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Pharmacological inhibition of focal adhesion kinase 1 (FAK1) and anaplastic lymphoma kinase (ALK) identified via kinome profile analysis attenuates lipopolysaccharide-induced endothelial inflammatory activation

Erna-Zulaikha Dayang [a], Matthijs Luxen [a,b], Timara Kuiper [a], Rui Yan [a,1], Savithri Rangarajan [c], Matijs van Meurs [a,b], Jill Moser [a,b], Grietje Molema [a,*]

[a] Department of Pathology and Medical Biology, Medical Biology Section, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands
[b] Department of Critical Care, University of Groningen, University Medical Center Groningen, Groningen, the Netherlands
[c] PamGene International B.V., 's-Hertogenbosch, the Netherlands

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ABSTRACT

Sepsis is a life-threatening condition often leading to multiple organ failure for which currently no pharmacological treatment is available. Endothelial cells (EC) are among the first cells to respond to pathogens and inflammatory mediators in sepsis and might be a sentinel target to prevent the occurrence of multiple organ failure. Lipopolysaccharide (LPS) is a Gram-negative bacterial component that induces endothelial expression of inflammatory adhesion molecules, cytokines, and chemokines. This expression is regulated by a network of kinases, the result of which *in vivo* enables leukocytes to transmigrate from the blood into the underlying tissue, causing organ damage. We hypothesised that besides the known kinase pathways, other kinases are involved in the regulation of EC in response to LPS, and that these can be pharmacologically targeted to inhibit cell activation. Using kinome profiling, we identified 58 tyrosine kinases (TKs) that were active in human umbilical vein endothelial cells (HUVEC) at various timepoints after stimulation with LPS. These included AXL tyrosine kinase (Axl), focal adhesion kinase 1 (FAK1), and anaplastic lymphoma kinase (ALK). Using siRNA-based gene knock down, we confirmed that these three TKs mediate LPS-induced endothelial inflammatory activation. Pharmacological inhibition with FAK1 inhibitor FAK14 attenuated LPS-induced endothelial inflammatory activation and leukocyte adhesion partly via blockade of NF-κB activity. Administration of FAK14 after EC exposure to LPS also resulted in inhibition of inflammatory molecule expression. In contrast, inhibition of ALK with FDA-approved inhibitor Ceritinib attenuated LPS-induced endothelial inflammatory activation via a pathway that was independent of NF-κB signalling while it did not affect leukocyte adhesion. Furthermore, Ceritinib administration after start of EC exposure to LPS did not inhibit inflammatory activation. Combined FAK1 and ALK inhibition attenuated LPS-induced endothelial activation in an additive manner, without affecting leukocyte adhesion. Summarising, our findings suggest the involvement of FAK1 and ALK in mediating LPS-induced inflammatory activation of EC. Since pharmacological inhibition of FAK1 attenuated endothelial inflammatory activation after the cells were exposed to LPS, FAK1 represents a promising target for follow up studies.

1. Introduction

Sepsis is a potentially lethal condition caused by a dysregulated host response to infection [1]. To date, there are no therapeutic strategies available to prevent sepsis-associated multiple organ failure. Lipopolysaccharide (LPS) is a Gram-negative bacterial component and an important sepsis mediator which induces inflammation [2] and impairs microvascular barrier integrity [3]. LPS triggers the expression of pro-inflammatory adhesion molecules, cytokines, and chemokines [4] by endothelial cells (EC) to facilitate leukocyte adhesion. This interaction next enables the leukocytes to transmigrate into the tissue where they contribute to organ injury [5–7]. Preventing leukocyte adhesion

* Corresponding author at: Dept. of Pathology and Medical Biology, Medical Biology section, University of Groningen, University Medical Center Groningen, Hanzeplein 1, 9713 GZ, Groningen, the Netherlands.
E-mail address: g.molema@umcg.nl (G. Molema).
[1] Present address: Bao'an Maternal and Child Health Hospital, College of Medicine, Jinan University, Shenzhen, China.

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and transmigration by attenuating endothelial inflammatory activation is considered an important approach in the pursuit of developing therapeutic strategies to prevent organ failure in patients with sepsis [8].

Endothelial cells recognise LPS via pattern recognition receptors, that include Toll-like receptor 4 (TLR4) [9,10] and retinoic-inducible gene (RIG-I) [11]. While it is known that LPS induces the expression of a variety of inflammatory molecules through these receptors, the intermediary steps of LPS signalling that lead to altered gene expression are not completely known. Tyrosine kinases (TKs) are important in signal transduction and were previously shown to facilitate TLR4-driven expression of inflammatory mediators by macrophages [12], epithelial cells [13], and EC [14]. LPS-mediated activation of TKs is linked to NF-κB-dependent [15], and NF-κB-independent [16] expression of inflammatory molecules. Pharmacological inhibition of TKs was shown to reduce the production of inflammatory mediators in LPS-activated human EC *in vitro* [17–19], and in murine experimental sepsis [20], which highlights the potential of TKs as druggable targets in alleviating sepsis-associated inflammation.

In this study, we investigated which TKs are involved in LPS signal transduction in human umbilical vein endothelial cells (HUVEC) by profiling the network of active kinases, or kinome, in time. Of the kinases identified to be activated by LPS, we further investigated AXL tyrosine kinase (Axl), focal adhesion kinase 1 (FAK1), and anaplastic lymphoma kinase (ALK) for their role in LPS-mediated signalling in EC, and their potential as pharmacological targets. We next focused on FAK1 and ALK, and established molecular mechanisms underlying their involvement in LPS-induced inflammatory activation of EC. In addition, we assessed whether simultaneous inhibition of FAK1 and ALK could enhance inhibition of LPS-induced endothelial inflammatory activation and leukocyte adhesion. Finally, we investigated whether pharmacological inhibition of FAK1 and/or ALK after initiation of the signalling cascade in response to LPS, which is more representative of a therapeutic setting, was still capable of attenuating endothelial inflammatory activation.

2. Materials and methods

2.1. Cell culture and stimulation

2.1.1. HUVEC

HUVEC (Lonza, Breda, the Netherlands) were cultured at the UMCG Endothelial Facility as described previously [21]. Cells from passage 4 were seeded at a density of 40,000 cells/cm^2 in 6- or 12-well plates one day before the experiment, unless indicated otherwise. For siRNA interference experiments, HUVEC were plated at a density of 20,000 cells/cm^2 one day prior to siRNA transfection. HUVEC were then incubated for 2 h or 4 h with LPS (*E. coli*, O26:B6;15,000 EU/g; Sigma-Aldrich, St. Louis, MO, USA, stock dissolved in 0.9 % w/v NaCl) dissolved in medium to a final concentration of 1 μg/mL, unless indicated otherwise.

2.1.2. HL-60

Immortalised HL-60 (kindly provided by Dr. G. Fey, University of Erlangen, Germany) were cultured in RPMI 1640 medium (Thermo Fisher Scientific, Waltham, MA, United States) supplemented with 10 % v/v fetal calf serum (FCS, Sigma-Aldrich). Cells from passage 5–8 were used in the functional adhesion study.

2.1.3. SK-N-MC

Immortalised SK-N-MC (human neuroblastoma cell line, kindly provided by Anita Niemarkt, University Medical Center Groningen, the Netherlands) were cultured in RPMI 1640 medium supplemented with 10 % v/v FCS and were used between passage 6–8.

2.2. Protein sample preparation

HUVEC were washed with ice-cold phosphate-buffered saline (PBS) and lysed in mammalian protein extraction reagent buffer (M-PER, #78501) containing 1 % v/v Halt™ Protease Inhibitor (#78415), and 1 % v/v Halt™ Phosphatase Inhibitor (#78420, all reagents from Thermo Fisher Scientific). The protein lysates were then centrifuged at 16,000 *g* at 4 °C for 15 min and the supernatants were stored at −80 °C until further analysis. For PamGene kinase arrays and Western blot analyses, protein concentrations of the samples were determined using the Pierce™ Coomassie Plus (Bradford) Assay Kit (#23236, Thermo Fisher Scientific).

2.3. Tyrosine kinase activity profiling

Protein samples (5 μg protein/array) were loaded onto Protein Tyrosine Kinase (PTK) PamChip arrays (PamGene, 's-Hertogenbosch, the Netherlands) and analysed using PamStation12 (PamGene). This flow-through microarray tracks the phosphorylation status of 196 peptides, which is used to predict TK activity (**Fig. S1**). Post-array analysis was performed using BioNavigator software (v.6.3.67.0, PamGene), according to the manufacturer's instructions. Experiments were performed with three biological replicates generated independently. To account for cell and batch-to-batch variation, peptide phosphorylation data were normalised using the Combining Batches of Gene Expression Microarray data (ComBat) as previously described [22]. Subsequently, Upstream Kinase Analysis was performed using a functional scoring algorithm available in the BioNavigator software to identify the 'specificity score', which was used to define TKs with the largest alterations in activity compared to unstimulated controls. These kinases were mapped onto a kinase phylogenetic tree using the KinMap Web-based tool (http://kinhub.org/kinmap/). Out of the top 25 TKs identified per time point, three TKs were selected for further analysis.

2.4. Pharmacological inhibition of tyrosine kinases

Ceritinib (PubChem CID 57379345, #S7083, SelleckChem, Houston, TX, USA) and BMS777-607 (PubChem CID 24794418, #S1561, SelleckChem) were dissolved in DMSO, while FAK14 (PubChem CID 78260, #SML0837, Sigma-Aldrich) was dissolved in ultrapure water, according to manufacturers' instructions. Stocks were stored at −80 °C until needed. HUVEC were pre-treated with the inhibitors diluted in medium at different concentrations 30 min before LPS stimulation, unless indicated otherwise. HUVEC morphology was found to be normal when HUVEC were treated with FAK14 and Ceritinib at concentrations equal to or lower than 4 μM, and with BMS777-607 at all concentrations studied.

