Ebola Virus GP Activates Endothelial Cells

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Ebola GP triggers apoptosis through an additional pathway other than the ICAM-1 induction pathways. Finally, the cytoskeletal signaling pathways may serve as important targets for the development of therapeutic drugs against EBOV disease.

Ebola GP ICAM-1 virus–host interactions cytoskeletal signaling focal adhesion kinase TNF-α host factor antivirals micropinocytosis Rho signaling pathway

1. Introduction

Ebola virus (EBOV), a member of the Filoviridae family, is an enveloped and negative-stranded RNA virus that causes severe hemorrhagic fever with high mortality rates in humans and other animals [1][2]. The genome of EBOV consists of seven genes encoding nucleoprotein (NP), RNA-dependent RNA polymerase (L), transmembrane glycoprotein (GP), matrix protein VP40, VP35, VP30, and VP24 [3][4]. Apart from the structural transmembrane GP, the transcription of the GP gene generates two nonstructural glycoproteins, the small soluble glycoprotein (ssGP) and the soluble glycoprotein (sGP), through transcriptional editing. The sGP, in turn, generates Δ-peptide as a result of its cleavage [5][6][7]. The cleavage of GP by the cellular metalloprotease TNFα-converting enzyme (TACE) generally occurs during EBOV infection, giving rise to shed GP, which structurally resembles the full-length GP and therefore can misdirect host-neutralizing antibodies directed against the full-length GP by presenting alternative epitopes [6][8][9].

GP can bind to multiple cell surface molecules, conferring on EBOV the ability to infect a wide range of cell types, including immune cells such as macrophages and dendritic cells, and other cells such as hepatocytes, epithelial cells, and endothelial cells (ECs) [10][11]. Vascular dysregulation leading to an increase in blood vessel permeability, loss of endothelial barrier integrity, and hemorrhage plays an important role in the severity of EBOV infection [1][12][13]. Despite their susceptibility to EBOV in vitro, ECs are considered late viral targets in vivo, and the molecular mechanisms underlying their impairment during EBOV infection remain elusive to date [8][9]. In addition to direct viral infection, the vascular endothelium can be targeted indirectly via mediators such as cytokines, which are released upon infection of primary target cells, including immune cells, as well as virus-encoded GPs, which in turn can target ECs either directly or through the activation of primary target cells [14][15][16][17]. Among the proposed working models, only GP and the soluble shed GP have been found to play a key role in the activation of ECs and a decrease in their barrier function. Neither viral infection and replication nor other virus-encoded GPs, such as sGP, Δ-peptide, and ssGP, were found to be necessarily involved in the activation of ECs [1][8][14][17][18]. As

activation markers, the upregulation of cell adhesion molecules (CAMs), including intercellular adhesion molecules-1 (ICAM-1), was detected at the transcriptional level [14] and then at the protein level [1]. To confirm that EC activation depends on EBOV GP but does not require viral replication, Wahl-Jensen et al. [1] generated virus-like particles (VLPs) formed by VP40 and GP or only VP40 and demonstrated that only VLPs formed by VP40 and GP were able to activate human umbilical vein endothelial cells (HUVECs). However, whether the EBOV GP-mediated activation and disruption of ECs could be the result of its direct interaction with the cells via some receptors or an indirect effect by some cellular mediators induced by EBOV GP remains unclear.

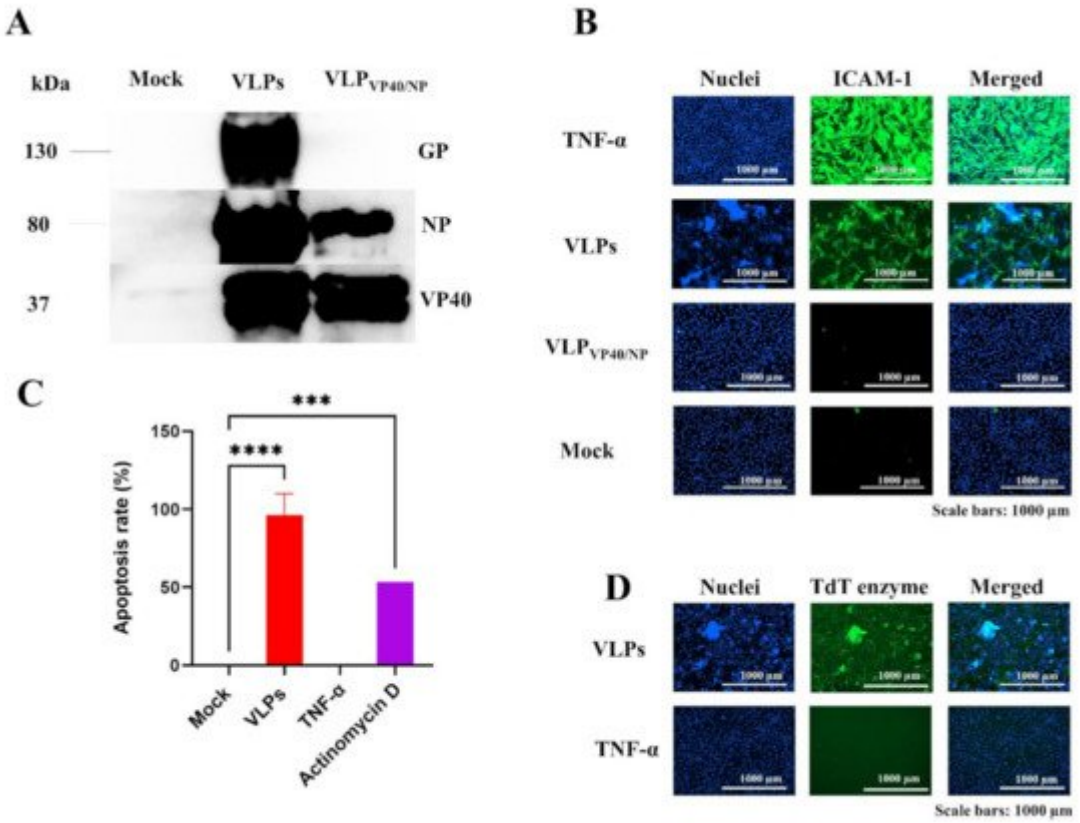
Existing evidence suggests that overexpression of ICAM-1 on ECs can lead to increased vascular permeability and loss of the endothelial barrier, which is mediated by the activation of the Rho/ROCK pathway [19][20] and rearrangement of the actin cytoskeleton [21][22]. The activation of the Rho/ROCK pathway by ICAM-1 has been reported to initiate a positive feedback loop that could result in the expression of more ICAM-1 and the recruitment of more leukocytes [23] and that subsequently lead to diverse vasculopathies [24][25]. Junaid et al. [13] recently reported that the inhibition of the Rho/ROCK pathway by RevitaCell Supplement suppressed Ebola VLP-induced permeability increase in HUVECs, suggesting a possible involvement of this molecular pathway in EBOV disease (EVD) pathogenesis.

