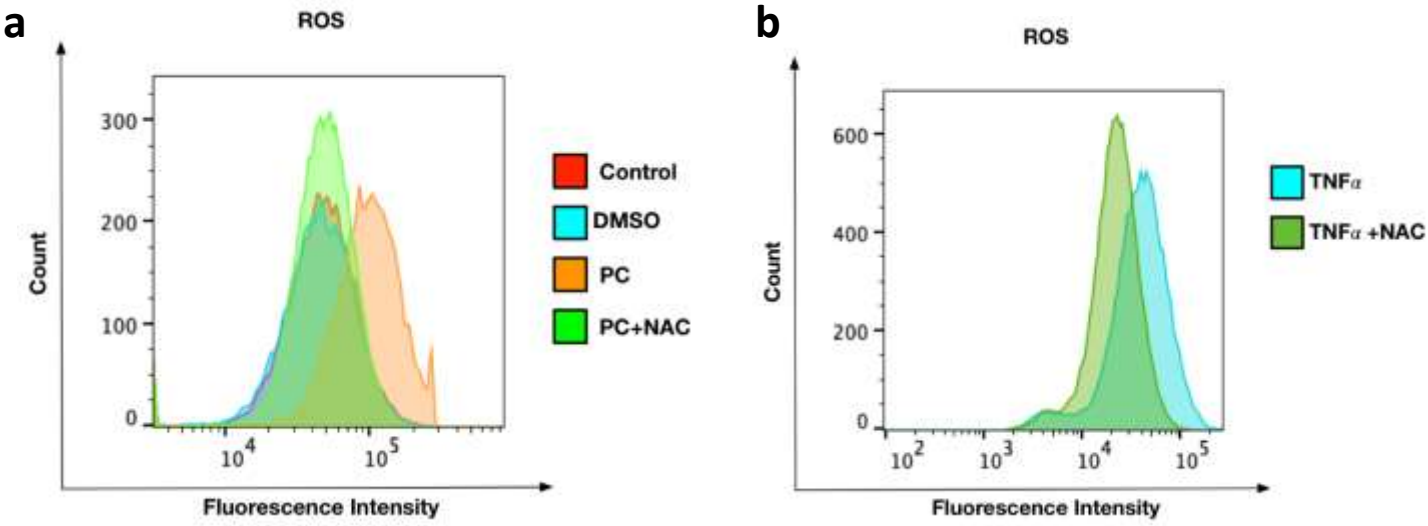
Supplementary Fig. 2:



Supplementary Fig. 2 ROS levels of endothelial cells treated with TNFα, TNFα and EMPA.

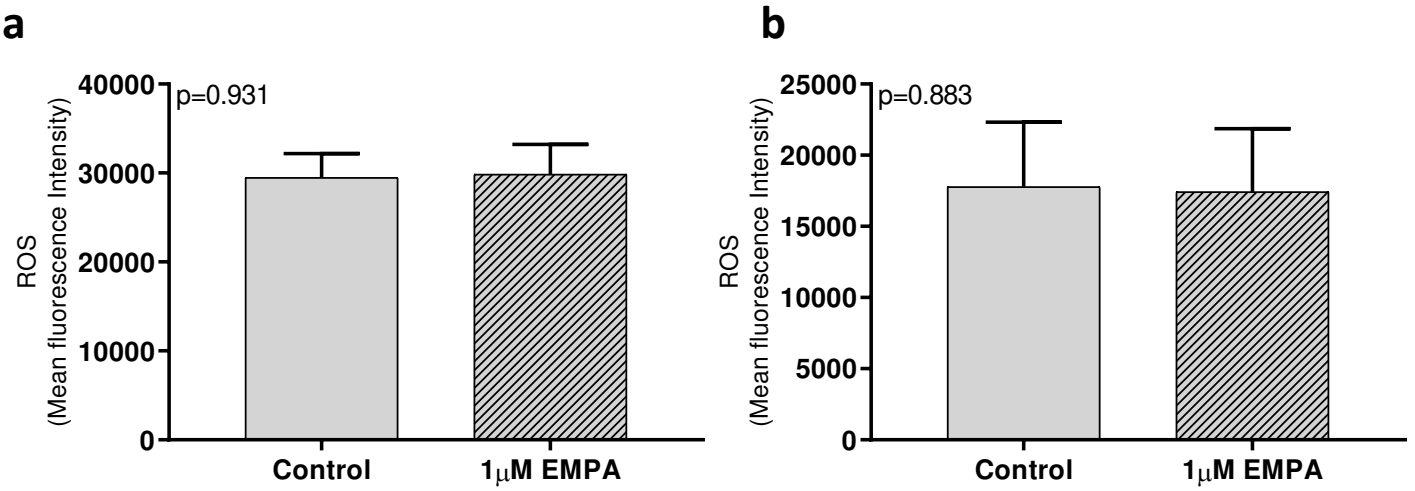
HCAECs or HUVECs were treated with 0.02% DMSO (control), 10 ng/mL TNFα or with 10 ng/mL TNFα with 1μM EMPA or DAPA for 6 h. ROS levels were measured using FACS in HCAECs (a, n=4) and HUVECs (b, n=7). A representative FACS measurement with all three conditions in HUVEC is shown (c). Data are presented as mean±SD. *p<0.05 vs. TNFα.

Supplementary Fig. 3



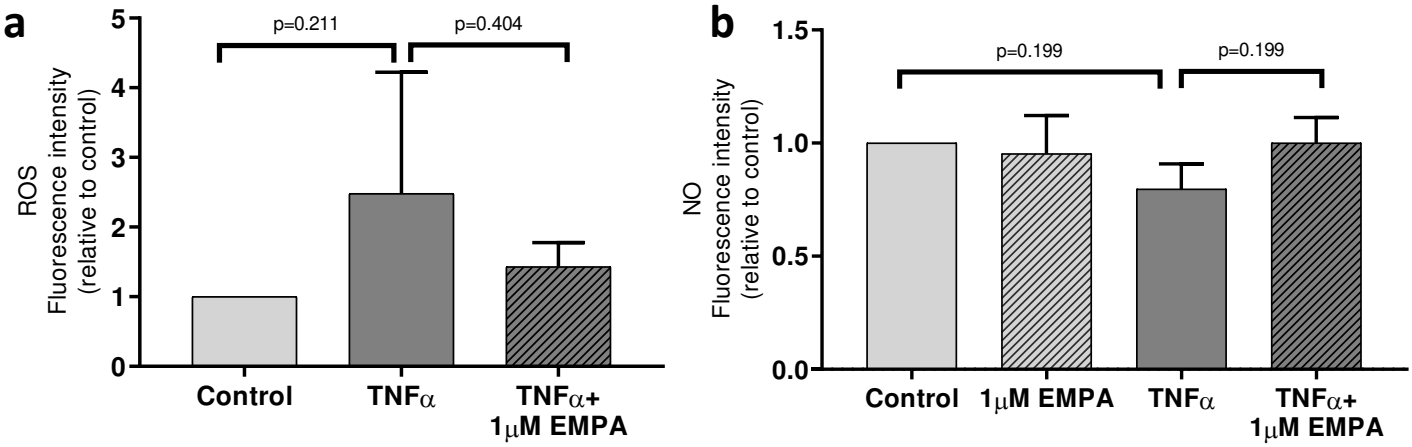
Supplementary Fig. 3 Pyocyanin- or TNFα-induced ROS formation is reversed by NAC. Anti-oxidant properties of 5 mM NAC were validated in 200 µM Pyocyanin (PC) treated HUVECs (a). Cells were pre-incubated with 5 mM NAC or vehicle for 2 h and subsequently exposed to 4 h TNF (10ng/mL) in the presence of NAC or vehicle (b).

Supplementary Fig. 4



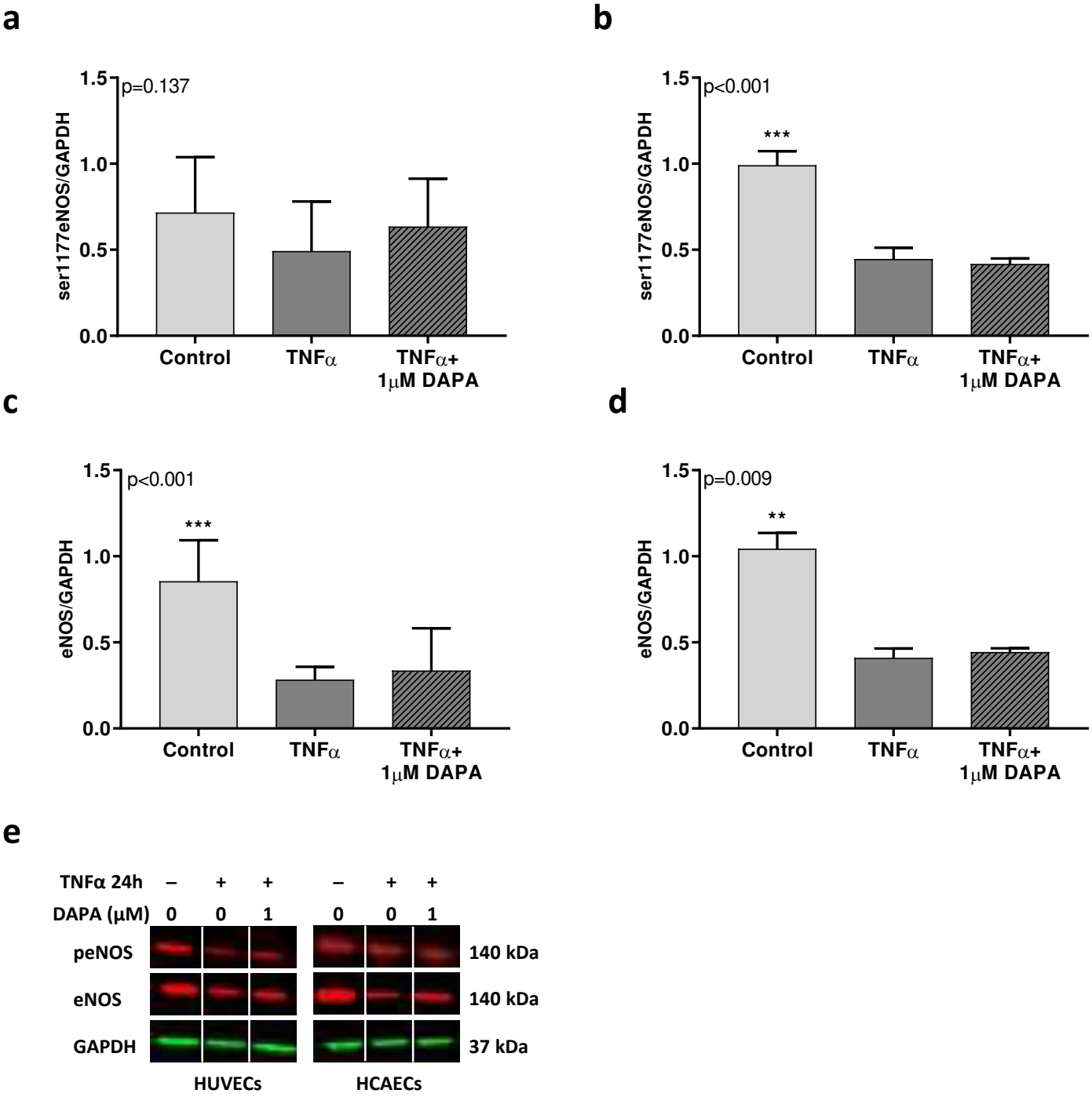
Supplementary Fig. 4 ROS levels in healthy ECs treated with EMPA. Healthy cells were treated with 0.02% DMSO (control) or 1μM EMPA and ROS levels were measured using FACS in HCAECs (a, n=3) and HUVECs (b, n=4). Data are presented as mean±SD.