2.5. Gene expression analysis by RT-qPCR

HUVEC were lysed in RLT® Plus buffer (Qiagen, Venlo, the Netherlands). Total RNA was isolated using the RNeasy® Plus Mini Kit (Qiagen), according to the manufacturer's protocols. RNA concentration (OD 260) and purity (OD260/OD280) were determined using a NanoDrop® ND-1000 UV–vis spectrophotometer (NanoDrop Technologies, Rockland, ME, USA). Samples with an OD260/OD280 ratio of ≥1.8 were included in the analysis. Synthesis of cDNA and qPCR were performed using Assay-on-Demand primers (Applied Biosystems, Table 1) as previously described [23]. The threshold cycle values (CT) for each sample were assessed in duplicate and averaged, with accepted standard deviation of the duplicates no higher than 0.5. All genes were normalised to the expression of the housekeeping gene GAPDH, yielding the ΔCT value. The relative mRNA level was calculated by $2^{-\Delta CT}$ [23].

Table 1
Assay-on-Demand primers used in this study for qPCR-based determination of mRNA levels of genes of interest. All primers were purchased from Applied Biosystems (Bleiswijk, the Netherlands).

Primer(s)	Assay ID
GAPDH	Hs99999905_m1
CD31	Hs99999905_m1
E-selectin	Hs00174057_m1
VCAM-1	Hs00365486_m1
ICAM-1	Hs00164932_m1
IL-6	Hs00174131_m1
IL-8	Hs00174103_m1
Cxcl6	Hs01124251_g1
Cxcl10	Hs00605742_g1
PTK2/FAK1	Hs01056457_m1
ALK	Hs00608284_m1

2.6. siRNA-mediated gene silencing of Axl, FAK1, and ALK

HUVEC were transfected with FlexiTube small interfering RNA (siRNA) sequences for human Axl, FAK1, and ALK (Qiagen, Table 2). AllStars negative control siRNA (Qiagen) was used as control. Transient transfection was performed using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA), according to the manufacturer's instructions. HUVEC medium was refreshed at 6 h and 46 h after transfection. Forty-eight hours after transfection, cells were challenged with LPS for 4 h, and subsequently analysed by RT-qPCR and Western blot to confirm knock down of the target genes, and investigate the effects of their absence on LPS-induced endothelial activation. Knock down of the genes did not diminish endothelial cell viability as assessed microscopically (results not shown).

2.7. Inflammatory adhesion molecule analysis by flow cytometry

HUVEC were briefly washed with sterile ice-cold PBS, trypsinised with trypsin-EDTA (0.025 % v/v), transferred into ice-cold buffer (5 % FCS v/v in PBS; FCS obtained from Biowest, Nuaillé, France) and centrifuged. Cells were then resuspended in 3 % v/v PE-conjugated mouse anti-human E-selectin, APC-conjugated mouse anti-human VCAM-1, FITC-conjugated mouse anti-human ICAM-1, and Brilliant Violet 421-conjugated mouse anti-human CD31 antibodies (#322606, #305810, #322720, and #303124, all from BioLegend, San Diego, CA, USA) in 5 % FCS in PBS for 30 min on ice in the dark. After washing with 5 % FCS in PBS, cells were analysed using a MACSQuant Analyser 10 flow cytometer (Miltenyi Biotech, Bergisch Gladbach, Germany). Data analysis was performed using Kaluza Flow analysis software (v.2.1, Beckman Coulter, Brea, CA, USA). Isotype control antibodies mouse IgG2aκ-PE (#2-4724-42, eBioscience, San Diego, CA, USA), mouse IgG1-APC (#IC002A, R&D System, Minneapolis, MN, USA), and mouse IgG1κ-BV421 (#400157, BioLegend) were used to correct for signals arising from non-specific binding.

2.8. Western blot analysis

Protein samples (10 μg protein/lane) were separated by SDS-PAGE on 10 % polyacrylamide gels and transferred to nitrocellulose membranes (Bio-Rad Laboratories, Utrecht, the Netherlands). Blots were incubated with blocking buffer (5 % w/v milk, Campina, Friesland, the Netherlands) in Tris-buffered saline (TBS) with 0.1 % v/v Tween-20 (TBST) for 1 h. The blots were subsequently incubated overnight at 4°C with primary antibodies (Table 3) diluted in 5 % milk in TBST for non-phospho-proteins or 5 % bovine serum albumin (BSA; Sigma-Aldrich) in TBST for phospho-proteins. After 15 min of washing with TBST, blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (Southern Biotech, Birmingham, AL, USA) diluted in blocking buffer for 1 h at room temperature (RT). After washing, detection was performed using Immobilon Forte Western HRP substrate (Millipore, Billerica, MA, USA). Images were taken using a GelDoc XR system (Bio-Rad). Western blot bands were quantified by densitometry and the background was subtracted using Image Lab software (v.5.2.1, Bio-Rad). After the proteins of interest were detected, the blots were rinsed in TBST, incubated with Restore™ PLUS Western Blot stripping buffer (#46430, Thermo Fisher Scientific) at RT for 30 min. Next, the blots were washed with TBST for 15 min, blocked with blocking buffer and incubated with the next primary and secondary antibodies, as described above.

Table 2
siRNA used for siRNA-based interference experiments. All siRNAs were purchased from Qiagen (Venlo, the Netherlands).

siRNA	Clone number
AllStars Negative Control	1027280
Axl	SI02626750 (Axl_13)
FAK1	SI02622130 (PTK2_10)
	SI00062461 (ALK_1)
ALK	SI00062475 (ALK_3)
	SI02632847 (ALK_5)

2.9. IL-6 and IL-8 quantification by ELISA

HUVEC supernatants were centrifuged at 1,500 rpm for 5 min at 4 °C and stored at −20 °C until further analysis. IL-6 and IL-8 concentrations in the supernatants were determined using IL-6 and IL-8 MAX Standard Set Human ELISA kits (BioLegend), according to the manufacturer's protocols.

2.10. Immunofluorescence microscopy

2.10.1. p65 and VE-cadherin expression

HUVEC were seeded at a density of 50,000 cells/cm^2 on sterile glass coverslips (Menzel-Gläser, Braunschweig, Germany) 72 h before the experiment. HUVEC were stimulated with LPS at 1 μg/mL for 30 min, 2 h, or 4 h. The cells were briefly washed with ice-cold PBS and fixed with 1 % v/v formaldehyde in PBS (Merck, Darmstadt, Germany) for 20 min on ice. After washing, the cells were permeabilised with 0.25 % v/v Triton X-100 in PBS (Sigma-Aldrich) for 5 min. The cells were washed again with PBS, and blocked with 3 % w/v BSA in PBS for 30 min. The cells were then incubated with primary antibodies, 2 μg/mL of rabbit anti-p65 (#8242, Cell Signaling Technology, Leiden, the Netherlands) or 0.2 μg/mL of rabbit anti-VE-cadherin (#2158, Cell Signaling Technology), diluted in washing buffer (PBS containing 0.5 % w/v BSA and 0.05 % v/v Tween-20) for 1 h at RT. Subsequently, cells were incubated with 8 μg/mL Alexa Fluor®555-conjugated donkey anti-rabbit secondary antibody (#A31572, Life Technologies) for 45 min at RT and mounted in

Table 3
Antibodies used for Western blot analyses. All antibodies were raised in rabbit, except for β-actin-specific antibody that was raised in mouse. GAPDH antibody was purchased from Santa Cruz Biotechnology (Heidelberg, Germany), while other antibodies were purchased from Cell Signaling Technology (Leiden, the Netherlands).

Antibody	#Cat no	Dilution (in 5 % milk or TBST)
GAPDH	sc25778	1:5,000
Total FAK	71433	1:1,000
Phospho-FAK Y397	3283S	1:1,000
Phospho-FAK Y576/577	3281	1:1,000
Phospho-FAK Y925	3284	1:1,000
Total-p65	8242	1:2,000
Phospho-p65 (S536)	3033	1:2,000
Total IκB-α	9242	1:1,000
Phospho-p38	4631	1:2,000
Total p38	9212	1:1,000
β-actin	3700	1:4,000

Aqua/Polymount medium containing DAPI (1.5 μg/mL; Polysciences, Warrington, PA, USA). Fluorescence images were taken with a Leica DM/RXA fluorescence microscope equipped with a Leica DFC450C digital camera (Leica, Microsystems Ltd., Germany) and Leica LAS V4.2 Image Overlay Software, or using a Leica DM4000B fluorescence microscope equipped with a Leica DFC345FX digital camera and Leica LAS V4.5 Image Software. All images were taken with equal exposure times.

2.10.2. ALK expression

HUVEC and SK-N-MC were transfected with siRNA as described above to knock down ALK. The cells were washed, fixed, permeabilised, blocked, and stained according to the protocols described above. Next, the cells were stained with primary antibody rabbit anti-ALK (6.52 μg/mL; #3633, Cell Signaling Technology) for 1 h. The secondary antibody incubation and fluorescence image acquisition were performed as described above.

