

# The Fibrin-derived Peptide FX06 Protects Human Pulmonary Endothelial Cells Against the COVID-19-Triggered Cytokine Storm

Zhiran Wang<sup>1</sup>, Dmitrii Lebedev<sup>1</sup>, Vadim Zhernovkov<sup>1</sup>, Sudharshan Rao<sup>1</sup>, Kevin Wu<sup>1</sup>, Lorcan Doyle<sup>1</sup>, Kieran Wynne<sup>1</sup>, Simeng Li<sup>1</sup>, Alfonso Blanco<sup>2</sup>, Margarita M. Mysior<sup>3</sup>, Jeremy Simpson<sup>3</sup>, Dimitri Scholz<sup>4</sup>, Petra Wülfroth<sup>5</sup>, Walter Kolch<sup>1</sup>, and Günther Eissner<sup>1</sup>

1. Systems Biology Ireland, School of Medicine, University College Dublin, Dublin, Ireland.

2. Flow Cytometry Core, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Dublin, Ireland.

3. College of Science, University College Dublin, Dublin, Ireland.

4. Imaging Core, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Dublin, Ireland.

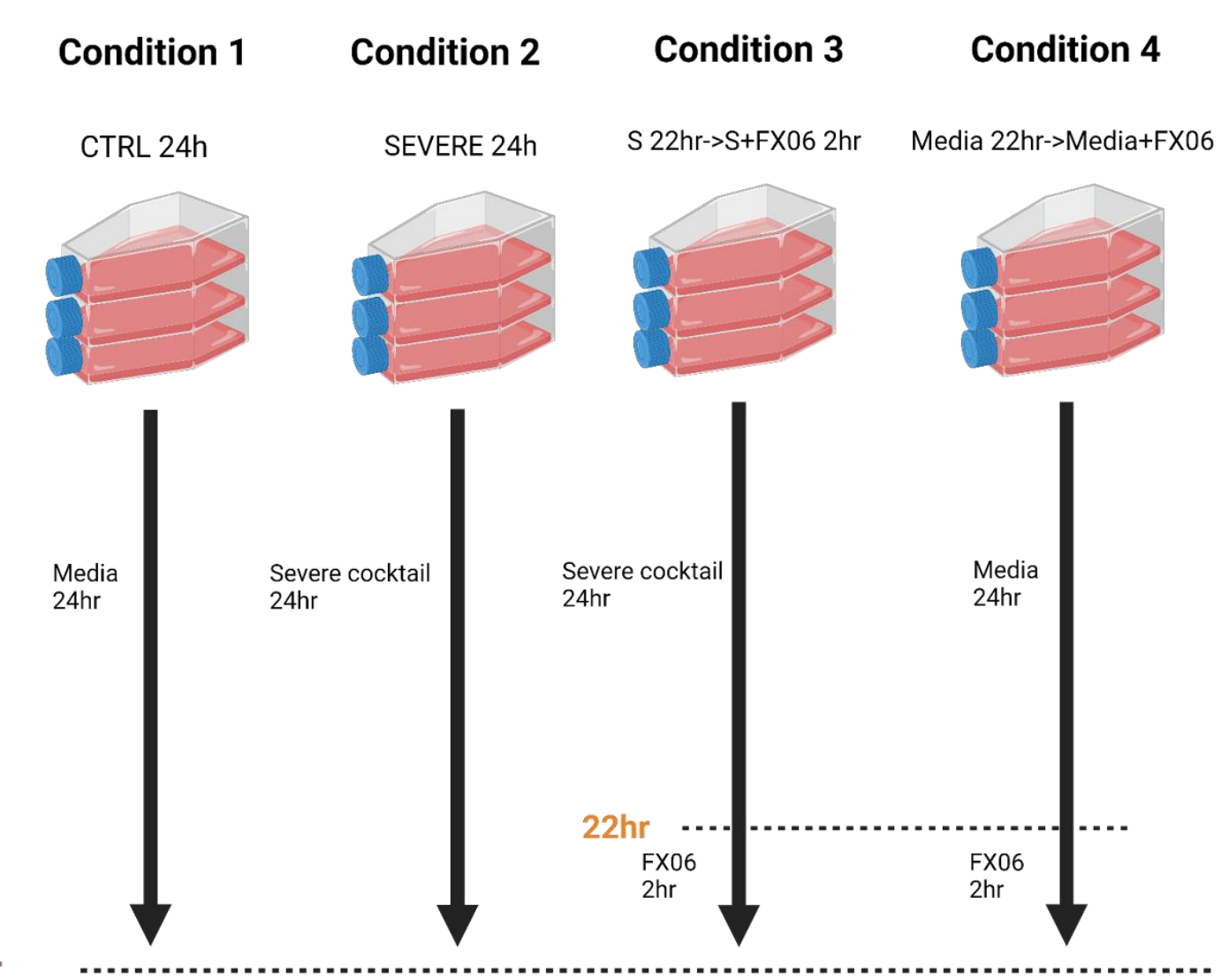
5. F4 Pharma, 1060 Vienna, Austria.



