

ORIGINAL ARTICLE

Basic Mechanisms in Allergic Disease

Staphylococcus aureus-derived extracellular vesicles induce monocyte recruitment by activating human dermal microvascular endothelial cells *in vitro*

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Summary

Background: Atopic dermatitis (AD) represents the most common inflammatory skin disorder in children showing massive infiltration of immune cells. The colonization of AD-afflicted skin by *Staphylococcus aureus* and *S. aureus*-derived extracellular vesicles (SEVs) has been associated with AD pathogenesis; however, the molecular mechanism underlying SEV-mediated inflammatory responses remains unclear.

Objective: We investigated how SEVs can mediate inflammatory responses in AD pathogenesis by examining the effect of SEVs on human dermal microvascular endothelial cells (HDMECs).

Methods: HDMECs were treated with SEVs, and the expression of cell adhesion molecules or cytokines was assessed using RT-qPCR, Western blot or cytokine array analyses. The receptor for SEVs and related signalling molecules in HDMECs were addressed and verified via gene knockdown or inhibitor experiments. The recruitment assay of human THP-1 monocytic cells on HDMECs was performed after SEV treatment in the presence or absence of the verified receptor or signalling molecule.

Results: SEVs, but not other gram-positive bacteria-derived extracellular vesicles, directly activated HDMECs by increasing the expression of cell adhesion molecules (E-selectin, VCAM1 and ICAM1) and that of IL-6, the inflammatory cytokine; consequently, they enhanced the recruitment of THP-1 monocytic cells to HDMECs. The SEV-induced HDMEC activation was dependent on Toll-like receptor 4 and the NF- κ B signalling pathway, which was rapidly activated within 1 hour post-treatment and followed by an upregulation of cell adhesion molecules and IL-6 at later time-points. Moreover, SEV-mediated HDMEC responses were more rapid and intense than those induced by the same protein concentrations of *S. aureus* extracts.

Conclusions & Clinical Relevance: SEVs as proinflammatory factors could mediate immune cell infiltration in AD by efficiently inducing endothelial cell activation and monocyte recruitment, which may provide insights into alleviating the *S. aureus*-mediated onset or progression of AD and its phenotypes.

1 | INTRODUCTION

Atopic dermatitis (AD) is the most common relapsing inflammatory skin disease affecting about 10%-20% of children and 1%-3% of adults worldwide. AD phenotypes are characterized by cutaneous hypersensitivity to environmental triggers through IgE-mediated sensitization, skin barrier dysfunction and immune dysregulation.¹ Its prevalence is largely dependent on multiple factors, including genetic and environmental factors such as pollutants and industrialization. So far, various genetic factors including single nucleotide polymorphisms or mutations have been associated with AD. Particularly, *FLG* mutations are found in up to 50% of patients with AD, indicating that dysregulated *FLG* expression represents a major predisposing factor for a dysfunctional epithelial barrier in the pathogenesis of AD.^{2,3}

Another important factor involved in the disruption of the epidermal barrier or in immune dysfunction is the colonization by *Staphylococcus aureus*. This infection represents a common feature of moderate to severe AD, showing overexpansion in more than 90% of patients with AD.⁴⁻⁶ In a birth cohort study, the colonization by *S. aureus* during the first 2 years of life was associated with the clinical onset of AD,⁷ implying that skin colonization by *S. aureus* may represent an important causal factor for AD development. In fact, *S. aureus* has been associated with the pathogenesis of AD, as it secretes enterotoxins, antigens and proteases that lead to the dysregulation of skin homeostasis by affecting keratinocytes and various immune cells.⁸

Recently, gram-positive bacteria including *S. aureus* have been shown to release extracellular vesicles (EVs) called membrane vesicles.^{9,10} Similar to the outer membrane vesicles of gram-negative bacteria, gram-positive bacteria-derived EVs are lipid bilayer-enclosed spherical vesicles of ~100 nm in diameter.^{9,11} These can mediate pathophysiological functions by inducing cellular inflammation and evoking host cell death.¹²⁻¹⁵ In particular, the application of *S. aureus*-derived EVs (SEVs) to tape-stripped mouse skin resulted in AD-like inflammation in the absence of live bacterial cells.^{12,16,17} Moreover, SEV-specific IgE serum levels were significantly increased in patients with AD,¹² suggesting that SEVs can evoke immune responses and promote AD; however, the molecular mechanism underlying SEV-mediated inflammatory responses remains unclear.

A prominent perivascular infiltrate containing immune cells represents another hallmark of AD during the acute and chronic phases.⁴ Moreover, microvascular endothelial cells participate in the host's innate immune response by expressing specific cytokines and cell adhesion molecules. This occurs through the cytokine cascade and therefore activates and subsequently increases the adherence of leucocytes to the endothelium and their movements towards extravascular tissues.¹⁸⁻²¹ Considering that *S. aureus* colonizes the epidermis and produces EVs that access the endothelium, we hypothesized that SEVs might directly stimulate microvascular endothelial cells and contribute to the pathogenesis of AD.

Here, we evaluated the ability of SEVs as proinflammatory factors to activate human dermal microvascular endothelial cells

(HDMECs) and recruit human monocytic THP-1 cells. We found that SEVs, but not other gram-positive bacteria-derived EVs, remarkably induced the expression of cell adhesion molecules and inflammatory cytokines in HDMECs and increased the adherence of THP-1 cells. Finally, we demonstrated that the SEV-evoked endothelial responses, mediated via Toll-like receptor 4 (TLR4)-dependent signalling pathways, occurred faster and were stronger than those evoked by *S. aureus* extracts.

2 | METHODS

2.1 | Materials

SP600125 and BAY 11-7082 were purchased from Calbiochem (La Jolla, CA), and chlorpromazine and nystatin were purchased from Sigma (St. Louis, MO). Antibodies for Western blotting were as follows: anti-P-p65, anti-P-JNK, anti-p65 and anti-JNK from Cell Signaling Technology (Danvers, MA); anti-ICAM1 and anti-VCAM1 from Santa Cruz Biotechnology (Santa Cruz, CA); and anti-E-selectin from R&D Systems (Minneapolis, MN).

2.2 | Cell culture

Human dermal microvascular endothelial cells (C-12210), derived from juvenile foreskin, were purchased from PromoCell GmbH (Heidelberg, Germany) and grown in Endothelial Cell Growth Medium MV2 (PromoCell GmbH) supplemented with 1% or 5% fetal calf serum and SupplementMix (C-39225; PromoCell GmbH) at 37°C in a humidified incubator (5% CO₂). Human monocytic cell line THP-1 was purchased from ATCC (ATCC TIB-202; Manassas, VA) and cultured in RPMI 1640 with or without phenol red media supplemented with 10% fetal bovine serum, 100 U/mL of penicillin and 100 µg/mL of streptomycin at 37°C in a 5% CO₂ incubator.

2.3 | Isolation of gram-positive bacterial EVs

Staphylococcus aureus (ATCC 6538) was grown in BD™ Tryptic Soy Broth medium (Franklin Lakes, NJ) for 18-24 hours at 32°C and then subcultured in the same condition after 1/100 dilution. *Propionibacterium acnes* (ATCC 6919) was cultured for 72 hours and inoculated into brain-heart infusion broth (Difco Laboratories, Detroit, MI) at a 1/100 dilution. *Lactobacillus plantarum* (Apsulloc 331261) was grown in MRS broth (BD Difco, Fisher Scientific, Waltham, MA) for 24 hours at 37°C under anaerobic conditions. Each bacterial culture was harvested by centrifugation at 3000×g for 10 minutes, and gram-positive bacterial EVs were isolated from the culture medium by ultracentrifugation at 150 000×g for 3 hours, as previously described.^{9,22} *S. aureus* extracts were prepared by sonication of the cells in phosphate-buffered saline (PBS) on ice for 30 seconds and then centrifugation at 13 000×g for 15 minutes to harvest the membrane fractions according to a previously reported method.²³ Protein concentrations in bacterial EVs or *S. aureus* extracts were measured

using a Pierce™ BCA Protein Assay kit (Thermo Fisher Scientific, Waltham, MA).

2.4 | Analysis and labelling of *S. aureus*-derived EVs

The size and distribution of SEVs were measured by dynamic light scattering using a Zetasizer Nano ZS (Malvern Instruments Limited, Malvern, UK) and analysed using the Dynamic V6 software. An average of three measurements was used. Isolated SEVs were labelled with 5 µmol/L DiI dye (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes, Eugene, OR) for 30 minutes at 37°C and then purified by density-gradient ultracentrifugation, as described in Supporting information. HDMECs were treated with DiI-labelled SEVs with or without endocytic inhibitors for 1 or 3 hours, and the DiI signal was analysed under a confocal microscope (LSM 700; Carl Zeiss, Jena, Germany). Confocal images were converted to JPG format using an image software (Zen 2.3; Carl Zeiss Microimaging GHBH, Jena, Germany). The signal intensity of fluorescence images was processed consistently by adjusting each image with brightness +40% and contrast 0% (Figure 1B) or brightness +20% and contrast +20% (Figure 2E).