2.11. Endothelial-leukocyte adhesion assay

2.11.1. HL-60 labelling

HL-60 cells were labelled with 10 μg/mL Hoechst 33342 (Life Technologies) for 10 min, washed and resuspended in RPMI 1640/1 % v/v FCS (Sigma-Aldrich). The viability of the resulting HL-60-Hoechst cells was assessed by flow cytometric forward-side scatter plots and was always >90 %.

2.11.2. HL-60-HUVEC adhesion

After 4 h of LPS exposure, HUVEC medium was removed and HUVEC monolayers were incubated for 1 h with approximately 300,000 HL-60 cells in 5 % FCS v/v in RPMI 1640. Subsequently, the non-adherent HL-60 cells were removed by washing with RPMI 1640 medium, and the remaining adherent HL-60 cells and HUVEC were trypsinised and resuspended in 5 % FCS v/v in PBS. Each sample was analysed using a MACSQuant Analyser (Miltenyi Biotech) in which the Hoechst-labelled HL-60 were identified using Vioblue channel. The number of HL-60 cells was divided by the total number of HL-60 cells added to the HUVEC cultures. The percentage of adherent HL-60 cells was compared between treatment groups.

2.12. Statistical analysis

Statistical analyses were performed by one-way ANOVA with Bonferroni post hoc correction, to compare multiple groups. Statistical analyses were performed using GraphPad Prism Software (v.8.2.1, GraphPad Prism Software Inc., San Diego, CA, USA). Differences were considered significant when $p < 0.05$.

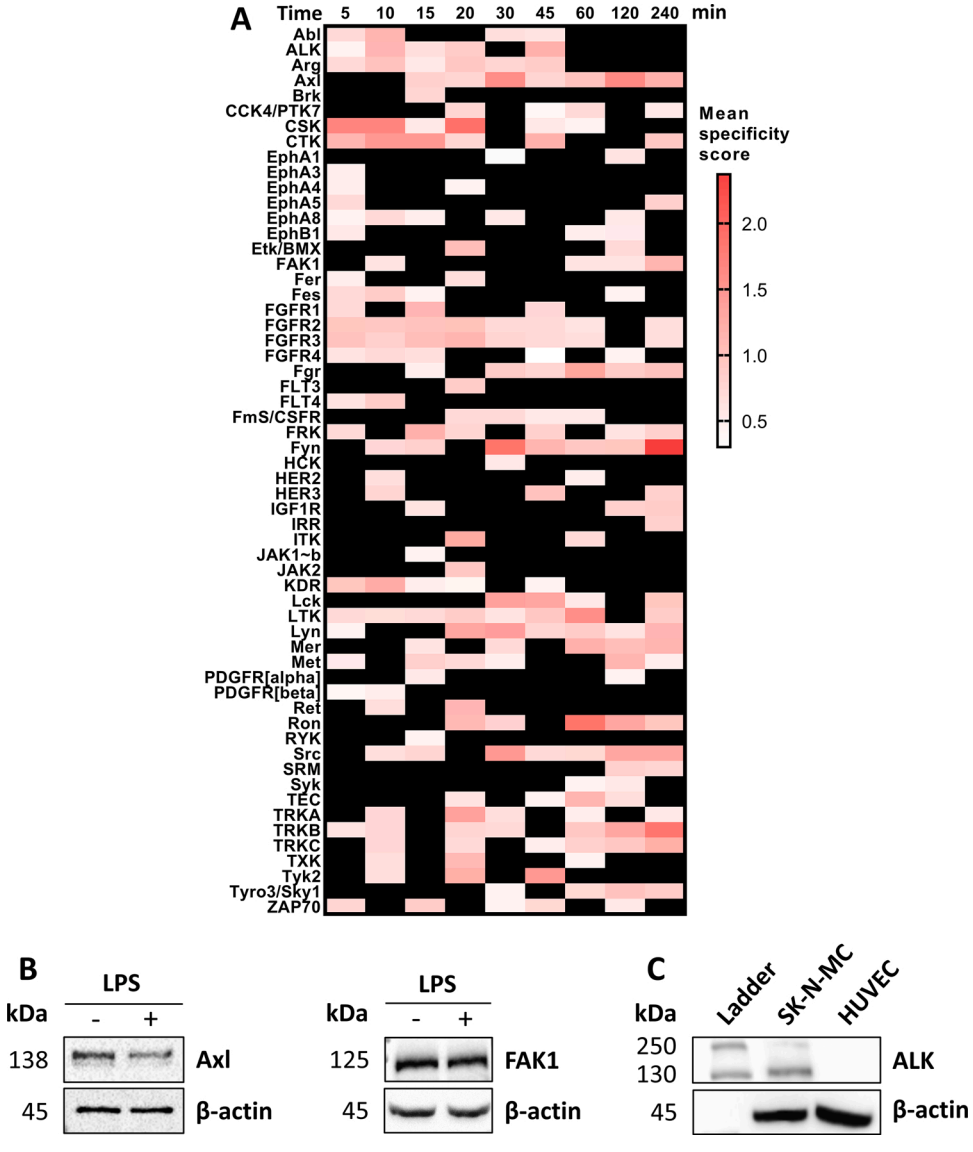


Fig. 1. Kinetics of tyrosine kinase activation pattern in LPS-activated endothelial cells. **(A)** Heatmap representing tyrosine kinases that are active over the time course of LPS stimulation (5–240 min) as identified by PamGene PTK kinase array technology. The prediction of TK activity is based on the pattern of peptide phosphorylation in LPS exposed HUVEC relative to unstimulated control and is represented as mean specificity scores (in red). TKs that were not in the top 25 activated kinases per time point compared to unstimulated control are represented in black. The heatmap is representative of three independent experiments. **(B)** Validation of Axl and FAK1 presence in quiescent and 4 h LPS-stimulated HUVEC by immunoblotting. β-actin was used as the loading control. The image is representative of three independent experiments. **(C)** Immunoblotting performed on SK-N-MC and HUVEC lysates to detect ALK. β-actin was used as the loading control. The image is representative of experiments performed in triplicate.

3. Results

3.1. Kinome analysis reveals various activated Tyrosine Kinases in LPS-exposed endothelial cells

The aim of our study was to identify potential new targets in the network of LPS-activated kinases in EC to interfere with sepsis-related endothelial activation. For this purpose, we assessed the activation of TKs in HUVEC exposed to LPS for different periods of time up to 240 min. Activated TKs per time point of LPS treatment were identified based on peptide phosphorylation patterns relative to unstimulated control, which is represented as mean specificity scores (Fig. 1**A**). The kinase activity profiling data revealed the involvement of various TKs in endothelial signalling with unique TK activity signatures at specific time points of LPS exposure. Kinase activity status is dynamic, as different (sets of) kinases were activated at different time points of activation **(Suppl. video 1)**. This suggests the existence of a complex network of dedicated TKs in LPS signal transduction in EC.

We selected the top 25 TKs showing the highest relative activity at each time point after LPS exposure (data not shown). Out of these top 25 TKs, fifty-eight unique TKs were found to be active in HUVEC exposed to LPS between 5–240 min (Fig. 1**A**). Of the 58 TKs identified, 29 TKs had mean specificity scores of more than 1 for at least one of the time points studied **(Fig. S1).** From this list of 29 TKs, we selected kinases that were shown in previous studies either to be involved in sepsis [24], or in inflammatory pathways in EC [16] with no known involvement in LPS-induced inflammatory signalling. Based on these criteria, we selected AXL tyrosine kinase (Axl), focal adhesion kinase 1 (FAK1), and anaplastic lymphoma kinase (ALK), and investigated whether these TKs were indeed expressed by (LPS-exposed) HUVEC. By immunoblotting, we found that Axl was reduced, whereas FAK1 was unaffected in HUVEC exposed to LPS for 4 h compared to unstimulated control (Fig. 1**B**), while ALK was not detected **(**Fig. 1**C).** Immunofluorescence detection, on the other hand, could detect ALK protein in both HUVEC and positive