## BACKGROUND

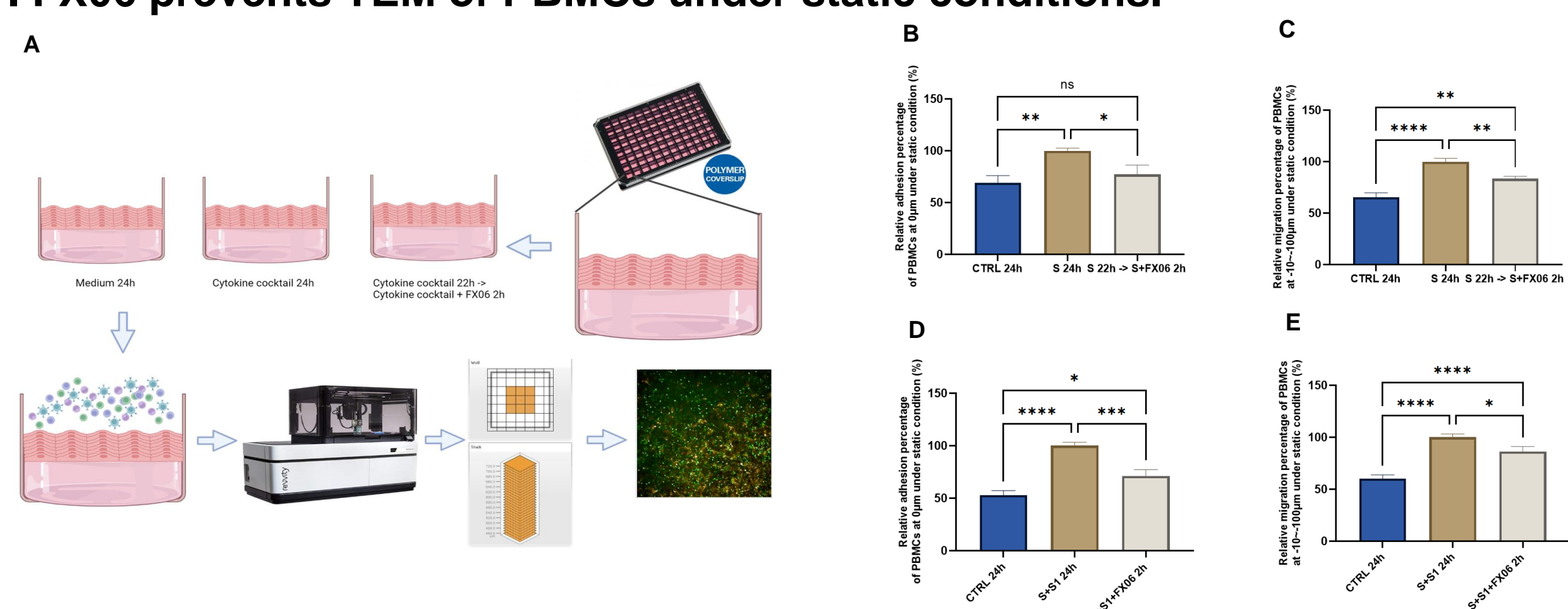
Coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has been a major health emergency since 2019. Endothelial dysfunction is a hallmark of COVID-19, leading to severe illness, i.e. multi-organ failure, coagulopathy, and death<sup>1</sup>. FX06, a fibrin-derived natural peptide, formerly known as B $\beta$ <sub>15-42</sub>, protects the vasculature in myocardial ischemia-reperfusion in animal models<sup>2</sup>. Therefore, it is a promising therapeutic candidate for endothelial complications such as capillary leak in COVID-19 and other forms of ARDS. The aim of this project is to investigate whether FX06 can attenuate COVID-19 cytokine-triggered inflammatory processes *in vitro*<sup>3-7</sup>.