2.5 | qRT-PCR analysis

Total RNAs were extracted using TRIzol (Invitrogen, Carlsbad, CA), and cDNA was synthesized using a Revertaid RT kit (Thermo Fisher Scientific) according to the manufacturer's instructions. qRT-PCR was performed using specific TaqMan probes and the 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Gene expression was determined using the $2^{-\Delta\Delta C_t}$ method, and all data were normalized to glyceraldehyde 3-phosphate dehydrogenase.

2.6 | Western blot analysis

Human dermal microvascular endothelial cells were lysed in RIPA buffer containing a phosphatase inhibitor cocktail (Roche, Basel, Switzerland). After centrifugation at 15 700×g for 10 minutes at 4°C, the supernatant was loaded onto a NuPAGE™ 4%-12% Bis-Tris Gel, and the protein bands were visualized using the Amersham Imager 600 system (GE Healthcare, Chicago, IL).

2.7 | Monocyte adhesion assay

The adhesion of THP-1 cells to HDMECs was assessed as previously described.²⁴ Briefly, HDMECs were pretreated with SEVs for 4 or 6 hours in 1% serum conditions. THP-1 cells were labelled with 5 µmol/L calcein-AM (Sigma) for 30 minutes, washed twice with PBS, and then applied onto HDMECs in 96-well plates under the medium conditions of RPMI 1640 without phenol red supplemented with 10% fetal bovine serum. After incubation for 1 hour, unbound cells were washed out with PBS, and fluorescence images were captured using an LSM 510 META confocal imaging system (Carl Zeiss, Oberkochen, Germany). The fluorescence intensity was determined using the Metamorph software (Molecular Devices, San Jose, CA).

2.8 | Cytokine array analysis

Human dermal microvascular endothelial cells were treated with *P. acnes*-derived EVs (PEVs) or SEVs in 1% serum conditions for 6 hours, and the conditioned media were applied to the cytokine array membranes (RayBiotech, Norcross, GA, Human Cytokine Array C3, CODE: AAH-CYT-3). The spot signals were developed according to the manufacturer's instructions, and each spot was analysed using densitometry and ImageJ software (National Institutes of Health Bethesda, MD, USA). The IL-6 levels in the culture supernatants were determined using a specific human ELISA kit for IL-6 (R&D Systems). To examine the effect of endocytic inhibitors on IL-6 levels, HDMECs were treated with 100 ng/mL SEVs in the presence of 10 µg/mL chlorpromazine, 10 µg/mL nystatin or DMSO for 6 hours.

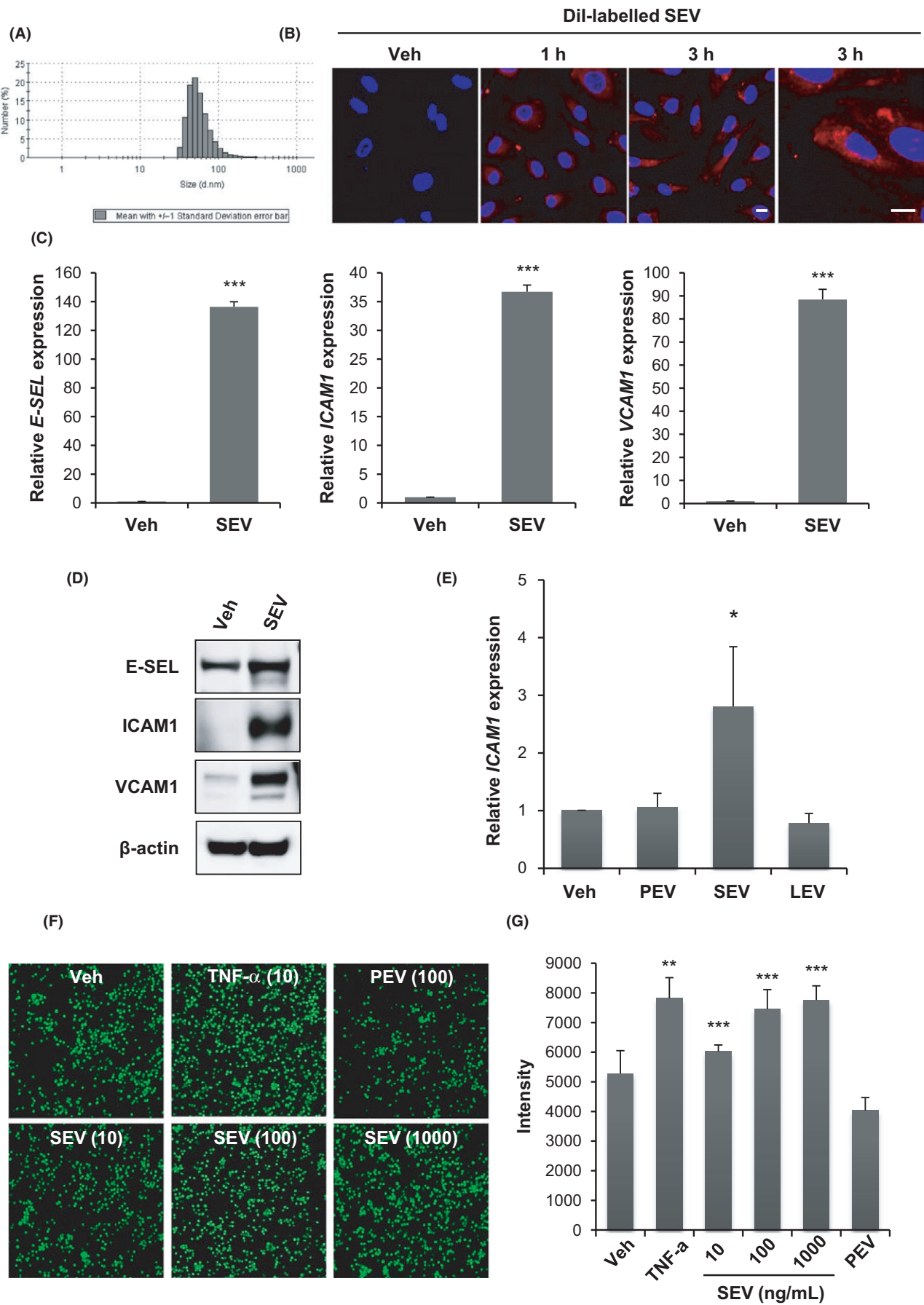
2.9 | siRNA transfection

Human dermal microvascular endothelial cells were transfected with siRNAs targeting human *p65* (SMARTpool siRNA; Dharmacon Inc., Lafayette, CO) or human *TLR4* or a non-targeting control siRNA using a Lipofectamine™ RNAiMAX kit (Thermo Fisher Scientific) according to the manufacturer's instructions.

2.10 | Statistical analysis

The data were analysed using the Student's *t* test and expressed as the mean ± standard deviation (SD). All experiments were performed at least three times, and representative results are shown.

FIGURE 1 *Staphylococcus aureus*-derived extracellular vesicles (SEVs) increase the expression of cell adhesion molecules in human dermal microvascular endothelial cells. A, SEVs analysed using dynamic light scattering. B, Confocal images of SEVs internalized into HDMECs. DAPI (blue) for the nuclei. Veh, HBS-treated. Scale bars, 10 µm. C, D, mRNA (C) or protein (D) levels for E-selectin, ICAM1, and VCAM1 in HDMECs treated with vehicle (HBS) or SEVs (1 µg/mL) for 4 h. mRNA levels were normalized to GAPDH, and β-actin was used as a loading control. E, ICAM1 expression in HDMECs treated with 100 ng/mL *P. acnes*-, *S. aureus*-, or *L. plantarum*-derived EVs for 4 h. Veh, HBS-treated. F, G, Fluorescent images of adherent THP-1 cells on HDMECs (F) and their quantification (G). HDMECs were pre-incubated with TNF-α (10 ng/mL), PEV (100 ng/mL), or the indicated SEV doses (10, 100, 1000 ng/mL) for 6 h and then co-cultured with calcein AM (green)-labelled THP-1 cells for an additional hour. Veh, HBS-treated. Magnification, ×100. Data in (C), (E) and (G) are expressed as the mean ± SD of three independent experiments (**P* < 0.05; ****P* < 0.001 compared to vehicle control)



3 | RESULTS

3.1 | *S. aureus*-derived EVs increased the expression of cell adhesion molecules in HDMECs

To test whether SEVs contribute to the perivascular infiltration of immune cells by directly stimulating HDMECs, we first isolated SEVs from *S. aureus* culture supernatants using a previously described combinational centrifugation method⁹ and analysed the size and distribution of SEVs using dynamic light scattering. The isolated SEVs had an average diameter of 186.6 ± 6.255 nm, although the average diameter of the main clusters appeared to be less than 100 nm (Figure 1A). When HDMECs were treated with SEVs labelled with the lipophilic compound dialkylcarbocyanine iodide, red fluorescence was observed within the cells even after 1 hour post-treatment; the signals became stronger over time (Figure 1B), indicating that SEVs were quickly internalized into HDMECs.

