**SUPPLEMENTARY MATERIAL**

# SARS-CoV-2 spike protein induces endothelial inflammation via ACE2 independently of viral replication

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## Supplementary Figure Legends

**Supplementary Fig. S1 – rS1p concentration-dependent effects on mRNA expression of pro-inflammatory markers in hMECs.** Human endothelial cells (hMEC) were exposed to increasing concentrations of rS1p (0.165, 0.33, 0.66, 1.32 μg/mL) for 5h and mRNA expression of IL-6 (A), TNFα (B), MCP-1 (C) and VCAM-1 (D) was assessed by RT-PCR (n=5). Data are expressed as ± SEM; * $p<0.05$ control (non-stimulated cells) vs. rS1p stimulated cells at different concentrations after 1-way ANOVA followed by Dunnett's post-hoc test.

**Supplementary Fig. S2 – rS1p increases pro-inflammatory gene expression and short term microparticle formation in hMECs.** Human endothelial cells (hMEC) were stimulated with rS1p (0.66 μg/mL) for 5h and 24h for assessment of TNFα (A), VCAM-1 (B), PAI-1 (C), thrombin (D), angiopoetin-2 (E) mRNA expression and short term microparticles formation (F) (n=6). Data are expressed as ± SEM; * $p<0.05$ control (Ctl) (non-stimulated cells) vs. rS1p stimulated cells after 1-way ANOVA followed by Tukey's post-hoc test.

**Supplementary Fig. S3 – rS1p does not change TGFβ and preproET-1 gene expression in hMECs.** Human endothelial cells (hMEC) were stimulated with rS1p (0.66 μg/mL) for 5h and 24h for assessment of TGFβ (A) and preproET-1 (B) mRNA expression (n=5). Data are expressed as ± SEM.

**Supplementary Fig. S4 – rS1p does not induce ROS production in hMECs.** Human endothelial cells (hMEC) were stimulated with rS1p (0.66 μg/mL) for 10 and 30 minutes for assessment of ROS (A) and $H_2O_2$ production (B) (n=5-6). Data are expressed as ± SEM.

**Supplementary Fig. S5 – rS1p does not change gene expression of antioxidants in hMECs.** Human endothelial cells (hMEC) were stimulated with rS1p (0.66 μg/mL) for 5h and 24h for assessment of SOD1 (A), catalase (B), glutathione peroxidase (GPX) (C), peroxiredoxin (PRDX) (D), heme oxygenase-1 (HO-1) (E) and thioredoxin (F) mRNA expression (n=6). Data are expressed as ± SEM.

**Supplementary Fig. S6 – rS1p increases gene expression of pro-inflammatory mediators and induces microparticle formation in hLECs.** Human endothelial cells (hLEC) were stimulated with rS1p (0.66 μg/mL) for 5h and 24h for assessment of IL-6 (A) and MCP-1 (B) mRNA expression; IL-6 (C) and MCP-1 (D) production; microparticles formation at long (E) and short term (F); and ROS (G) and H2O2 (H) production (n=5-6). Data are expressed as ± SEM; * $p<0.05$ control (Ctl) (non-stimulated cells) vs. rS1p stimulated cells after student's t-test (C, D, E) or 1-way ANOVA followed by Tukey's post-hoc test.

**Supplementary Fig. S7 – rS1p increases pro-inflammatory markers gene expression and microparticle formation in hAECs.** Human endothelial cells (hAEC) were stimulated with rS1p (0.66 μg/mL) for 5h and 24h for assessment of IL-6 (A) and MCP-1 (B) mRNA expression; IL-6 (C) and MCP-1 (D) production; microparticles formation at long (E) and short term (F); and ROS (G) and H2O2 (H) production (n=5-6). Data are expressed as ± SEM; * $p<0.05$ control (Ctl) (non-stimulated cells) vs. rS1p stimulated cells after student's t-test (C, D, E) or 1-way ANOVA followed by Tukey's post-hoc test.

**Supplementary Fig. S8 – rS1p increases pro-inflammatory markers gene expression and microparticle formation in hPECs.** Human endothelial cells (hPEC) were stimulated with rS1p (0.66 μg/mL) for 5h and 24h for assessment of IL-6 (A) and MCP-1 (B) mRNA expression; IL-6 (C) and MCP-1 (D) production; microparticles formation at long (E) and short term (F); and ROS (G) and H2O2 (H) production (n=5-6). Data are expressed as ± SEM; * $p<0.05$ control (Ctl) (non-stimulated cells) vs. rS1p stimulated cells after student's t-test (C, D, E) or 1-way ANOVA followed by Tukey's post-hoc test.

**Supplementary Fig. S9 – rS1p effects in pro-inflammatory markers mRNA expression after treatment with the ACE2 inhibitor MLN-4760 in human endothelial cells.** Human endothelial cells (hMEC) were stimulated with rS1p (0.66 μg/mL) for 5h for assessment of IL-6 (A), MCP-1 (B), PAI-1 (C) and VCAM-1 (D) mRNA expression in the presence or absence of MLN-4760 (440 pmol), an ACE2

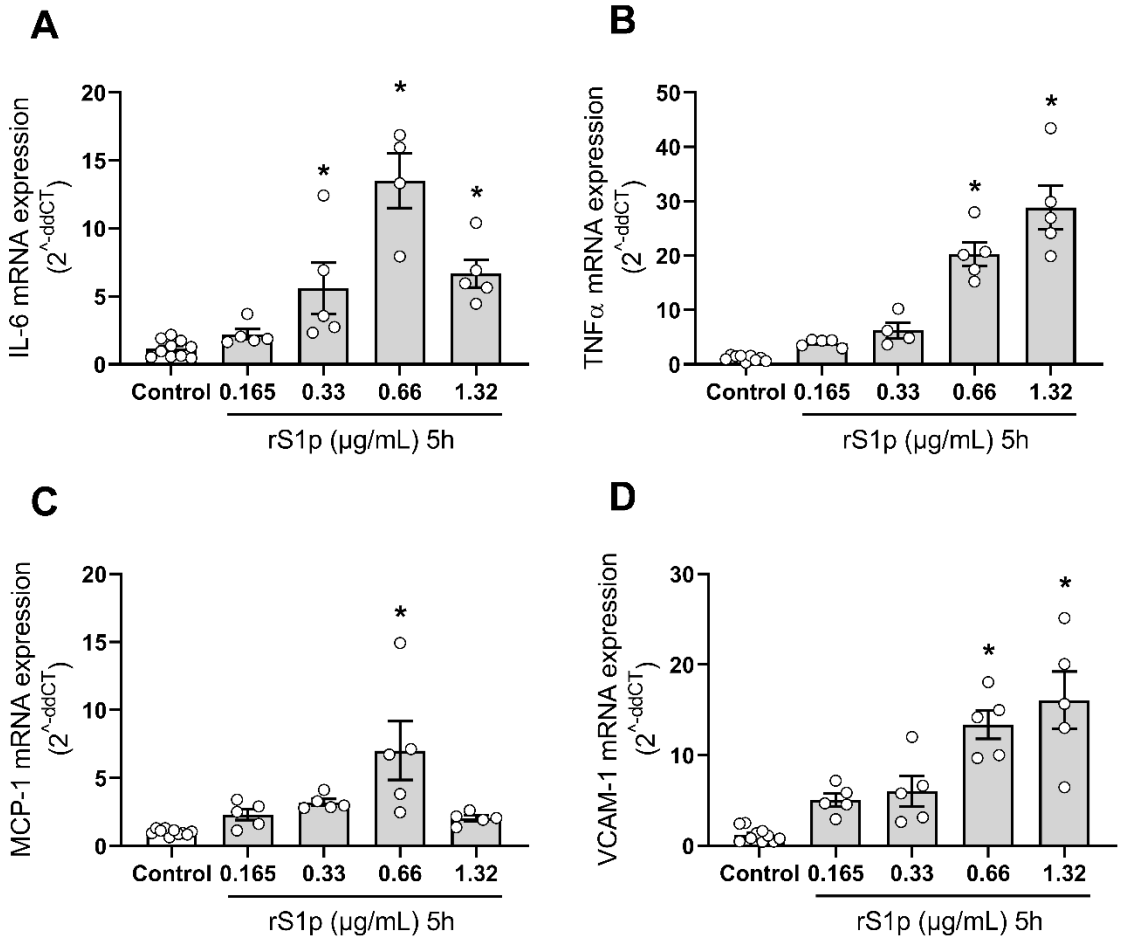
inhibitor (n=10-11). Data are expressed as ± SEM; * $p<0.05$ control (Ctl) (non-stimulated cells) vs. rS1p stimulated cells; † $p<0.05$ rS1p stimulated cells vs. rS1p stimulated cells treated with MLN-4760 after 1-way ANOVA followed by Tukey's post-hoc test.

**Supplementary Fig. S10 – rS1p effects in pro-inflammatory markers mRNA expression after treatment with the ACE2 activator DIZE in human endothelial cells.** Human endothelial cells (hMEC) were stimulated with rS1p (0.66 μg/mL) for 5h for assessment of IL-6 (A), MCP-1 (B) and TNFα (C) mRNA expression in the presence or absence of DIZE (190 pmol), an ACE2 activator (n=10). Data are expressed as ± SEM; * $p<0.05$ control (Ctl) (non-stimulated cells) vs. rS1p stimulated cells in the presence or absence of DIZE after 1-way ANOVA followed by Tukey's post-hoc test.