Supplementary Fig. 5



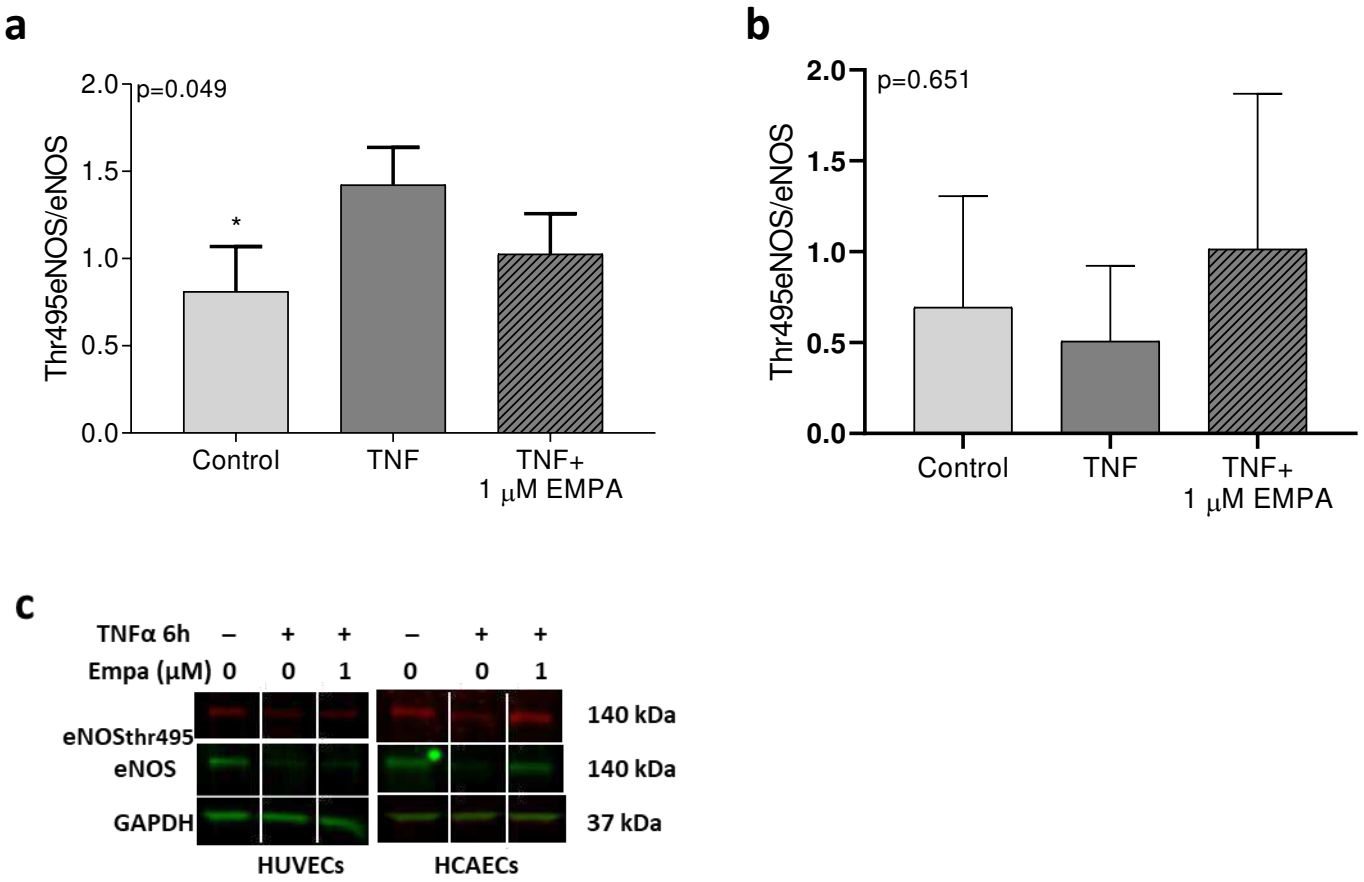
Supplementary Fig. 5 ROS (a) and NO (b) levels in HUVEC measured by live cell imaging. Three independent experiments were performed for ROS and NO. Cells were treated with 0.02% DMSO (control), 10 ng/mL TNFα or 10 ng/mL TNFα with 1 μM EMPA and for NO measurements with EMPA only. Data are presented as mean±SD.

Supplementary Fig. 6



Supplementary Fig. 6 Expression of $eNOS_{Ser1177}$ and total eNOS in endothelial cells treated with TNFα and DAPA. Cells were treated with 0.02% DMSO (control), 10 ng/mL TNFα or with 10 ng/mL TNFα with 1 μM DAPA. $eNOS_{Ser1177}$ levels were determined after 24 h TNFα stimulation in HCAECs (a, n=6-8) and HUVECs (b, n=5). Total eNOS levels were determined after 24 h TNFα stimulation in HCAECs (c, n=6-8) and HUVECs (d, n=5). Representative images of $eNOS_{Ser1177}$ and total eNOS western blots (h). GAPDH was used as internal control. Data are presented as mean±SD. *p<0.05, **p<0.01, ***p<0.001 vs. TNFα

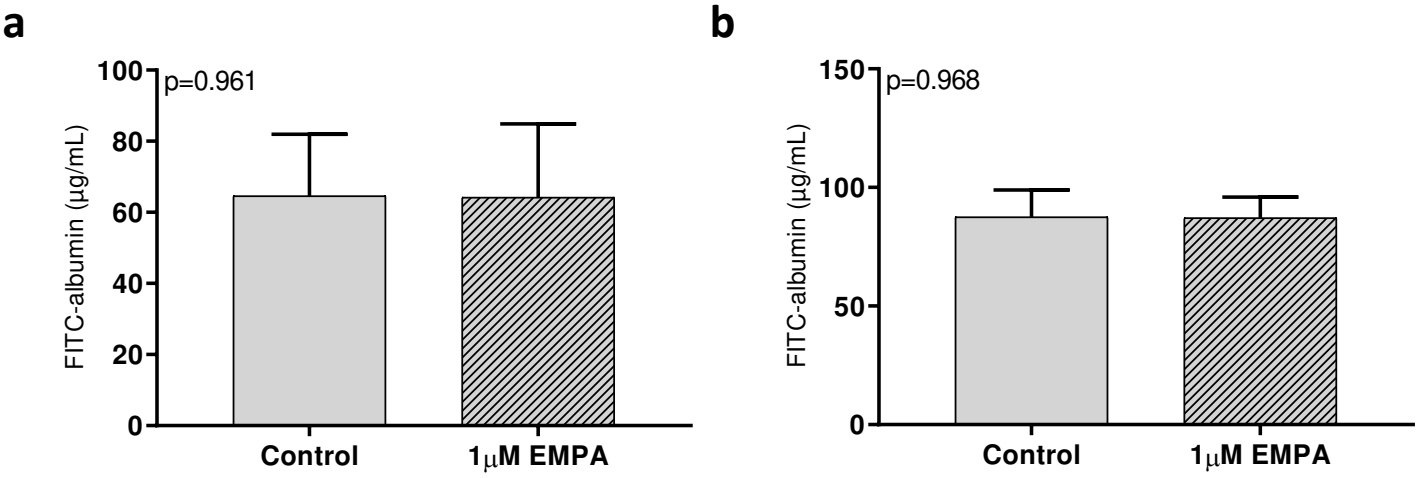
Supplementary Fig. 7



Supplementary Fig. 7:

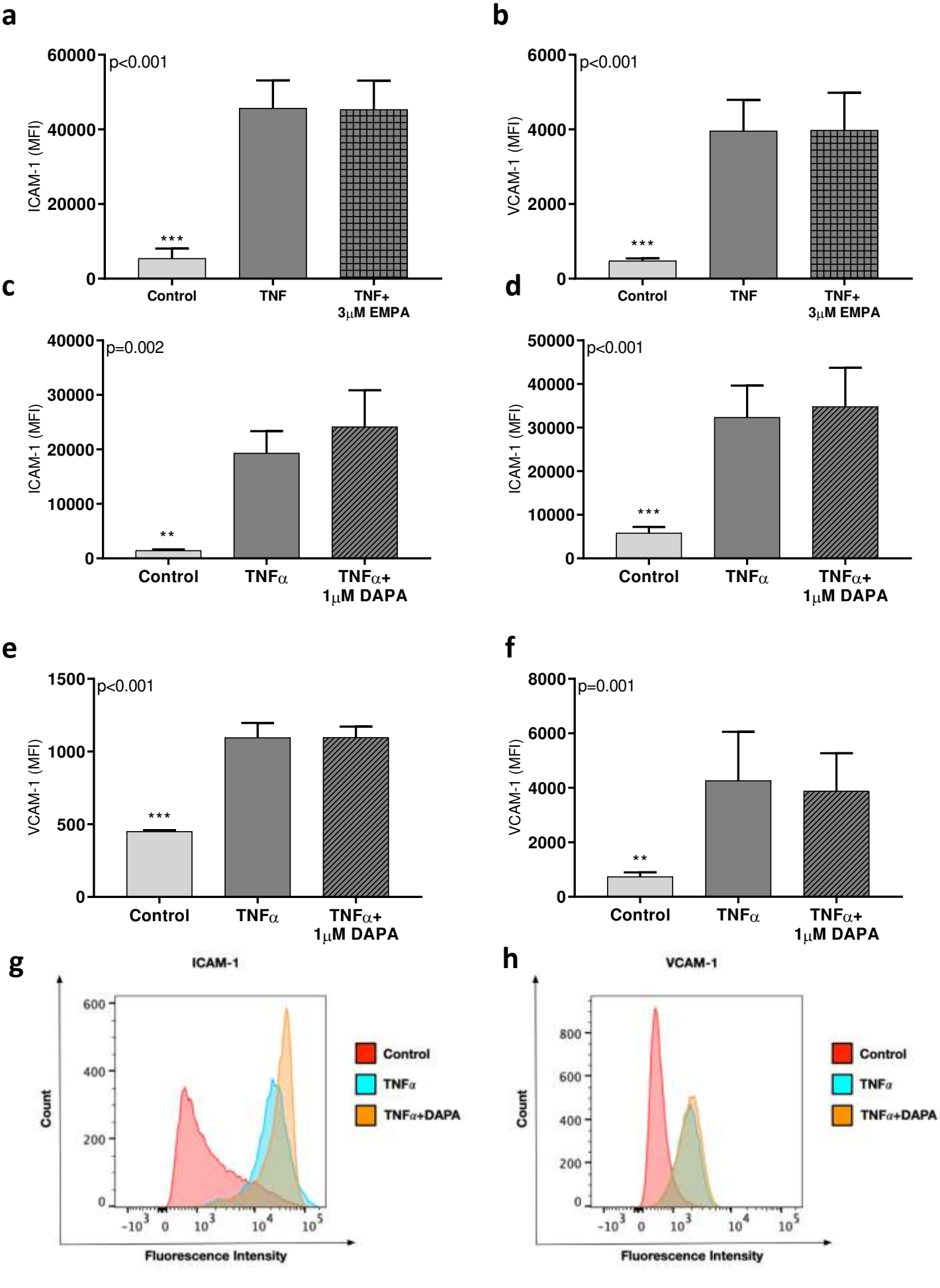
Protein expression of $eNOS_{thr495}$ / total eNOS in endothelial cells treated with TNFα and EMPA. Cells were treated with 0.02% DMSO (control), 10 ng/mL TNFα or 10 ng/mL TNFα with 1 μM EMPA. $eNOS_{thr495}$, total eNOS and GAPDH levels were determined after 6 h TNFα stimulation in HCAECs (a) and HUVECs (b), both n=3. Data are presented as mean±SD. *p<0.05 vs. TNFα

Supplementary Fig. 8



Supplementary Fig. 8 Endothelial cell permeability of healthy cells subjected to EMPA. Permeability was assessed in a trans-well assay by FITC-labelled albumin leakage in HCAECs (a, n=12 trans-wells/condition from 4 different cell batches) and HUVECs (b, n=4 trans-wells/condition). Data are presented as mean±SD.

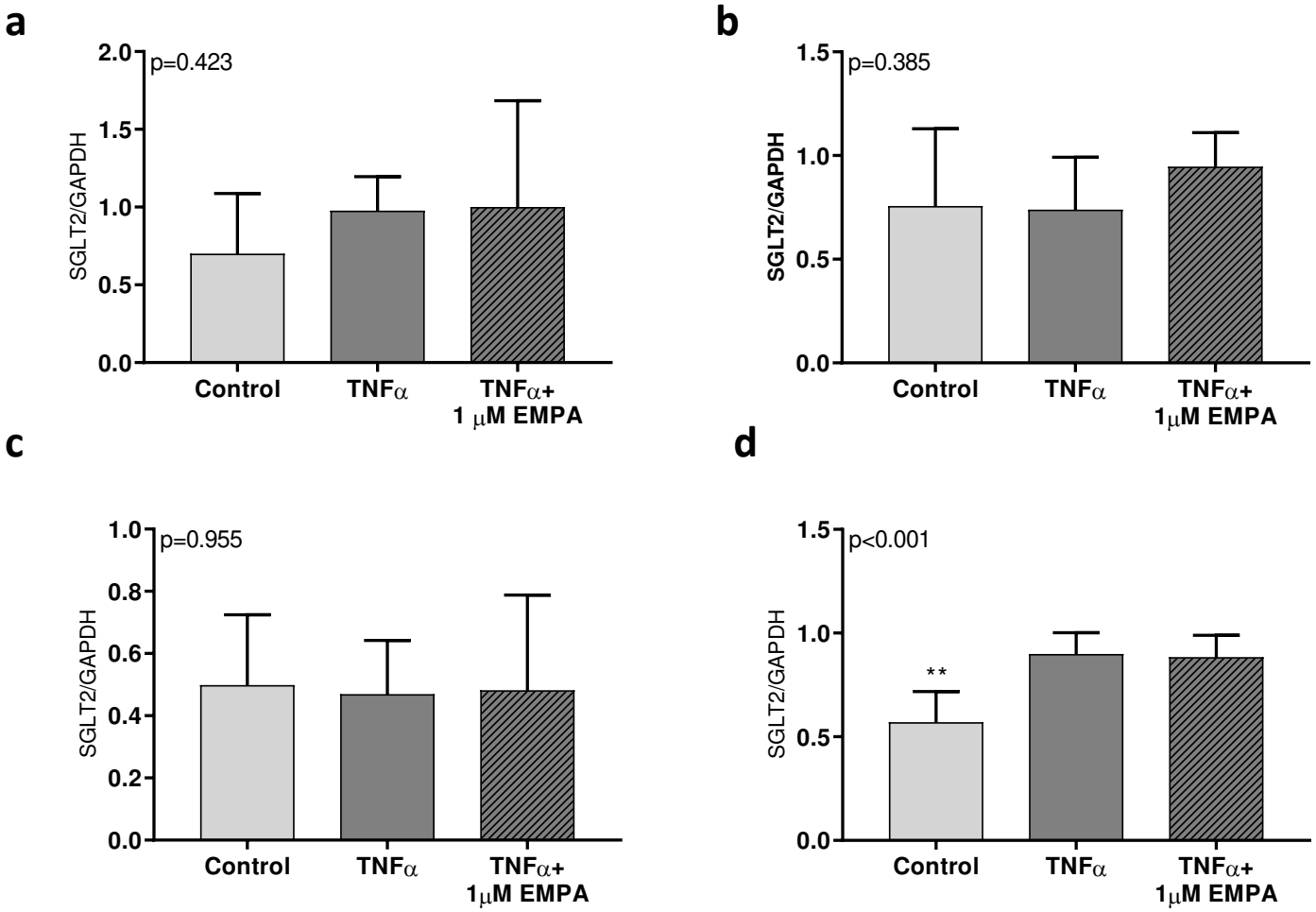
Supplementary Fig. 9

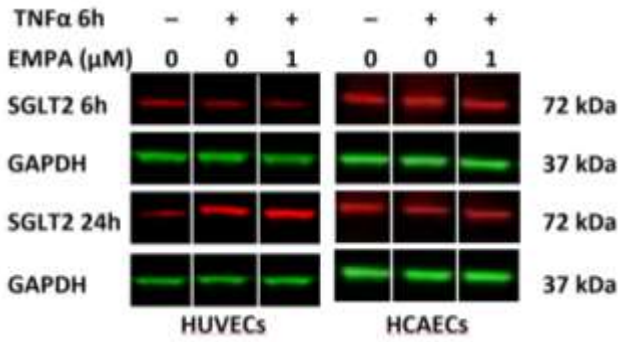


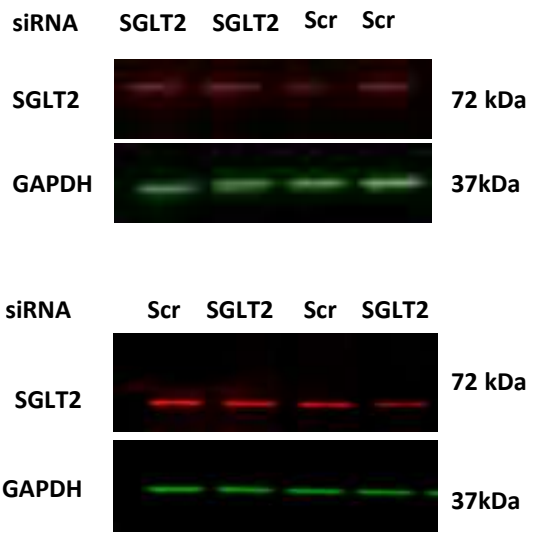
Supplementary Fig. 9

ESM Fig 9 Expression of adhesion molecules of endothelial cells treated with TNFα and high dose EMPA and DAPA. Cells were treated with 0.02% DMSO (control), 10 ng/mL TNFα or with 10 ng/mL TNFα with 3 µM EMPA or with 1 µM DAPA. ICAM-1 levels were determined after 4 h TNFα stimulation in HUVECs (a, n=5). VCAM-1 levels were determined after 4 h TNFα stimulation in HUVECs (b, n=5). ICAM-1 and VCAM-1 levels were determined after 24 h TNFα stimulation in HCAECs (c+e, n=3) and HUVECs (d+f, n=6). Representative FACS measurements of ICAM-1 and VCAM-1 for all three conditions in HUVEC is shown (g+h). Data are presented as mean±SD. ***p<0.001 vs. TNFα