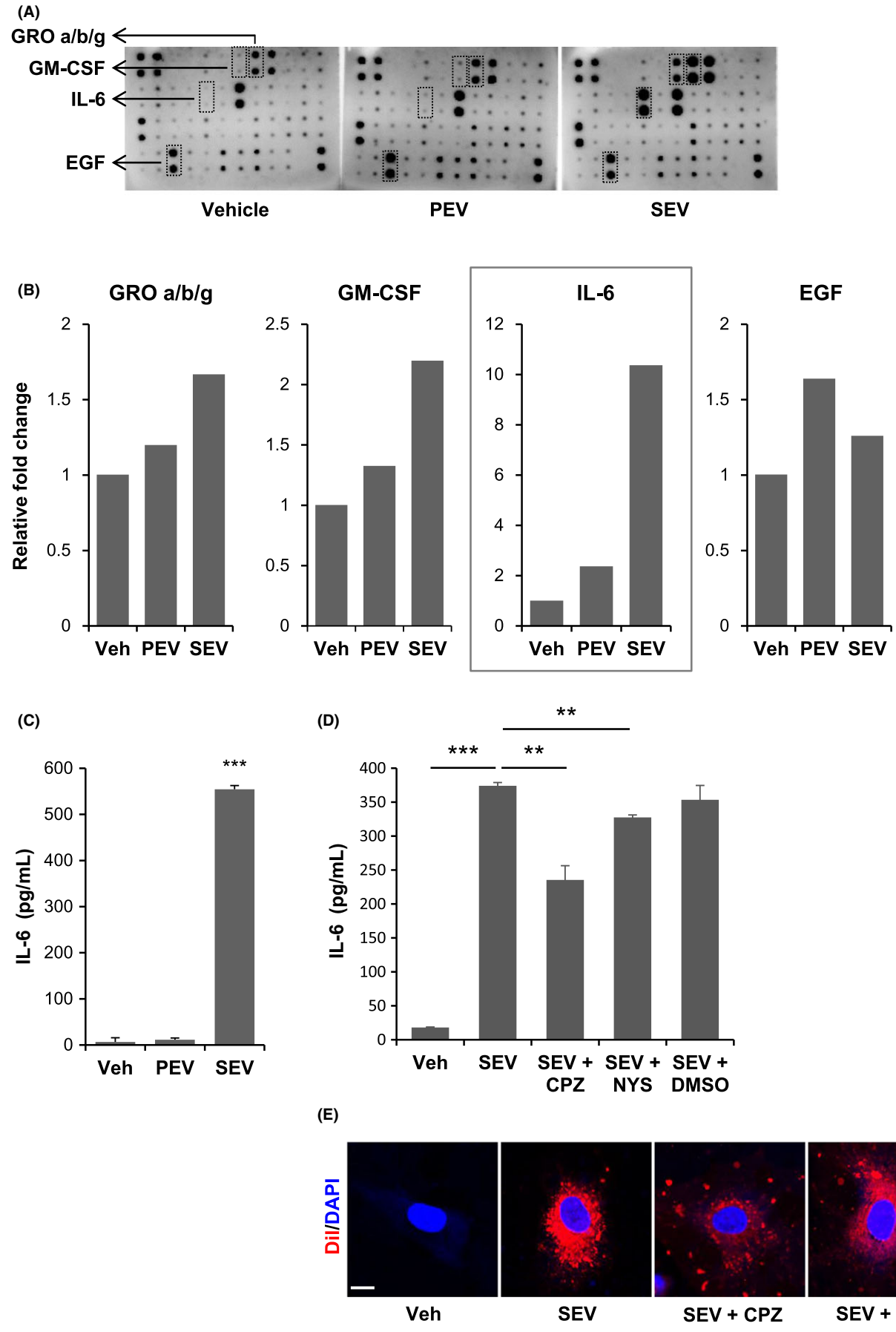
We observed no significant cytotoxicity in HDMECs when administering up to $1 \mu\text{g/mL}$ of SEVs under 1% serum conditions (Figure S1). Therefore, we examined the effect of SEVs on HDMECs at doses ranging from 10 to 1000 ng/mL. SEVs did not influence tube formation or endothelial cell migration (Figure S2). Instead, the SEV treatment remarkably increased the expression of cell adhesion molecules such as E-selectin, ICAM1, and VCAM1 both at the mRNA and protein levels within 4 hours post-treatment (Figure 1C,D). These SEV-mediated effects were reproducible and dose-dependent, regardless of whether the cells were cultured under 1% or 5% serum conditions (Figure S3). Interestingly, SEVs at $1 \mu\text{g/mL}$ showed an effect similar to that of administration of TNF- α (Figure S3), a positive control for the inflammatory activation of endothelial cells.²⁴ Furthermore, the SEV-induced transcriptional upregulation of adhesion molecules began as early as 90 minutes and increased until 6 hours post-treatment, although VCAM1 expression decreased at 6 hours when compared to that at 4 hours (Figure S4). For the established SEV treatment conditions (100 ng/mL in 1% serum-containing media for 4–8 hours), our results suggested that SEVs could function as main proinflammatory factors in HDMECs and that the SEV-induced endothelial responses occur promptly upon exposure. Interestingly, unlike other gram-positive bacteria-derived EVs (ie, PEVs and *L. plantarum*-derived EVs), only SEVs induced the expression of ICAM1 (Figure 1E). This implied that bacterial EVs could reflect the species-specific pathogenicity or virulence of parent cells towards target cells.

To further investigate the upregulation of cell adhesion molecules on HDMECs following SEV treatment, we performed a monocyte recruitment assay using the fluorescently labelled human monocytic cell line THP-1. Whereas the recruitment of monocytes is considered essential for the effective clearance of various infections, recruited monocytes also contribute to the pathogenesis of inflammatory disorders.²⁵ We assessed the numbers of THP-1 cells that attached to HDMECs before or after stimulation with TNF- α or various concentrations of SEVs. In agreement with the increased expression of cell adhesion molecules (Figures S3 and 1C,D), THP-1 cells significantly adhered to TNF- α -treated or SEV-treated HDMECs in a dose-dependent manner, when compared to vehicle-treated or PEV-treated HDMECs (Figure 1F,G). Previously, a high number of monocytes were reported to adhere to TNF- α -exposed HDMECs.²⁴ SEVs alone did not change (CD18) or decrease (CD11 α , C49D) the expression of receptors for cell adhesion molecules in THP-1 cells at an early time-point, and the levels of these receptors became similar at a later time-point between the two groups (Figure S5a–c). Interestingly, the expression of differentiation markers for macrophage lineage from monocytic cells was markedly increased at an early (ICAM1) or at a later (CD14) time-point with a considerable reduction in CCR2, which is supposed to be decreased upon monocyte differentiation at both time-points (Figure S5d–f), implying that the exposure of THP-1 cells to SEVs for a short time may be enough to induce the differentiation of THP-1 cells, but has a little effect on the expression of receptors for adhesion molecules. Taken together, these results suggested that SEVs can specifically and quickly induce the expression of representative cell adhesion molecules in HDMECs, thereby increasing the recruitment of monocytes on endothelial cells.

3.2 | SEVs increased the expression of the IL-6 proinflammatory cytokine in HDMECs

We further investigated whether SEVs can induce the release of inflammatory cytokines, most of which are released from skin and immune cells upon *S. aureus* infection.^{12,15,26} We performed a cytokine array analysis using conditioned media from HDMECs at 6 hours post-SEV treatment. Among 42 analysed cytokines, only four, that is, GRO $\alpha/\beta/\gamma$, GM-CSF, IL-6, and EGF, were upregulated in SEV-treated HDMECs when compared to the vehicle- or PEV-treated groups; in particular, IL-6 expression exhibited the largest increase following SEV administration (Figure 2A,B). The

FIGURE 2 *Staphylococcus aureus*-derived extracellular vesicles increase the expression of IL-6 in HDMECs. HDMECs were treated with *Propionibacterium acnes*-derived EVs (PEVs) or SEVs for 6 h, and the culture supernatants were analysed using a cytokine array and an ELISA kit for IL-6. A, Cytokine array analysis. Red boxes denote the cytokines that were increased in SEV-treated cells compared to vehicle (HBS)- or PEV-treated groups. B, Denoted spots on the blot were analysed using densitometry. C, The secreted IL-6 protein levels were assessed in culture supernatants of SEV-treated HDMECs using an ELISA kit. The data are expressed as the mean $\pm$ SD of three independent experiments ($***P < 0.001$ compared to vehicle control). Veh, HBS-treated. D, SEVs were used to treat HDMECs supplemented with vehicle (HBS), 10 $\mu\text{g/mL}$ chlorpromazine (CPZ), 10 $\mu\text{g/mL}$ nystatin (NYS) or DMSO for 6 h. The secreted IL-6 protein levels were determined using an IL-6 ELISA kit. The data are expressed as the mean $\pm$ SD of four samples from two biological replicates ($***P < 0.001$; $**P < 0.05$). E, The DiI-labelled SEVs were used to treat HDMECs for 3 h. The DiI signal (red) and DAPI (blue) were observed under confocal microscopy. Veh, HBS-treated. Scale bar, 10 μm



SEV-induced IL-6 release from HDMECs was further validated using a quantitative IL-6 ELISA assay and showed a robust increase compared to that in the vehicle- or PEV-treated groups (Figure 2C). Furthermore, this effect was markedly reduced by co-treatment of chlorpromazine, a clathrin-dependent endocytosis inhibitor (Figure 2D). Although nystatin, a caveolae-dependent endocytosis inhibitor, also reduced the effect, chlorpromazine revealed a more potent inhibitory activity in both endocytosis of Dil-labelled SEVs and SEV-induced IL-6 release (Figure 2D,E), suggesting that the entry of SEVs into HDMECs is mainly mediated by clathrin-dependent endocytosis.