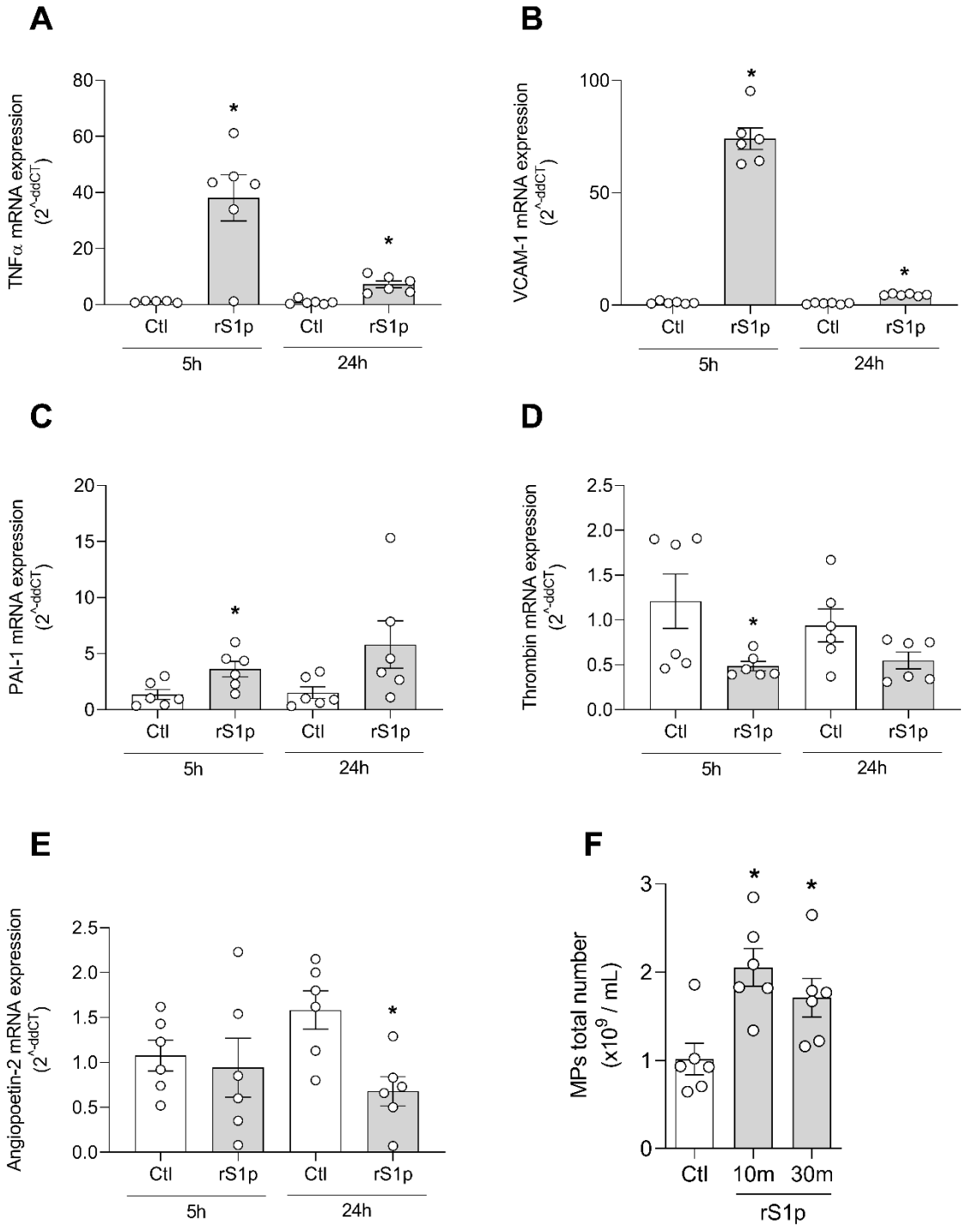
**Supplementary Fig. S11 – characterization of ACE2 co-immunoprecipitation coupled mass spectrometry protein identification and label free proteomics.** Optimisation of anti-ACE2 antibody for co-immunoprecipitation (A). Antibodies were used from Abcam (ab15348) and proteintech (66699-1). Lane labelling: M - protein ladder, L - protein lysate load, U - unbound fraction, 1 to 3 - washes, E - elute. Western blot with primary anti-ACE2 (Abcam), secondary anti-rabbit Alexa800. Native/non-denaturing protein isolation was done to maintain the protein-protein interaction. This led to identification of more soluble ACE2 fractions (bands below 70kDa) along with glycosylated and high molecular weight ACE2 (bands above 100kDa). Co-immunoprecipitation of Anti-ACE2 (Abcam) (B). Lane labelling: M - protein ladder, 1 - protein lysate load, 2 - unbound fraction, 3 to 6 - washes, 7- elute, 8 - elution from beads with anti-ACE2 (negative control). Final protein load amount was of 10% of each fraction (except elution fraction). 4-12% Bis tris gel, nitrocellulose membrane with 5% BSA was used. ClueGO enriched gene ontology term for biological processes (C). Each circle represents a group of gene (identified in ACE2 Co-IP) that belong to either a particular biological function. Size of the circle represents the enrichment significance (cut off Bonferroni FDR <0.5, at least 3 gene per group)

**Supplementary Fig. S12 – rS1p or ACE2 siRNA does not alter ET-1 and angiopoetin-2 levels in human endothelial cells.** Human endothelial cells (hMEC) were stimulated with rS1p (0.66 μg/mL) for 24h for assessment of ET-1 (A) and angiopoetin-2 (B) production in the presence or absence of ACE2 siRNA (n=8). Data are expressed as ± SEM.

**SUPPLEMENTARY FIGURE S1**



**SUPPLEMENTARY FIGURE S2**



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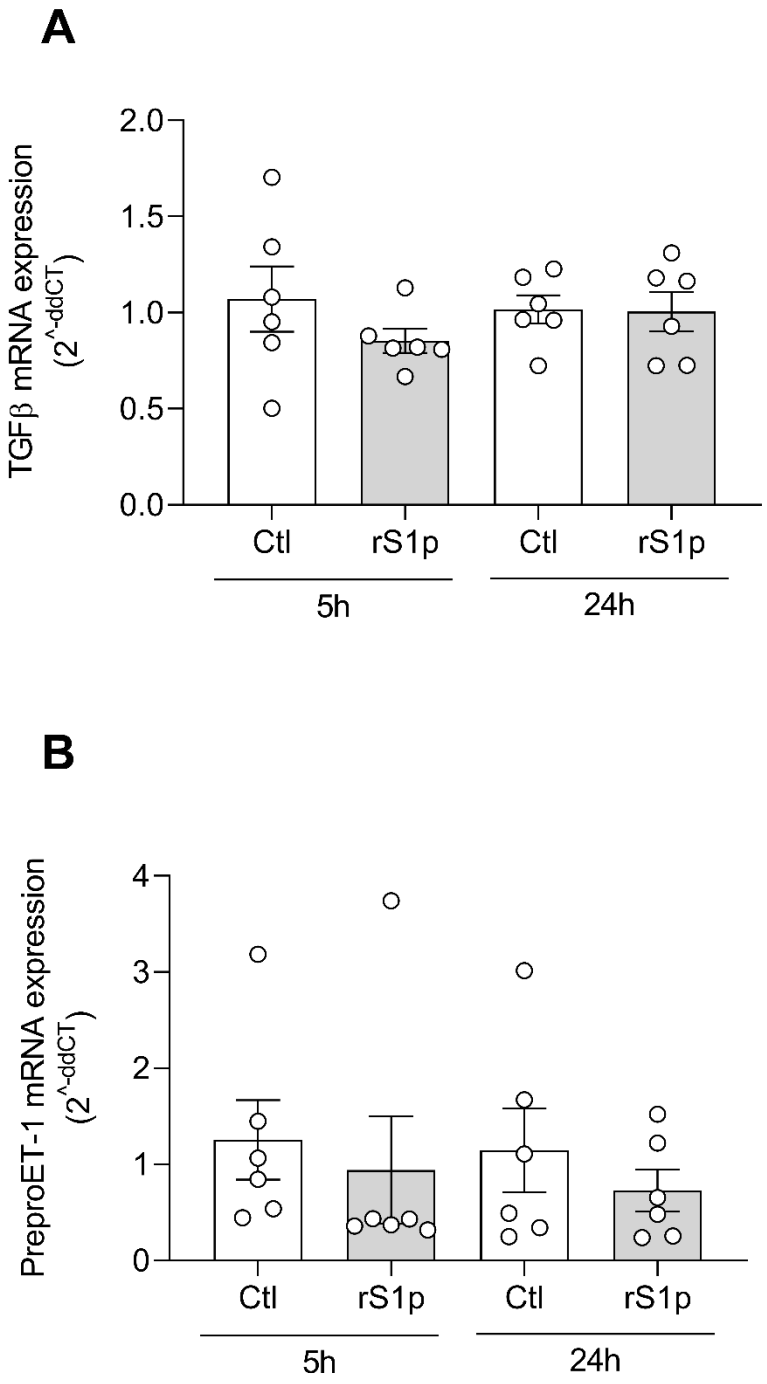

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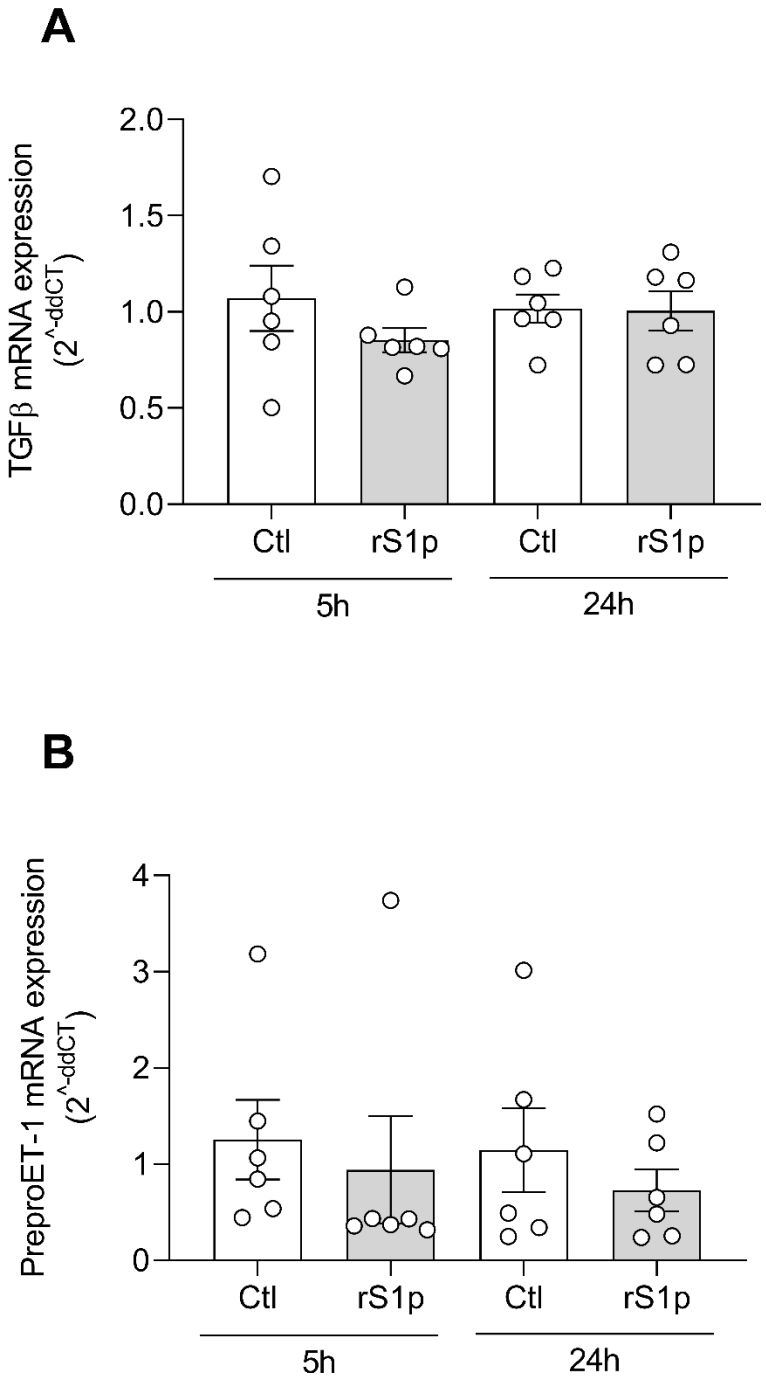