Supplementary Fig. 10







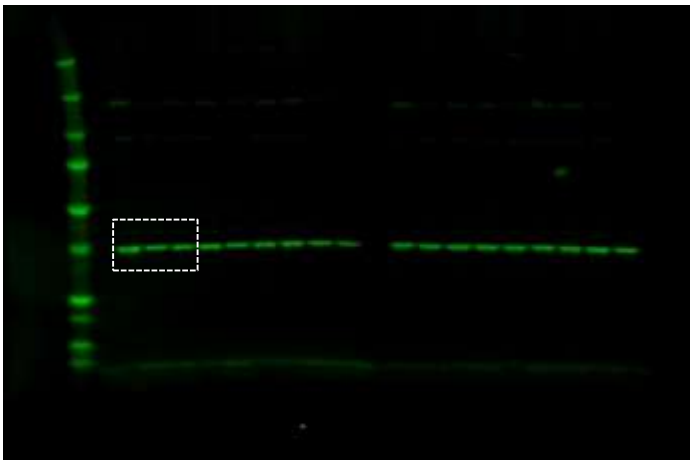
g

Sample	Cq (ACTB)	*N0 (ACTB)	Cq (SLC5A2)	*N0 (SLC5A2)
HCAEC	15,52	1,12E-04	ND	ND
HEK-293	17,02	3,42E-05	ND	ND

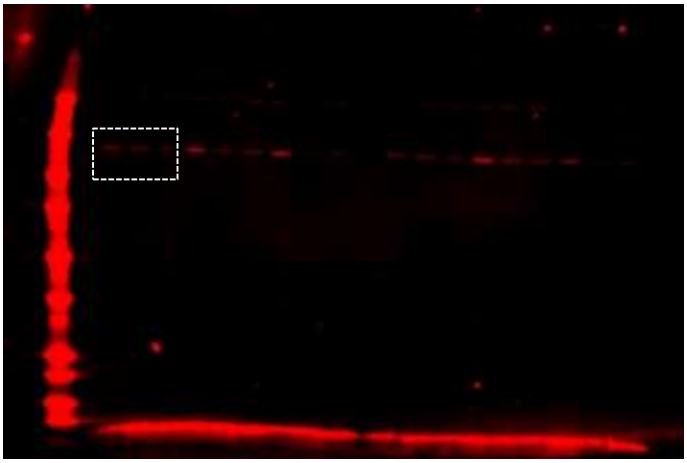
Supplementary Fig. 10 Protein expression of SGLT2 in endothelial cells treated with TNFα and EMPA. Cells were treated with 0.02% DMSO (control), 10 ng/mL TNFα or with 10 ng/mL TNFα with 1μM EMPA. SGLT2 levels were determined after 6 h and 24 h TNFα stimulation in HCAECs (respectively a+c, n=6 and c, n=12) and HUVECs (respectively b+d, n=6). Representative images of SGLT2 western blots (e). GAPDH was used as internal control. Detection of SGLT2 protein in endothelial cells exposed to SGLT2 or negative control siRNA and scrambled (scr) RNA (f) in HCAECs (n=2, upper panel) and HUVECs (n=2, lower panel). qPCR table showing no SGLT2 (SLC5A2) mRNA in HCAECs and HEK-293,*N0 is defined as the starting concentration for each sample. This is expressed in arbitrary fluorescence units.
(g). Data are presented as mean±SD. **p<0.01 vs. TNFα

Supplementary Fig. 11

a



b



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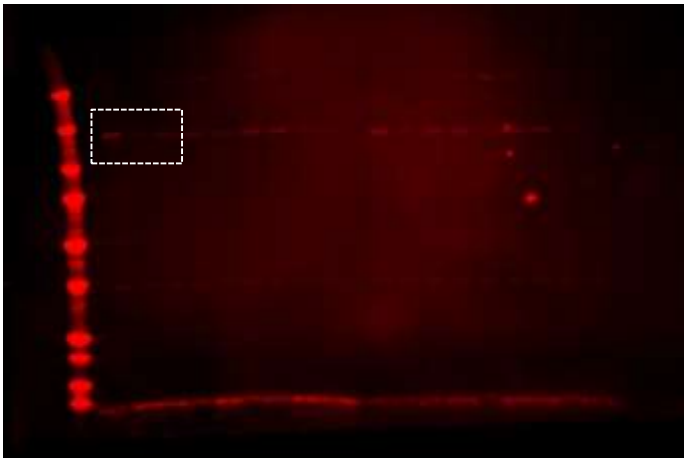


Supplementary Fig. 11: Full-length blot of for ESM Figure 6e HCAECs

Panel a GAPDH in HCAECs, bands 37 kDa

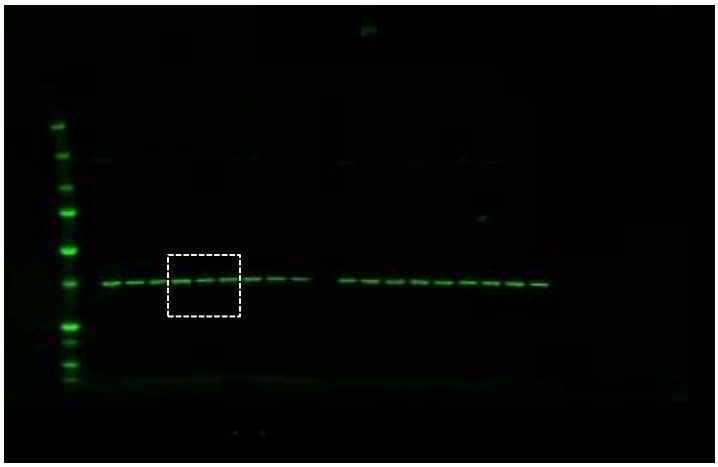
Panel b: eNOS in HCAECs, bands 140 kDa

Panel c: peNOS$_{Ser1177}$ in HCAECs, bands 140 kDa

From left to right in bands in dashed areas are included in ESM fig 6e: control, TNF, TNF+DAPA.

Supplementary Fig. 12

a



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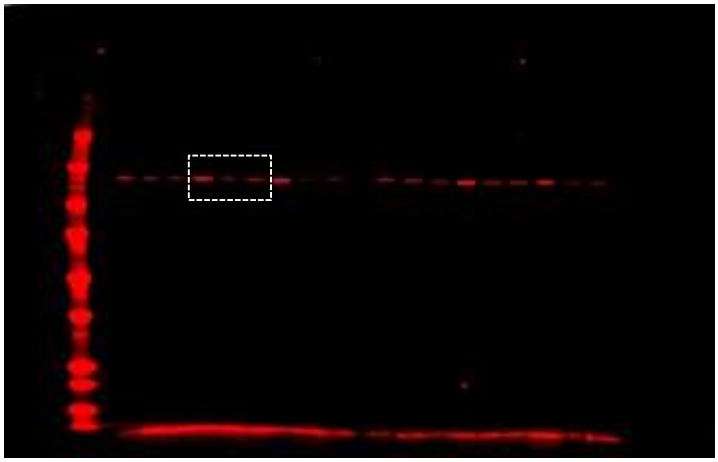

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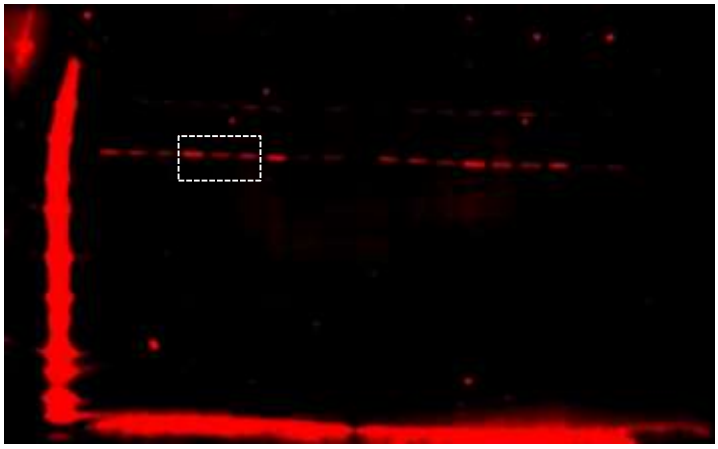


Supplementary Fig. 12: Full-length blot of for ESM Figure 6e HUVECs

Panel a: GAPDH in HUVECs, bands 37 kDa

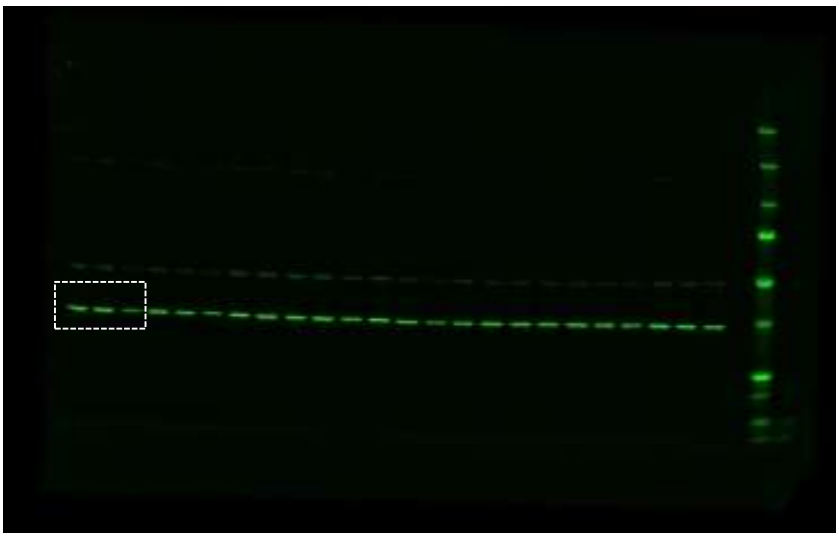
Panel b: eNOS in HUVECs, bands 140 kDa

Panel c: eNOS$_{Ser1177}$ in HCAECs, bands 140 kDa

From left to right bands in dashed areas are included in ESM fig 6e: control, TNF, TNF+DAPA.