3.3 | SEVs induced the activation of HDMECs via TLR4 signalling

Similar to skin cells or leucocytes, endothelial cells also express TLRs that can be activated by bacteria-derived pathogenic materials and enhance the expression of cytokines and cell adhesion molecules upon activation.²⁷⁻²⁹ We examined whether HDMEC TLRs can mediate SEV-induced cellular responses. To determine the major type of TLRs expressed by HDMECs, we analysed the absolute expression levels of each TLR mRNA using droplet digital PCR. HDMECs expressed high levels of *TLR4* and low levels of *TLR2* among the six tested TLRs (*TLR1*, 2, 3, 4, 5 and 9) (Figure S6a). To investigate whether TLR4 is mainly responsible for the SEV-mediated activation of endothelial cells, we pretreated HDMECs with specific small interfering RNAs (siRNAs) against *TLR4* to attenuate the endogenous TLR4 expression and evaluated the expression of cell adhesion molecules after SEV treatment. The knockdown efficiency by *TLR4* siRNAs was approximately 90%, when compared to *TLR4* expression in the scrambled control siRNA-treated group (Figure S6b). The SEV treatment did not change *TLR4* mRNA levels independent of the TLR4 knockdown (Figure S6c). In accordance with the TLR4 knockdown, the upregulation of *E-selectin*, *ICAM1* and *VCAM1* mRNA levels in HDMECs following SEV treatment was remarkably reduced (Figure 3A); this was accompanied by a reduction in protein levels (Figure 3B). TLR4 also modulated IL-6 mRNA and protein levels, whereas this was abolished following TLR4 knockdown (Figure 3C, D). Therefore, TLR4 is a crucial receptor for the SEV-mediated activation of endothelial cells.

3.4 | SEVs induced the activation of HDMECs via the NF- κ B signalling pathway

Adhesion molecules like E-selectin, ICAM1, and VCAM1 and the IL-6 cytokine possess the NF- κ B response element and JUN-N-terminal kinase (JNK)-activator protein 1 binding site in their proximal promoter region.³⁰⁻³² Hence, we explored whether the SEV-mediated activation of HDMECs is dependent on the NF- κ B and JNK signalling pathways, which are also involved in TLR-mediated signalling. We monitored the activation of NF- κ B or JNK by analysing protein phosphorylation up to 6 hours post-SEV treatment. The levels of phosphorylated p65, a subunit of NF- κ B, increased

immediately after SEV treatment and were sustained until 6 hours post-treatment (Figure 4A). The phosphorylation of JNK increased relatively late when compared to that of NF- κ B, showing a clear increase from 1 hour post-treatment (Figure 4A). Interestingly, among cell adhesion molecules, E-selectin showed the earliest increase at 5 minutes, followed by VCAM1 and then ICAM1 at 4 hours post-treatment; the highest protein levels for VCAM1 and ICAM1 were observed at 6 hours post-treatment (Figure 4A). To evaluate whether both signalling pathways are involved in the upregulation of cell adhesion molecules and the release of IL-6, we treated HDMECs with SEVs in the presence of specific inhibitors: BAY 11-7082 and SP600125 for NF- κ B and JNK, respectively. The inhibitory activity of each compound was confirmed by analysing the protein levels of each phosphorylated form (Figure S7a). Treatment with BAY 11-7082 dramatically reduced the SEV-mediated expression of cell adhesion molecules and IL-6 at both the protein and mRNA levels (Figures 4B,C and S7b). Treatment with SP600125 also moderately reduced the SEV-induced expression of E-selectin, ICAM1 and IL-6; however, its effect on VCAM1 protein levels was not significant when compared to that in the vehicle-treated control (Figures S7b and 4B,C). This suggested that JNK signalling may be less involved in the SEV-mediated HDMEC activation process as opposed to NF- κ B signalling. We further confirmed the key role of NF- κ B in the SEV-induced activation of endothelial cells by knocking down p65 NF- κ B. We validated the knockdown by analysing p65 mRNA expression (Figure S7c) and showed that this leads to a clear reduction in the RNA and protein expression of adhesion molecules and the release of IL-6 (Figures S7d and 4D,E). Collectively, these results demonstrated that TLR4-NF- κ B represents a major signalling pathway for the activation of HDMECs by SEVs.

3.5 | SEVs induced the adherence of monocytes to HDMECs via TLR4 and NF- κ B signalling

Considering the TLR4- and NF- κ B-dependent upregulation of adhesion molecules and IL-6 in HDMECs following SEV treatment, we further determined the effects of these signalling molecules on the SEV-induced monocyte recruitment. The transfection of HDMECs with either *TLR4* or *p65* siRNAs significantly attenuated the basal or SEV-induced THP-1 adhesion to HDMECs, when compared to that in the scrambled control-treated groups (Figure 5A,B). These results suggested that TLR4-NF- κ B signalling is important for mediating the adherence of immune cells to HDMECs and that SEVs can recruit monocytes onto endothelial cells via this signalling pathway.

3.6 | SEVs were more potent than *S. aureus* extracts in activating HDMECs

Bacteria-derived EVs are more cytotoxic to host cells than purified toxins,¹¹ suggesting that EVs are efficient carriers of bioactive substances. We compared the potency of SEVs and *S. aureus* extracts in activating endothelial responses by treating HDMECs with the same