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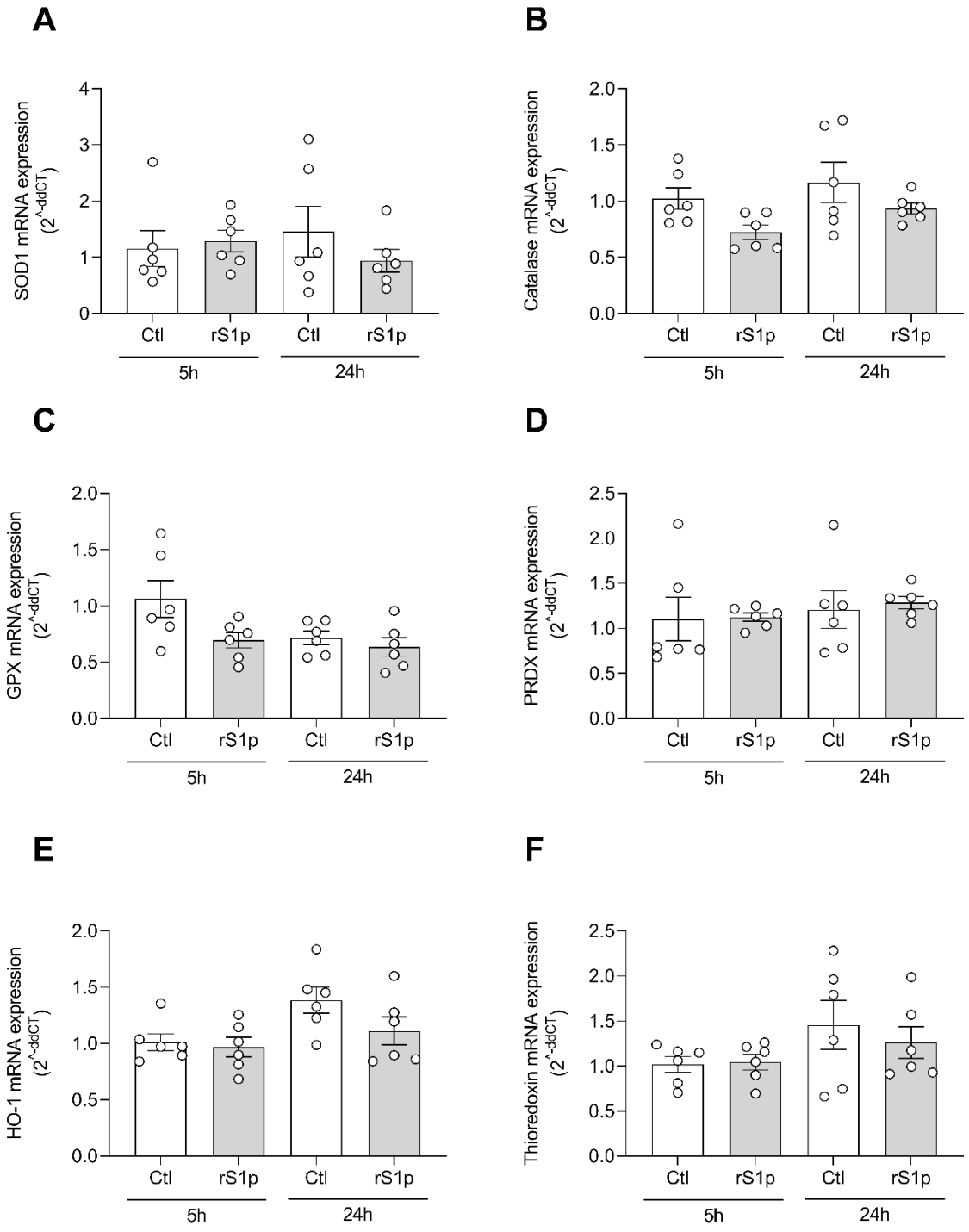

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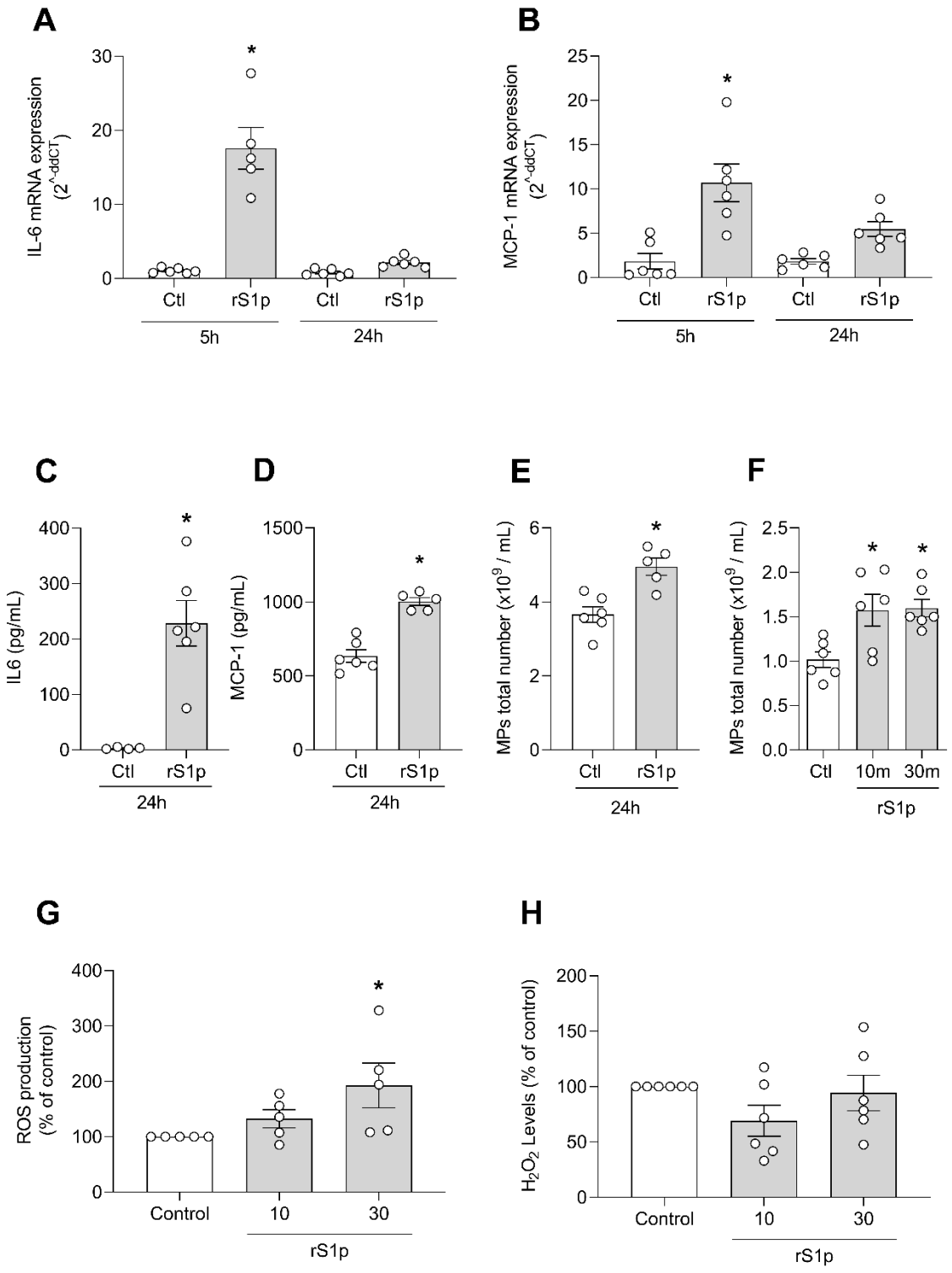

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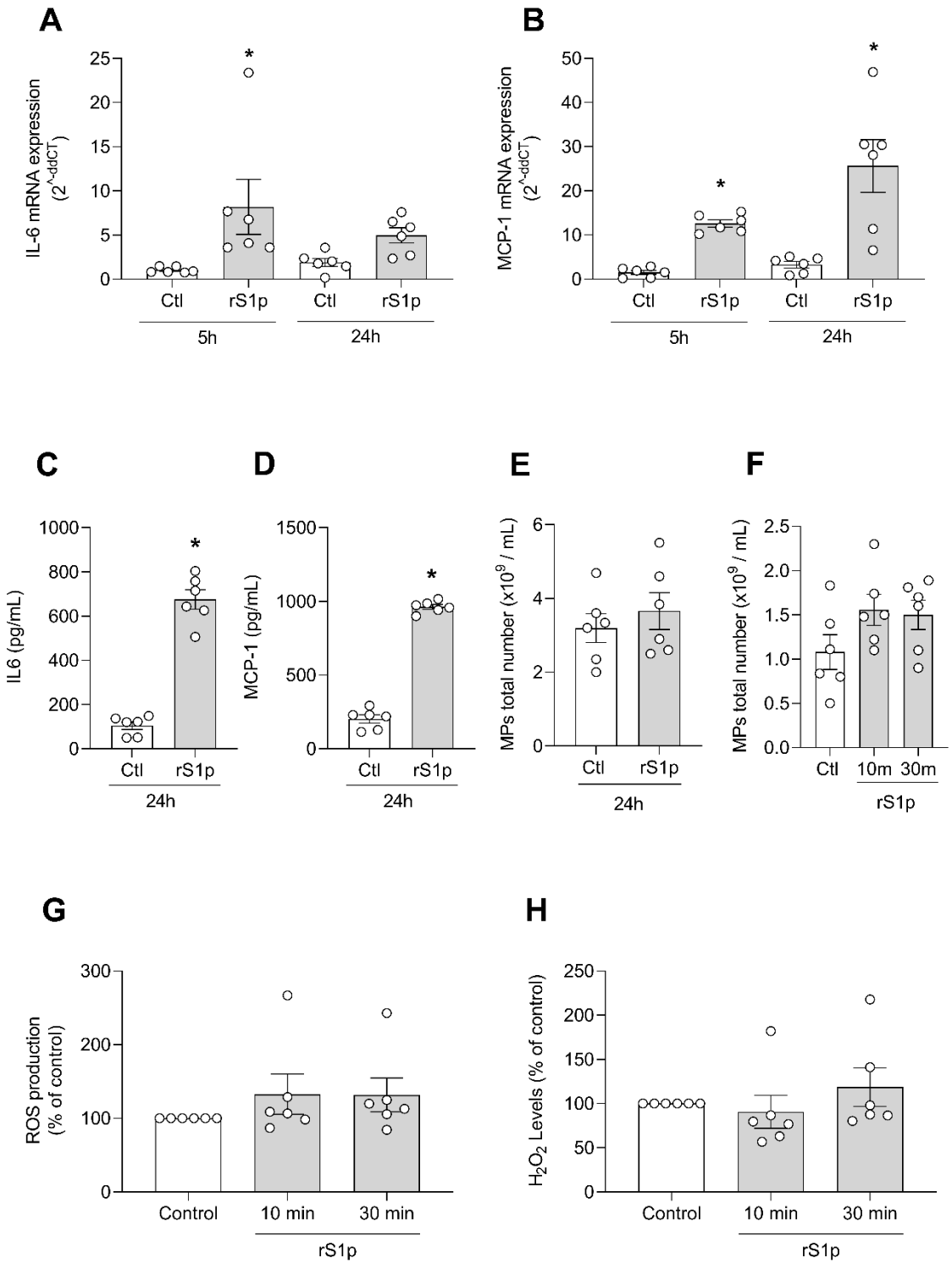