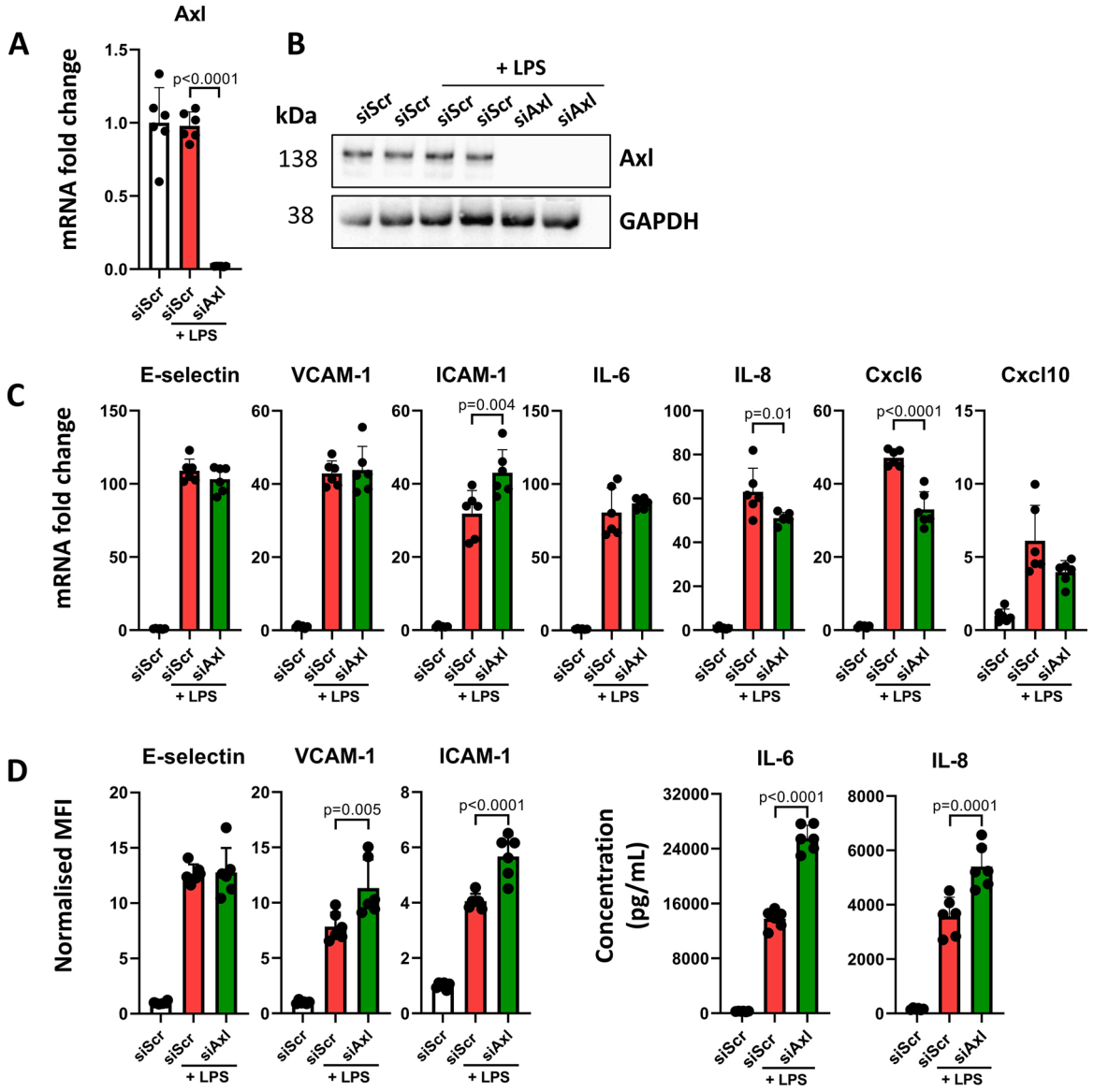


Fig. 2. Axl is involved in LPS-induced expression of inflammatory molecules.
Effect of siRNA-based Axl knock down on **(A)** mRNA and **(B)** protein levels of Axl compared to scrambled siRNA (siScr) as control. GAPDH was used as the loading control. The image is representative of three independent experiments. Effect of Axl knock down on LPS-induced **(C)** mRNA and **(D)** protein levels of the inflammatory molecules as determined by RT-qPCR respectively flow cytometry and ELISA. HUVEC were exposed to LPS for 4 h (**A-D**). Each experimental condition represents the mean ± SD of six replicates from one independent experiment. The graphs shown are from one representative experiment.

control SK-N-MC (Fig. 4**A**). These data demonstrate that the three TKs chosen for follow up are expressed in HUVEC.

3.2. Axl, FAK1, and ALK partly mediate LPS-induced expression of inflammatory molecules

Axl, FAK1, and ALK were shown to be present and activated in HUVEC at several time points of LPS activation. To investigate to which extent these TKs control endothelial expression of LPS-induced inflammatory molecules, we knocked them down using siRNA and determined the effects on inflammatory gene and protein expression. Knock down of Axl, FAK1, and ALK was successful in diminishing their mRNA (Fig. 2**A,**3 **A**) and protein levels (Fig. 2**B,**3 **B,**4 **A**). Following LPS exposure of cells lacking Axl, mRNA levels of IL-8 and Cxcl6 were reduced, and those of ICAM-1 were increased (Fig. 2**C**). While protein levels of VCAM-1, ICAM-1, IL-6, and IL-8 were increased (Fig. 2**D**), none of the other genes assessed, i.e. E-selectin, VCAM-1, IL-6, and Cxcl10, were affected by absence of Axl. FAK1 knock down resulted in reduced mRNA and protein expression of E-selectin, reduced VCAM-1, IL-6, IL-8, and Cxcl6 mRNA expression, and reduced IL-6 and IL-8 protein production in response to LPS (Fig. 3**C,D**). ALK knock down, lastly, resulted in diminished mRNA expression of all genes studied, and attenuated protein expression of only E-selectin and VCAM-1 (Fig. 4 **B, C**). These data show that FAK1, ALK, and to a lesser extent Axl, play a role in mediating LPS-induced expression of inflammatory molecules in EC.

3.3. Pharmacological inhibition of Axl, FAK1, and ALK attenuates LPS-induced expression of endothelial inflammatory molecules

Since Axl, FAK1, and ALK mediate the expression of inflammatory molecules in EC, we further investigated whether these kinases could be pharmacologically inhibited to reduce LPS-induced endothelial activation. FAK14, a FAK1 inhibitor, and Ceritinib, an ALK inhibitor, attenuated, in a concentration-dependent manner, mRNA and protein

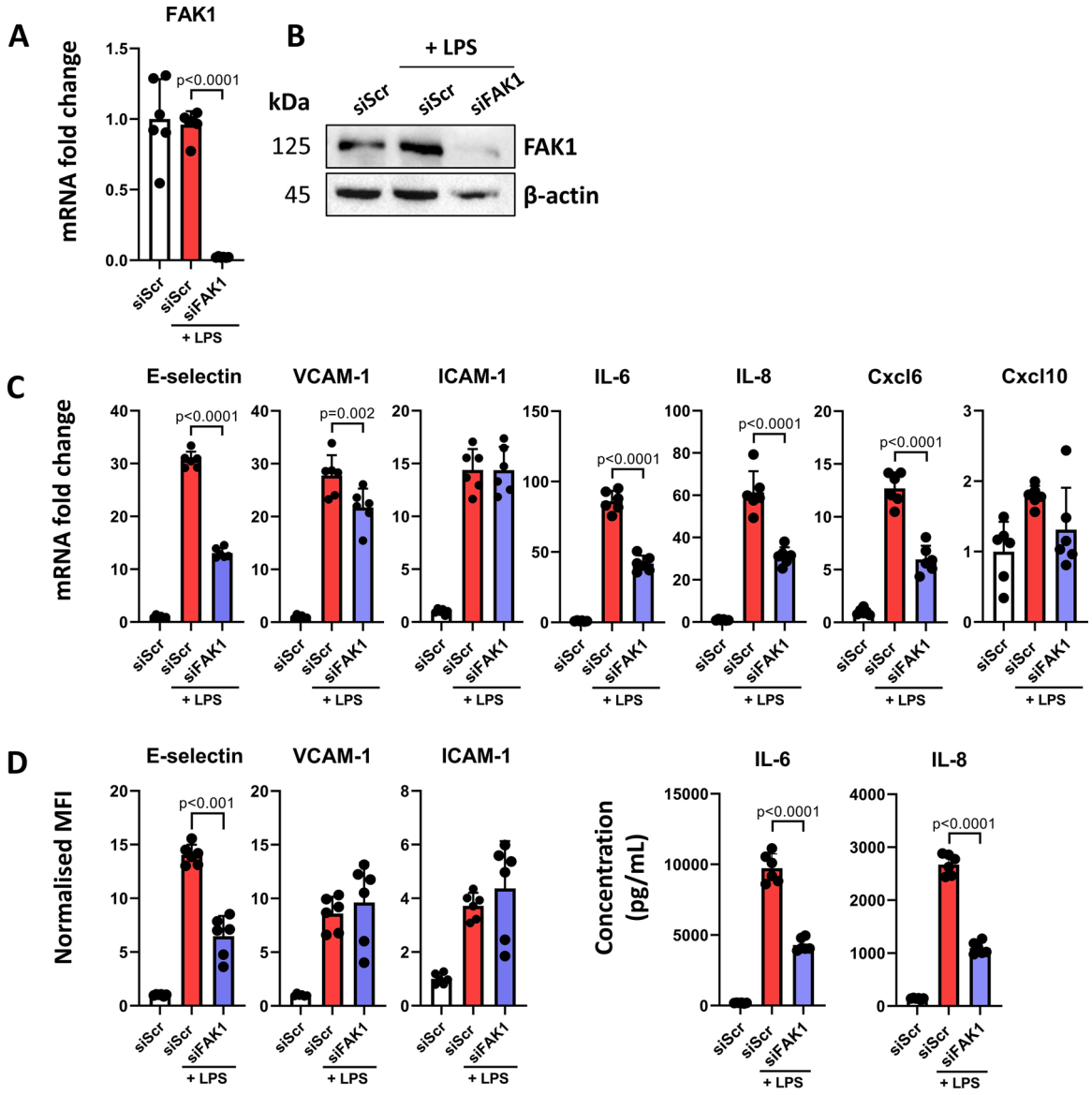


Fig. 3. FAK1 is involved in LPS-induced expression of inflammatory molecules.
Effect of siRNA-based FAK1 knock down on LPS-induced **(A)** mRNA and **(B)** protein levels of FAK1 compared to scrambled siRNA (siScr) as control. β-actin was used as the loading control. The image is representative of three independent experiments. Effect of FAK1 knock down on **(C)** mRNA and **(D)** protein levels of the inflammatory molecules as determined by RT-qPCR respectively flow cytometry and ELISA. HUVEC were exposed to LPS for 4 h (**A-D**). Each experimental condition represents the mean ± SD of six replicates from one independent experiment. The graphs shown are representative of three independent experiments.