Supplementary Fig. 13

a



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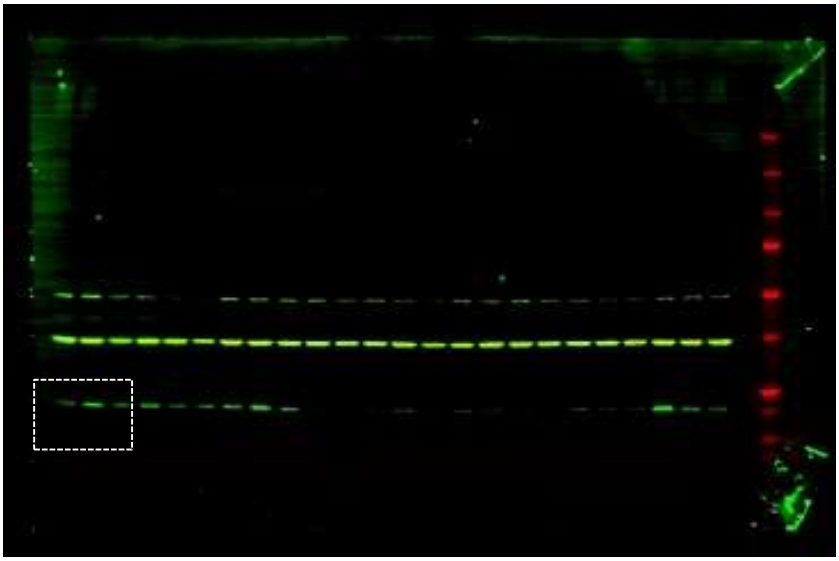


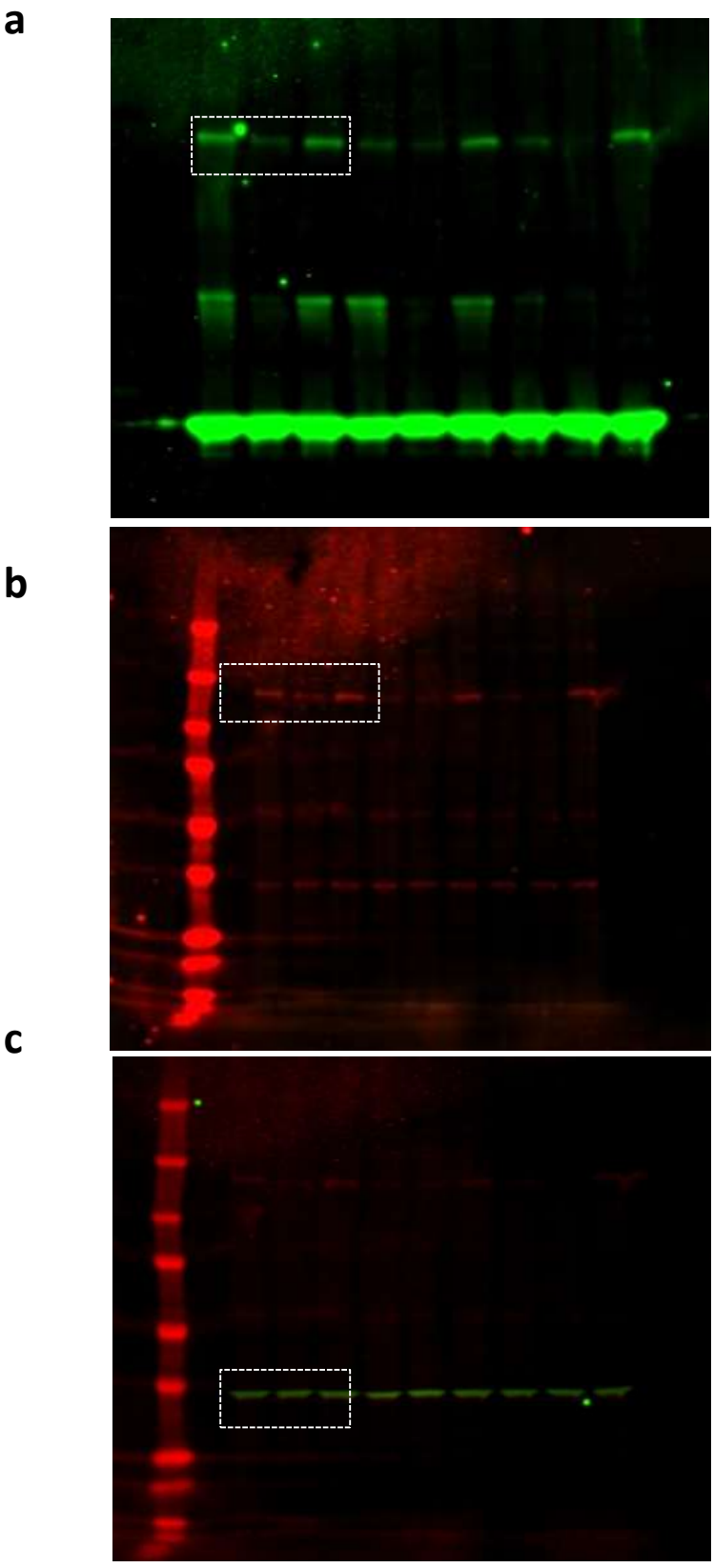
Supplementary Fig. 13: Full-length blot for Figure 4c HCAECs

Panel a: GAPDH in HCAECs, Bands at 37 kDa

Panel b: Cav-1 in HCAECs, Bands at 21 kDa

From left to right bands in dashed areas are included in fig 4c control, TNF, TNF+EMPA.

Supplementary Fig. 14:



Supplementary Fig. 14: Full-length blot for ESM Figure 7c HCAECs

Panel a: eNOS, bands 140 kDa

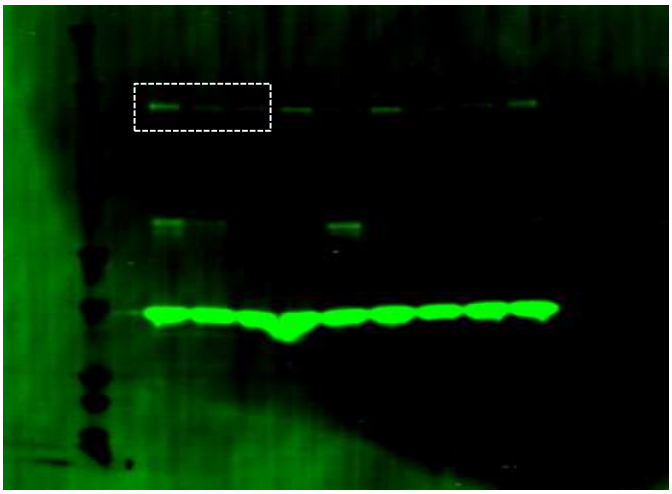
Panel b: eNOS$_{Thr495}$, bands 140 kDa

Panel c: GAPDH, bands 37 kDa

From left to right bands in dashed areas are included in ESM fig 7c: control, TNF, TNF+EMPA.

Supplementary Fig. 15:

a



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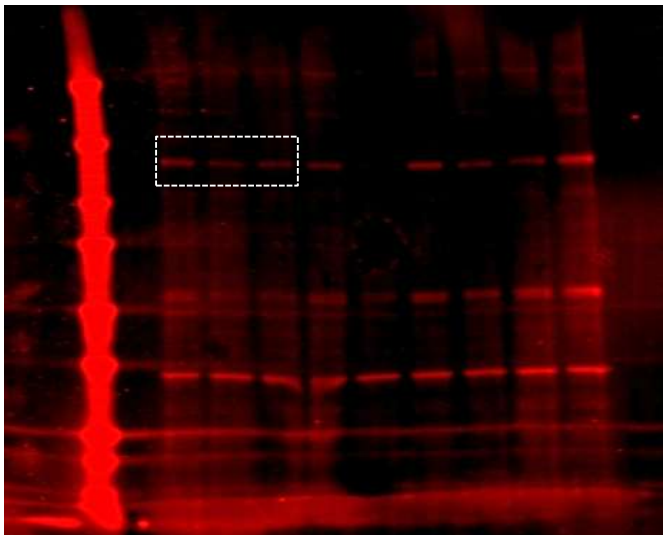

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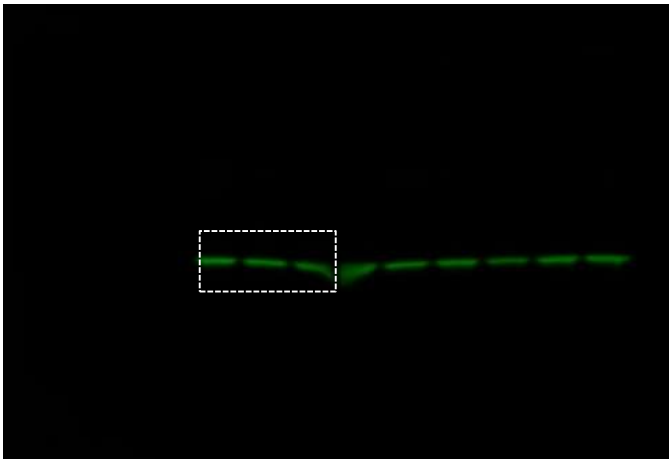


Supplementary Fig. 15: Full-length blot for ESM Figure 7c HUVECs

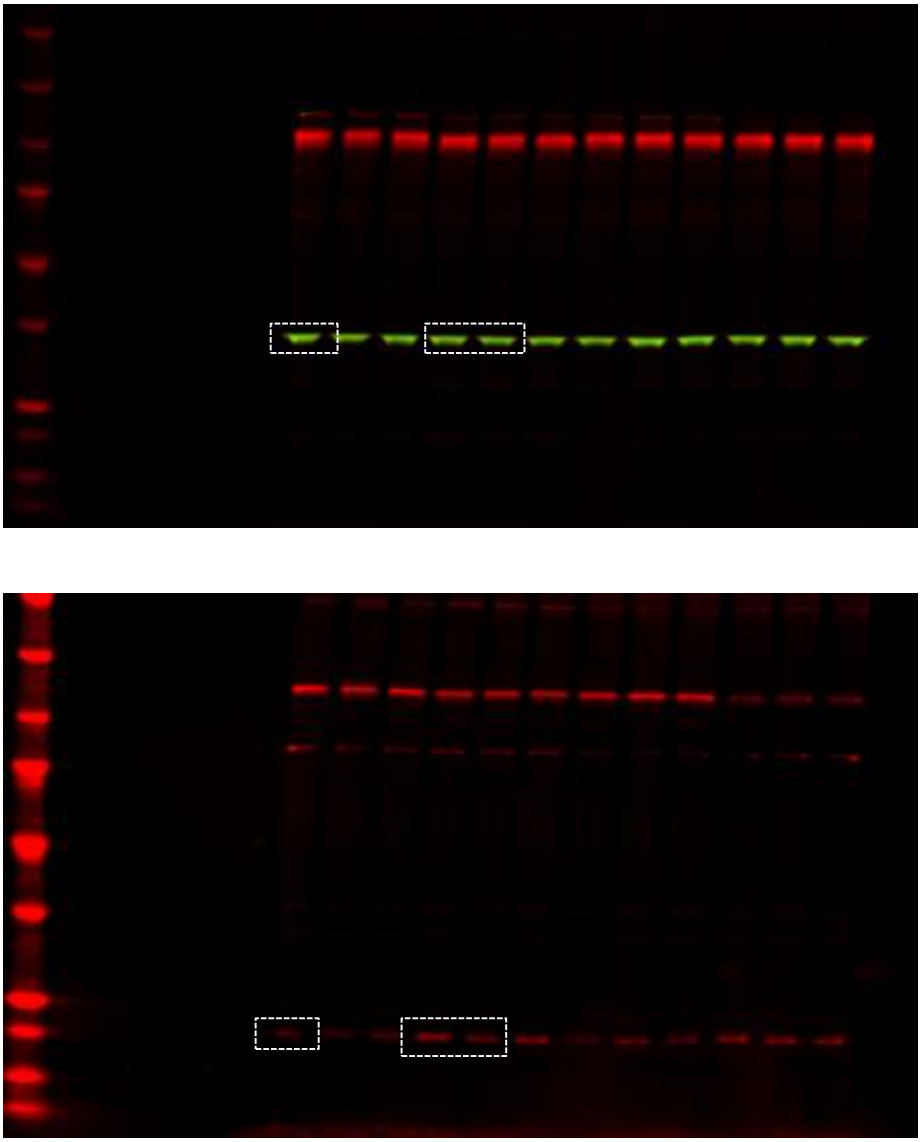
Panel a: eNOS, bands 140 kDa

Panel b: eNOS$_{Thr495}$, bands 140 kDa

Panel c: GAPDH, bands 37 kDa

From left to right bands in dashed areas are included in ESM fig 7 c: control, TNF, TNF+EMPA.