In the present study, we hypothesized that suppressing the Rho/ROCK pathway and some cytoskeletal signaling molecules would prevent the overexpression of ICAM-1 and associated ECs disruption observed during EBOV infection. First, we confirmed the upregulation of ICAM-1 expression in HUVECs after exposure to Ebola VLPs bearing GP as well as TNF-α, but not Ebola VLPs lacking GP. In contrast to TNF-α treatment, Ebola VLPs bearing full-length GP induced ICAM-1 overexpression at late time points. We also found that only Ebola VLPs treatment induced significant cytotoxicity, mainly apoptosis. Moreover, screening of the cytoskeletal signaling inhibitors library identified focal adhesion kinase (FAK) inhibitors as potent inhibitors of ICAM-1 mediated by both TNF-α and Ebola VLPs bearing GP. Our results suggest that EBOV GP stimulates ECs to induce endothelial activation and dysfunction with the involvement of host cytoskeletal signaling molecules, which represent potential therapeutic targets for EBOV infection.

2. Current Insights

Dysfunction and injury of ECs constitute the major outcomes of EBOV infection and play key roles in disease development. Previous studies have indicated that EBOV GP is the main determinant of EC activation and cytotoxicity [1][17]. However, the mechanisms underlying the interactions between EBOV GP and ECs are largely unknown. In this study, we found that, similar to TNF-α, Ebola VLPs bearing GP (referred to as VLPs) could activate ECs, as confirmed by the upregulation of ICAM-1 expression on the cell surface (**Figure 1**B). In addition to the induction of ICAM-1 expression, the exposure of ECs to VLPs led to the appearance of cytopathic effects (**Figure 1**B), which were due to apoptosis, while TNF-α activation of ECs was not associated with apoptosis and subsequent cytopathic effects (**Figure 1**C–E). In contrast to VLPs, GP-deficient VLPs (referred to as $VLP_{VP40/NP}$) did not activate ECs, as shown by the absence of ICAM-1 expression (**Figure 1**B), suggesting that GP is required for vascular activation and disruption. These results are consistent with those of previous studies indicating that GP

is the main determinant of vascular injury and cytotoxicity and that viral replication is not required for the vascular dysfunction observed during EBOV infection.