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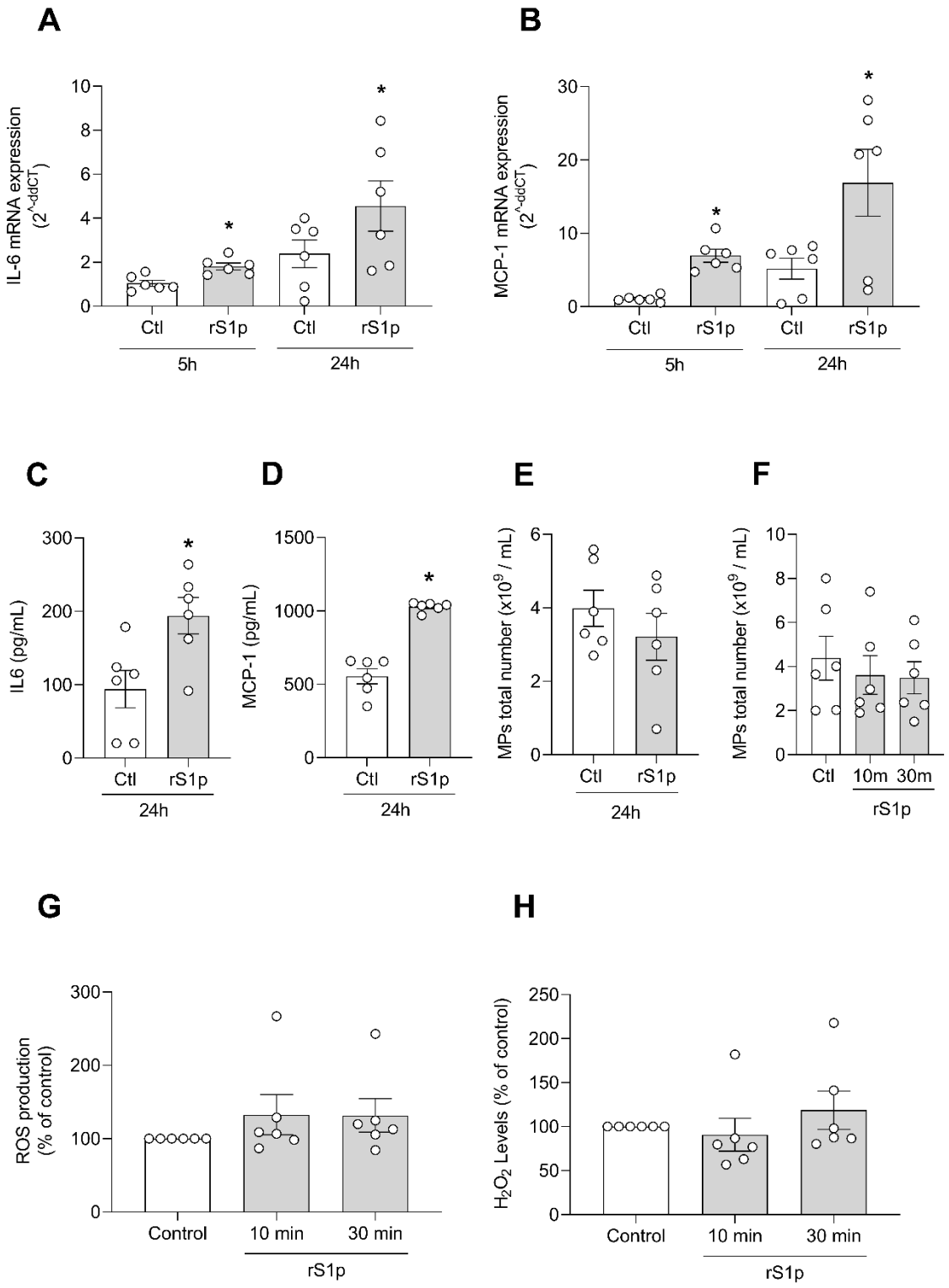

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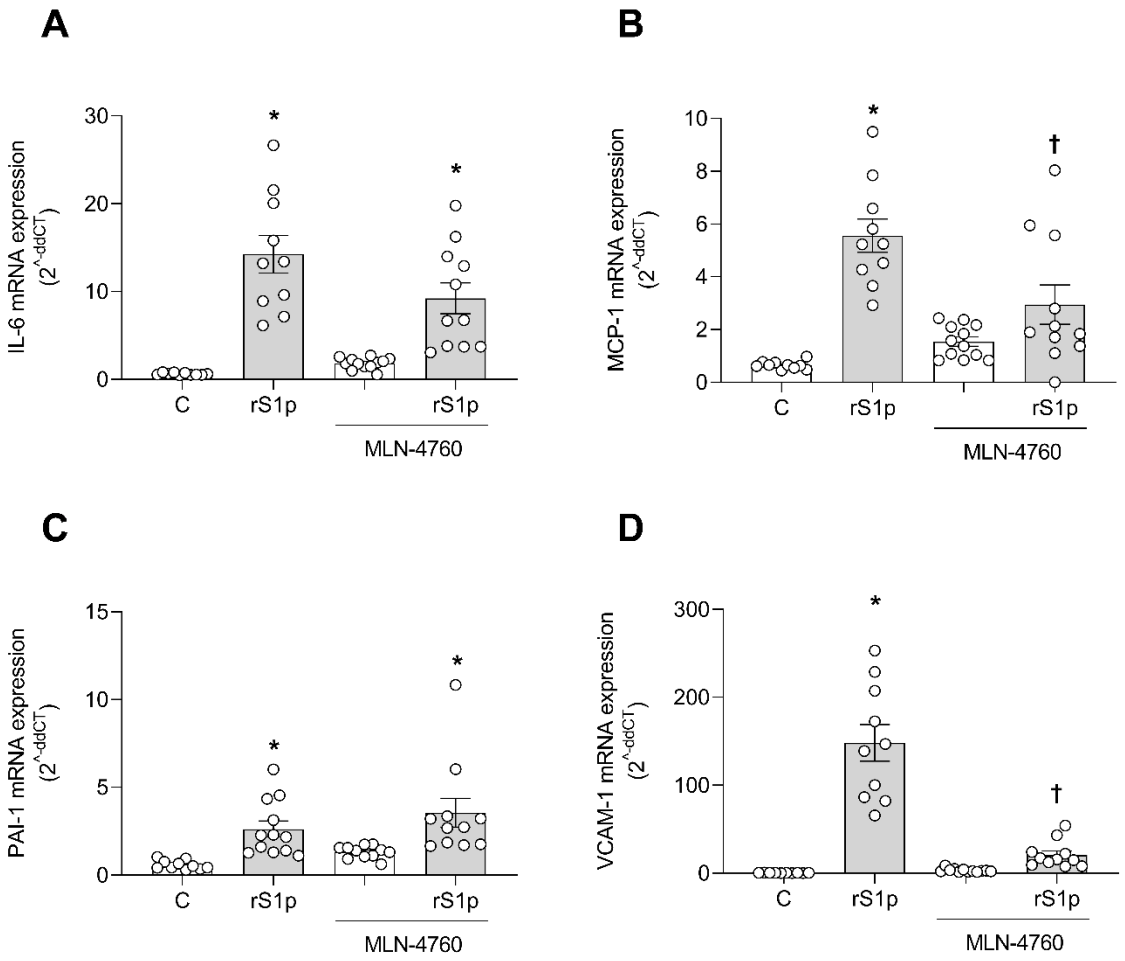

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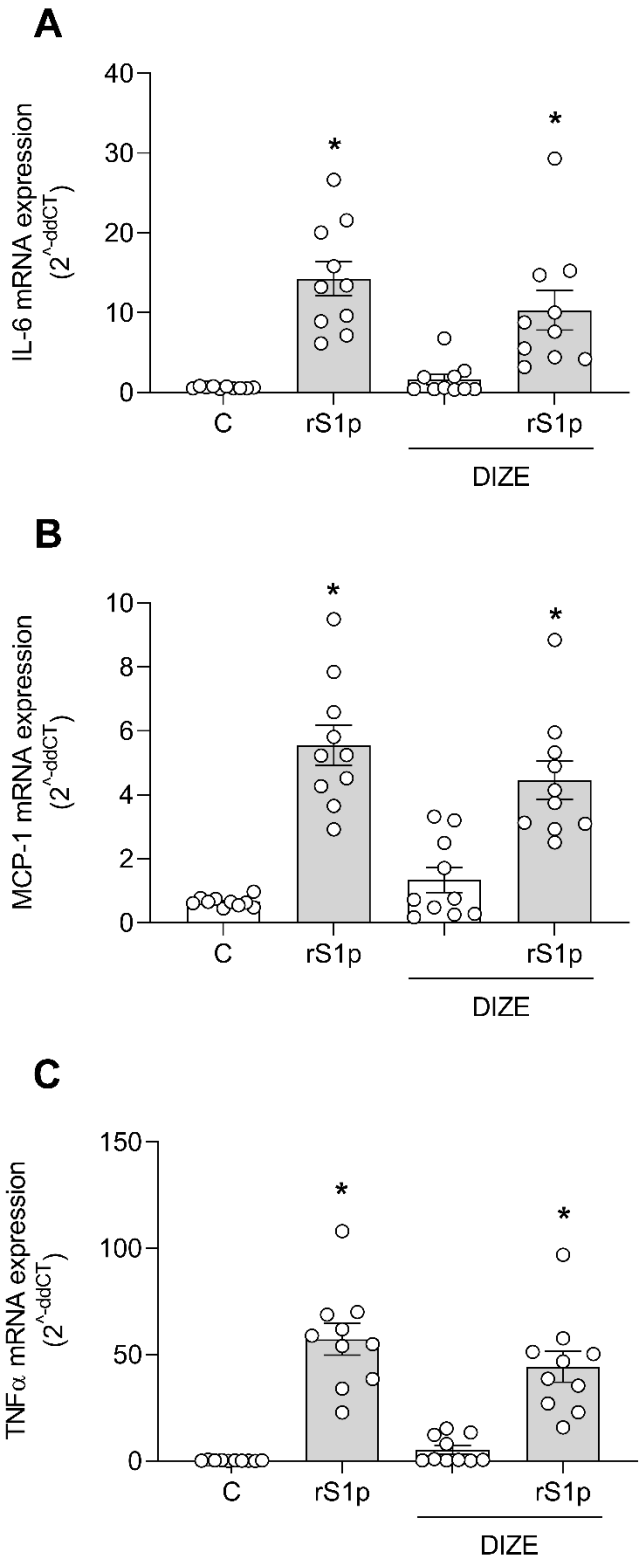