Supplementary Fig. 16



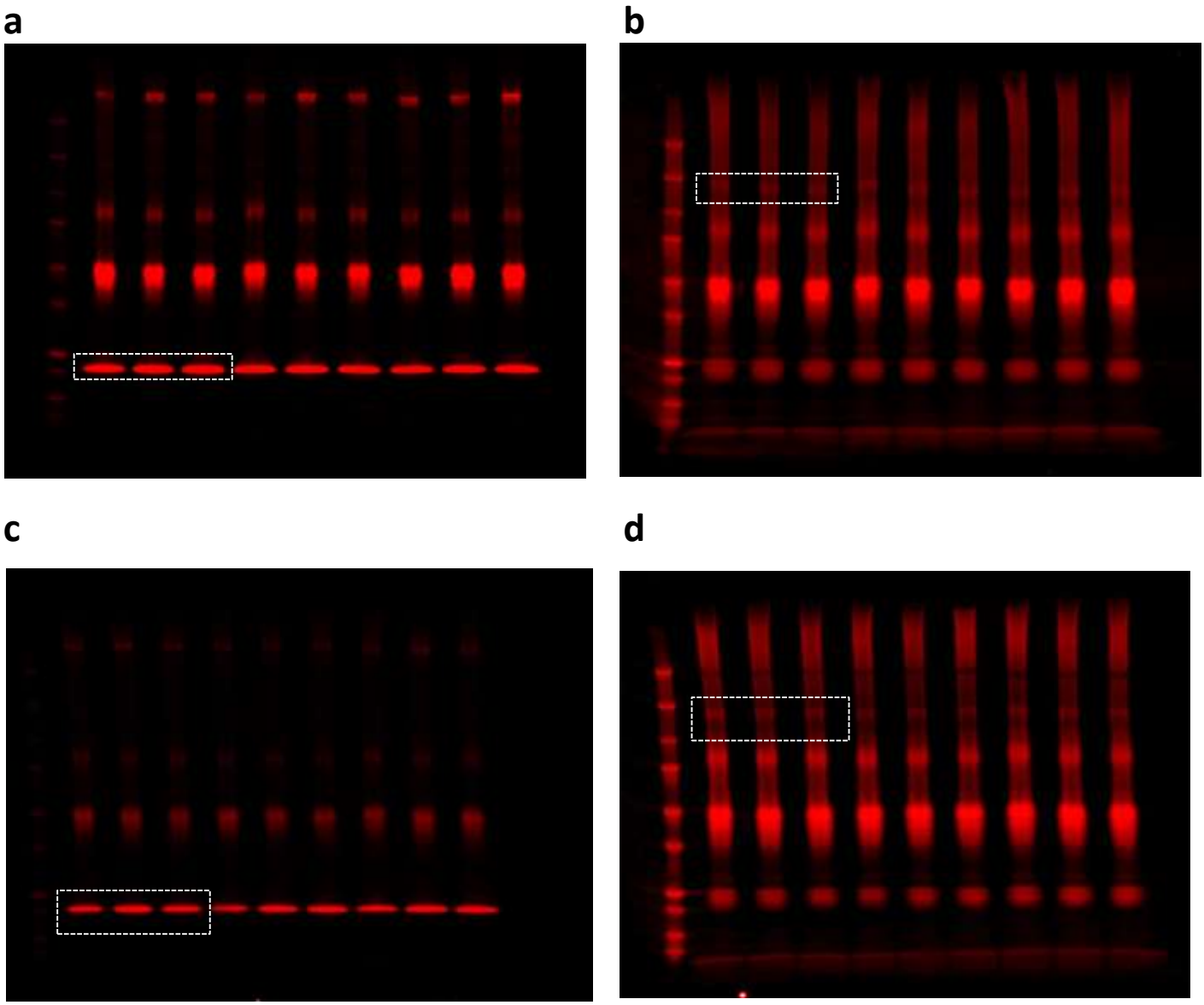
Supplementary Fig. 16: Full-length blot for Figure 4c HUVECs

Panel a: GAPDH in HUVECs, Bands at 37 kDa

Panel b: Cav-1 in HUVECs, Bands at 21 kDa

From left to right bands in dashed areas are included in figure 4c : control, TNF, TNF+EMPA.

Supplementary Fig. 17:



Supplementary Fig. 17: Full-length blot for Figure 4f HCAECs and HUVECs

Panel a: Cav-1 in HCAECs, Bands at 21 kDa

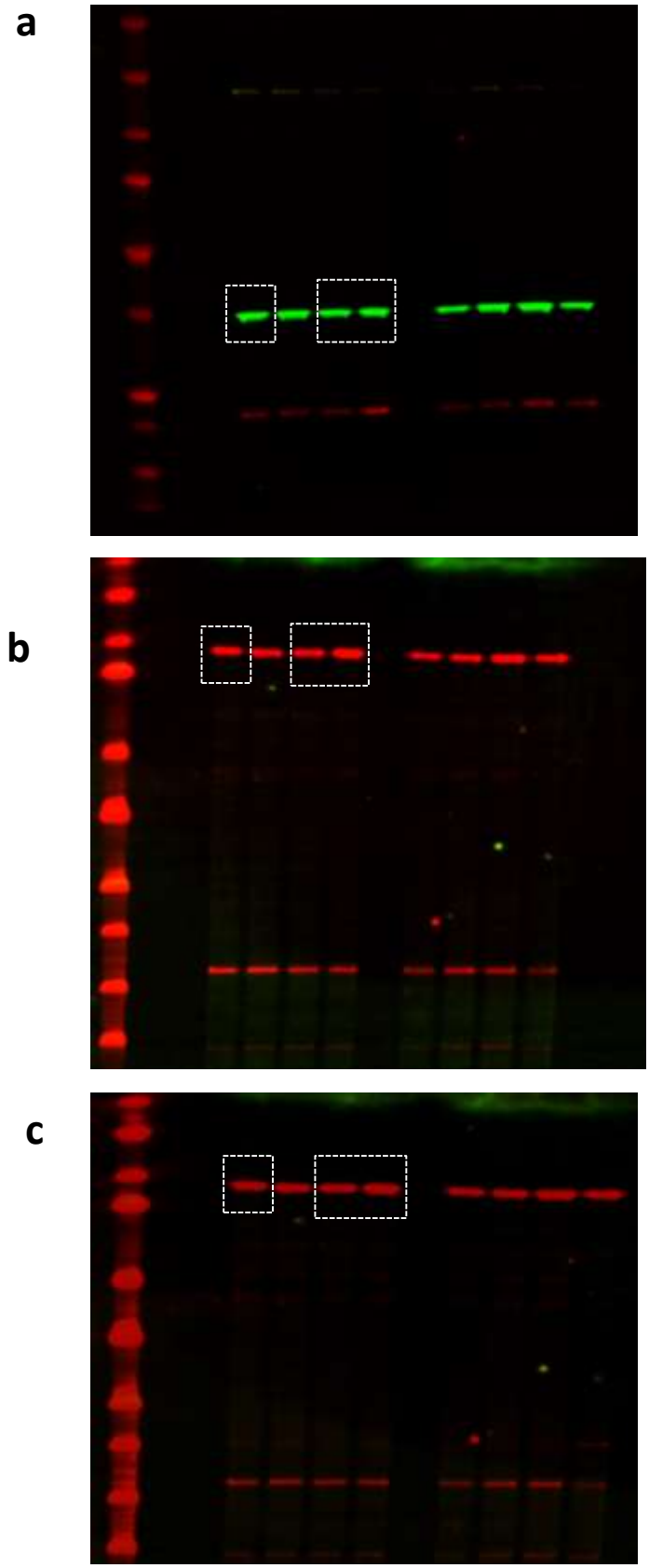
Panel b: eNOS in HCAECs, Bands at 140 kDa

Panel c: Cav-1 in HUVECs, Bands at 21 kDa

Panel d: eNOS in HUVECs, Bands at 140 kDa

From left to right bands in dashed areas are included in figure 4f: control, TNF, TNF+EMPA.