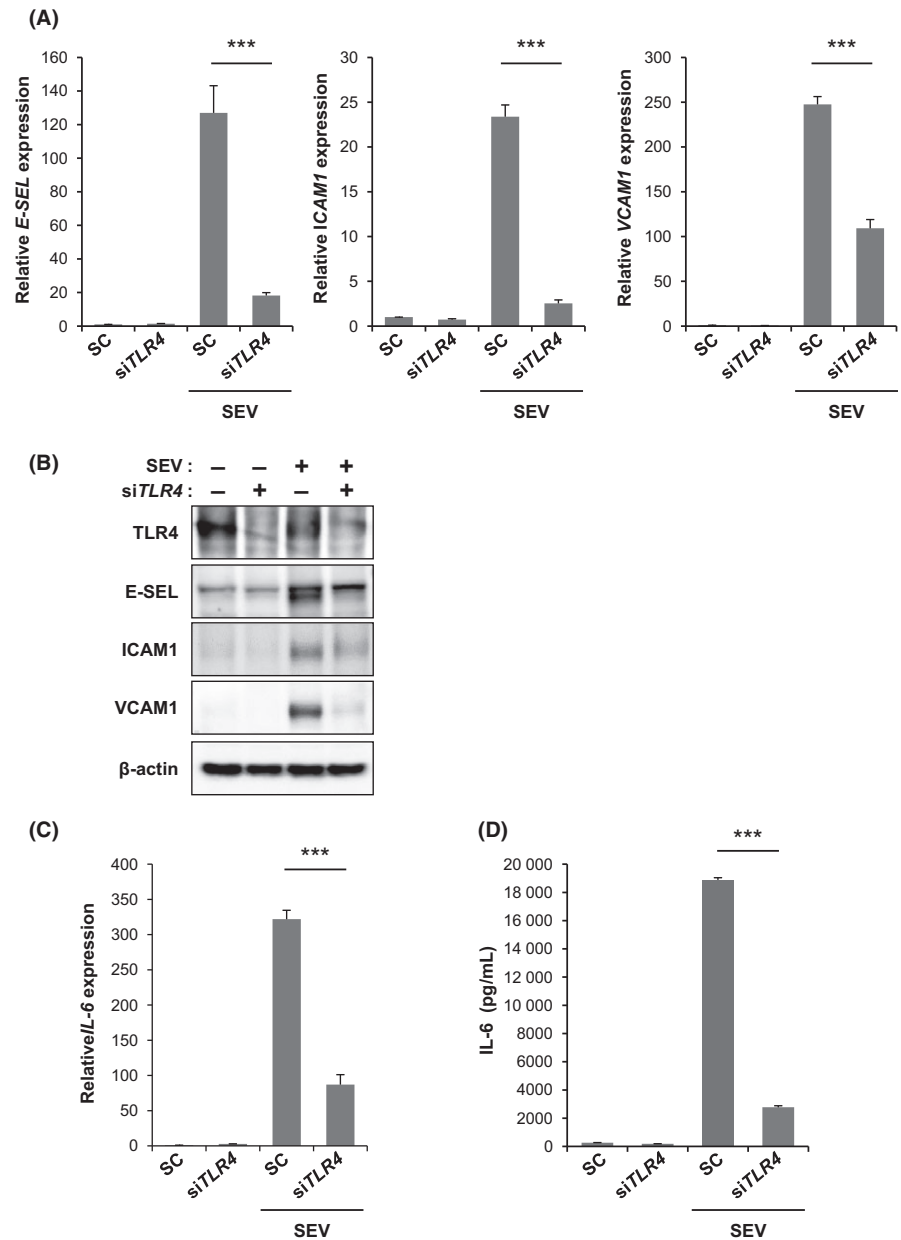


FIGURE 3 *Staphylococcus aureus*-derived extracellular vesicles induce the activation of HDMECs via TLR4 signalling. HDMECs were transfected with scrambled control (SC) or TLR4 siRNAs (siTLR4) for 48 h and then treated with SEVs (100 ng/mL) for an additional 6 h. The mRNA (A, C) and protein expression levels (B, D) of E-selectin, ICAM1, VCAM and IL-6 were determined using qRT-PCR or Western blotting, using the respective primers or antibodies and an ELISA kit for IL-6. The data are expressed as the mean $\pm$ SD of three independent experiments (** $P < 0.001$)

amount of proteins. Next, we determined the expression levels of phosphorylated p65 and cell adhesion molecules over time. An increased phosphorylation of p65 was clearly observed at 1 hour post-treatment with SEVs, which was followed by an increase in the expression of E-selectin, VCAM1 and ICAM1 in this order at later time-points (Figure 6A). Interestingly, *S. aureus* extracts increased the expression of these proteins at a much later time-point, that is, 48 hours post-treatment, at which the degree of HDMEC activation by SEVs vs *S. aureus* extracts was reversed (Figure 6A). Similarly, IL-6 expression increased from 4 hours post-SEV treatment and gradually decreased thereafter; however, the effect of *S. aureus* extracts was only apparent at 48 hours post-treatment (Figure 6B). The SEV-induced IL-6 release was accordingly and significantly increased when compared to that triggered by *S. aureus* extracts throughout the entire time course, leading to a substantial difference in the

accumulated IL-6 amounts at 48 hours between the two groups (Figure 6C). To determine the concentration of *S. aureus* extracts that induces activation of HDMECs similar to that induced by 100 ng/mL of SEVs, cells were treated with various protein concentrations of *S. aureus* extracts for 6 hours, and the expression of cell adhesion molecules and IL-6 was analysed using qRT-PCR and ELISA (Figure S8). Interestingly, *S. aureus* extracts 10-fold more concentrated than the SEV dose were less effective in activating the transcription of cell adhesion molecules, although a 100-fold higher concentration exhibited a higher efficacy than SEVs (Figure S8a). Similar results were obtained regarding IL-6 mRNA and protein levels (Figure S8b). *S. aureus* extracts also exerted their effects in a TLR4-dependent manner, as the *S. aureus* extract-induced VCAM1 expression disappeared following treatment with TLR4 siRNA (Figure S9). Because of the low efficacy of *S. aureus* extracts, other adhesion

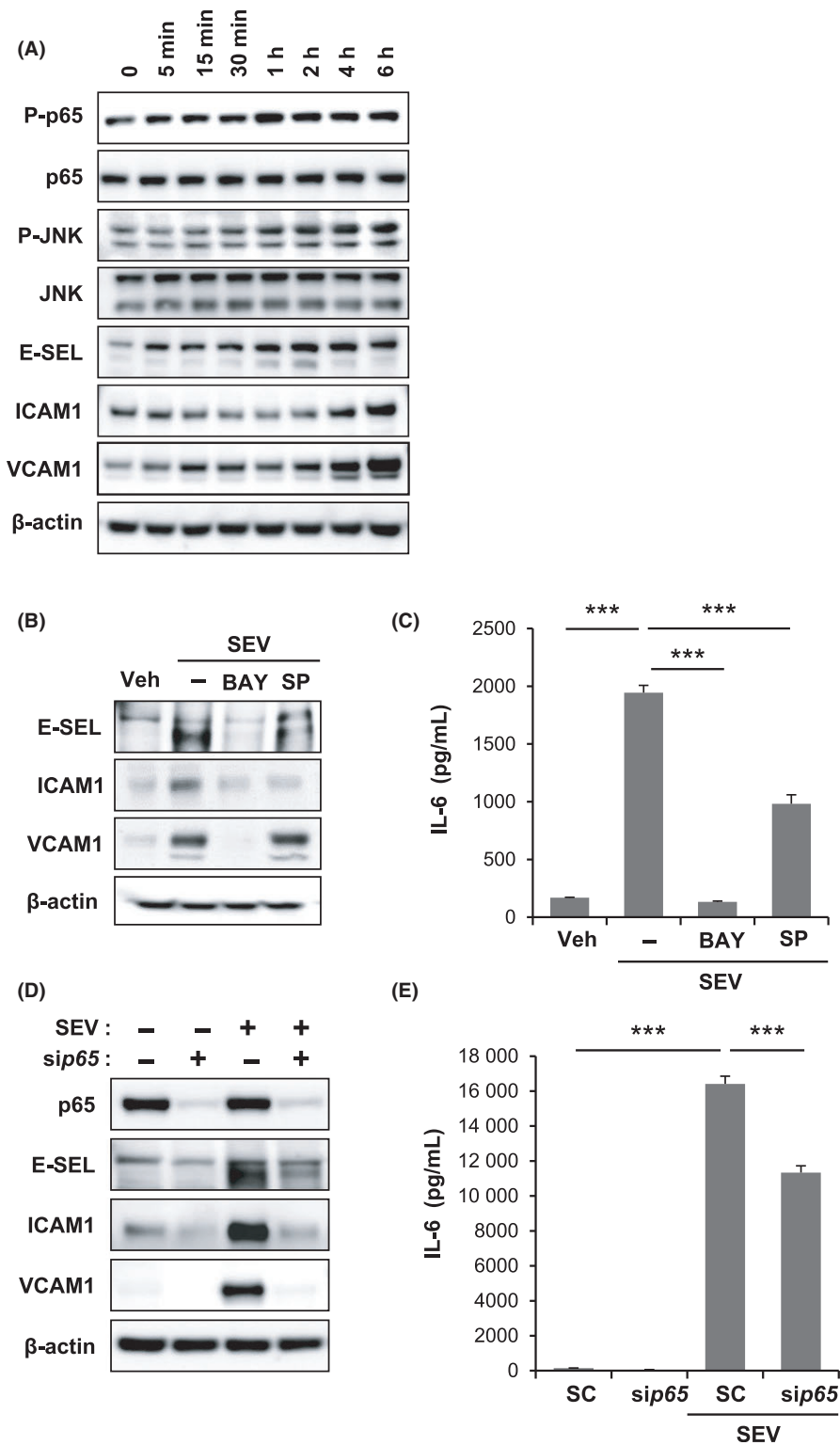


FIGURE 4 *Staphylococcus aureus*-derived extracellular vesicles induce the activation of HDMECs via the NF-κB signalling pathway. A, Total or phosphorylated protein levels for p65 (p65 vs P-p65) and JNK (JNK vs P-JNK), and adhesion molecules in HDMECs treated with SEVs (100 ng/mL) and harvested at the indicated time-points. B, C, Protein levels of adhesion molecules (B) and IL-6 (C) in HDMECs pretreated with inhibitors for 1 h and incubated with SEV (100 ng/mL) for an additional 6 h. Veh, HBS-treated; -, DMSO-treated; BAY, BAY 11-7082; SP, SP600125. D, E, Protein levels of adhesion molecules (D) and IL-6 (E) in HDMECs transfected with scrambled control (SC) or p65 siRNAs (sip65) for 48 h and treated with SEV (100 ng/mL) for 6 h. β-actin was used as a loading control. Data in (C) and (E) are expressed as the mean ± SD of three independent experiments (***P < 0.001)

molecules such as E-selectin and ICAM1 appeared not to be affected by the TLR4 knockdown. These results suggested that SEVs, as potent proinflammatory factors, trigger a more rapid and intense induction of cell adhesion molecules and inflammatory cytokines than *S. aureus* extracts in HDMECs and could contribute to the infiltration of immune cells in hyperinflammatory skin disorders, such as AD.