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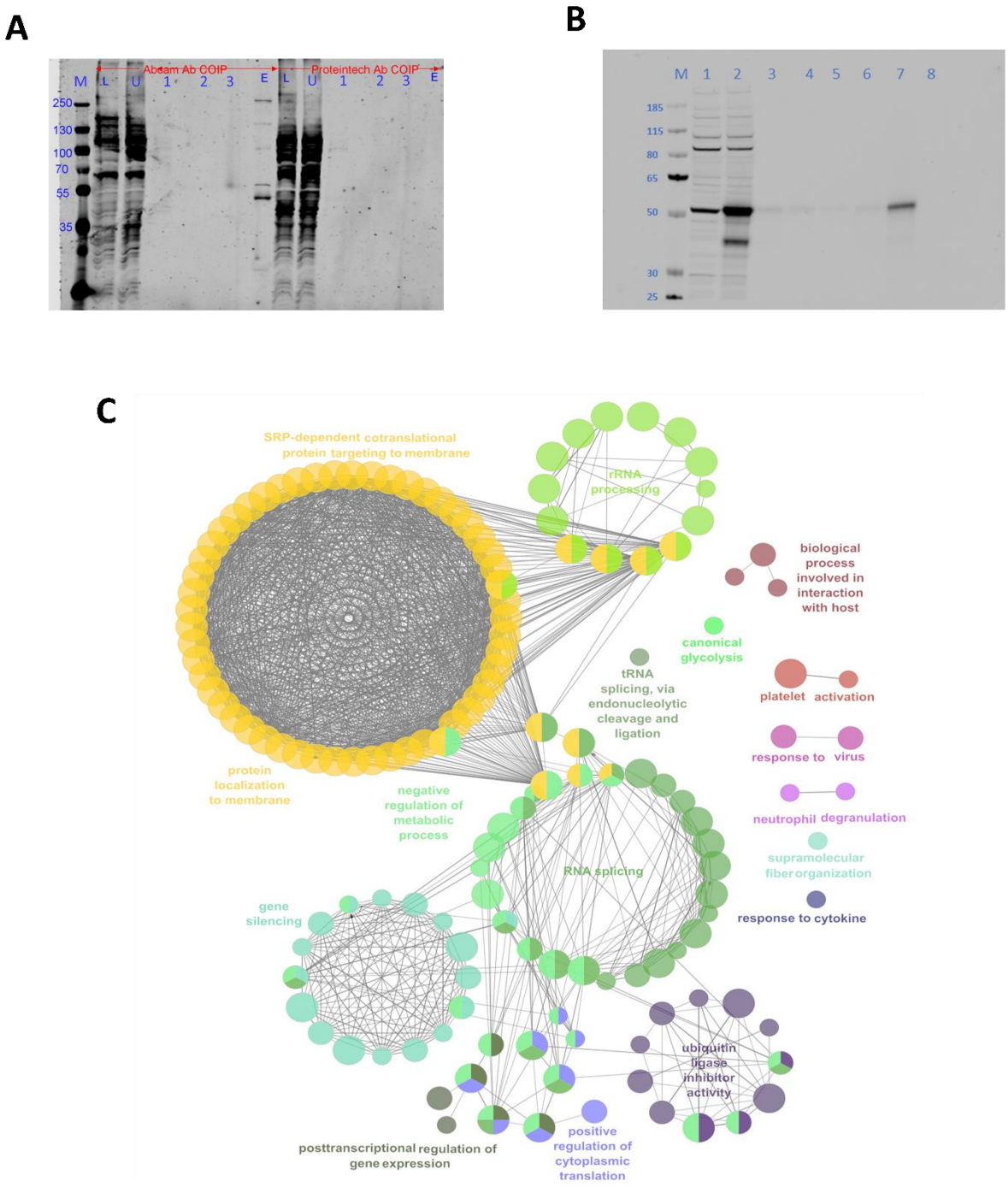

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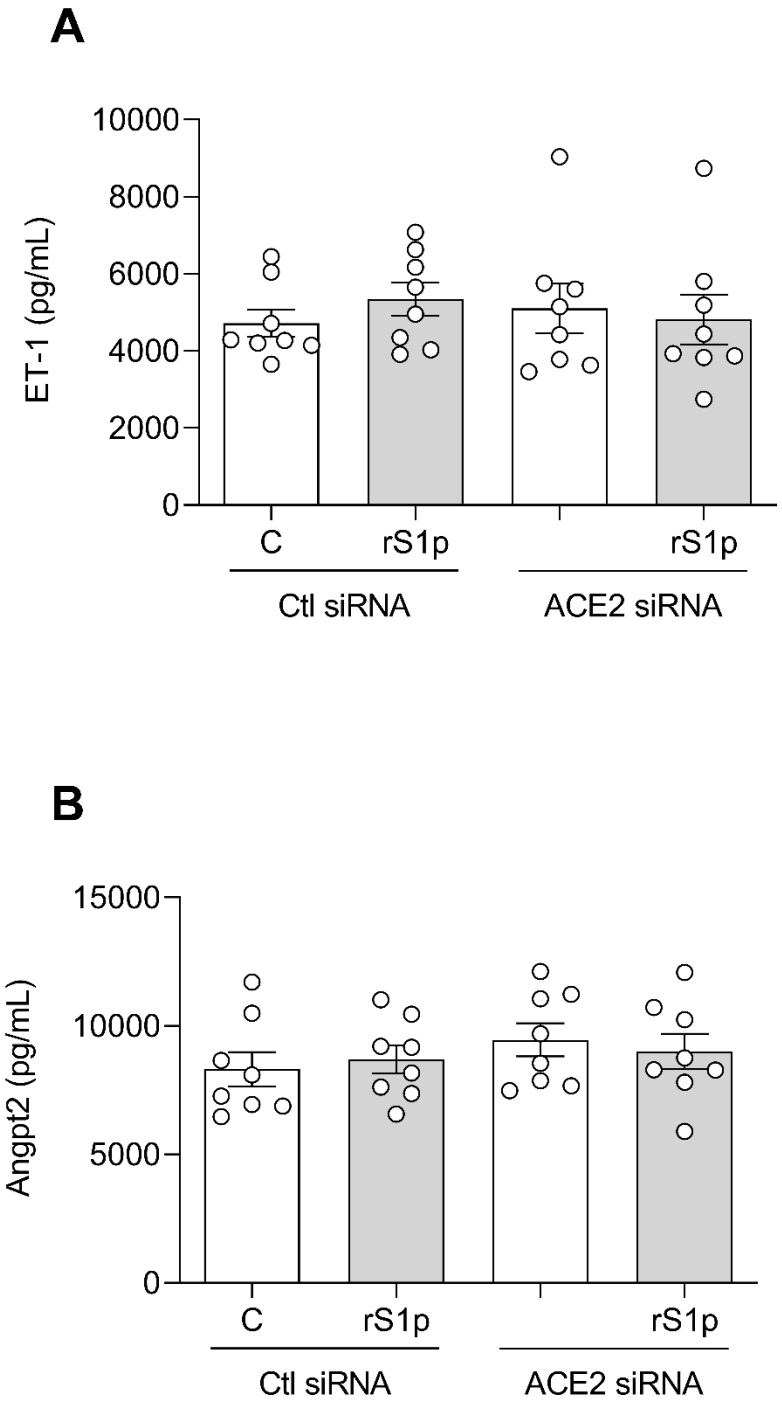
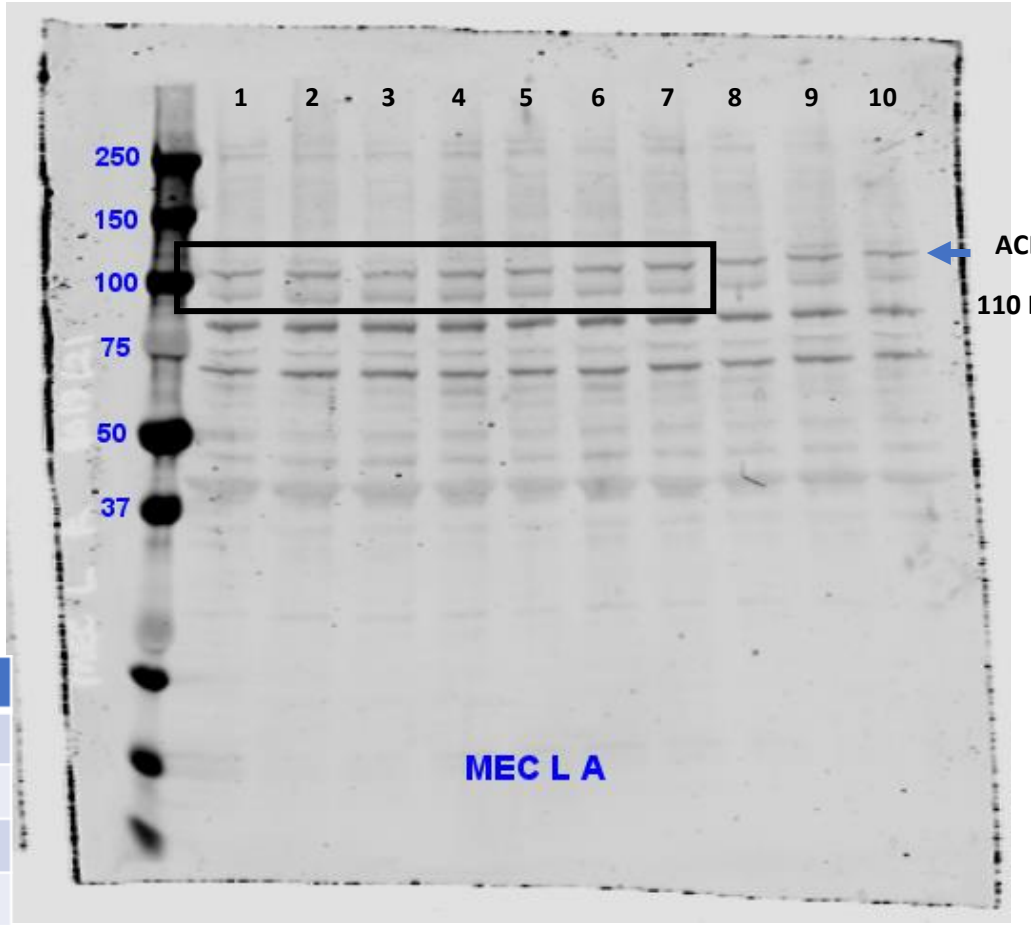
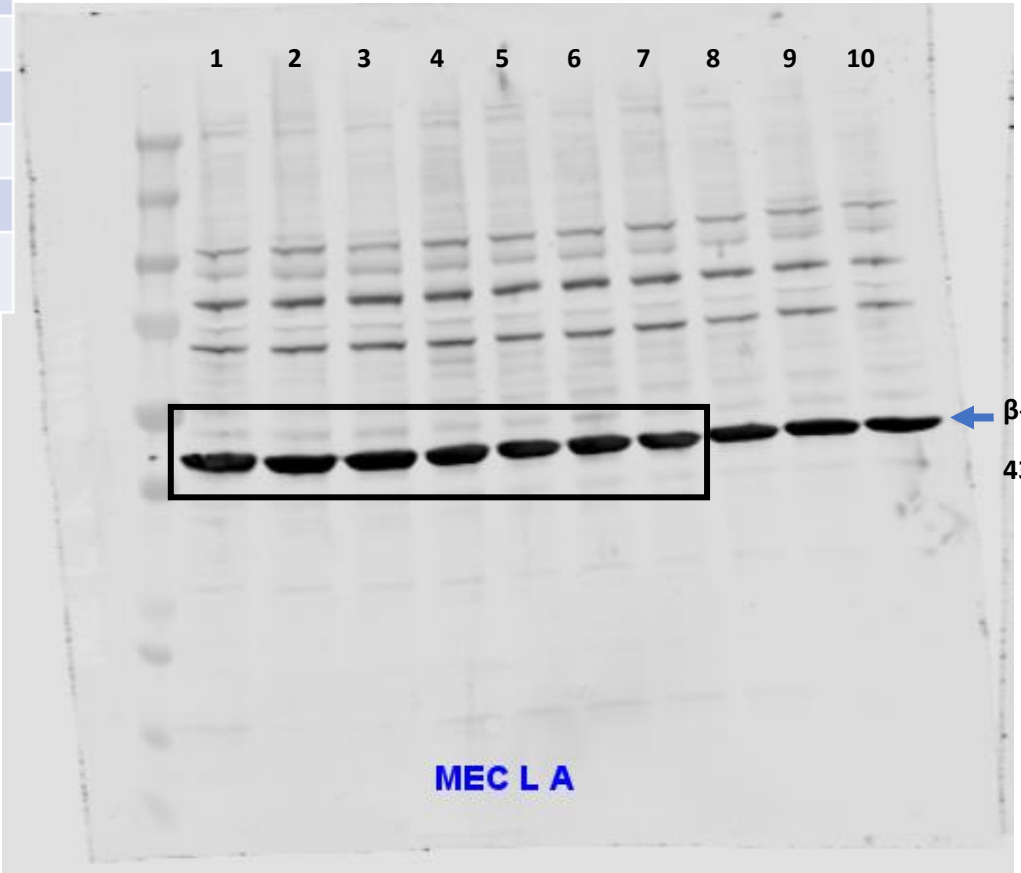