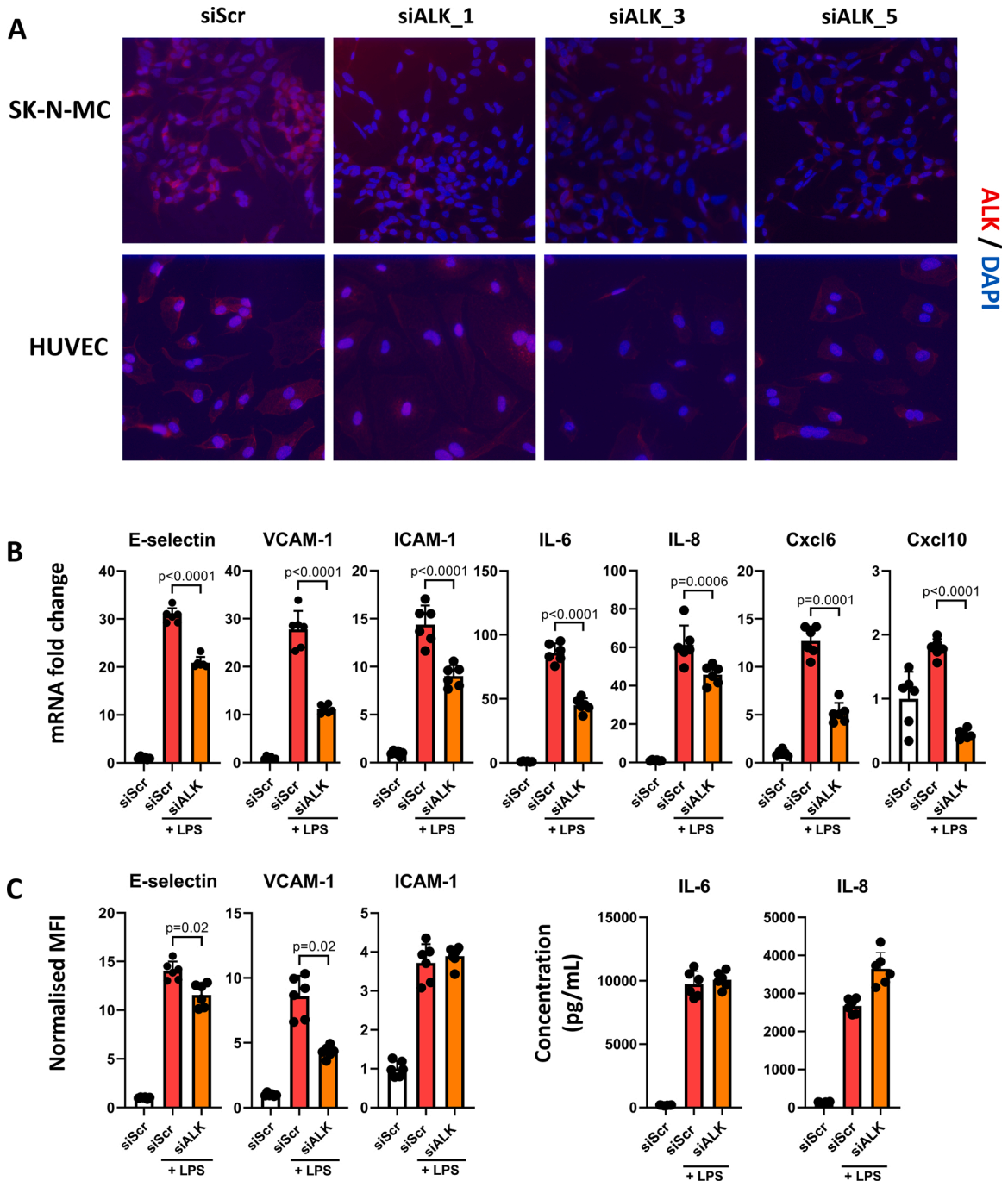


Fig. 4. ALK is involved in LPS-induced expression of inflammatory molecules.
(A) Effect of siRNA-based ALK knock down on ALK protein in HUVEC and SK-N-MC as positive control as determined by immunofluorescence staining. The images show ALK (red) and DAPI nuclear staining (blue). Effect of ALK knock down on LPS-induced **(B)** mRNA and **(C)** protein levels of the inflammatory molecules as determined by RT-qPCR respectively flow cytometry and ELISA. HUVEC were exposed to LPS for 4 h (**B, C**). Each experimental condition represents the mean ± SD of six replicates from one independent experiment. The graphs shown are representative of three independent experiments.

expression of E-selectin, VCAM-1, ICAM-1, IL-6, and IL-8 at 2 h (Fig. 5**A, B**) and 4 h **(Fig. S3A)** after start of LPS exposure (Fig. 5**B, S3B).** Treatment with Axl inhibitor BMS777–607 also inhibited mRNA expression of E-selectin, VCAM-1, ICAM-1, and IL-6 in a concentration-dependent manner (Fig. 5**A).** In contrast, protein levels of the inflammatory adhesion molecules were unaffected (Fig. 5**B).** The expression of CD31, an endothelial adhesion molecule which is constitutively expressed, was not affected either at mRNA or protein level following kinase inhibitor treatment (Fig. 5**A, B).** Taken together, these results strengthen the conclusion that FAK1, ALK, and to a lesser degree Axl, control part of the LPS inflammatory signalling pathway in EC and are thus therapeutic targets of interest.

3.4. FAK1 inhibition, but not ALK inhibition, attenuates NF-κB activation in LPS-stimulated HUVEC

Since LPS-mediated endothelial activation is largely NF-κB driven [11,21], and several TKs have been linked to NF-κB activation [15,20], we investigated whether pharmacological inhibition of FAK1 and ALK would alter NF-κB activation status in LPS-stimulated EC.

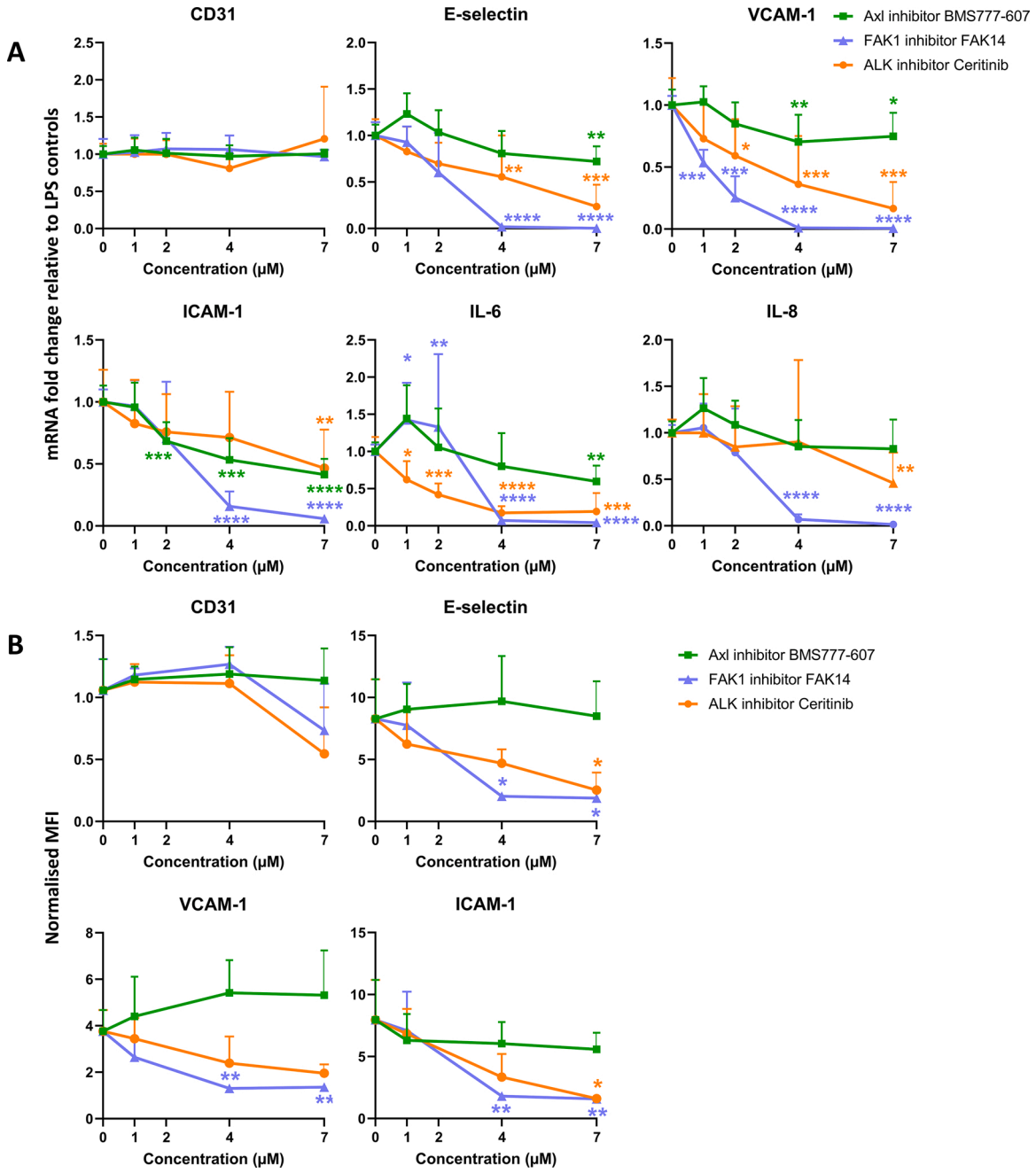
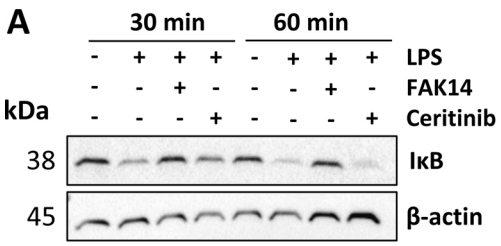


Fig. 5. Pharmacological inhibition of Axl, FAK1, and ALK diminishes LPS-induced expression of inflammatory molecules.
(A) Effect of BMS777-607, FAK14, and Ceritinib on the mRNA levels of CD31, E-selectin, VCAM-1, ICAM-1, IL-6, and IL-8 in HUVEC after 2 h of LPS exposure as determined by RT-qPCR. Each experiment represents the mean ± SD of three replicates. The graphs shown are representative of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. **(B)** Effect of BMS777-607, FAK14, and Ceritinib on CD31, E-selectin, VCAM-1, and ICAM-1 protein expression by HUVEC after 4 h of LPS exposure as determined by flow cytometry. *$p < 0.05$, **$p < 0.01$. In **(A)** and **(B)**, drugs were added 30 min before LPS exposure start.