## EXPERIMENT DESIGN



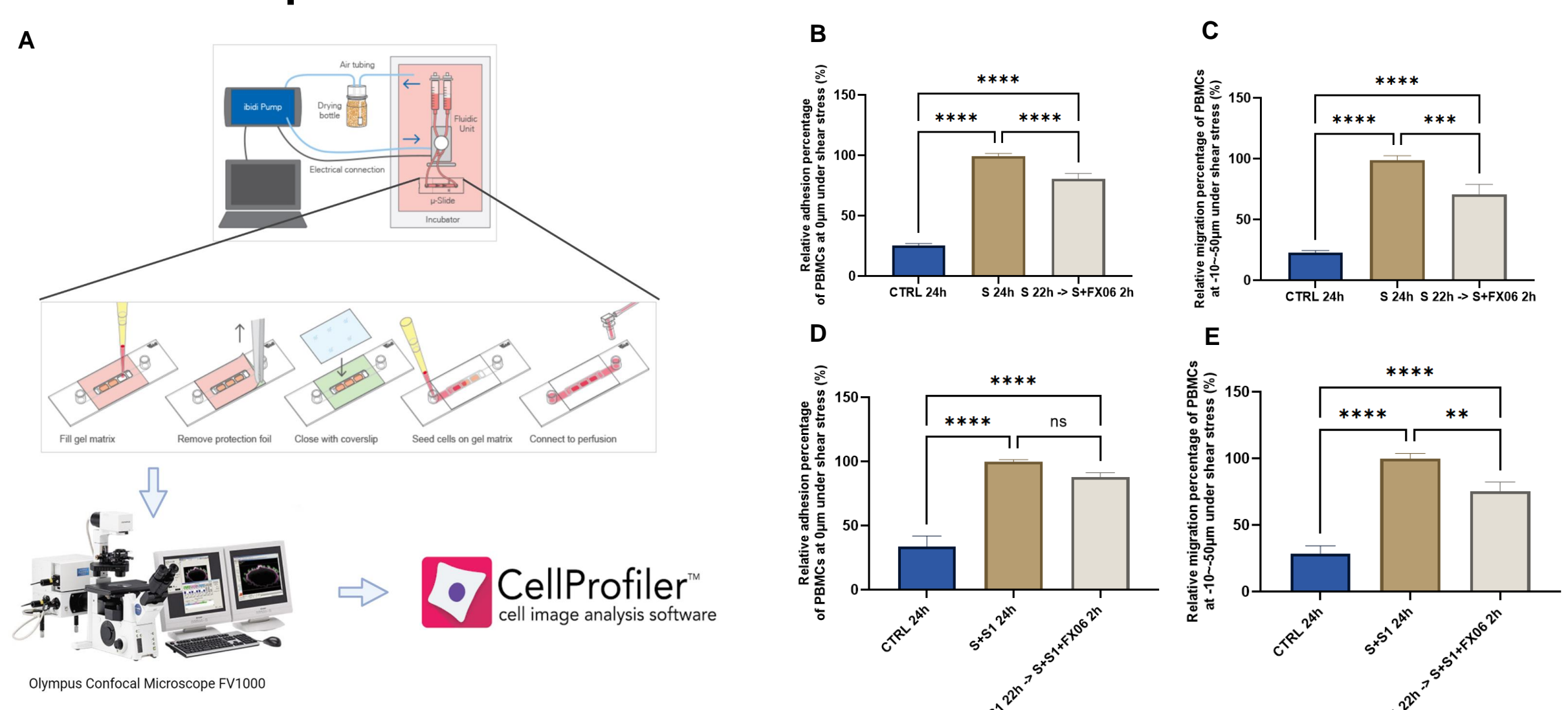
## RESULTS

### 1. FX06 prevents TEM of PBMCs under static conditions.



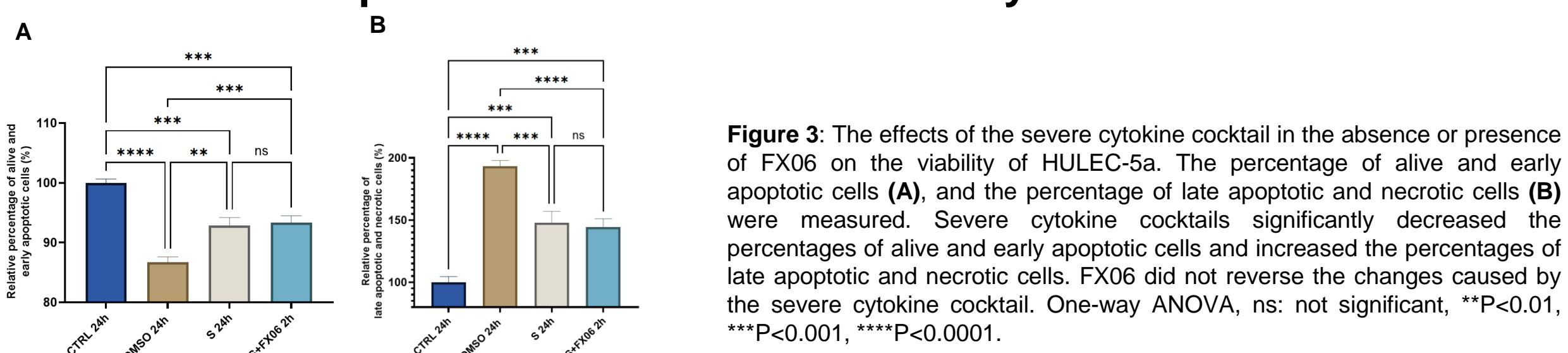
**Figure 1:** The effects of the severe cytokine cocktail and FX06 on TEM through EC monolayer. (A) The workflow of TEM assay under static conditions. PBMC adhesion (0  $\mu$ m) (B and D) and PBMCs migration (~10–100  $\mu$ m) (C and E) under static conditions in ECs treated with the severe cytokine cocktail, or the severe cytokine cocktail and S1. FX06 reduced the increased TEM caused by the severe cytokine cocktail or the combination of the severe cytokine cocktail and S1, in ECs cultured under static conditions. One-way ANOVA, ns: non-significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

### 2. FX06 shows protective effects under shear stress.



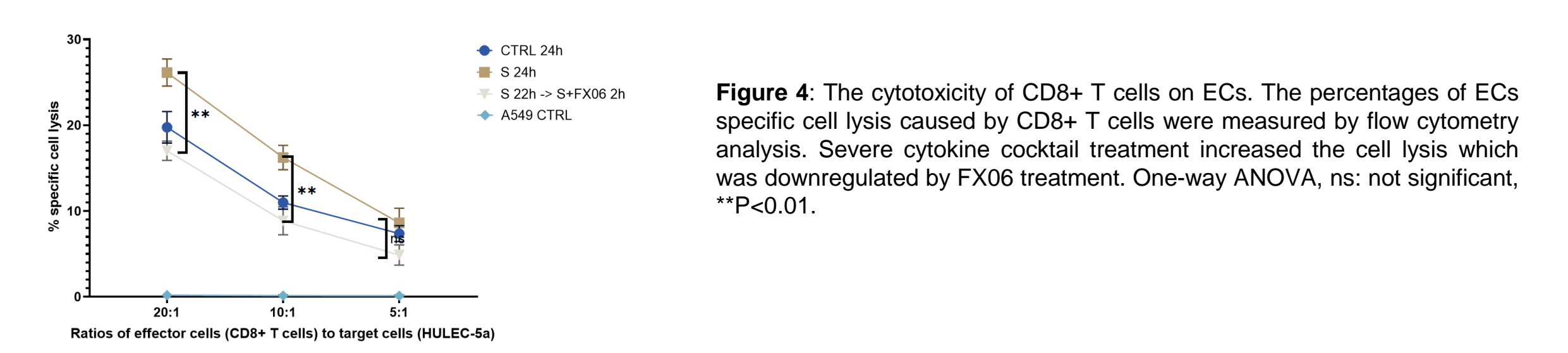
**Figure 2:** The effects of the severe cytokine cocktail and FX06 on TEM through EC monolayer. (A) The workflow of TEM assay under shear stress. PBMC adhesion (0  $\mu$ m) (B and D) and PBMCs migration (~10–50  $\mu$ m) (C and E) under shear stress in ECs treated with the severe cytokine cocktail, or the severe cytokine cocktail and S1. FX06 reduced the increased TEM caused by the severe cytokine cocktail or the combination of the severe cytokine cocktail and S1, in ECs cultured under shear stress. One-way ANOVA, ns: non-significant, \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