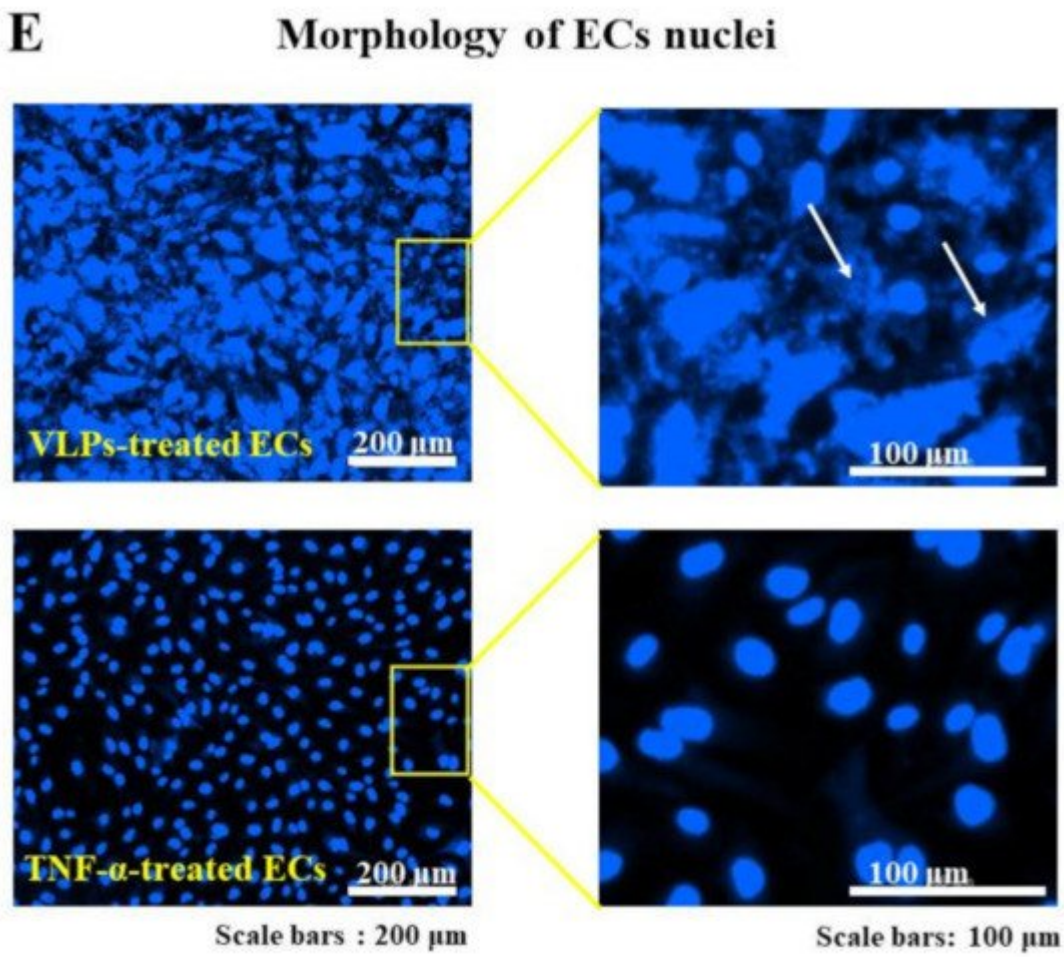


Figure 1. Ebola VLPs activate ECs and induce apoptosis. (**A**) The different VLPs preparations were purified from supernatants of transfected HEK293T cells and characterized using Western blotting with monoclonal antibodies (anti-GP, anti-VP40, and anti-NP). Supernatants from non-transfected cells were used as the negative control (Mock). Characterization with anti-GP indicated the absence of GP in $VLP_{VP40/NP}$. (**B**) ECs were exposed to VLPs (10× dilution) or $VLP_{VP40/NP}$ (10× dilution) for 48 h. Following the treatment, cells were fixed and permeabilized, and the activation was detected by immunofluorescence analysis using a monoclonal antibody directed against ICAM-1. The cells were then stained with Hoechst 33358 for imaging (magnification, ×10). Human recombinant TNF-α and supernatants from non-transfected cells (mock) were used as the positive and negative controls, respectively. Only cells treated with VLPs and TNF-α induced the expression of ICAM-1. Unlike TNF-α, VLPs-mediated upregulation of ICAM-1 expression was associated with cytopathic effects on ECs. Neither $VLP_{VP40/NP}$ nor the mock control induced the upregulation of ICAM-1 expression. (**C**) The apoptotic rate was quantified using the fluorescence intensity of the nuclear staining with the TdT enzyme. Data represent the percent values of apoptotic cells derived from the normalization to the mock-treated and are expressed as mean ± SD (n = 2) from two independent experiments. *** p = 0.0006 and **** p < 0.0001 compared to the mock. The apoptotic rate in VLPs-, TNF-α- and Actinomycin D (4 µg/mL)-treated cells were compared to that in the cells treated with media (mock), applying one-way ANOVA, followed by Dunnett's multiple comparisons test. (**D**) Images of nuclear staining from VLPs- and TNF-α-treated cells are shown, with VLPs-treated cells exhibiting apoptotic morphology in comparison to the TNF-α-treated cells. (**E**) Close up images showing the morphology of ECs nuclei after treatment

with VLPs and TNF-α. White arrows indicate disrupted and aggregated nuclei in contact with VLPs. VLPs: virus-like particles; ECs: endothelial cells; ICAM-1: intercellular adhesion molecules 1.

The induction of ICAM-1 expression on the surface of ECs and the associated apoptotic cell death occurred in a VLPs dose-dependent manner (**Figure 2**A), and the level of the observed cytotoxicity was found to be directly dependent on the level of EBOV GP. Previously, it has been demonstrated that the cytotoxicity of EBOV is significantly increased by the overexpression of GP [26], which has been further supported by Ray et al. [27], who reported that the expression of EBOV GP in recombinant adenovirus (Ad-Ebola GP)-transduced primary human cardiac microvascular endothelial cells and HUVECs induced apoptotic cell death with related cytopathic effects in a dose-dependent manner, demonstrating the importance of GP expression level in the cytotoxic effect through apoptosis induction.