**SUPPLEMENTARY FIGURE S13 - ORIGINAL BLOTS – FIGURE 3B – ACE2**

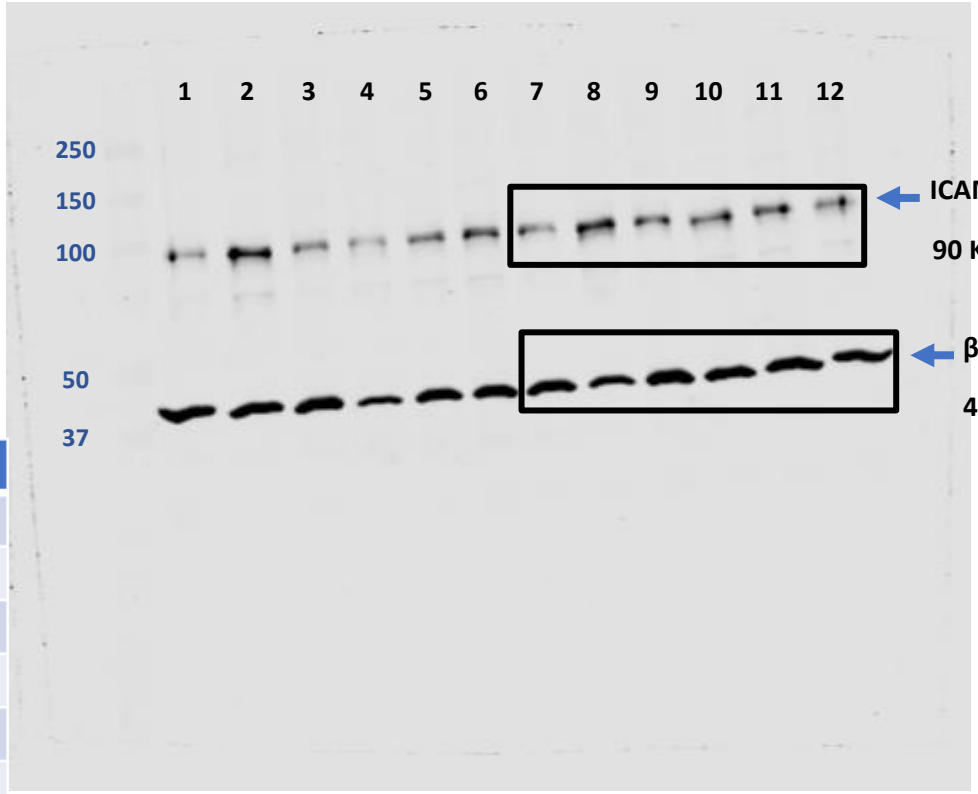


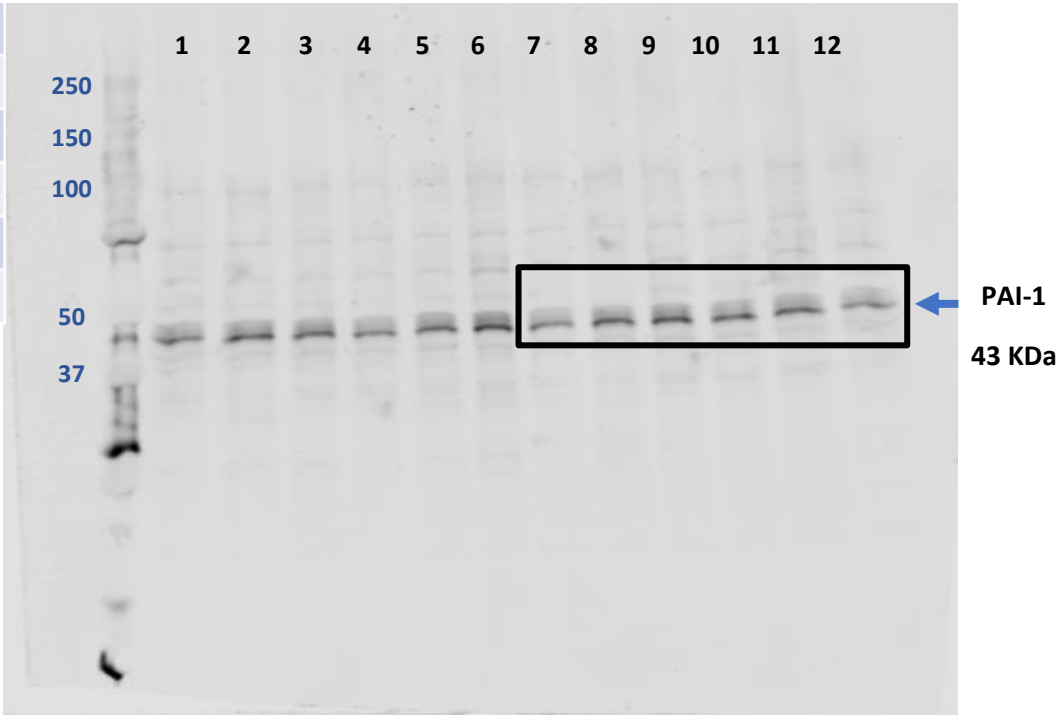

| # | Sample ID |
|---|---|
| 1 | Control |
| 2 | Control |
| 3 | rS1p |
| 4 | rS1p |
| 5 | MLN-4760 |
| 6 | rS1p + MLN-4760 |
| 7 | rS1p + MLN-4760 |
| 8 | DIZE |
| 9 | rS1p + DIZE |
| 10 | rS1p + DIZE |



**SUPPLEMENTARY FIGURE S14 – ORIGINAL BLOTS - FIG 3F/3G – ICAM-1 AND PAI-1**

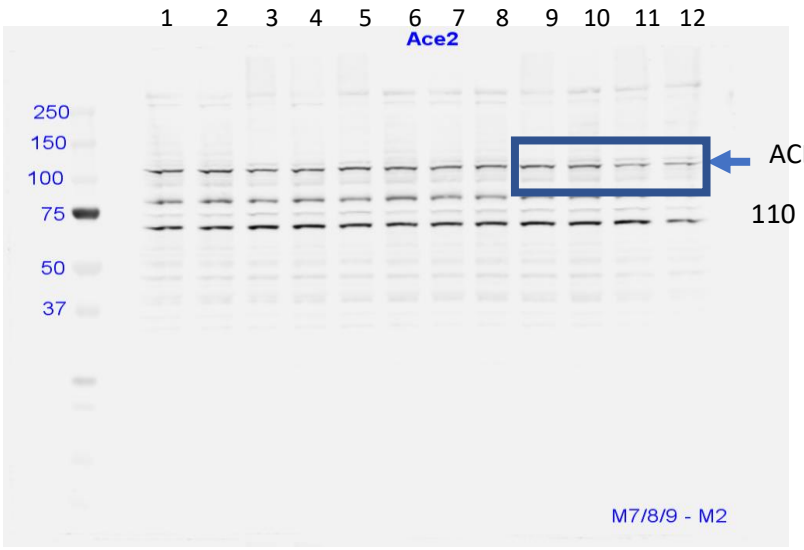
| # | Sample ID |
|---|---|
| 1 | Control |
| 2 | rS1p |
| 3 | Control |
| 4 | rS1p |
| 5 | MLN-4760 + control |
| 6 | MLN-4760 + rS1p |
| 7 | Control |
| 8 | rS1p |
| 9 | Control |
| 10 | rS1p |
| 11 | MLN-4760 + control |
| 12 | MLN-4760 + rS1p |



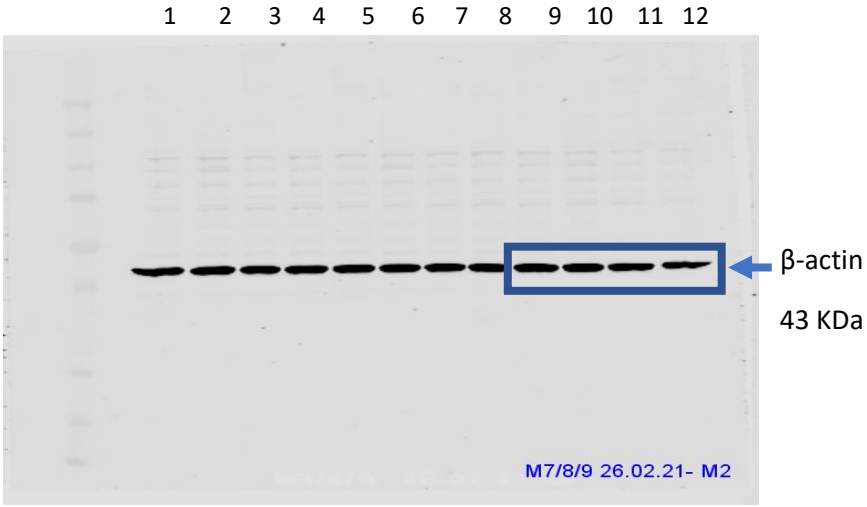



**SUPPLEMENTARY FIGURE S15 - ORIGINAL BLOTS – FIGURE 4A – ACE2**

| | | | |
|---|---|---|---|
| 1 | Ctl | Ctl siRNA | |
| 2 | S1 24h | | MEC1 |
| 3 | Ctl | ACE2 siRNA | exp7 |
| 4 | S1 24h | | |
| 5 | Ctl | Ctl siRNA | |
| 6 | S1 24h | | MEC2 |
| 7 | Ctl | ACE2 siRNA | exp8 |
| 8 | S1 24h | | |
| 9 | Ctl | Ctl siRNA | |
| 10 | S1 24h | | MEC3 |
| 11 | Ctl | ACE2 siRNA | exp9 |
| 12 | S1 24h | | |