### 3. FX06 does not protect the endothelium from cytokine-induced cell death.



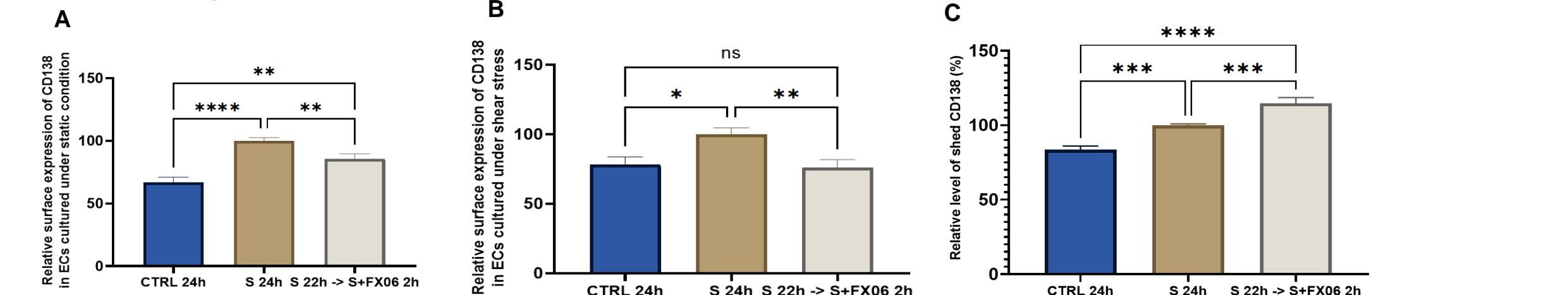
**Figure 3:** The effects of the severe cytokine cocktail in the absence or presence of FX06 on the viability of HULEC-5a. The percentage of viable and early apoptotic cells (A), and the percentage of late apoptotic and necrotic cells (B) were measured. Severe cytokine cocktails significantly decreased the percentages of viable and early apoptotic cells and increased the percentages of late apoptotic and necrotic cells. FX06 did not reverse the changes caused by the severe cytokine cocktail. One-way ANOVA, ns: not significant, \*\* $P < 0.01$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

### 4. Protective Effect of FX06 on Reducing Cytotoxicity of CD8+ T Cells



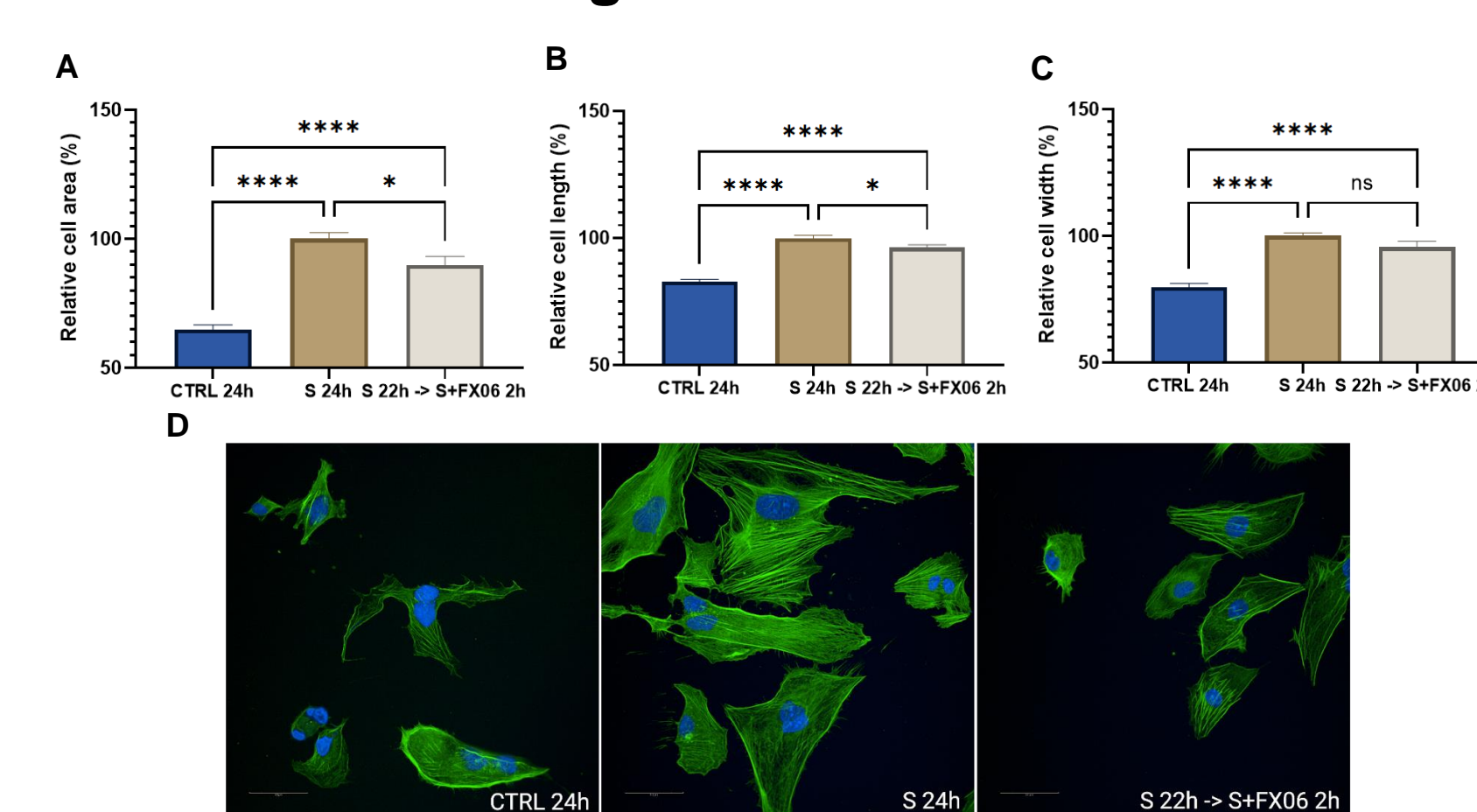
**Figure 4:** The cytotoxicity of CD8+ T cells on ECs. The percentages of ECs specific cell lysis caused by CD8+ T cells were measured by flow cytometry analysis. Severe cytokine cocktail treatment increased the cell lysis which was downregulated by FX06 treatment. One-way ANOVA, ns: not significant, \*\* $P < 0.01$ .