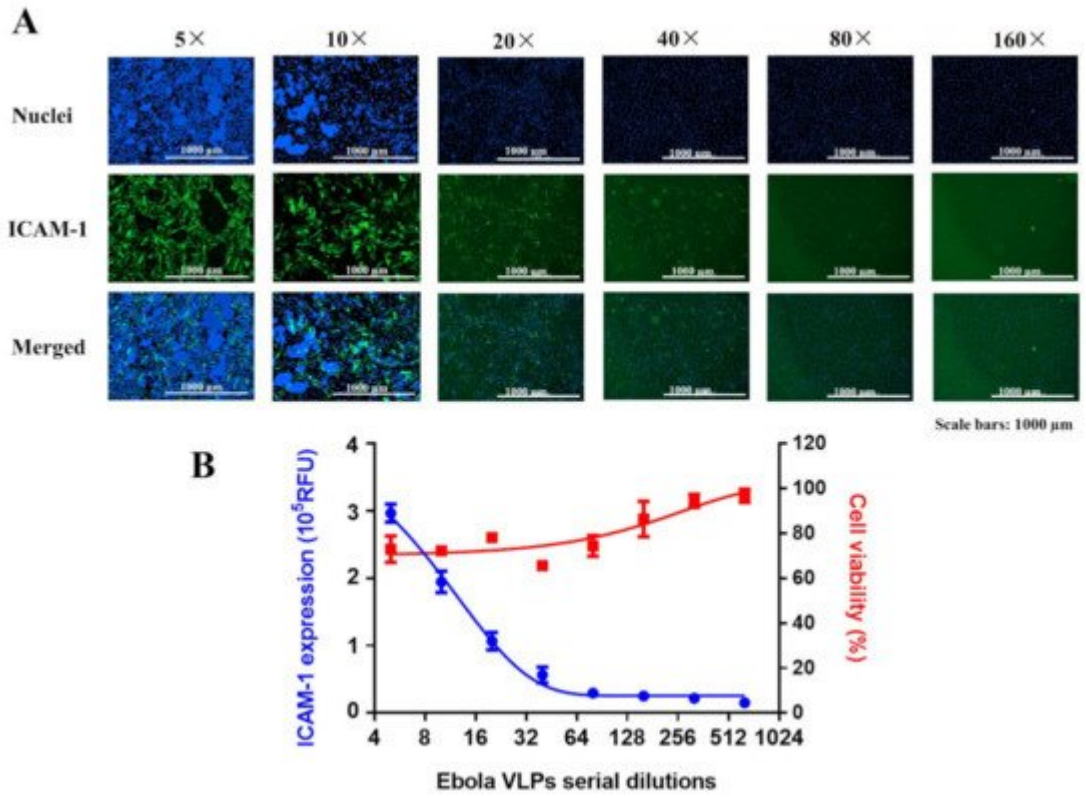


Figure 2. Ebola VLPs activate ECs in a dose-dependent manner. (**A**,**B**) ECs were treated with serial dilutions of VLPs for 48 h, fixed, and permeabilized for detection by immunofluorescence using an antibody directed against ICAM-1. Cell viability was determined using the Cell Counting Kit-8 assay. (**A**) Immunofluorescence images are shown for each VLPs dilution factor. (**B**) Dose-dependent curves showing induced ICAM-1 expression (blue) and cell viability (red). Data are representative of two independent experiments (*n* = 3). VLPs: virus-like particles; ECs: endothelial cells; ICAM-1: intercellular adhesion molecules 1.

In agreement with these findings, we showed that ECs treated with 10× diluted VLPs, which contained approximately 9.2 µg/mL of GP, exhibited a strong cytopathic effect than that of ECs treated with 160× diluted VLPs, which contained a low amount of GP (**Figure 2**).

The relevance of EBOV GP level on the induction of ECs apoptosis and disruption suggests a direct correlation between disease severity and the presence of a high level of shed GP and high viral load in the serum of EVD patients, as reported elsewhere [28].

ICAM-1 expression is constitutively low at the surface of ECs and may be upregulated in response to stimuli, such as viral infection, oxidative stress, and pro-inflammatory cytokines, including interleukin-1β (IL1β), TNF-α, and interferon-γ (IFN-γ) [29][30]. Kinetic studies of ICAM-1 expression upon activation with TNF-α or VLPs revealed a difference in kinetics patterns. TNF-α-mediated upregulation of ICAM-1 occurred early around 6 h post-treatment and reached a peak at 12 h before decreasing around 48 h, with no cytopathic effect observed in ECs over time (**Figure 3**C). Previous studies have shown that TNF-α can activate the endothelium and modulate its barrier function in tissue-culture models and in vivo while maintaining a relatively intact morphology [2]. This observation was confirmed in our study, where the activation of the endothelium by TNF-α was not associated with a direct induction of ECs disruption and the appearance of cytopathic effects. In contrast, VLP-mediated upregulation of ICAM-1 appeared at a later time point (48 h post-treatment), with cells presenting injury and typical apoptotic morphology. A correlation between the levels of ECs disruption and the expression of ICAM-1 was observed (**Figure 3**B). While the treatment of ECs with TNF-α did not affect cell viability and growth, ECs exposure to VLPs increased the survival rate of the cells by approximately 200% from 6 h, with a peak at 12 h before decreasing from 48 h (**Figure 3**B, graph at the right). To the best of our knowledge, our study is the first to show that Ebola VLPs upregulate host cell proliferation within several hours following treatment before inducing apoptosis. We speculate that the EBOV VLP-induced abnormal cell proliferation might be the result of the cell stress response, which in turn has triggered the subsequent apoptosis after failing to cope with the insult caused by VLPs [31]. More studies to understand the interplay between the GP on the VLPs, the ECs proliferation, and the apoptosis induction are needed.