Supplementary Fig. 18



Supplementary Fig. 18: Full-length blot of for Figure 5g HCAECs

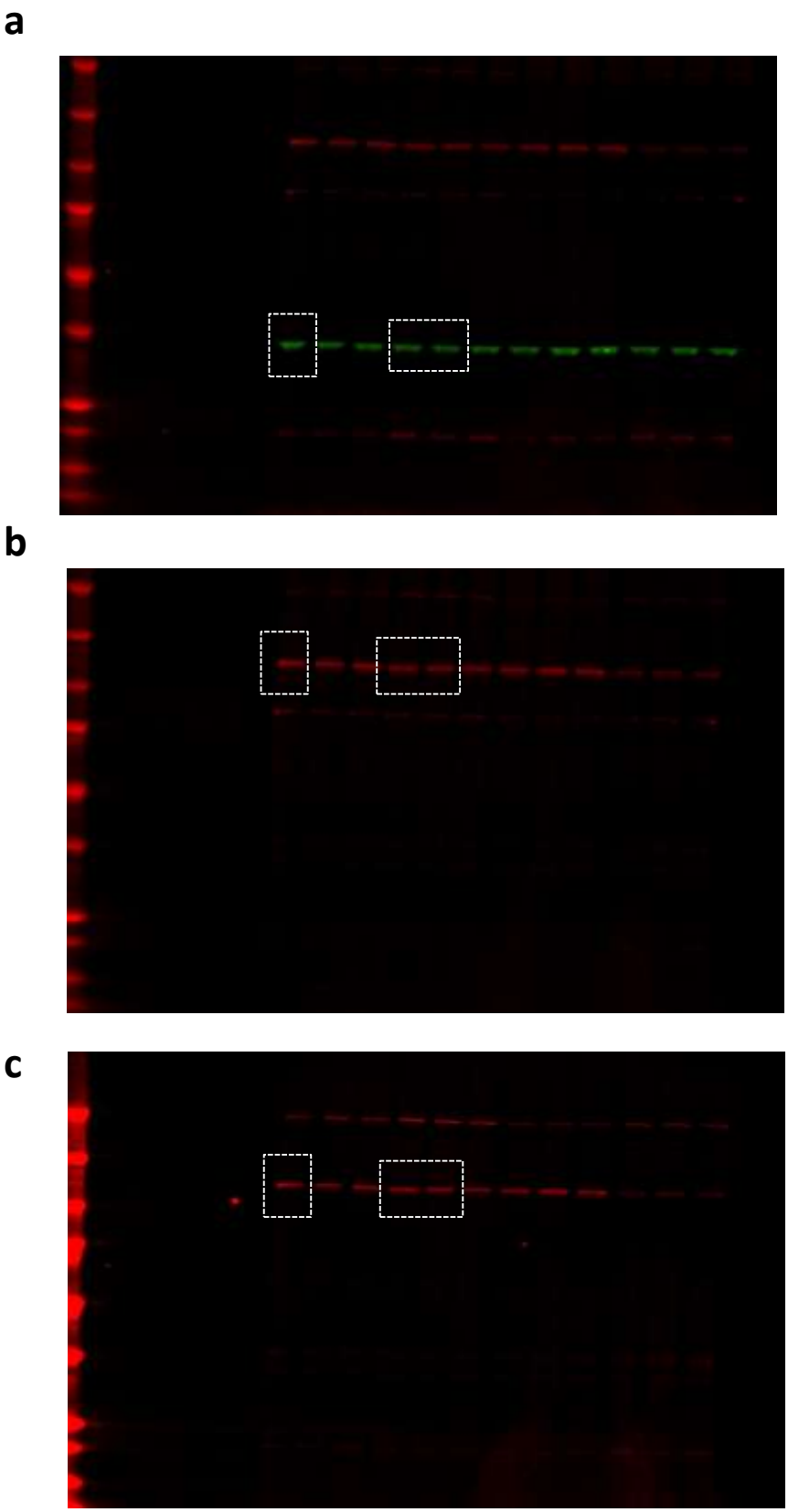
Panel A: GAPDH in HCAEC, bands 37 kDa

Panel B: eNOS in HCAECs, bands 140 kDa

Panel C: eNOS$_{Ser1177}$ in HCAECs, bands 140 kDa

From left to right bands in dashed areas are included in fig 5g: control, TNF, TNF+EMPA.

Supplementary Fig. 19



Supplementary Fig. 19: Full-length blot of for Figure 5g HUVECs

Panel A: GAPDH in HUVECs, bands 37 kDa

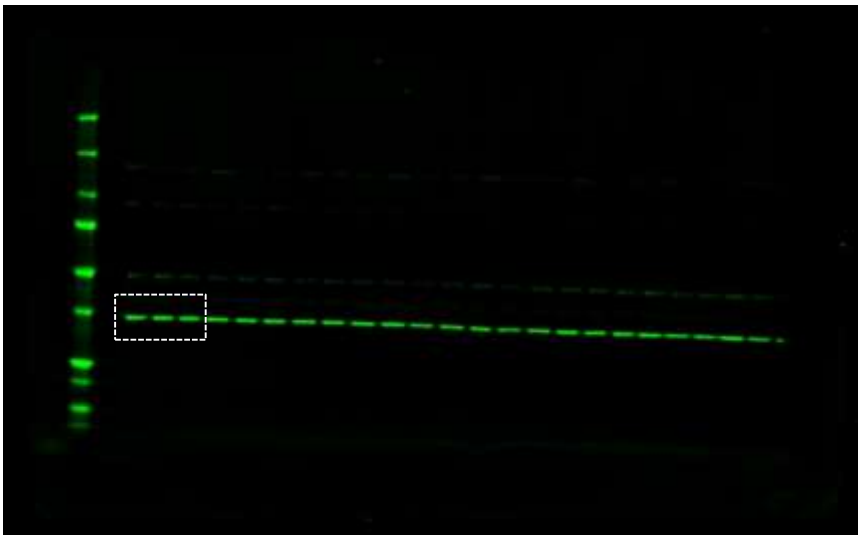
Panel B: eNOS in HUVECs, bands 140 kDa

Panel C: eNOS$_{Ser1177}$ in HUVECs, bands 140 kDa

From left to right bands in dashed areas are included in fig 5g: control, TNF, TNF+EMPA.

Supplementary Fig. 20

a



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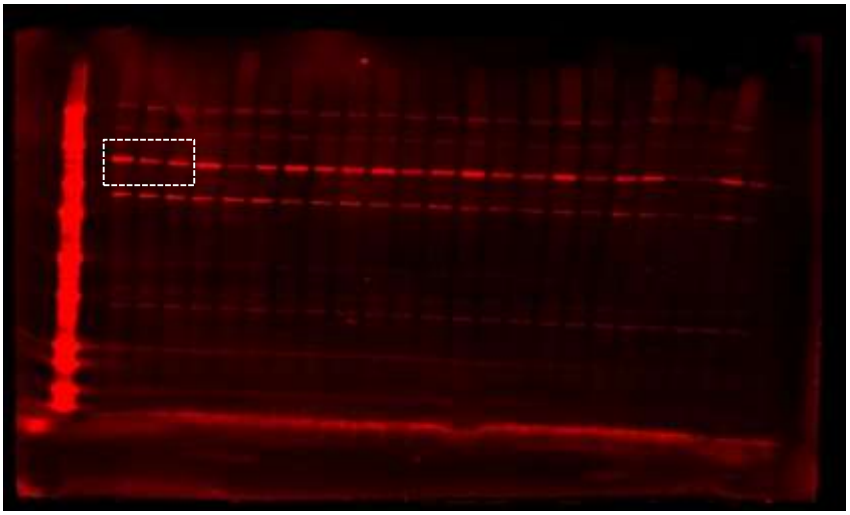
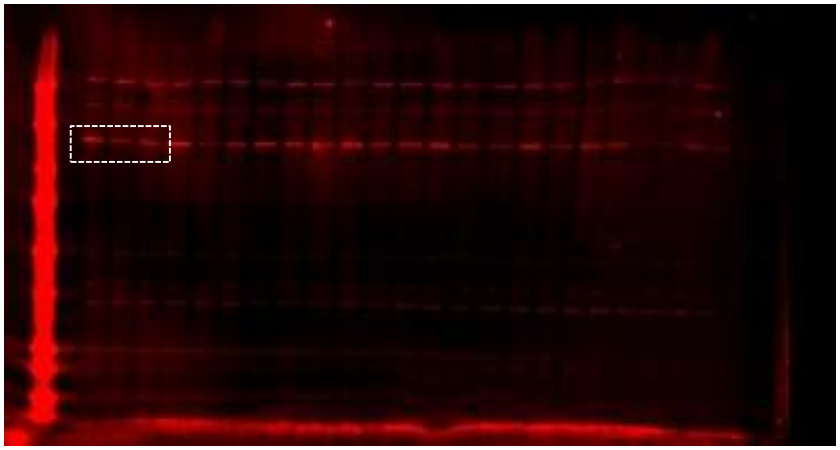


c



ESM Fig 20: Full-length blot of for Figure 6g HCAECs

Panel a: GAPDH in HCAECs, bands 37 kDa

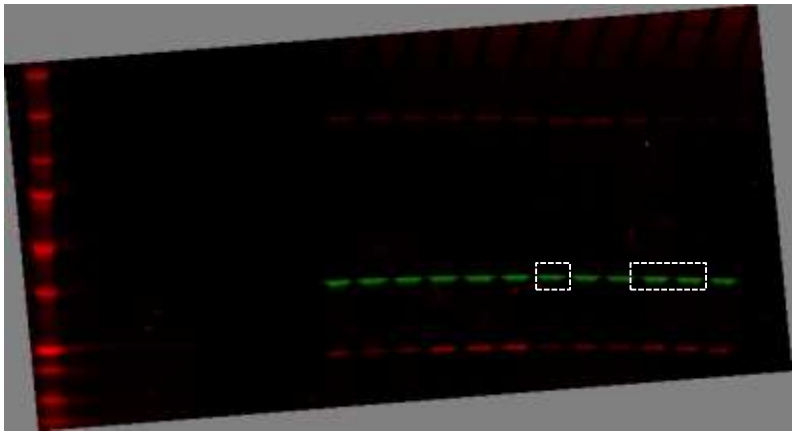
Panel b: eNOS in HCAECs, bands 140 kDa

Panel c: peNOS$_{Ser1177}$ in HCAECs, bands 140 kDa

From left to right bands in dashed areas are included in fig 6g: control, TNF, TNF+EMPA.