Immunoblotting of HUVEC lysates showed reduced IκB-α protein following 30 and 60 min of LPS stimulation compared to unstimulated controls (Fig. 6**A).** LPS-induced IκB-α protein degradation was inhibited following treatment with FAK14, but not following Ceritinib treatment **(**Fig. 6**A).** In support of these findings, immunofluorescent staining revealed nuclear p65 accumulation in 19 % of HUVEC 30 min after start of LPS exposure, which was almost completely inhibited by FAK14, but not Ceritinib treatment **(**Fig. 6**B, S2).** We conclude that attenuation of LPS-induced expression of inflammatory molecules by FAK1 inhibition, but not ALK inhibition, is partly mediated by inhibition of the NF-κB pathway.

3.5. Inhibition of both FAK1 and ALK reduces LPS-induced inflammatory activation of HUVEC in an additive manner

Next, we investigated whether the combination of FAK14 and Ceritinib would result in enhanced inhibition of LPS-induced inflammatory activation of HUVEC. The combination of drugs reduced E-selectin, VCAM-1, ICAM-1, IL-6, and IL-8 mRNA (Fig. 7**A**) and protein levels (Fig. 7**B**), the additive effect being concentration-dependent without affecting cell viability (data not shown). Additionally, the combination of FAK14 and Ceritinib at 2 μM (FAK-CER) further inhibited the expression of E-selectin, VCAM-1, and ICAM-1 both at mRNA (**Fig. S4A**) and protein levels **(Fig. S4B)** compared to single drug treatment.



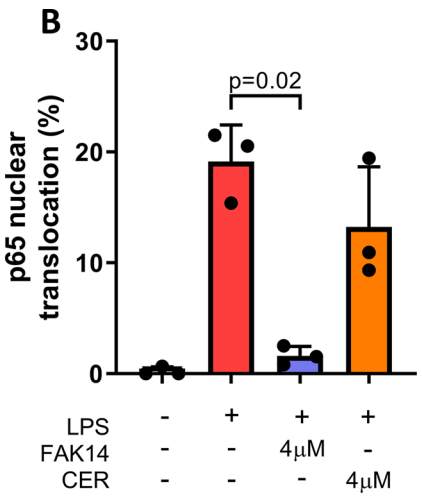


Fig. 6. Inhibition of FAK1, but not ALK, attenuates activation of the NF-κB pathway in LPS-activated HUVEC.
(A) Effect of FAK14 and Ceritinib on IκB protein levels in HUVEC determined by immunoblotting after LPS exposure. β-actin was used as the loading control. The figure represents three independent experiments. **(B)** Effect of FAK14 and Ceritinib (CER) on p65 nuclear translocation in HUVEC exposed to LPS for 30 min. The nuclear p65 translocation percentage was determined per 300 cells. Each group represents the mean ± SD of three independent experiments. Drugs were added 30 min before LPS administration at 4 μM in **(A)** and **(B)**.

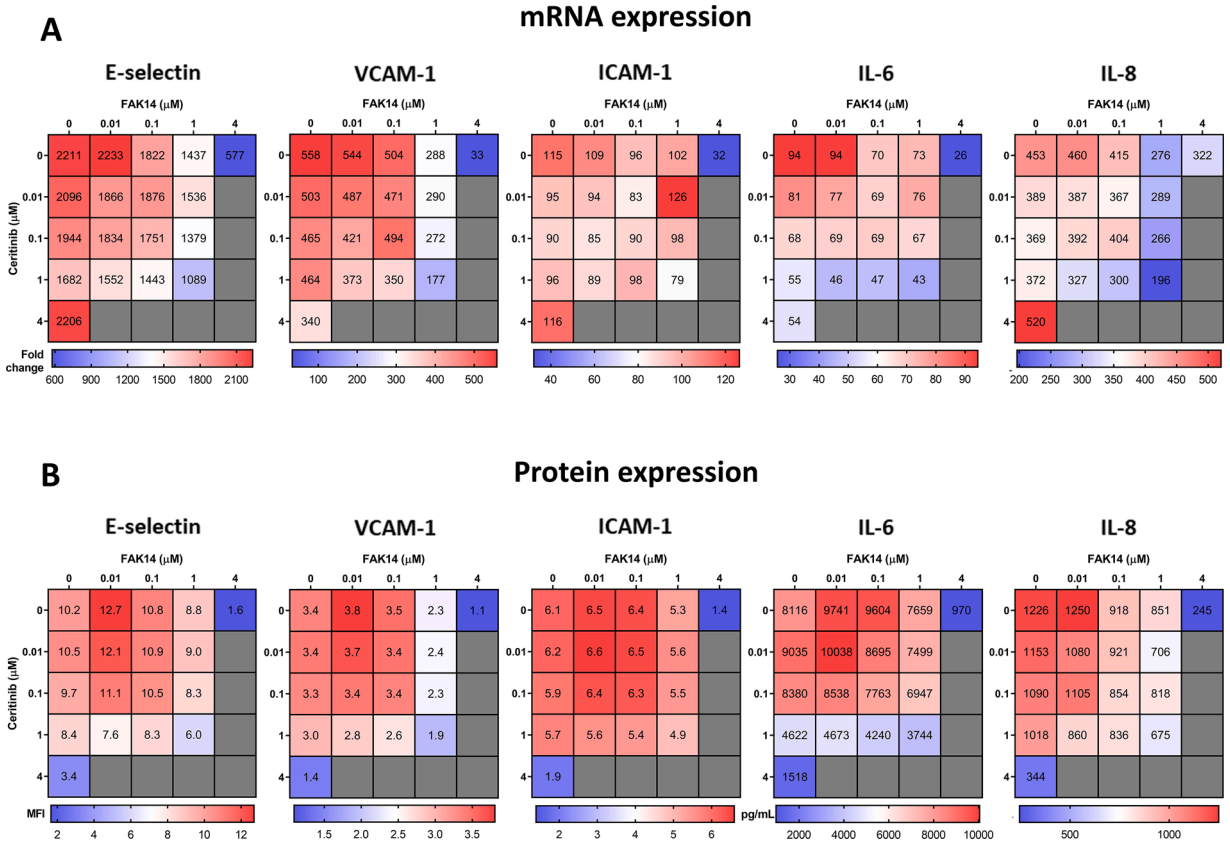


Fig. 7. Combined pharmacological inhibition of FAK1 and ALK enhances inhibition of LPS-induced expression of inflammatory molecules compared to single FAK1 or ALK inhibition.
(A) Effect of combined FAK14 and Ceritinib treatment (0.01–1 μM) on E-selectin, VCAM-1, ICAM-1, IL-6, and IL-8 mRNA expression by LPS-activated HUVEC determined by RT-qPCR. Values report fold change relative to unstimulated controls. Each group represents the mean of three independent experiments. **(B)** Effect of combined FAK14 and Ceritinib treatment (0.01–1 μM) on protein levels of LPS-activated HUVEC as determined by flow cytometry and ELISA. In **(A)** and **(B)** the drugs were added 30 min before the start of LPS stimulation. HUVEC were exposed to LPS for 4 h **(A, B)** . Each group represents the mean of three independent experiments. The combinations of drug concentrations that were not studied are represented in grey.

Cytokine/chemokine mRNA and protein levels were also lower following FAK-CER treatment, except for IL-8 which was only affected at protein level (**Fig. S4A, S4C**). CD31 mRNA and protein levels were not affected by the combination treatment (**Fig. S4A, S4B**). These results suggest that FAK1 and ALK control different signal transduction pathways activated in EC by LPS.

3.6. FAK14, but not Ceritinib, reduces HL-60 adhesion to LPS-activated endothelial cells

Adhesion molecules expressed by LPS-activated EC facilitate tethering, rolling, and adhesion of leukocytes [25]. Therefore, we investigated the functional consequences of FAK1 and ALK inhibition on HL-60 leukocyte adhesion to LPS-activated HUVEC using flow cytometry.

FAK14, but not Ceritinib, significantly reduced HL-60 adhesion to LPS-activated HUVEC at 4 μM (Fig. 8**A**). In contrast, combination treatment with FAK14 and Ceritinib, both at 2 μM, did not affect HL-60 adherence to the LPS-activated HUVEC (Fig. 8**A**).