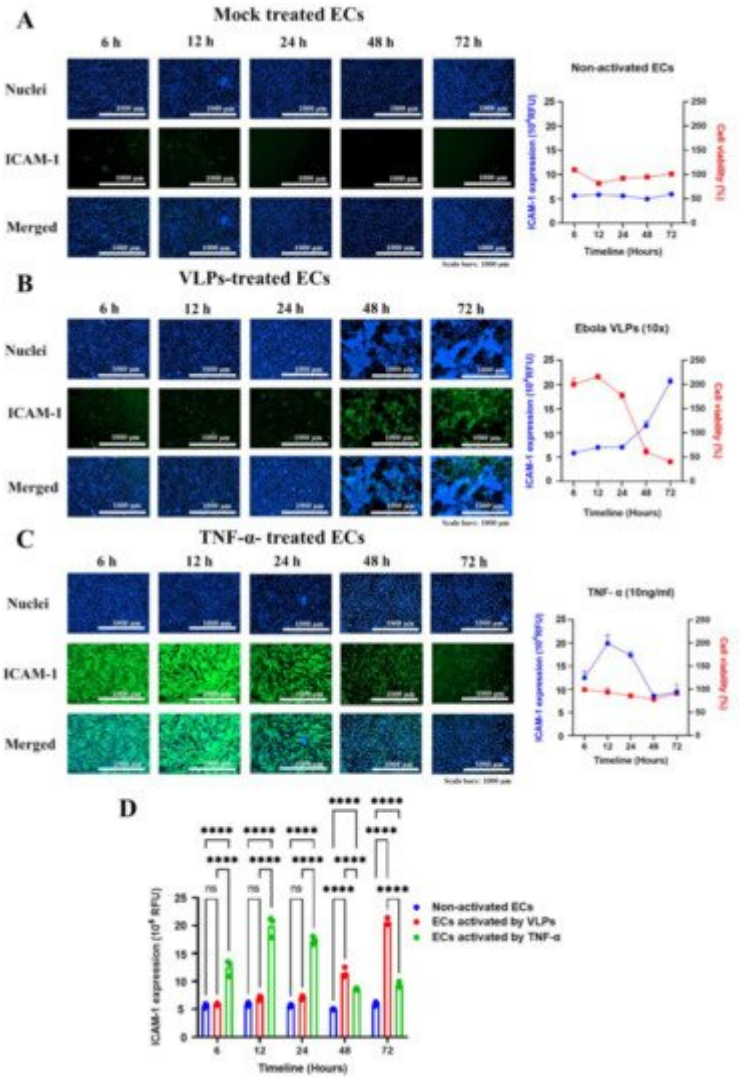


Figure 3. Kinetics of ICAM-1 protein expression. (**A**–**C**) ECs were exposed to VLPs (10× dilution), TNF-α (10 ng/mL), or cell medium only (mock) for 6, 12, 24, 48, and 72 h. Following the treatment at each designated time point, cells were fixed and permeabilized for the detection of ICAM-1 protein expression by immunofluorescence using antibodies directed against ICAM-1. Related cell viability at each time point was evaluated using the CCK-8 assay. (**A**) No detection of ICAM-1 expression (left) and no change in viability was observed in non-activated ECs (right). (**B**) A significant increase in ICAM-1 protein expression levels was shown with VLPs treatment from 48 h post-treatment. The cell viability was inversely proportional to the ICAM-1 protein expression and was reduced drastically from 48 h post-treatment. (**C**) TNF-α-treated cells presented an early signal of ICAM-1, with maximal levels of expression at 12 h post-treatment and a further decrease over time. (**D**) The summary graph of the kinetics of ICAM-1 protein expression. The results are presented as averages, and error bars indicate standard deviation (n = 3). Statistical significance is shown: **** $p < 0.0001$. VLPs and TNF-α-treated cells were compared, and both were also compared with the non-activated cells applying two-way ANOVA and followed by Tukey's multiple comparisons test. ICAM-1: intercellular adhesion molecules 1; ECs: endothelial cells; VLPs: virus-like particles.

The level of ICAM-1 expression in VLP-treated ECs was not upregulated before 48 h of incubation. Connolly-Andersen et al. observed the same kinetic pattern for ICAM-1 upregulation at the transcriptional level following the activation of HUVECs with various doses of Crimean-Congo hemorrhagic fever virus (CCHFV) [30]. Wahl-Jensen et al. reported the upregulation of ICAM-1 at 12 and 24 h at the level of gene transcription after treatment of HUVECs with Ebola VLPs consisting of GP and VP40 and at the protein level after the infection of HUVECs with live EBOV [1]. Whether the expression observed at the cell surface was a basal ICAM-1 expression, as no cytopathic effect was reported together with the ICAM-1 upregulation in HUVECs, needs to be clarified. One of the limitations of our assay was the evaluation of ECs activation only with the induction of ICAM-1 upregulation, which cannot provide more details such as ECs contraction and ultrastructural changes. Transmission electron microscopy of ECs undergoing apoptosis in the presence of the VLPs in comparison with the mock-treated ECs would provide a deep understanding of the VLPs' effect on ECs.

The involvement of the Rho/ROCK pathway in the induction of hyperpermeability in HUVECs treated with Ebola VLPs [13] led us to hypothesize that the inhibition of the Rho/ROCK pathway would suppress TNF-α- and VLP-induced ICAM-1 upregulation, as well as VLP-induced apoptotic disruption in ECs. Our hypothesis was verified for only the suppression of ICAM-1 expression (**Figure 4**A,B). The kinetic patterns of TNF-α and VLP activities had an impact on the modulation of the Rho/ROCK pathway, as seen by RevitaCell suppression of TNF-α-induced ICAM-1 at 12 h (**Figure 4**B) and VLP-induced ICAM-1 at 48 h (**Figure 4**A). Several studies have reported that the inhibition of Rho/ROCK does not reverse the increase in EC permeability for several hours after TNF-α exposure and stimulation [32][33][34]. Consistent with these reports, the inhibition of the Rho/ROCK pathway in our study did not influence TNF-α-induced ICAM-1 expression upregulation at 48 h after treatment. Furthermore, the inhibition of the Rho/ROCK pathway led to the abnormal cell proliferation of ECs exposed to VLPs at 12 h of incubation, suggesting a probable interaction with downstream effectors or pathways that might be specific to the VLPs and responsible for the observed apoptosis.