Supplementary Fig. 21:

a



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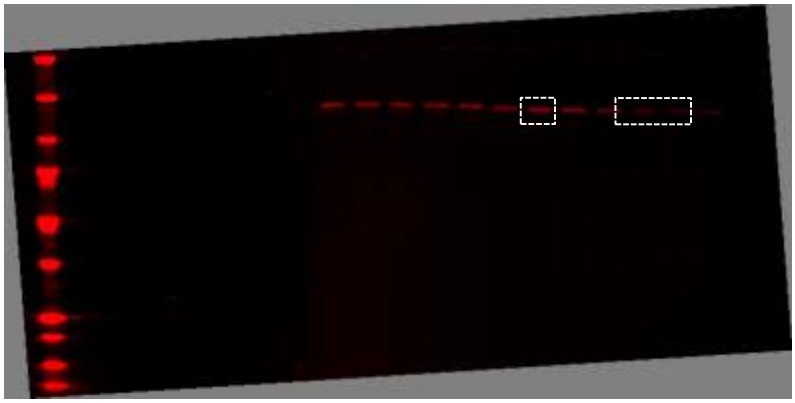

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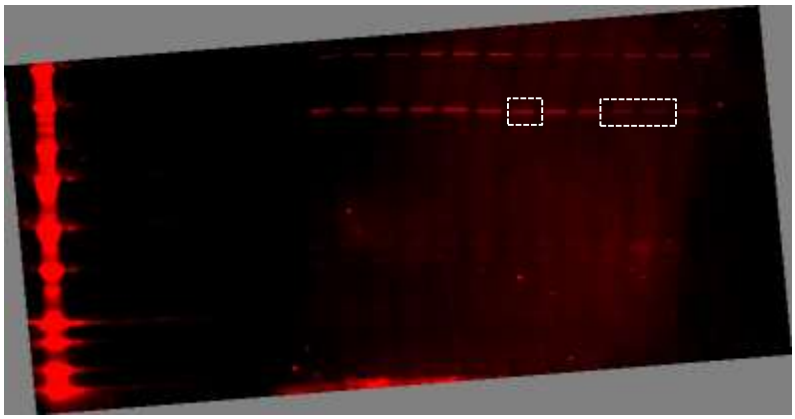


Supplementary Fig. 21: Full-length blot of for Figure 6g HUVECs

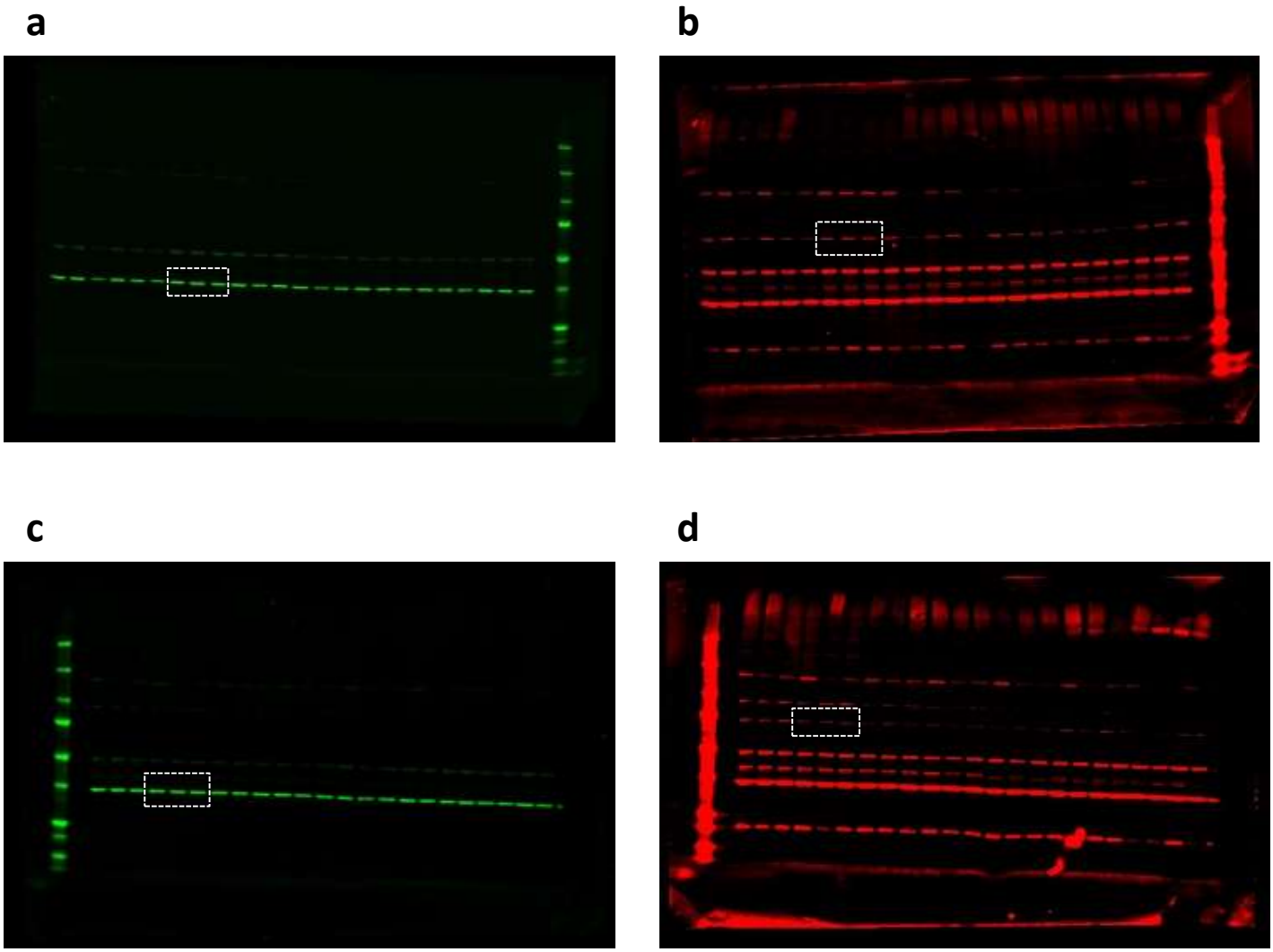
Panel a: GAPDH in HUVECs, bands 37 kDa

Panel b: eNOS in HUVECs, bands 140 kDa

Panel c: peNOS$_{Ser1177}$ in HUVECs, bands 140 kDa

From left to right bands in dashed areas are included in fig 6g: control, TNF, TNF+EMPA.

Supplementary Fig. 22



Supplementary Fig. 22: Full-length blot for ESM Figure 10e HCAECs

Panel a: GAPDH 6 h TNF, HCAECs, bands 37 kDa

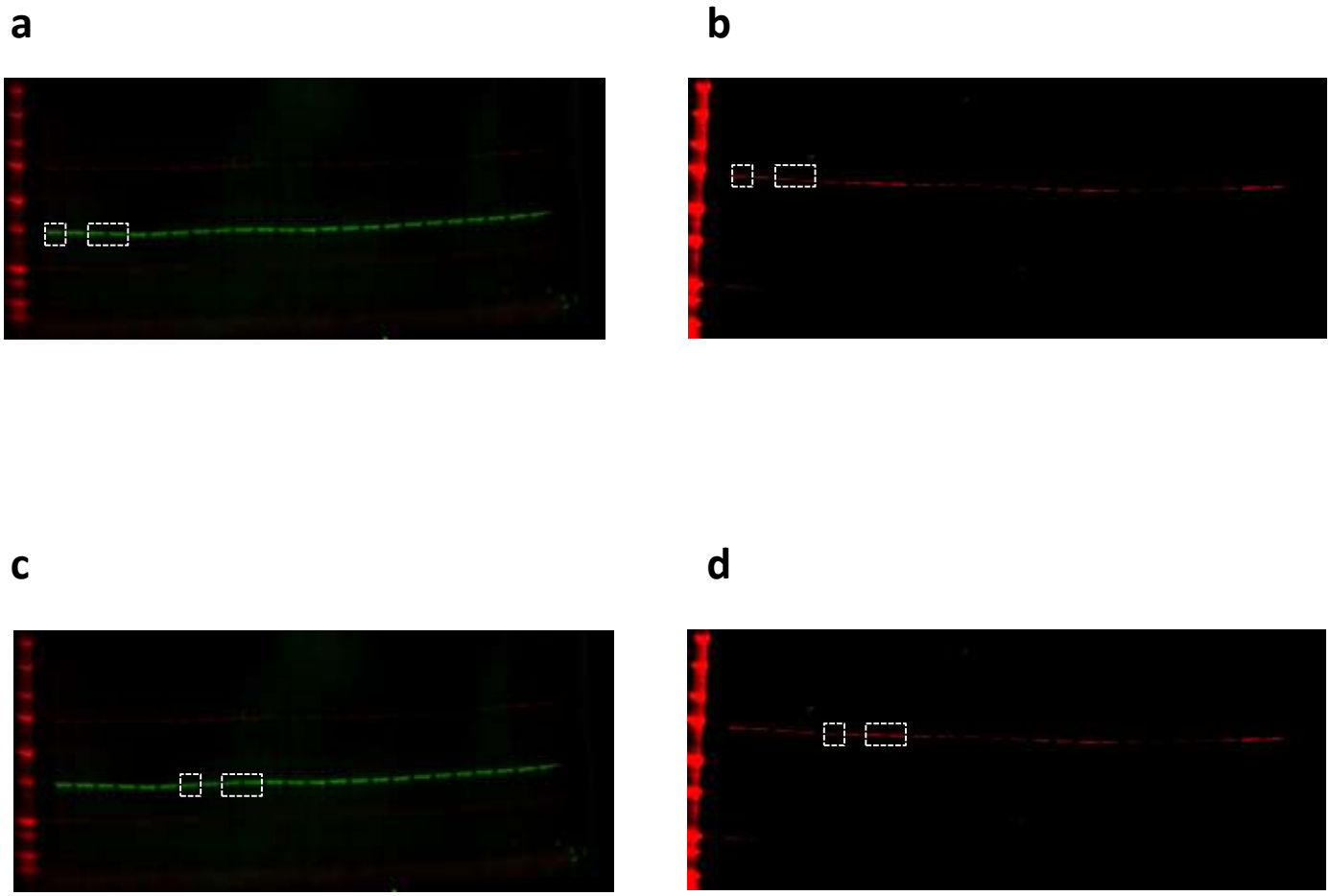
Panel b: SGLT2 6 h TNF, HCAECs, bands ~72 kDa

Panel c: GAPDH 24 h TNF, HCAECs, bands 37 kDa

Panel d: SGLT2 24 h TNF, HCAECs, bands ~72 kDa

From left to right in dashed areas are included in ESM fig 10e: control, TNF, TNF+EMPA.

Supplementary Fig. 23



Supplementary Fig. 23: Full-length blot for ESM Figure 10e HUVECs

Panel a: GAPDH 6 h TNF, HUVECs, bands 37 kDa

Panel b: SGLT2 6 h TNF, HUVECs, bands ~72 kDa

Panel c: GAPDH 24 h TNF, HUVECs, bands 37 kDa

Panel d: SGLT2 24 h TNF, HUVECs, bands ~72 kDa

From left to right in dashed areas are included in ESM fig 10e: control, TNF, TNF+EMPA.