LPS-induced loss of VE-cadherin, an endothelial junction molecule, leads to loss of endothelial integrity [26]. We hence investigated the effect of FAK1 and ALK inhibition on LPS-induced gap formation and on VE-cadherin expression by HUVEC. LPS stimulation of HUVEC induced gap formation (Fig. 8**B**) which could not be prevented by FAK14 treatment. In contrast, Ceritinib-treated monolayers were devoid of gaps, which was comparable to unstimulated control (Fig. 8**B**). Of note, total VE-cadherin protein levels were unaffected in both FAK14 and Ceritinib-treated HUVEC as determined by immunoblotting (Fig. 8**C**). These results suggest that a FAK1-mediated, but not ALK-mediated, inflammatory pathways play a role in leukocyte adhesion to LPS-activated EC, while ALK is implied in junctional gap formation.

3.7. Administration of FAK14, but not Ceritinib, after the start of LPS exposure reduces endothelial inflammatory activation

To investigate whether FAK1 or ALK inhibition can reduce inflammatory activation when drugs are administered after the start of LPS

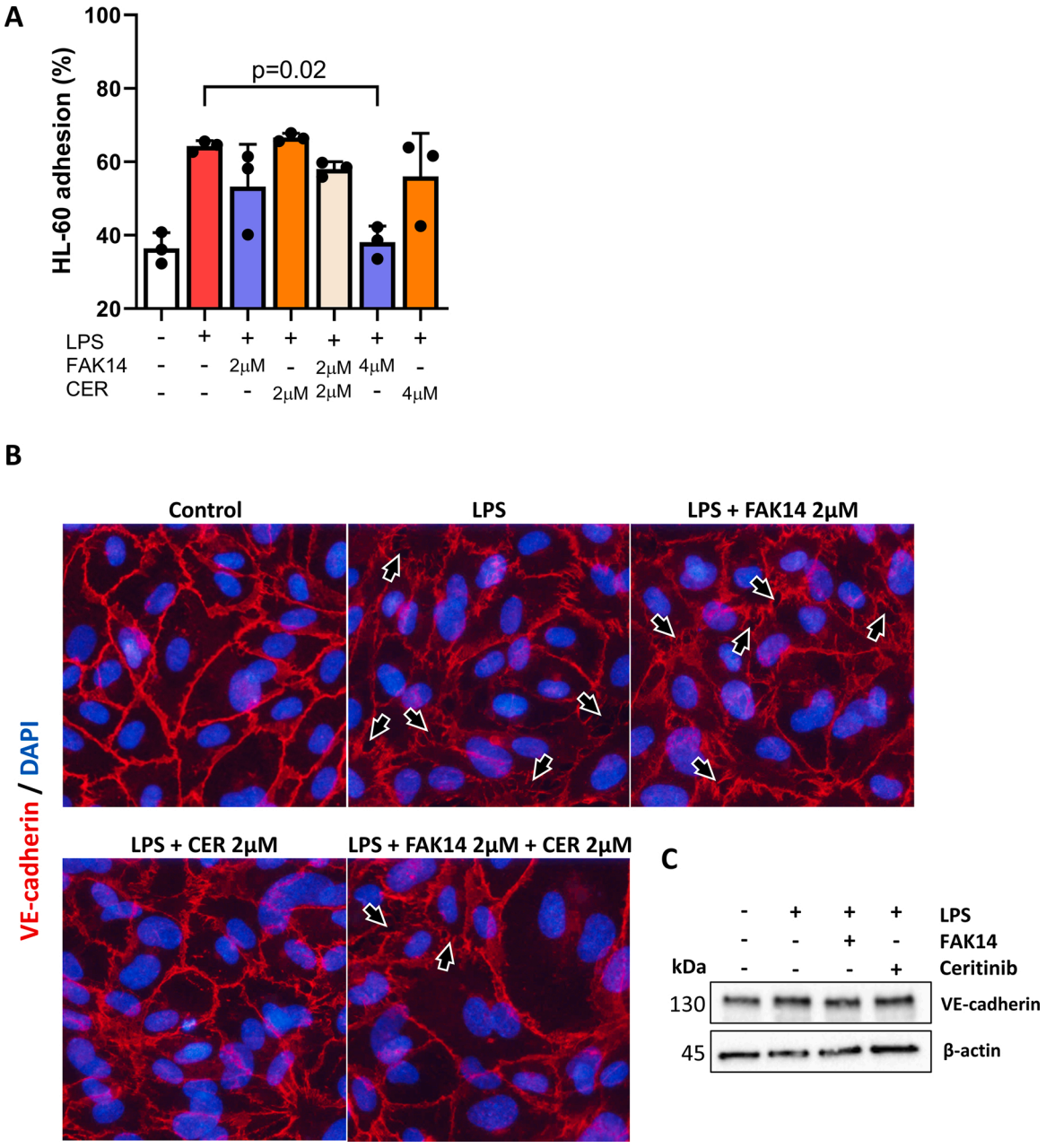


Fig. 8. Effects of FAK1 and ALK inhibition on leukocyte adhesion to LPS-activated endothelial cells and on VE-cadherin.
(A) Effect of FAK14, Ceritinib (CER) and combined drug treatment on HL-60 leukocyte adhesion to LPS-activated HUVEC. Each group represents the mean ± SD of three replicates in one experiment. The graphs shown are representative of three independent experiments.
(B) Effect of FAK14, Ceritinib (CER) and combined drug treatment on VE-cadherin localisation in LPS-activated HUVEC. The images show VE-cadherin (red) and DAPI nuclear staining (blue). Gap formation between endothelial cells is annotated with arrows. Only intact HUVEC monolayers were evaluated for presence of junctional gap formation. Images were captured with equal exposure times. Original magnification is x400. **(C)** Effect of FAK14 and Ceritinib treatment at 4 μM on VE-cadherin protein level by HUVEC as determined by immunoblotting. β-actin was used as loading control. Image is representative of three independent experiments. HUVEC were exposed to LPS for 4 h **(A)** or 2 h **(B, C)**. Drugs were added 30 min prior to LPS administration in **(A)**, **(B)**, and **(C)**.

exposure, we treated HUVEC with FAK14, Ceritinib, or the combination of these drugs, at 10, 45, or 90 min after LPS administration (Fig. 9**A**). FAK14 treatment reduced mRNA expression of VCAM-1, ICAM-1, Cxcl6, and Cxcl10 (Fig. 9**B**), and protein expression of E-selectin, VCAM-1, (Fig. 9**C**), and IL-6 (Fig. 9**D**) when administered 10-45 min after LPS administration. In contrast, Ceritinib administration after the start of LPS exposure did not inhibit the expression of inflammatory molecules at any time point studied (Fig. 9**B-D**). Taken together, only inhibition of FAK1 signalling after LPS-induced signal transduction has started is capable of alleviating endothelial inflammatory activation.

4. Discussion

Sepsis is a dysregulated host response to infection, that can quickly escalate to organ failure, leading to intensive care admission, organ support and eventually death. LPS is recognised by EC via TLR4 and RIG-