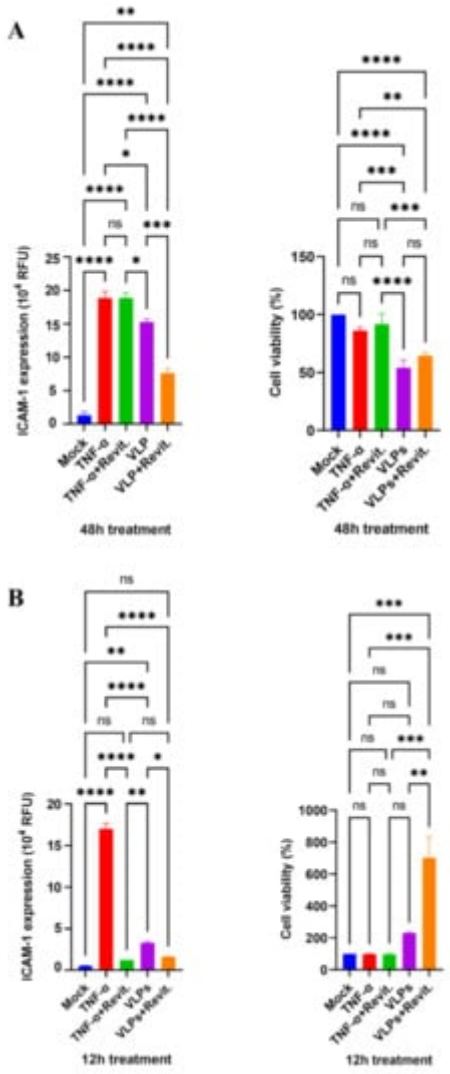


Figure 4. Effect of RevitaCell on VLPs- and TNF-α-induced ICAM-1 upregulation. (**A**) ECs were pre-treated for 1 h with RevitaCell (1×) and then treated with VLPs (10×) or TNF-α (10 ng/mL). Forty-eight hours after treatment, cells were fixed and immunostained for fluorescence measurement. For each condition, the percentage of ICAM-1 expression was calculated as the ratio of the expression level in the wells pre-treated with RevitaCell to the expression level in wells treated with either VLPs or TNF-α only. Cell viability was also assessed using the cell counting kit-8 according to the manufacturer's recommendations. Data for ICAM-1 expression and cell viability in the presence of RevitaCell are shown, representing mean ± SD of triplicate results from two independent experiments. (**B**) To study the effect of RevitaCell at early incubation time, a similar pre-treatment with RevitaCell (1×) was performed. Fluorescence was measured 12 h after the addition of VLPs (10×) or TNF-α (10 ng/mL). EC viability was similarly assessed 12 h after appropriate treatments. Data show relative VLPs- or TNF-α-mediated ICAM-1 expression levels with or without RevitaCell (left), as well as cell viability under corresponding treatment (right). One-way ANOVA followed by Tukey post-test was applied to compare data, which are presented as mean ± SD of triplicate results. Statistical significance is shown: **** Extremely significant ($p < 0.0001$), *** Extremely significant ($p = 0.0001$ to 0.001), ** Very significant ($p = 0.001$ to 0.01), * Significant ($p = 0.01$ to 0.05), ns not significant ($p \geq 0.05$). VLPs: virus-like particles; ECs: endothelial cells; ICAM-1: intercellular adhesion molecules 1.

Since the Rho/ROCK pathway controls EC dysfunction through the regulation of cytoskeleton organization [23], we screened the inhibitors of host cytoskeleton signaling pathways and identified FAK inhibitors as potent inhibitors of ICAM-1 upregulation mediated by both TNF-α and VLPs (**Figure 5**B,C). Among the nine FAK inhibitors analyzed for their dose-response activity, BI-4464 was the most effective against VLPs-induced ICAM-1 expression, while Defactinib (VS-6063) showed potency against both VLPs- and TNF-α-induced ICAM-1 expression (**Figure 6**).