### 5. FX06 might assist in maintaining the normal barrier function of ECs by altering the surface expression of Syndecan-1 (SDC1/CD138).



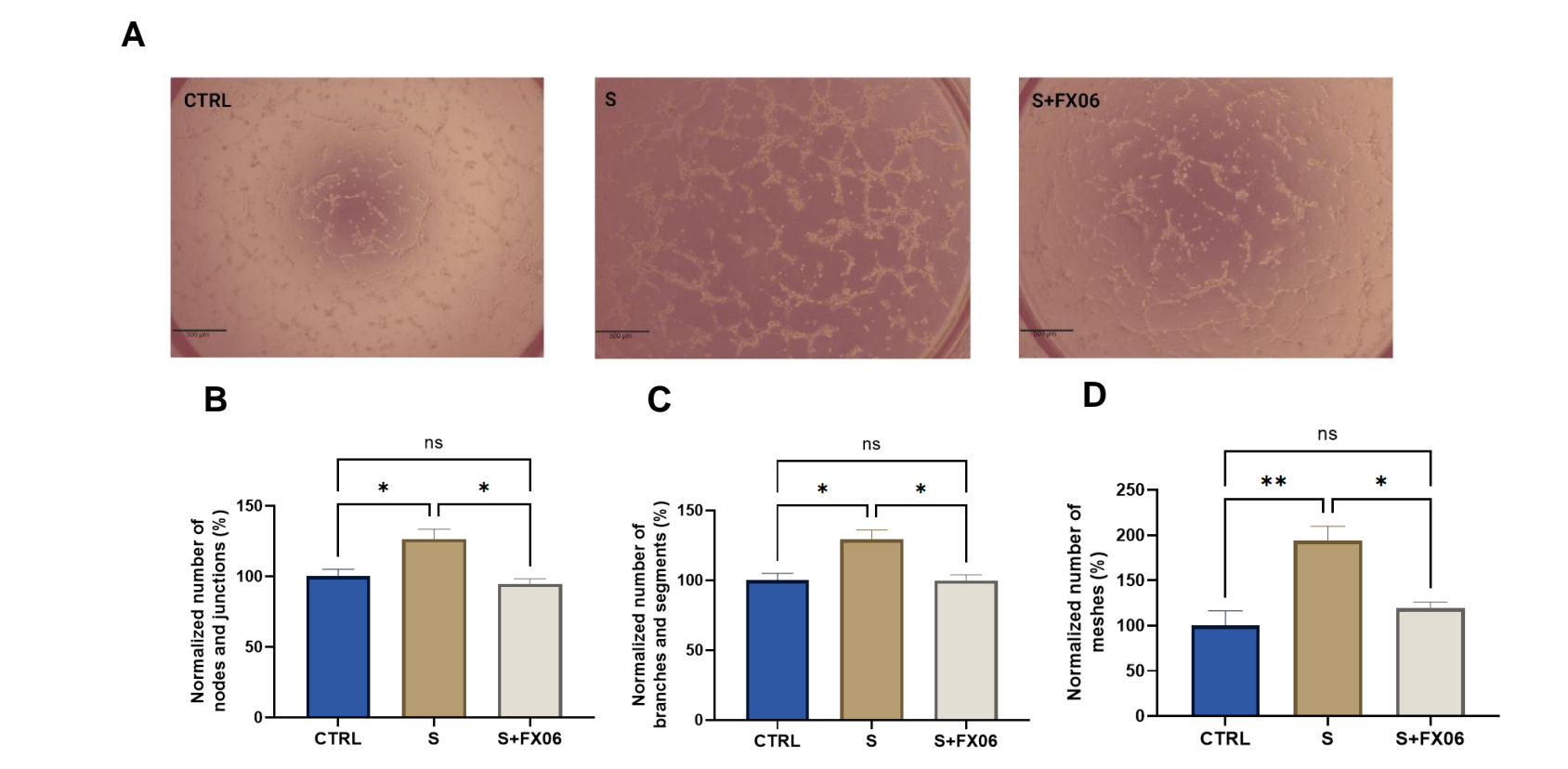
**Figure 5:** The changes of CD138 on ECs. Under both static (A) and shear stress (B) conditions, the severe cytokine cocktail caused more surface expression of CD138, and FX06 reversed the surface expression level of CD138 back to control levels. Figure C shows the level of shed CD138. The severe cytokine cocktail induced more shed CD138, whereas FX06 treatment caused even more. One-way ANOVA, ns: not significant, \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\*\* $P < 0.0001$ .

### 6. FX06 reverses morphology changes caused by the severe cytokine cocktail. And FX06 shortens the F-actin fiber length.