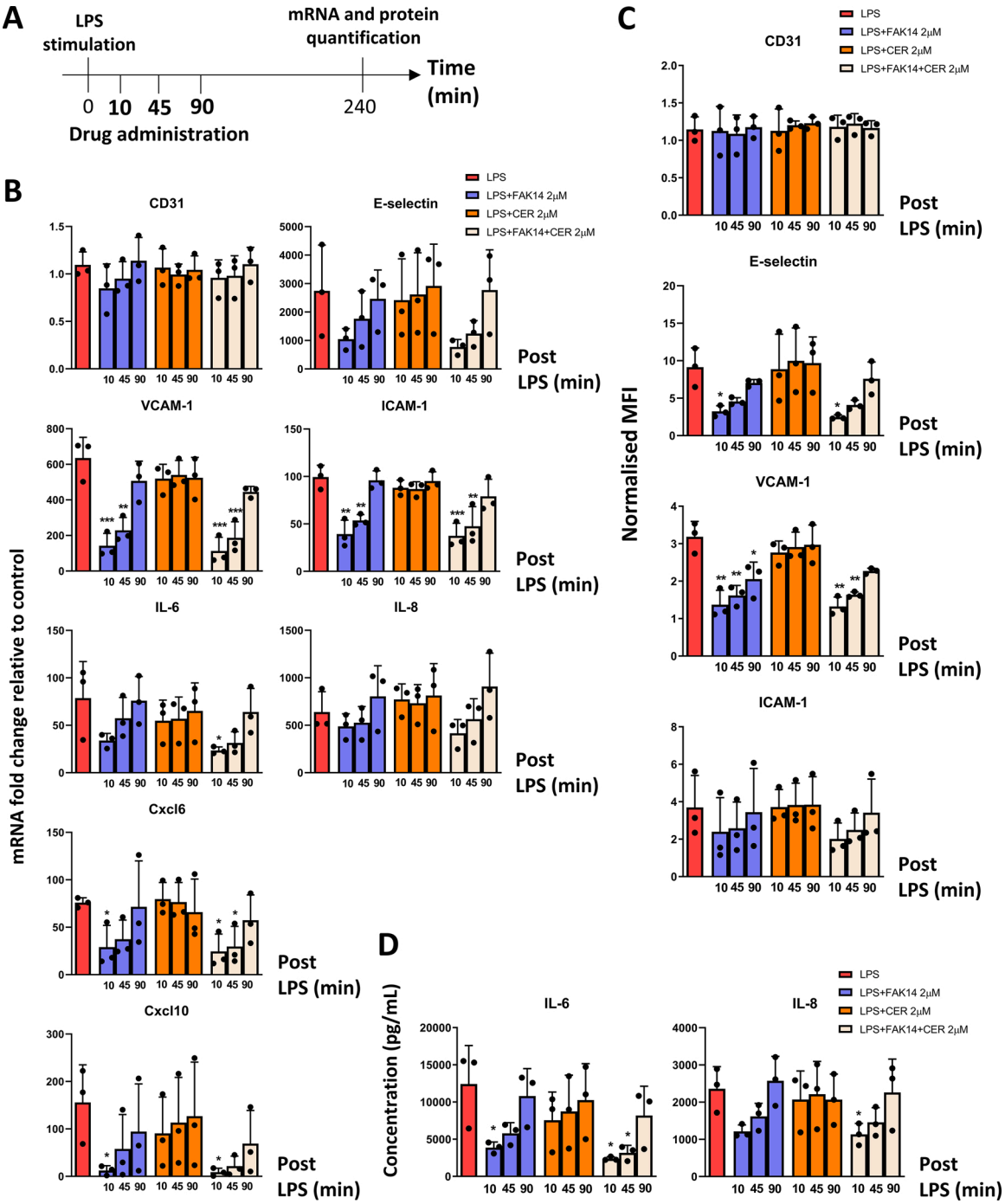


Fig. 9. Inhibition of FAK1, but not ALK, after LPS administration suppresses expression of inflammatory molecules by HUVEC.
(A) Schematic overview of the experiment. **(B)** Effect of time of FAK14 and Ceritinib administration after the start of HUVEC exposure to LPS on E-selectin, VCAM-1, ICAM-1, IL-6, IL-8, Cxcl6, and Cxcl10 mRNA expression as determined by RT-qPCR. Each group represents the mean ± SD of three independent experiments. **(C)** The effect of FAK14 and Ceritinib administration after the start of HUVEC exposure to LPS on CD31, E-selectin, VCAM-1, and ICAM-1 protein expression as determined by flow cytometry, and **(D)** on IL-6 and IL-8 protein production as determined by ELISA. HUVEC were exposed to LPS for 4 h **(B-D)**. Each group represents the mean ± SD of three independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

I which results in increased expression of surface adhesion molecules, cytokines, and chemokines. These molecules facilitate leukocyte recruitment into tissues, and therapeutic strategies aiming to reduce their expression are expected to alleviate organ failure in sepsis patients. Tyrosine kinases are involved in intracellular signalling processes by forming extensive networks that transduce signals to change the transcription of target genes. The current study aimed to identify TKs involved in endothelial LPS signalling and investigate whether these kinases represent useful pharmacological targets to attenuate LPS-induced endothelial inflammatory activation. Kinase activity profiling revealed previously known, and, most importantly, previously unknown kinases to be involved in LPS signalling in EC. siRNA-based knock down of Axl, FAK1, and ALK confirmed a role for these three kinases in endothelial inflammatory activation. Pharmacological inhibition of FAK1 and ALK furthermore demonstrated that these two kinases can be considered druggable targets to interfere with endothelial inflammatory signalling triggered by LPS.

FAK1 is a TK that regulates cell adhesion, migration, proliferation, and survival [27]. Apart from its prominent role in the regulation of physiological signalling processes, FAK1 also controls the expression of inflammatory molecules in mouse fibroblasts, EC, and cancer cells [16, 28]. The role of FAK1 in TNFα- and IL-1β-induced endothelial expression of inflammatory molecules was previously established [29] yet its role in LPS-mediated inflammatory activation has not been reported before. In our study, we showed that FAK1 inhibition with FAK14, a specific FAK1 inhibitor, almost completely abrogated LPS-induced endothelial expression of inflammatory molecules. Although FAK14 was previously shown to mechanistically inhibit FAK1 activity via modulation of its auto-phosphorylation site Y397 in breast cancer cells [30], in HUVEC we did not observe consistent reduction of FAK1 phosphorylation levels at this site following inhibition with FAK14 at 4 μM (data not shown). At the same time, in our experiments FAK14 treatment always resulted in consistent inhibition of expression of inflammatory molecules following LPS exposure. Therefore, our data suggest that the anti-inflammatory effect of FAK14 in LPS-activated EC was independent of Y397 phosphorylation status. Other phosphorylation sites of FAK1, such as Y861 [31], could be involved in LPS-induced activation of FAK1, but their role in LPS-induced inflammatory signalling remains to be clarified as this was beyond the scope of our current study.

Our siRNA-based knock down studies showed that FAK1 knock down attenuated LPS-induced E-selectin, but not VCAM-1 and ICAM-1 protein expression. In contrast, pharmacological inhibition with FAK14 attenuated LPS-induced expression of all three proteins. This difference might be explained by FAK14 also targeting other kinases than FAK1, e.g. Pyk2 [29]. Another possible explanation is that FAK14 inhibits the activity of both the kinase and non-kinase domains of FAK1. These domains were previously shown to mediate inflammatory activation of HUVEC independent of each other [16,32]. Inhibition of FAK1 with an inhibitor that targets its auto-phosphorylation site blocked IL-4-induced VCAM-1 expression to similar extents as seen in IL-4-activated HUVEC expressing dominant negative FAK-related non-kinase [32]. These findings suggest also an active role of the non-kinase domain of FAK1 in mediating inflammatory activation. This involvement could also be occurring in our experiments, although FAK14 has only been described to target the autophosphorylation site of FAK1 in the kinase domain [30]. As our study indicates an inhibitory effect of FAK14 on LPS-induced inflammation independent of modulation of the FAK1 Y397 phosphorylation site, further research is required to elucidate the underlying molecular mechanisms.

In our study, we found that FAK14 inhibited LPS-induced expression of inflammatory molecules in EC via NF-κB signalling. Preliminary data indicates that p38 MAPK activity increases following FAK14 treatment **(Fig. S5)**, which was also reported in the study on FAK1 inhibition of TNFα-mediated endothelial activation [29]. As FAK1 inhibition attenuates NF-κB-induced inflammatory signal transduction, the increase in p38 MAPK activity could be the result of compensatory mechanisms in HUVEC in response to FAK1 inhibition. Administration of FAK14 after the start of LPS exposure furthermore partly blocked LPS-induced inflammatory signalling, which implies that FAK1 is involved further downstream in the LPS signalling cascade. This notion is supported by the identification of FAK1 activity by kinome profiling at later time points of LPS exposure.

ALK, originally discovered as a fusion protein in anaplastic, large-cell non-Hodgkin's lymphoma [33], was identified to mediate IFN-β-driven inflammation in murine experimental sepsis [24]. Here, we show that siRNA-based ALK knock down inhibited LPS-induced expression of endothelial inflammatory molecules. Likewise, pharmacological inhibition of ALK with Ceritinib, an FDA-approved drug currently used to treat ALK-positive non-small cell lung carcinoma [34], exerted similar inhibitory effects as ALK knock down. Despite consistent reduction of expression of inflammatory molecules following both Ceritinib treatment and siRNA-based knock down of ALK, we could not detect ALK mRNA in HUVEC. In contrast, ALK protein was detected in HUVEC by immunofluorescent staining, and its expression diminished following knock down using ALK-specific siRNA. This finding indicates that ALK is expressed in HUVEC, even though this could not be shown by Western blot.

Apart from ALK, Ceritinib has been described to target other kinases, such as RSK1, FAK1, and IGF-1R [35]. Therefore, in our study it is possible that Ceritinib reduced the expression of inflammatory molecules in LPS-activated EC by inhibiting TKs other than ALK. Regardless of whether Ceritinib in our study setup inhibited endothelial activation via ALK or via other kinase(s), this target pathway of inflammatory gene transcription was not controlled by NF-κB, and, as shown in preliminary studies, not by p38 MAPK either (**Fig. S5**). Attenuation of LPS-induced expression of inflammatory molecules by Ceritinib was without any functional consequence for leukocyte-endothelial adhesion *in vitro*. Strikingly, Ceritinib lost its capacity to inhibit LPS-induced expression of inflammatory molecules when it was administered to EC after the start of LPS exposure. This may suggest that ALK, or other kinase target(s) of Ceritinib, play a crucial role in EC in the early initiation of LPS-mediated inflammatory signalling, but not in relaying the signal further downstream after initiation of the LPS signalling cascade.

In conclusion, our study used a kinase activity profiling approach to identify druggable TKs that are involved in endothelial LPS signalling. We here present data that demonstrate that two TKs, FAK1 and ALK, are involved in the regulation of LPS-induced endothelial inflammatory activation, which is attenuated by pharmacological inhibition of these two kinases. To our knowledge, this is the first time that FAK14 and Ceritinib have been evaluated for their inhibitory effects on LPS-mediated inflammatory activation of EC. While Ceritinib did not influence endothelial activation when administered after the start of exposure of HUVEC to LPS, FAK14 attenuated the expression of endothelial inflammatory molecules even up to 45 min after LPS administration. Follow up studies in relevant experimental animal models of sepsis will enhance our understanding of the pharmacological opportunities of FAK1 as a target to inhibit endothelial inflammatory responses. It is crucial for these studies to take endothelial heterogeneity into account [36], as EC in different organs and different microvascular segments may respond differently to FAK1 inhibition. It is imperative to also ensure that inhibition of FAK1 will not negatively influence normal functions of microvascular segments in organs that are not affected by the disease, nor negatively influence cell types other than the target cells. The outcomes of these studies could indicate potential benefit of pharmacological inhibition of kinases in EC with existing inhibitors, such as FAK14, to prevent sepsis-associated multiple organ failure.

Declaration of Competing Interest

The authors report no declarations of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.biopha.2020.111073.

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