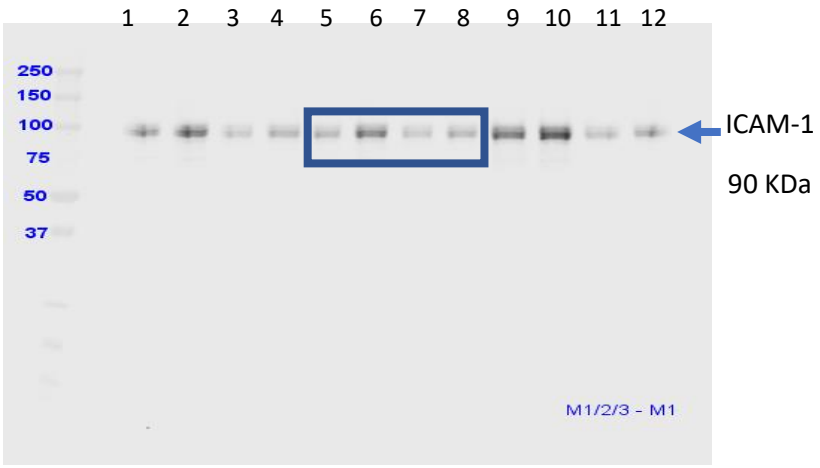



| | | | |
|---|---|---|---|
| 1 | Ctl | Ctl siRNA | |
| 2 | S1 24h | | MEC1 |
| 3 | Ctl | ACE2 siRNA | exp7 |
| 4 | S1 24h | | |
| 5 | Ctl | Ctl siRNA | |
| 6 | S1 24h | | MEC2 |
| 7 | Ctl | ACE2 siRNA | exp8 |
| 8 | S1 24h | | |
| 9 | Ctl | Ctl siRNA | |
| 10 | S1 24h | | MEC3 |
| 11 | Ctl | ACE2 siRNA | exp9 |
| 12 | S1 24h | | |

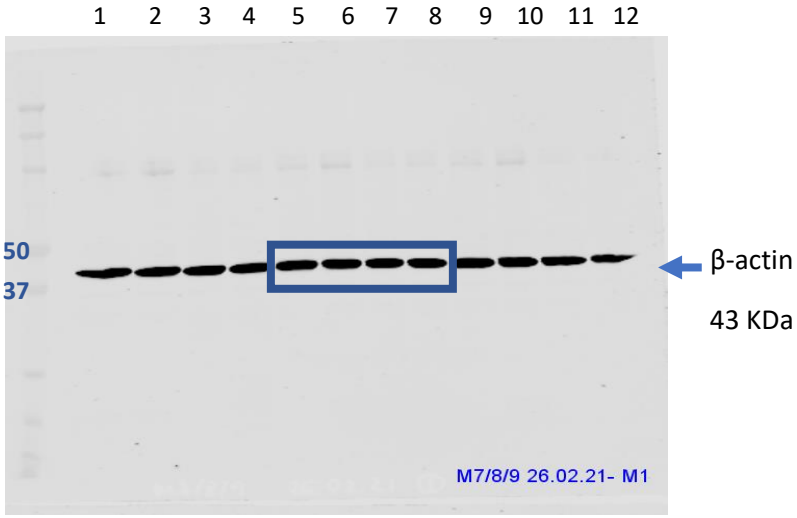


**SUPPLEMENTARY FIGURE S16 - ORIGINAL BLOTS – FIGURE 4D – ICAM-1**

| | | | |
|---|---|---|---|
| 1 | Ctl | Ctl siRNA | |
| 2 | S1 24h | | MEC1 |
| 3 | Ctl | ACE2 siRNA | exp7 |
| 4 | S1 24h | | |
| 5 | Ctl | Ctl siRNA | |
| 6 | S1 24h | | MEC2 |
| 7 | Ctl | ACE2 siRNA | exp8 |
| 8 | S1 24h | | |
| 9 | Ctl | Ctl siRNA | |
| 10 | S1 24h | | MEC3 |
| 11 | Ctl | ACE2 siRNA | exp9 |
| 12 | S1 24h | | |

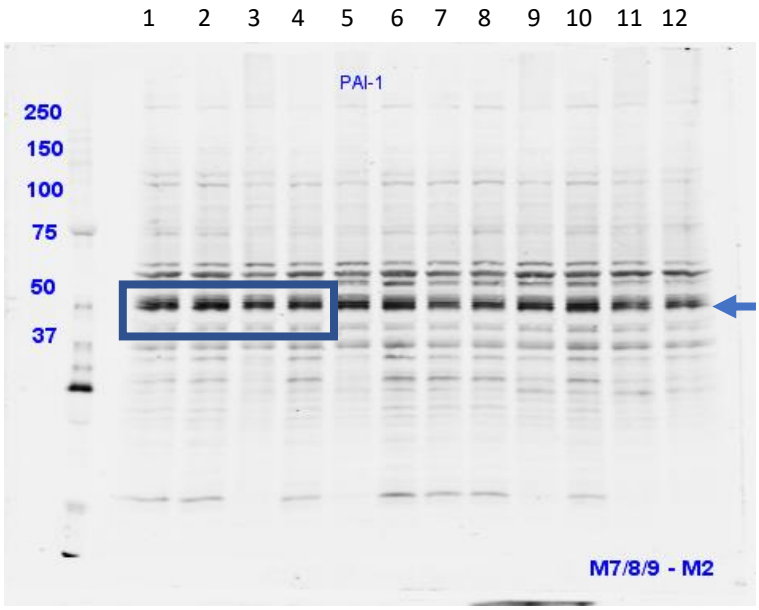



| | | | |
|---|---|---|---|
| 1 | Ctl | Ctl siRNA | |
| 2 | S1 24h | | MEC1 |
| 3 | Ctl | ACE2 siRNA | exp7 |
| 4 | S1 24h | | |
| 5 | Ctl | Ctl siRNA | |
| 6 | S1 24h | | MEC2 |
| 7 | Ctl | ACE2 siRNA | exp8 |
| 8 | S1 24h | | |
| 9 | Ctl | Ctl siRNA | |
| 10 | S1 24h | | MEC3 |
| 11 | Ctl | ACE2 siRNA | exp9 |
| 12 | S1 24h | | |

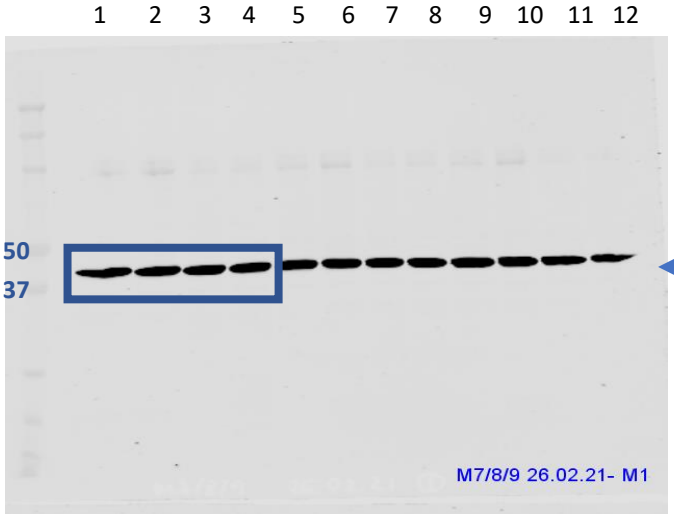


**SUPPLEMENTARY FIGURE S17 - ORIGINAL BLOTS – FIGURE 4E – PAI-1**

| | | | |
|---|---|---|---|
| 1 | Ctl | Ctl siRNA | |
| 2 | S1 24h | | MEC1 |
| 3 | Ctl | ACE2 siRNA | exp7 |
| 4 | S1 24h | | |
| 5 | Ctl | Ctl siRNA | |
| 6 | S1 24h | | MEC2 |
| 7 | Ctl | ACE2 siRNA | exp8 |
| 8 | S1 24h | | |
| 9 | Ctl | Ctl siRNA | |
| 10 | S1 24h | | MEC3 |
| 11 | Ctl | ACE2 siRNA | exp9 |
| 12 | S1 24h | | |

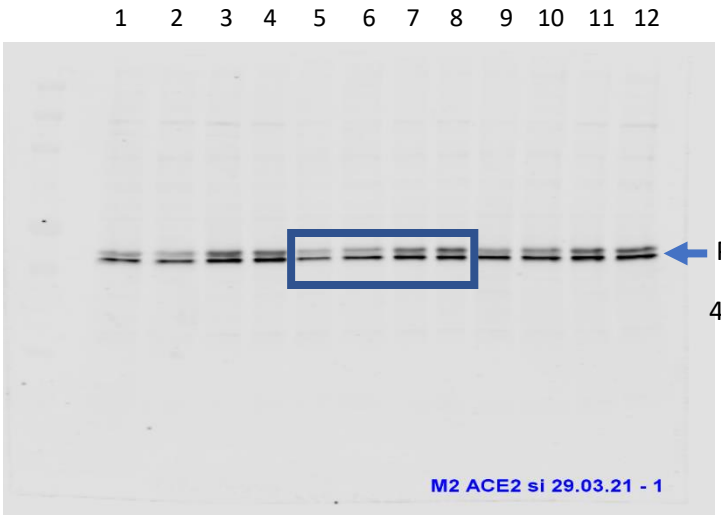



| | | | |
|---|---|---|---|
| 1 | Ctl | Ctl siRNA | |
| 2 | S1 24h | | MEC1 |
| 3 | Ctl | ACE2 siRNA | exp7 |
| 4 | S1 24h | | |
| 5 | Ctl | Ctl siRNA | |
| 6 | S1 24h | | MEC2 |
| 7 | Ctl | ACE2 siRNA | exp8 |
| 8 | S1 24h | | |
| 9 | Ctl | Ctl siRNA | |
| 10 | S1 24h | | MEC3 |
| 11 | Ctl | ACE2 siRNA | exp9 |
| 12 | S1 24h | | |