4 | DISCUSSION

While SEV-mediated responses in immune cells and keratinocytes have been previously investigated,¹⁵ the effects of SEVs on endothelial cells remain unclear. We demonstrated that SEVs activate HDMECs rapidly and effectively by increasing the expression of

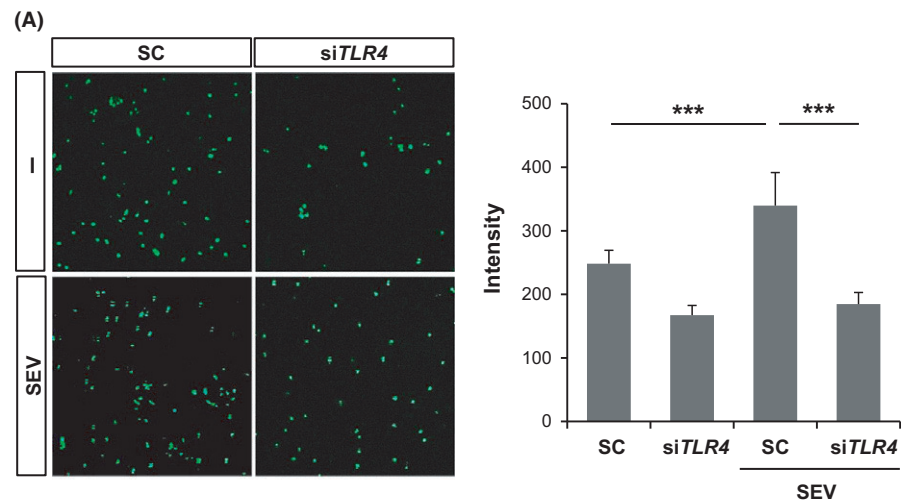
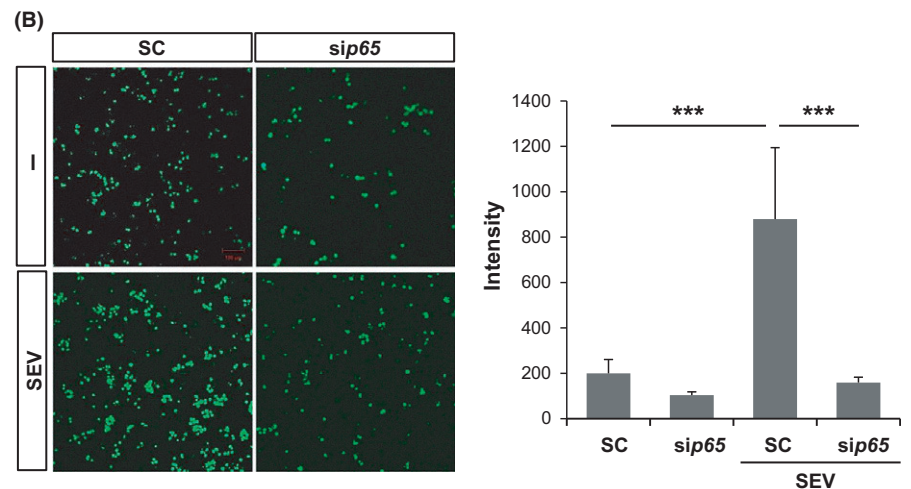


FIGURE 5 *Staphylococcus aureus*-derived extracellular vesicles induce the adherence of monocytes to HDMECs via TLR4 and NF- κ B signalling. HDMECs were transfected with scrambled control (SC), TLR4 (A), or *p65* (B) siRNAs for 48 h, treated with vehicle (-) or SEV (100 ng/mL) for an additional 6 h, and then cocultured with calcein AM-labelled THP-1 cells for 1 h. The fluorescence microscopy images show THP-1 cells (green) attached to HDMECs for each treatment condition; the fluorescence intensity was quantified using a fluorometer. Magnification, $\times 100$. The data are expressed as the mean $\pm$ SD of three independent experiments (** $P < 0.001$)



cell adhesion molecules and inflammatory cytokines through TLR4-NF- κ B signalling and subsequently induce monocyte adherence to HDMECs (Figure 6D). Thus, to our knowledge, this is the first report demonstrating the direct effects of SEVs on the human dermal microvascular endothelium.

Staphylococcus aureus is a representative pathogenic microorganism that is closely associated with the onset and progression of AD.^{4,7} Accordingly, antibiotic therapy is prescribed for patients with AD; however, this is less effective because of the re-colonization with the same toxin-secreting organism when drugs are no longer prescribed, the emergence of antibiotic-resistant strains, or the disturbance of beneficial commensal microbiomes.^{5,26,33} Therefore, alternative therapeutic strategies have been developed to regulate rather than eradicate microbes in patients with AD using specific monoclonal antibodies against virulent toxins, vaccines, probiotics or substances derived thereof.

Several *S. aureus*-derived factors have been speculated to be involved in immune responses and AD phenotypes including superantigen, staphylococcal enterotoxin B, staphylococcal alpha toxin (α -hemolysin), lipoteichoic acid, peptidoglycan and staphylococcal protein A (SPA).³⁴⁻⁴¹ However, SPA or superantigen did not show any significant correlation with skin inflammation or severity of AD,^{42,43}

suggesting that there is a molecular complex in *S. aureus*-mediated AD pathogenesis. In addition to virulence-associated factors, *S. aureus* spontaneously releases EVs. SEVs have been considered as a potent vehicle that deliver such virulent factors to the epidermis. Based on proteomic analysis, SEVs contain more than 100 proteins including α -hemolysin, SPA and β -lactamases.⁹ Recently, it was reported that EVs derived from gram-negative bacteria essentially deliver lipopolysaccharide into the cytosol via endocytosis and trigger caspase-11 activation,⁴⁴ suggesting that EV-associated pathogen-associated molecular patterns (PAMPs) have greater capacity than their free form for cytosolic access. By transporting bacterial products including virulence-associated factors and PAMPs to the recipient cells via a receptor-ligand interaction and/or endocytosis, SEVs may mediate immune system evasion, host cell invasion and pathogenesis.⁴⁵ Moreover, EVs can deliver bacterial products at high concentration locally and offer them specific access to otherwise inaccessible cellular and tissue compartments. With these intrinsic properties, SEVs can evoke a rapid and strong reaction in HDMECs compared to *S. aureus* extract possibly by increasing cytosolic access of PAMPs or DAMPs such as tri- or di-acyl lipopeptides by interacting with TLRs, especially TLR4, thereby delivering them at high concentration intracellularly. In addition, SEVs can directly transport virulence-associated factors intracellularly, which