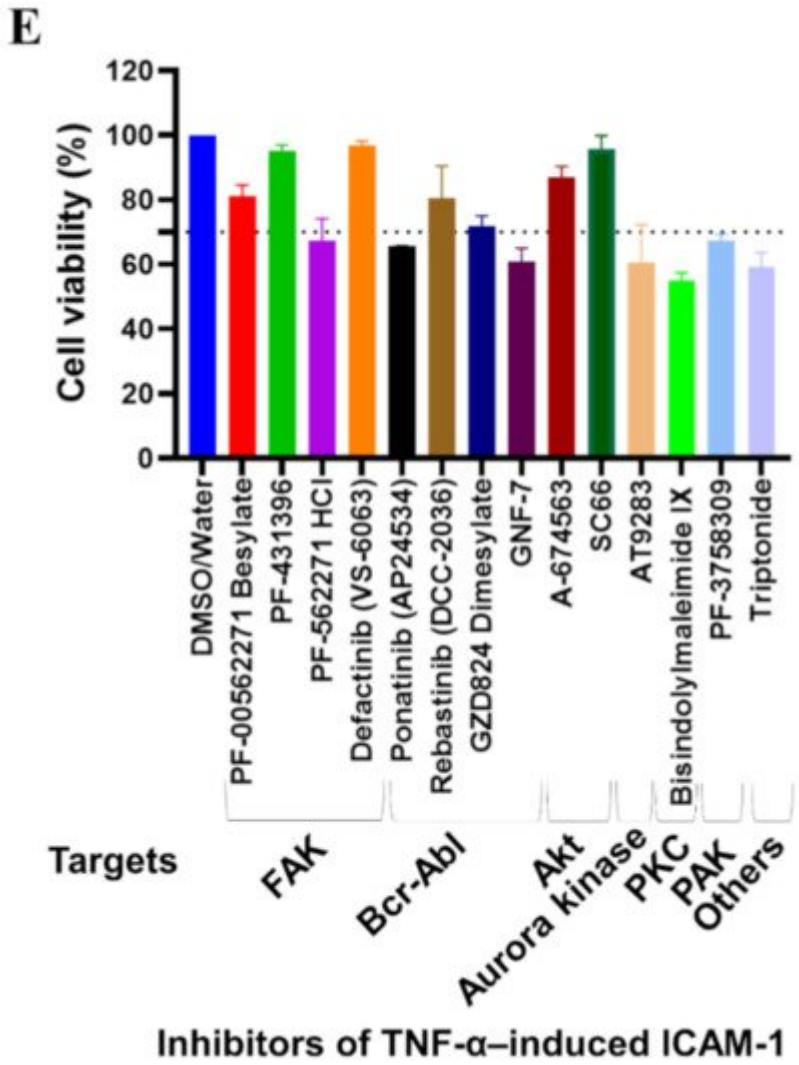
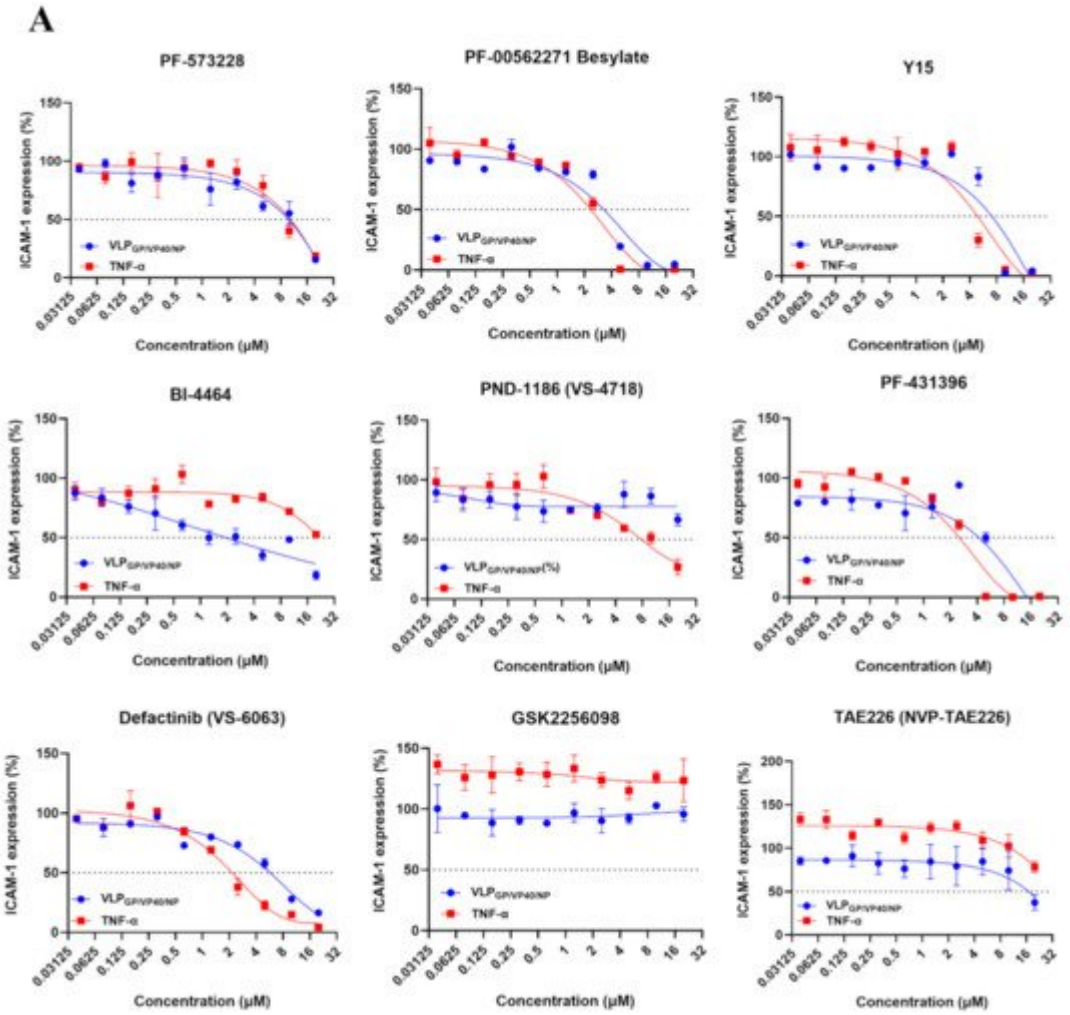


Figure 5. Screening of host cytoskeletal signaling compounds inhibiting VLPs-mediated endothelial cells activation (**A**) Illustration of the screening strategy. The screening was conducted on VLPs- or TNF-α-treated cells. Compounds (*n* =179) were evaluated for their effects on the inhibition of ICAM-1 expression. The fluorescence corresponding to the expression of ICAM-1 was measured 12 and 48 h post-treatment for TNF-α- or VLPs-treated cells, respectively, following immunostaining. The protective effects of the compounds were assessed through nuclear staining and imaging. RevitaCell Supplement was used as the positive control. Compounds exhibiting 70% inhibition of ICAM-1 expression were considered effective inhibitors, with 28 and 14 of them inhibiting VLPs- and TNF-α-induced ICAM-1 expression, respectively. (**B**) The target host proteins for VLPs-induced ICAM-1 inhibitors and (**C**) TNF-α-induced ICAM-1 inhibitors are shown. (**D**) The inhibitors of VLPs-induced ICAM-1 expression targeting FAK and (**E**) those of TNF-α-induced ICAM-1 expression targeting FAK, Akt, and Bcr-Abl showed less

than 30% cytotoxicity (limit shown by grid lines at 70% cell viability). VLPs: virus-like particles; ECs: endothelial cells; ICAM-1: intercellular adhesion molecules 1; Akt: Ak strain transforming kinase; FAK: Focal adhesion kinase; Bcr-Abl: breakpoint cluster region-abelson.