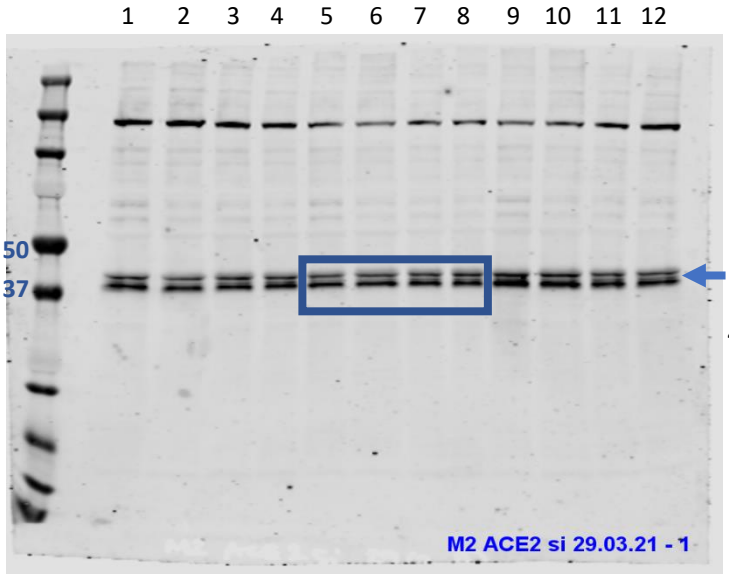


**SUPPLEMENTARY FIGURE S18 - ORIGINAL BLOTS – FIGURE 5A – ERK1/2**

| | | | M2 |
|---|---|---|---|
| 1 | Ctl | Ctl siRNA | |
| 2 | S1 24h | | |
| 3 | Ctl | ACE2 siRNA | exp4 |
| 4 | S1 24h | | |
| 5 | Ctl | Ctl siRNA | |
| 6 | S1 24h | | |
| 7 | Ctl | ACE2 siRNA | exp5 |
| 8 | S1 24h | | |
| 9 | Ctl | Ctl siRNA | |
| 10 | S1 24h | | |
| 11 | Ctl | ACE2 siRNA | exp6 |
| 12 | S1 24h | | |

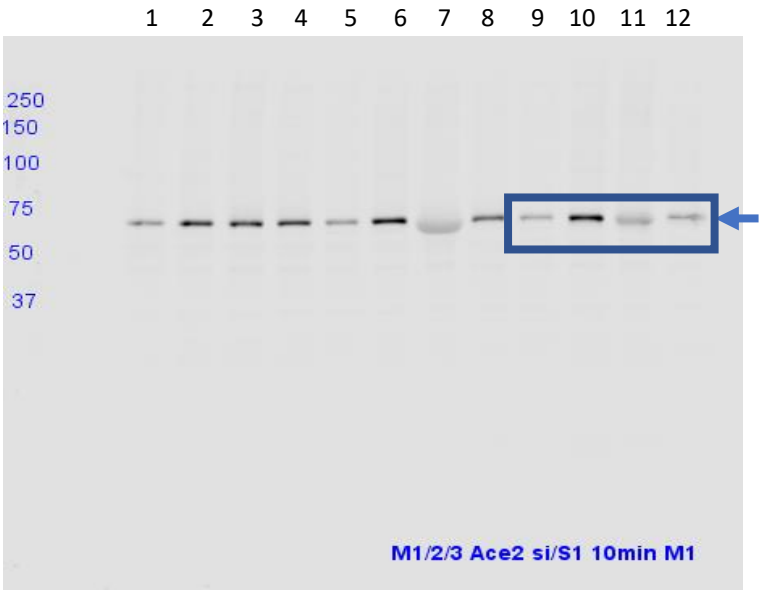



| | | | M2 |
|---|---|---|---|
| 1 | Ctl | Ctl siRNA | |
| 2 | S1 24h | | |
| 3 | Ctl | ACE2 siRNA | exp4 |
| 4 | S1 24h | | |
| 5 | Ctl | Ctl siRNA | |
| 6 | S1 24h | | |
| 7 | Ctl | ACE2 siRNA | exp5 |
| 8 | S1 24h | | |
| 9 | Ctl | Ctl siRNA | |
| 10 | S1 24h | | |
| 11 | Ctl | ACE2 siRNA | exp6 |
| 12 | S1 24h | | |

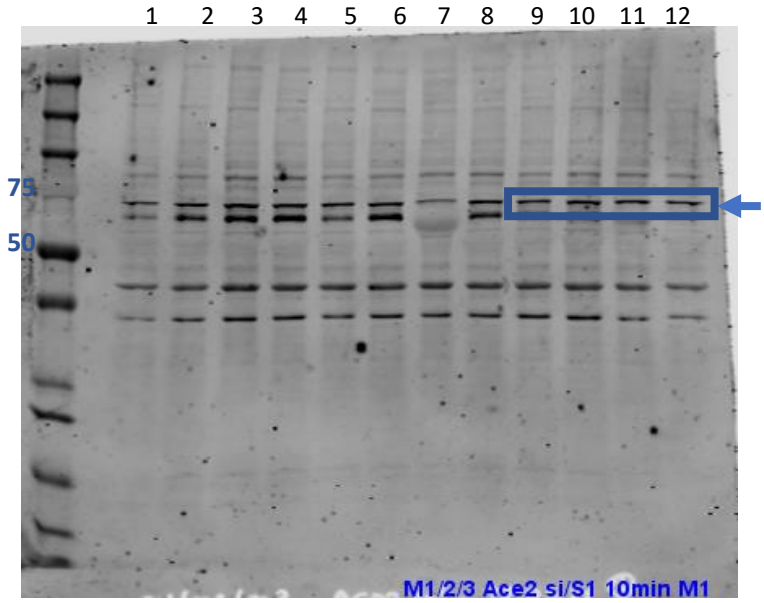


**SUPPLEMENTARY FIGURE S19 - ORIGINAL BLOTS – FIGURE 5B – NFkB**

| | | | M1/2/3 |
|---|---|---|---|
| 1 | Ctl | Ctl siRNA | |
| 2 | S1 24h | | |
| 3 | Ctl | ACE2 siRNA | exp1 |
| 4 | S1 24h | | |
| 5 | Ctl | Ctl siRNA | |
| 6 | S1 24h | | |
| 7 | Ctl | ACE2 siRNA | exp2 |
| 8 | S1 24h | | |
| 9 | Ctl | Ctl siRNA | |
| 10 | S1 24h | | |
| 11 | Ctl | ACE2 siRNA | exp3 |
| 12 | S1 24h | | |

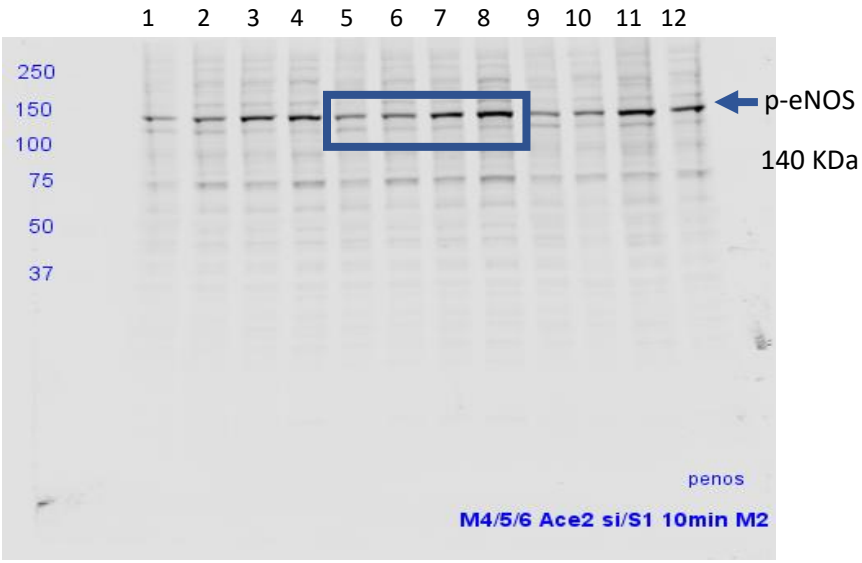



| | | | M1/2/3 |
|---|---|---|---|
| 1 | Ctl | Ctl siRNA | |
| 2 | S1 24h | | |
| 3 | Ctl | ACE2 siRNA | exp1 |
| 4 | S1 24h | | |
| 5 | Ctl | Ctl siRNA | |
| 6 | S1 24h | | |
| 7 | Ctl | ACE2 siRNA | exp2 |
| 8 | S1 24h | | |
| 9 | Ctl | Ctl siRNA | |
| 10 | S1 24h | | |
| 11 | Ctl | ACE2 siRNA | exp3 |
| 12 | S1 24h | | |

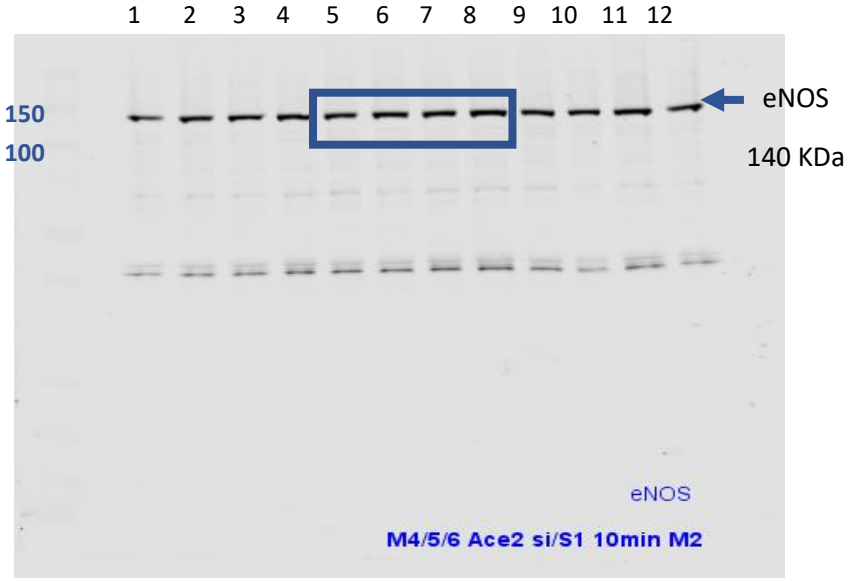


**SUPPLEMENTARY FIGURE S20 - ORIGINAL BLOTS – FIGURE 5C – eNOS**

| | | | M4/5/6 |
|---|---|---|---|
| 1 | Ctl | Ctl siRNA | |
| 2 | S1 24h | | |
| 3 | Ctl | ACE2 siRNA | exp4 |
| 4 | S1 24h | | |
| 5 | Ctl | Ctl siRNA | |
| 6 | S1 24h | | |
| 7 | Ctl | ACE2 siRNA | exp5 |
| 8 | S1 24h | | |
| 9 | Ctl | Ctl siRNA | |
| 10 | S1 24h | | |
| 11 | Ctl | ACE2 siRNA | exp6 |
| 12 | S1 24h | | |




| | | | M4/5/6 |
|---|---|---|---|
| 1 | Ctl | Ctl siRNA | |
| 2 | S1 24h | | |
| 3 | Ctl | ACE2 siRNA | exp4 |
| 4 | S1 24h | | |
| 5 | Ctl | Ctl siRNA | |
| 6 | S1 24h | | |
| 7 | Ctl | ACE2 siRNA | exp5 |
| 8 | S1 24h | | |
| 9 | Ctl | Ctl siRNA | |
| 10 | S1 24h | | |
| 11 | Ctl | ACE2 siRNA | exp6 |
| 12 | S1 24h | | |



**SUPPLEMENTARY FIGURE S21 - ORIGINAL BLOTS – FIGURE 5D – ACE2 expression in Hek293 cells**