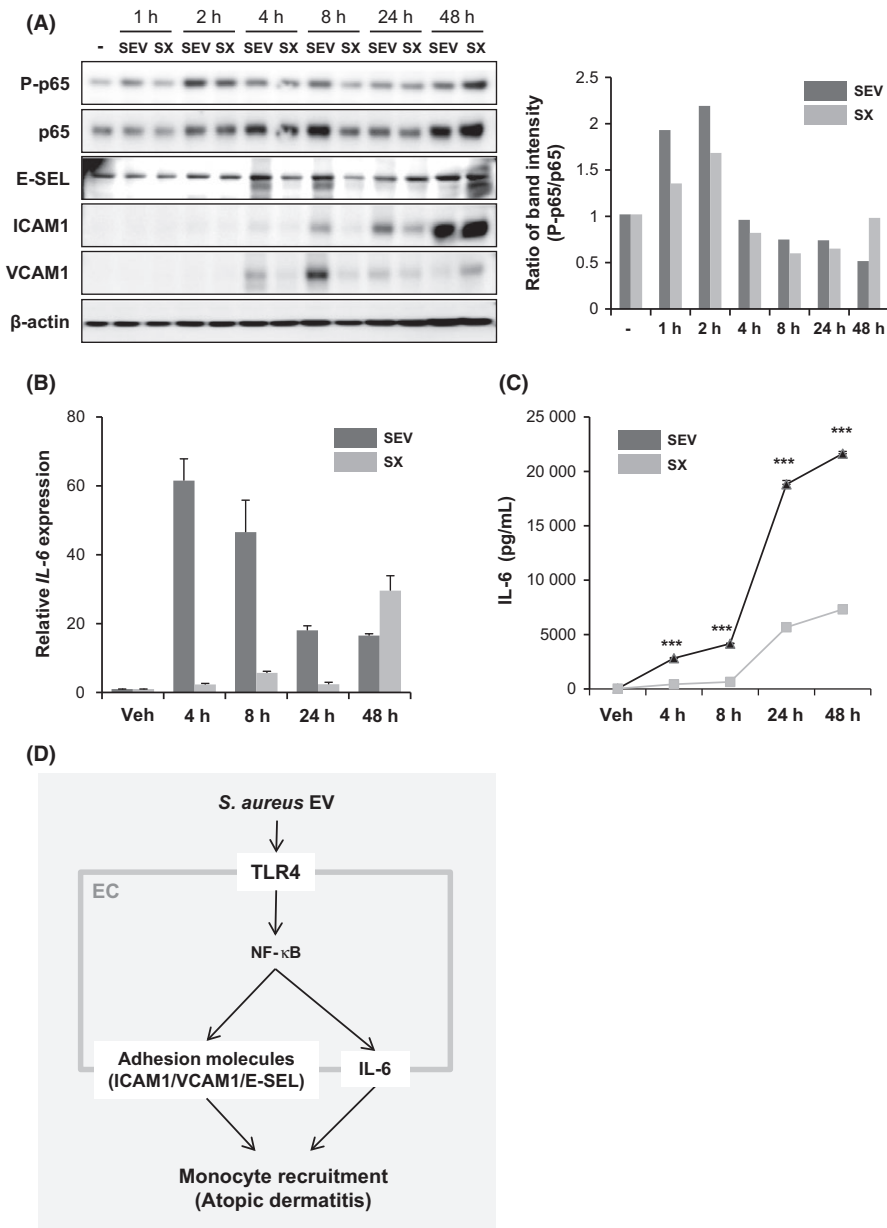


FIGURE 6 *Staphylococcus aureus*-derived extracellular vesicles are more potent than *S. aureus* extracts in activating HDMECs. A-C, HDMECs treated with 100 ng/mL of SEVs or *S. aureus* extracts (SXs) were harvested at the indicated time-points. A, The protein levels of total (p65) or phosphorylated p65 (P-p65) and those of adhesion molecules were analysed by Western blotting (left panel). The ratio of the band intensity between the phosphorylated and total p65 protein was evaluated by densitometry after normalization using β -actin (right panel). β -actin was used as a loading control. -, HBS-treated. B, C, mRNA (B) and protein (C) levels of IL-6 at the indicated time-points post-treatment with SEVs or SXs, analysed by qRT-PCR and ELISA, respectively. The data are expressed as the mean $\pm$ SD of three independent experiments (*** P < 0.001 compared to SX at each time-point). Veh, HBS-treated. D, Schematic diagram for SEV-mediated endothelial cell activation via TLR4-NF- κ B signalling and the consequent monocyte recruitment in atopic dermatitis

otherwise are freely dispersed and degraded extracellularly. SEVs may also overcome the limitation of *S. aureus* to access blood vessels from the epidermis and activate microvascular endothelial cells in vivo. Taken together, SEVs represent potent pathogenic substances that evoke skin inflammatory responses.

Even though EVs derived from bacteria display common structural properties and functions in inter-cellular communication, considering the different efficacies in evoking cellular responses in HDMECs among gram-positive bacteria-derived EVs (Figures 1E,F,G and 2), differences in endotoxin composition (triacylated or diacylated lipopeptides) or in cell or receptor type-dependent EV-mediated mechanisms may occur. Whether there are differences among gram-positive bacteria-derived EVs in the production of proinflammatory cytokines and/or monocyte differentiation remains to be investigated.

The pathogenicity and accessibility of SEVs onto endothelial cells may be a direct reason for the phenomena shown in previous reports where topical application of SEVs to AD-like mouse skin lesions or tape-stripped mouse skin or mouse airway exposure to SEVs induced massive infiltration of inflammatory cells, resulting in eczematous dermatitis or pulmonary inflammation, respectively.^{12,15,16} Therefore, the release of SEVs and SEV-mediated cellular responses may represent another regulatory target for therapy for chronic inflammatory disorders such as AD and asthma; by reducing SEV release or inhibiting the SEV-mediated endothelial activation, the pathogenesis of inflammatory disorders may be attenuated.

In addition to the upregulation of cell adhesion molecules in HDMECs, the secretion of IL-6 was increased by SEV treatment. This result is in agreement with previous reports claiming that SEV treatment increased the production of IL-6 by other cell types such

as dermal fibroblasts, alveolar macrophages, airway epithelial cells or HaCaT cells in vitro.^{12,15,46} In addition, the application of SEVs in tape-stripped or AD-like mouse skins or mouse airway exposure to SEVs induced Th1, Th17, and/or Th2-mediated immune responses in vivo.^{12,15,46} Given the key role of IL-6 in priming Th17 differentiation, an important pathologic network in AD lesions,^{3,47-51} the SEV-stimulated IL-6 release from HDMECs may contribute to the progression of AD, in part via polarizing naïve T cells towards Th17; however, the direct involvement of SEV-stimulated IL-6 in Th17 differentiation remains to be elucidated.

Among TLRs, pattern recognition receptors for host defence expressed in the skin and immune cells, TLR2 has emerged as the main receptor for *S. aureus* in various cell types by recognizing tri- or di-acyl lipopeptides and forming heterodimers with TLR1 or TLR6.^{5,52-55} The progression of AD is associated with polymorphisms in human TLR2.^{56,57} As components constituting the innate immune system, endothelial cells also express various TLRs (TLRs 1-10) as well as NOD-like or RIG-I like receptors, but their basal expression levels, except those of TLR3 and TLR4, are relatively low compared with those of immune cells.²⁷⁻²⁹ Furthermore, TLR2 expression is known to be robustly induced upon exposure to microbial and host factors; therefore, TLR4 at baseline and TLR2 thereafter can be speculated to continuously or simultaneously respond to pathogen-associated or danger-associated molecular patterns on endothelial cells. Based on the absolute copy numbers of TLR transcripts, TLR4, and not TLR2, was predominantly expressed at baseline in HDMECs (Figure S6), in agreement with previous reports.²¹ Moreover, TLR4 was significantly involved in the *S. aureus*- or SEV-mediated activation of endothelial cells via NF- κ B (Figures 3, 5 and S9). While TLR4 mediated bacteria-induced endothelial cell activation in the physiological relevant human umbilical vein endothelial cell model⁵⁸ and was considered critical for host survival in an *S. aureus*-induced murine brain abscess model,⁵⁹ the involvement of TLR4 in *S. aureus*-mediated inflammatory skin disorders has not been elucidated. Therefore, the inhibitory regulation of the TLR4-NF- κ B signalling pathway in HDMECs will aid in attenuating the *S. aureus*-mediated AD pathogenesis, even though we do not rule out the possibility of involvement of other TLRs, particularly TLR2, in SEV-mediated endothelial activation. In summary, our results suggested that lipid bilayer-enclosed and nano-sized SEVs efficiently induce endothelial cell activation by increasing the expression of cell adhesion molecules and inflammatory cytokines and the consequent monocyte adherence to HDMECs. Therefore, inhibiting the release of EVs from *S. aureus* without eradicating the microbes and impeding the SEV-mediated endothelial activation using neutralizing antibodies or inhibitors may offer alternative approaches for alleviating the *S. aureus*-mediated onset or progression of AD and its phenotypes.

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CONFLICT OF INTEREST

JK, B-HB, E-JC, HGL, TRL, and E-GC are employees of AmorePacific Corporation.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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Supplementary Information

Staphylococcus aureus*-derived extracellular vesicles induce monocyte recruitment by activating human dermal microvascular endothelial cells *in vitro

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Supplementary Methods

Labeling SEVs with DiI

Isolated SEVs were incubated with 5 μ M DiI dye (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; Molecular Probes, Eugene, OR) for 30 min at 37°C. To purify DiI-labeled SEVs, 50% iodixanol (Axis-Shield PoC AS, Oslo, Norway) was placed at the bottom of an ultracentrifuge tube, overlaid with 10% iodixanol and DiI-labeled SEVs, and then ultracentrifuged at 100,000 $\times g$ for 2 h at 4°C. DiI-labeled SEVs were obtained from the interface of the 50% and 10% iodixanol layers

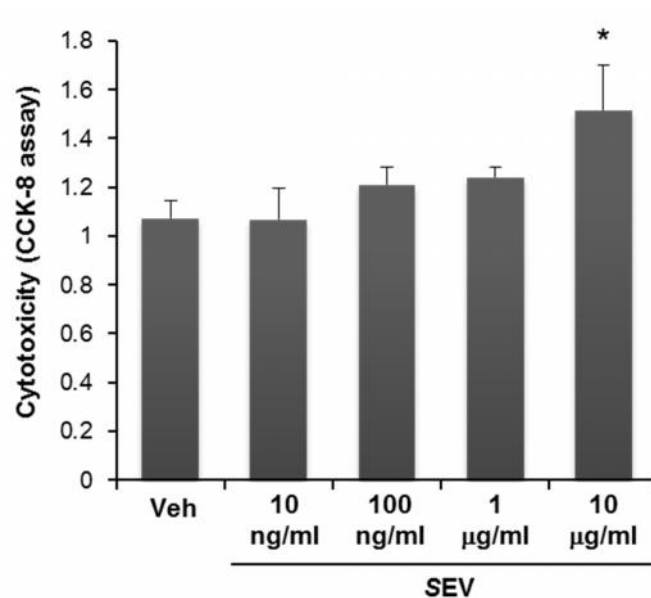
Tube formation assay

In brief, 250 μ l of growth factor-reduced Matrigel (BD Biosciences) was pipetted into the wells of a 24-well tissue culture plate and polymerized for 30 min at 37°C. HUVECs and HDMECs were incubated in EBM-2 medium supplemented with 1% FBS (EBM-2/1% FBS) for 6 h and dissociated with 0.25% trypsin. The cells were resuspended in EBM-2/1% FBS, plated onto the layer of Matrigel at a density of 0.8×10^5 cells/well (HDMECs) or 1.2×10^5 cells/well (HUVECs), and were treated with SEVs at various concentrations. The images for tube formation were taken over time under phase-contrast microscope.