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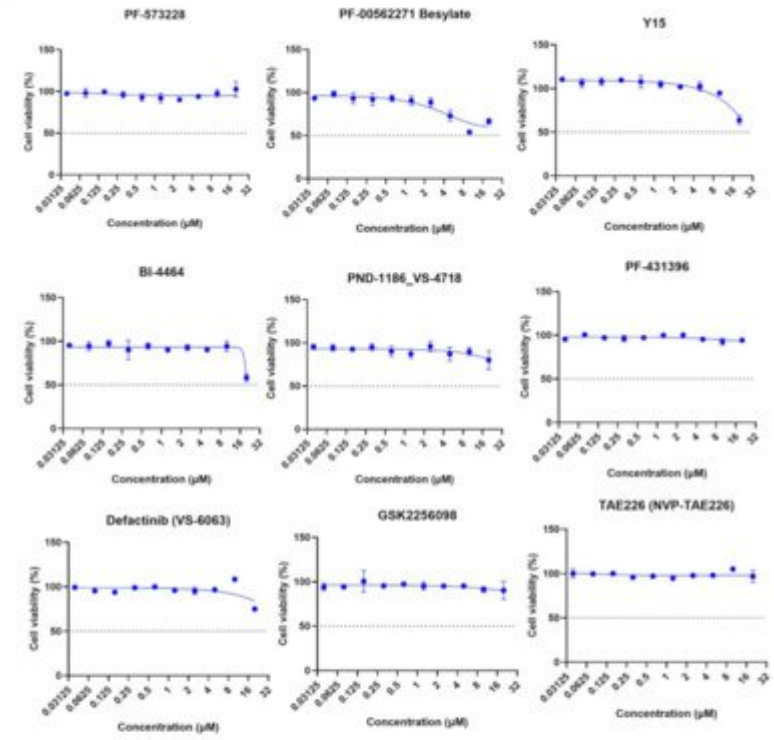

C

Compound name	CC_{50} (μM)	VLPs treated ECs		TNF-α treated ECs	
		IC_{50} (μM)	SI	IC_{50} (μM)	SI
PF-573228	>20	10	>2	10.5	>1.9
PF-00562271 Besylate	≥20	3.6	≥5.5	2.3	≥8.6
Y15	≥20	6.2	≥3.2	3.8	≥5.2
BI-4464	≥20	0.9	≥22.2	20	1
PND-1186 (VS-4718)	≥20	≥20	≥1	8.1	≥2.4
PF-431396	>20	4	>5	2.3	>8.6
Defactinib (VS-6063)	≥20	2.1	≥9.5	0.9	≥22.2
GSK2256098	>20	>20	>1	>20	>1
TAE226 (NVP-TAE226)	>20	17.9	>1.1	>20	>1

Figure 6. Dose-response curves of potent FAK inhibitors. (**A**) ECs were pretreated for 1 h at 37 °C with 10 increasing doses (0.0390625, 0.078125, 0.1562, 0.3125, 0.625, 1.25, 2.5, 5, 10, and 20 μM) of PF-573228, PF-00562271 Besylate, Y15, BI-4464, PND-1186 (VS-4718), PF-431396, Defactinib (VS-6063), GSK2256098, and TAE226 (NVP-TAE226) before the addition of TNF-α (10 ng/mL) or VLPs (10×) for 12 and 48 h incubation, respectively. Cells were then subjected to immunofluorescence assay for ICAM-1 evaluation. ICAM-1 inhibition percentage of each compound corresponds to the relative ICAM-1 expression induced by VLPs or TNF-α in the presence of the compound, which was normalized to the relative ICAM-1 expression induced by VLPs or TNF-α only. In another set of experiments, (**B**) ECs were treated for 48 h with the same concentration range of the above-mentioned compounds and subjected to cytotoxicity analysis. Data represent the mean ±SD (error bars) of triplicate results from two independent experiments. (**C**) The IC_{50} and CC_{50} values for each compound are shown with their corresponding SI (CC_{50}/IC_{50}). FAK: Focal adhesion kinase; ECs: endothelial cells; VLPs: virus-like particles; ICAM-1: intercellular adhesion molecules 1.

Unexpectedly, FAK inhibitors did not prevent Ebola VLPs-induced apoptosis in ECs, suggesting the possible existence of an additional host signaling pathway, acting either independently of FAK, or at triggering GP-mediated apoptosis at the upstream of FAK pathway associated with ICAM-1 induction. Further investigation of other

identified compounds targets, including HSP and Bcr-Abl, may be key to unraveling the molecular mechanisms of GP-induced apoptosis. To the best of our knowledge, this is the first study identifying FAK as a therapeutic target for EVD pathogenesis. FAK is overexpressed in various cancers and has been found to play key roles in normal and cancer cellular functions, such as survival and proliferation [35]. Notably, it has been reported as an anticancer target. As some of our identified compounds are in clinical test phases, the FAK inhibitors constitute potential therapeutic drugs or lead compounds for further development, which target disease pathogenesis related to endothelial barrier dysfunction in severe cases of EVD.

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