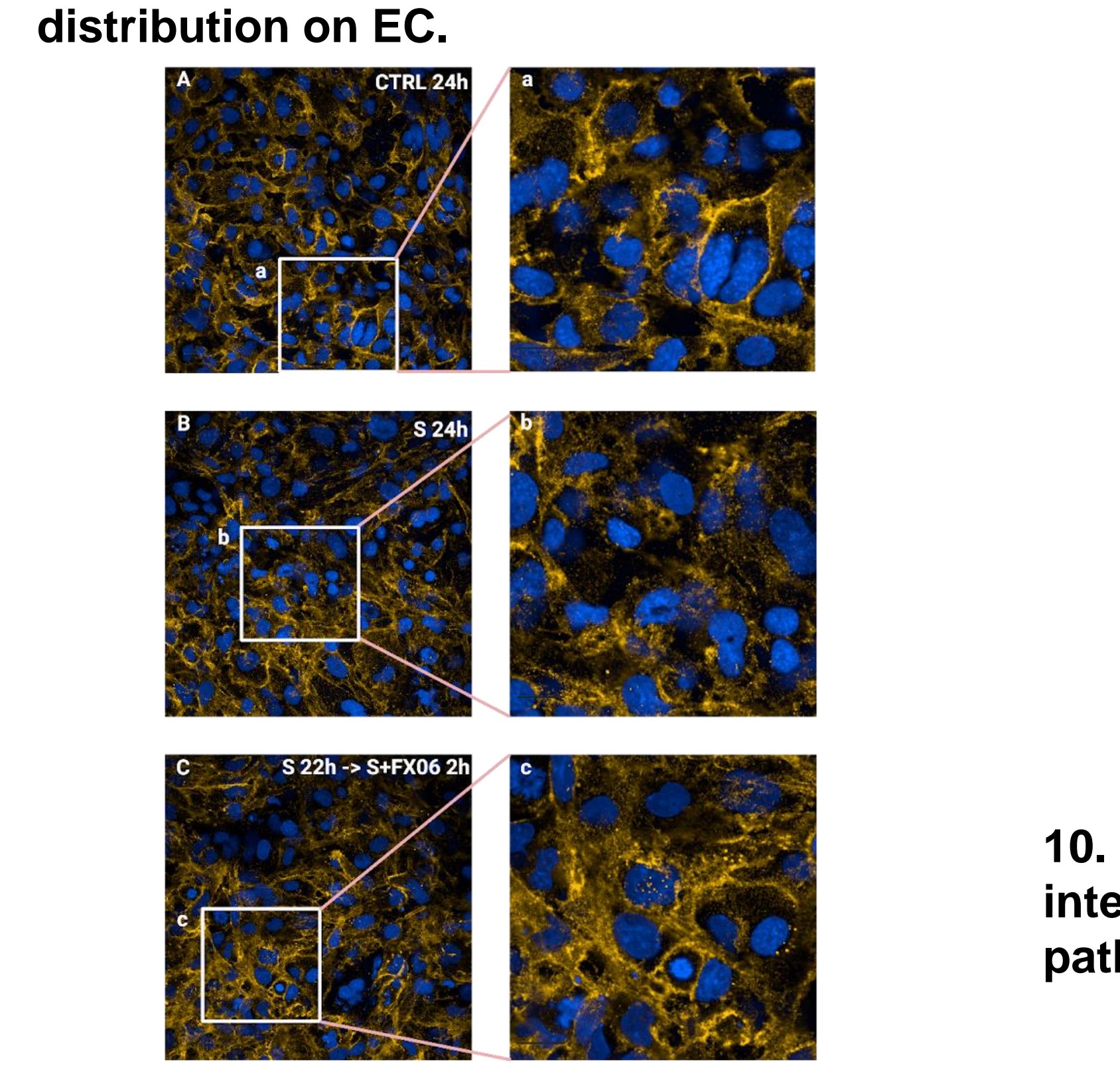
**Figure 6:** The morphological changes of ECs. The cell area (A), cell length (B), and cell width (C) were increased by the severe cytokine cocktail. FX06 decrease the cell area and cell length back to normal. One-way ANOVA, ns: not significant, \* $P < 0.05$ , \*\*\*\* $P < 0.0001$ . ECs were incubated with anti-F-actin antibodies, Alexa Fluor 488 Phalloidin (green), DAPI (blue) was utilised to stain nuclei. Images were taken with a 40x objective (D). Scale bar: 50  $\mu$ m.

### 7. FX06 reduces enhanced angiogenesis activity induced by the severe cytokine cocktail.



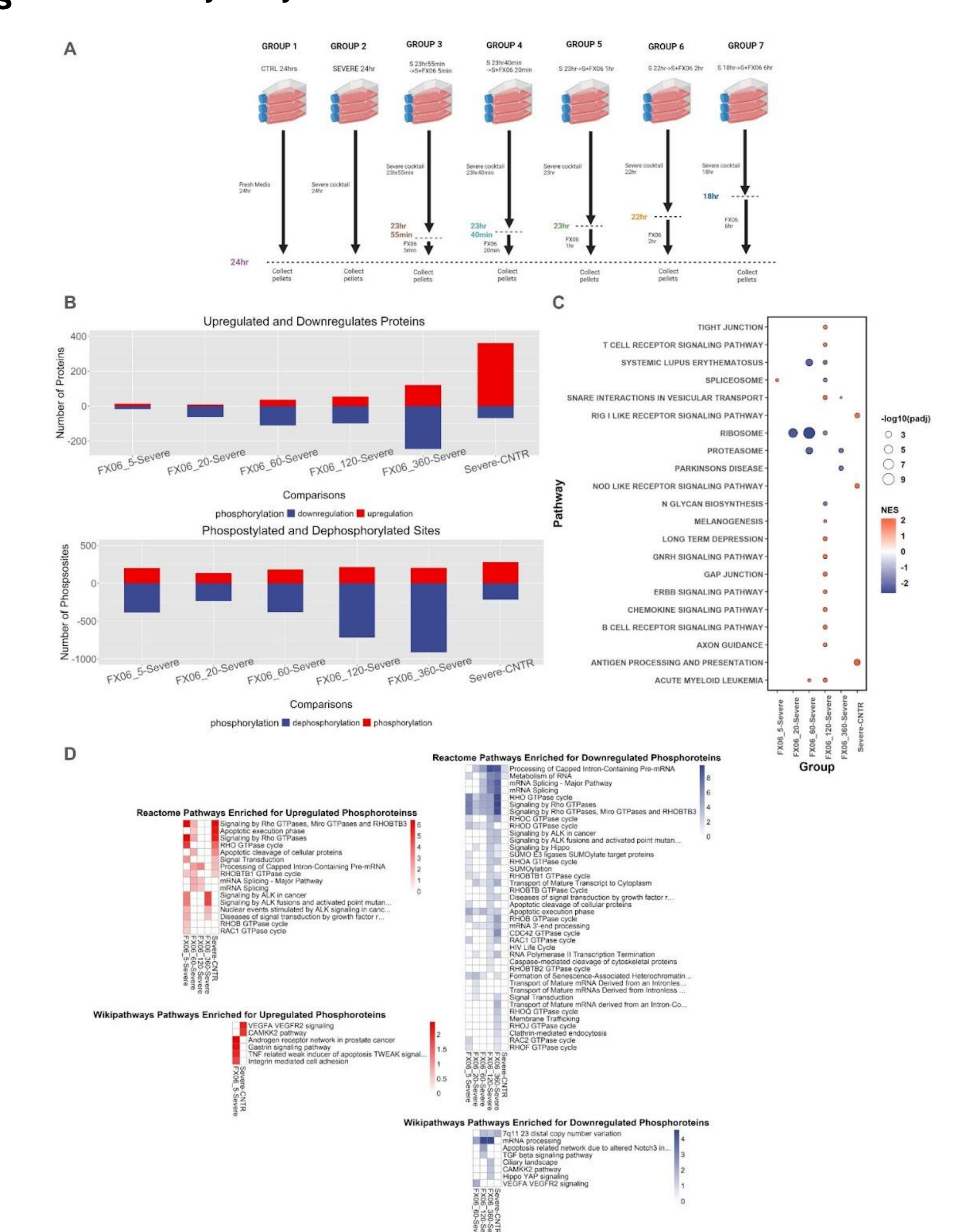
**Figure 7:** The effects of the severe cytokine cocktail and the severe cytokine cocktail with the presence of FX06 on angiogenesis activities in ECs. Images from (A) were taken from Zeiss Primovert microscope with a 4x objective. Scale bar: 500  $\mu$ m. FX06 normalized the total number of nodes and junctions (B), the number of meshes (C), and the total number of segments and branches (D) that were enhanced by the severe cytokine cocktail. One-way ANOVA, ns: not significant, \* $P < 0.05$ , \*\* $P < 0.01$ .

### 8. FX06 restores continuous VE-cadherin/CD144 distribution on EC.



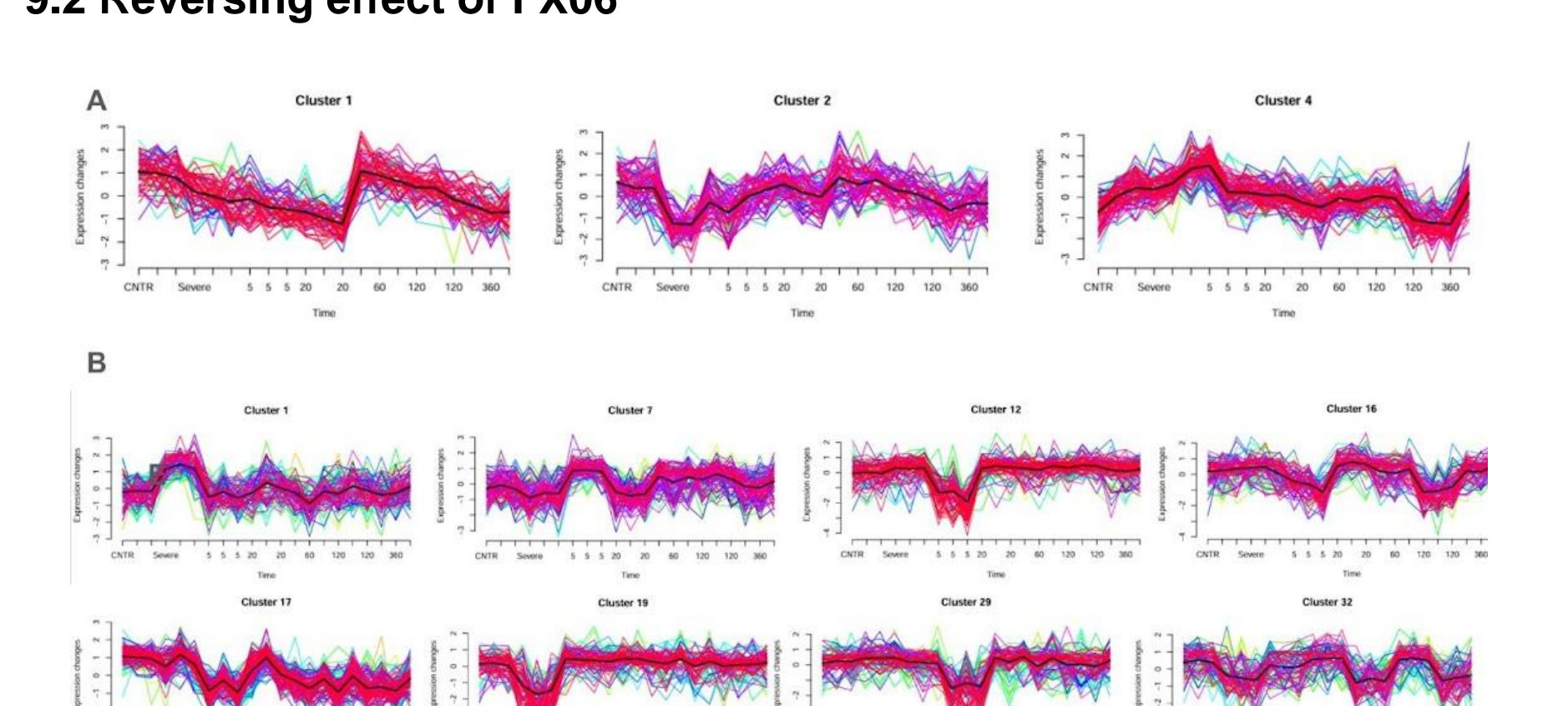
**Figure 8:** The effects of the severe cytokine cocktail and the severe cytokine cocktail with the presence of FX06 on VE-cadherin distribution in ECs. ECs were incubated with anti-VE-cadherin antibodies and stained with Alexa Fluor 546 (yellow). DAPI (blue) was utilised to stain nuclei. FX06 Images were taken with a Phenix Opera Confocal Microscope with magnification 40. Scale bar: 50  $\mu$ m. White boxes highlight the zoomed areas (7.a, 7.b and 7.c). Zoomed scale bar: 18.75  $\mu$ m.

### 9. Proteomics and phosphoproteomics data analysis. 9.1 Pathway analysis



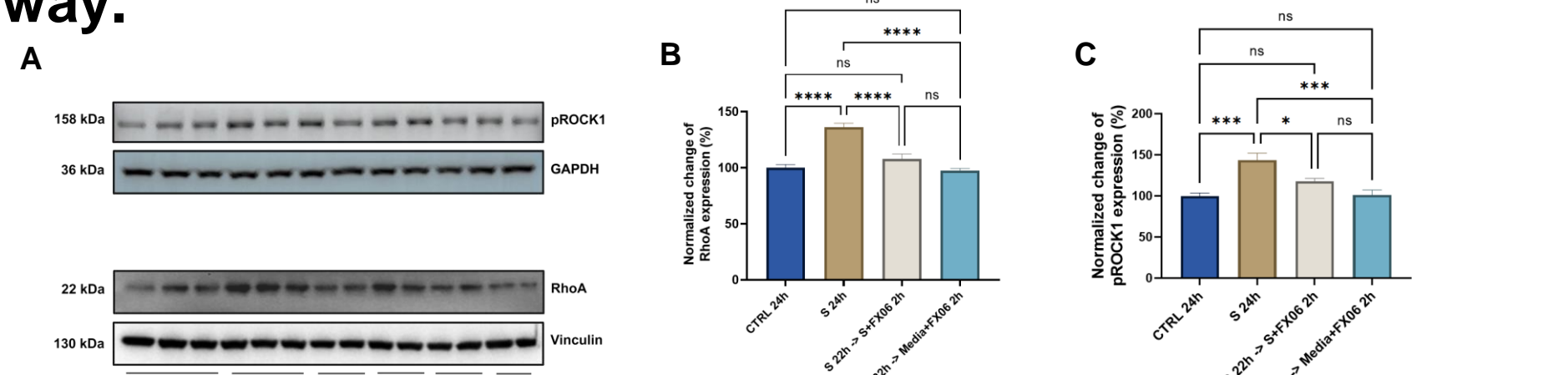
**Figure 9:** (A) The experimental design. (B) Box plots with the overall number of upregulated and downregulated proteins (top) and phosphosites (bottom) for each cell group comparison. The red part of the boxes represents upregulated proteins, while the blue part shows the number of downregulated proteins. (C) Bubble plot showed there FX06 induces an increase in the activity of Tight Junction, Gap Junction, Gonadotropin-releasing hormone signaling and ERBB signaling. (D) Proteins upregulated by the severe cytokine cocktail were associated with Interferon Signaling, Cytokine Signaling, Interleukin-1 Signaling, Immune System, SARS-CoV-1 Infection, and Antigen Presentation pathways. FX06 induced phosphoproteomics related to RhoA and RhoG GTPase cycles, and RhoGTPase signaling.

### 9.2 Reversing effect of FX06



**Figure 10:** (A) FX06 demonstrates the ability to reverse the abundance of proteins altered by the cytokine cocktail, restoring them to the levels close to control. (B) 8 clusters had changes in protein phosphorylation that were reversed by FX06.

### 10. FX06 may restore the integrity of the vascular wall by interfering with the RhoA-ROCK-1 signal transduction pathway.



**Figure 11:** 2h-treatment of FX06 decreased the RhoA expression (B) and phospho-ROCK1 (C) that was enhanced by the severe cytokine cocktail. One-way ANOVA, ns: not significant, \* $P < 0.05$ , \*\*\* $P < 0.001$ , \*\*\*\* $P < 0.0001$ .

## Conclusions & Outlook

- FX06 shows great potential in reducing vascular leakage to protect the endothelium against the proinflammatory effect of COVID-19-triggered cytokines.
- The protective mechanism of action of FX06 is NOT due to anti-apoptotic or anti-necrotic effects.
- FX06 rather reduces inflammation-driven processes by tightening up junctions between ECs, decreasing angiogenic activity, limiting cell contraction, and stimulating endothelial recovery-related transcription factors.
- FX06 interferes with the proinflammatory RhoA-ROCK-1 and VEGF-related signal transduction pathways to restore vascular integrity. Further studies are currently conducted to fully elucidate its mechanism of action.

## REFERENCES

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