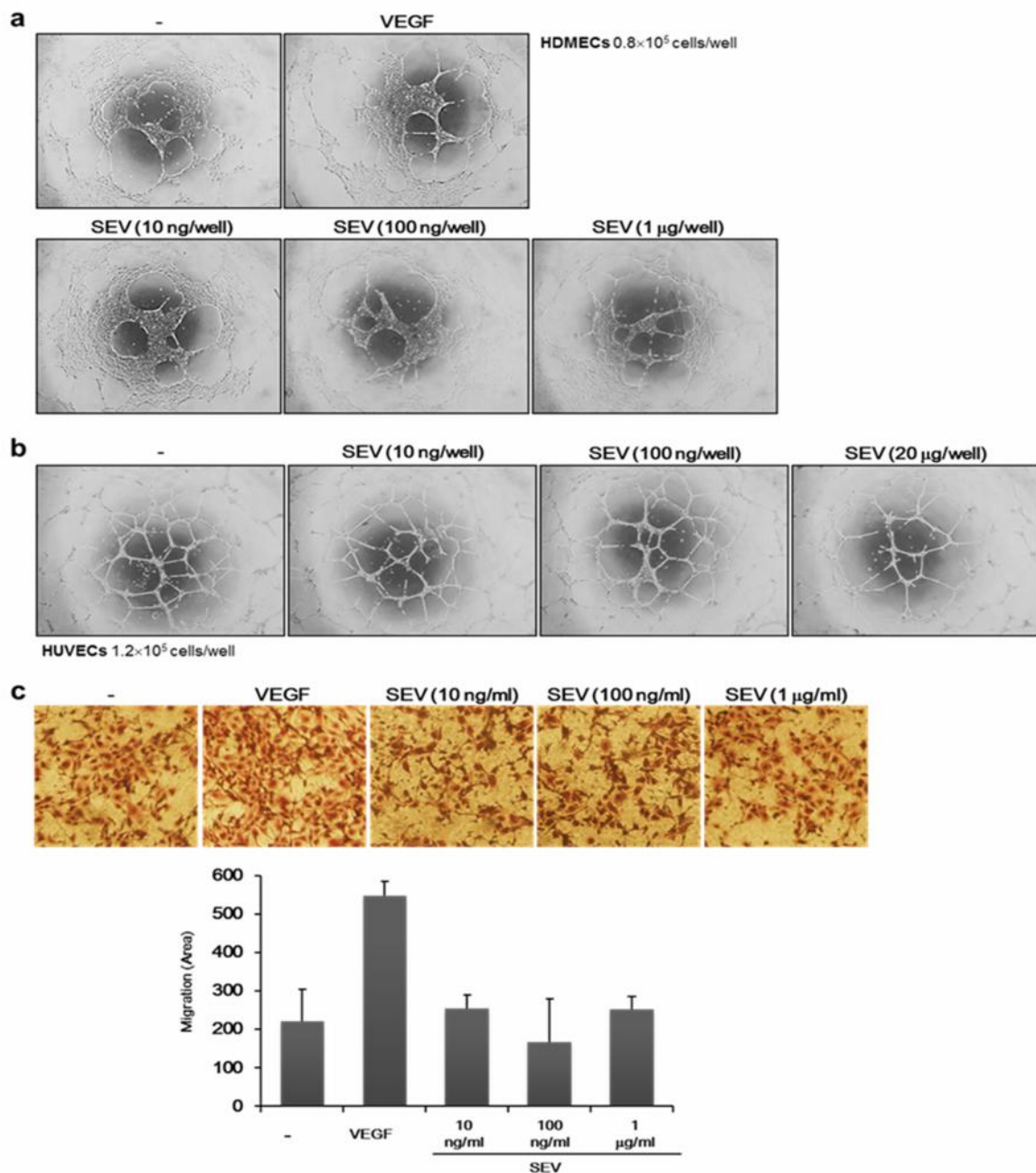
Endothelial cell migration assay

HDMEC migration was evaluated using a modified Boyden chamber assay (Corning Costar, Acton, MA). Briefly, HDMECs were serum-starved for 6 h in EBM-2/1% FBS, dissociated using 0.25% trypsin, and re-suspended in EBM-2/1% FBS. The cells (1×10^5 in 100 μ l) were placed in the upper portion of the gelatin-coated Boyden chamber. SEVs at various concentrations (10, 100, 1000 ng/mL) or VEGF (10 ng/mL) were added into the lower compartment. After a 6-h incubation at 37°C, the bottom of the filter was fixed with methanol and stained with hematoxylin and eosin. The migrated HDMECs on the bottom of the filter were counted in random high-power ($\times 200$) microscope fields.

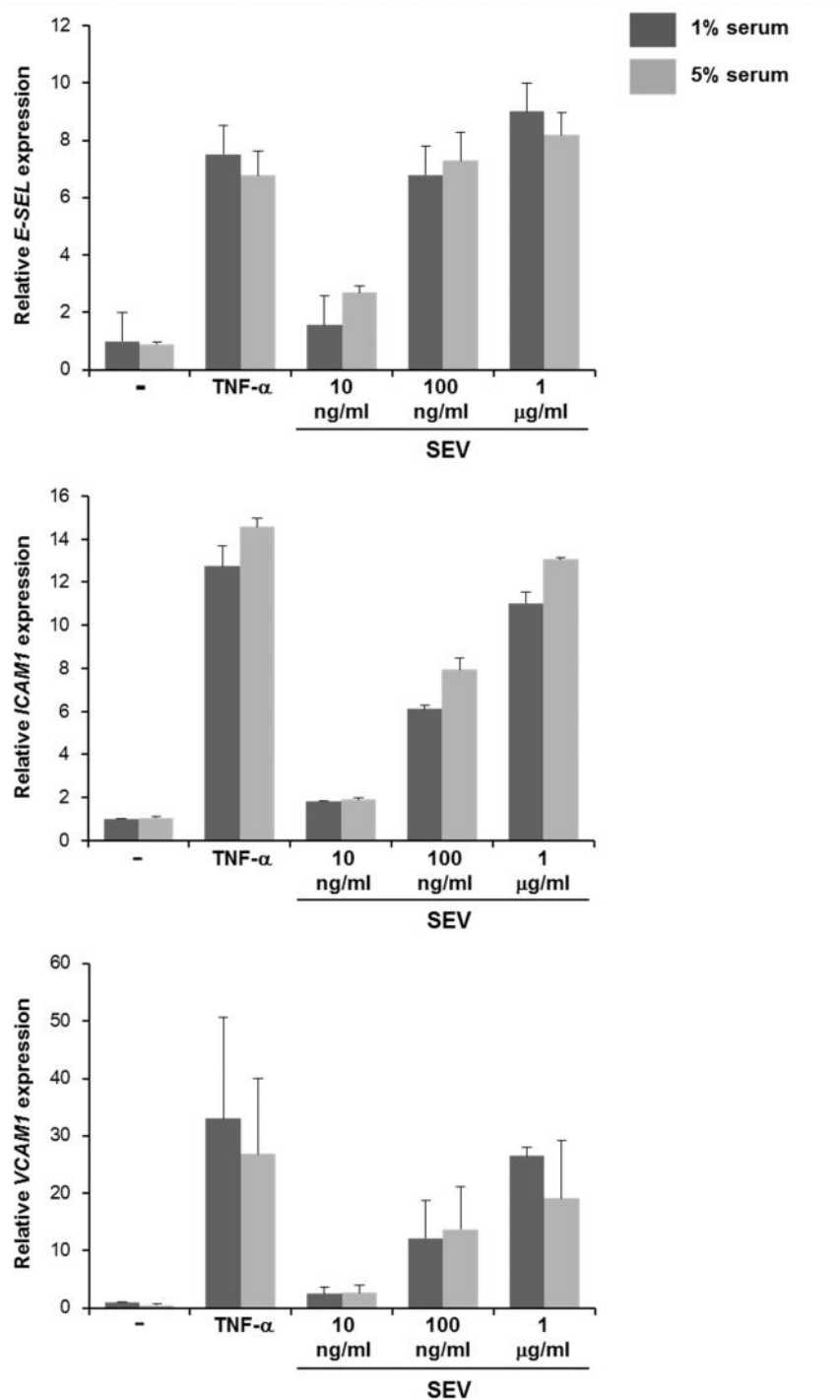
Supplementary Figures



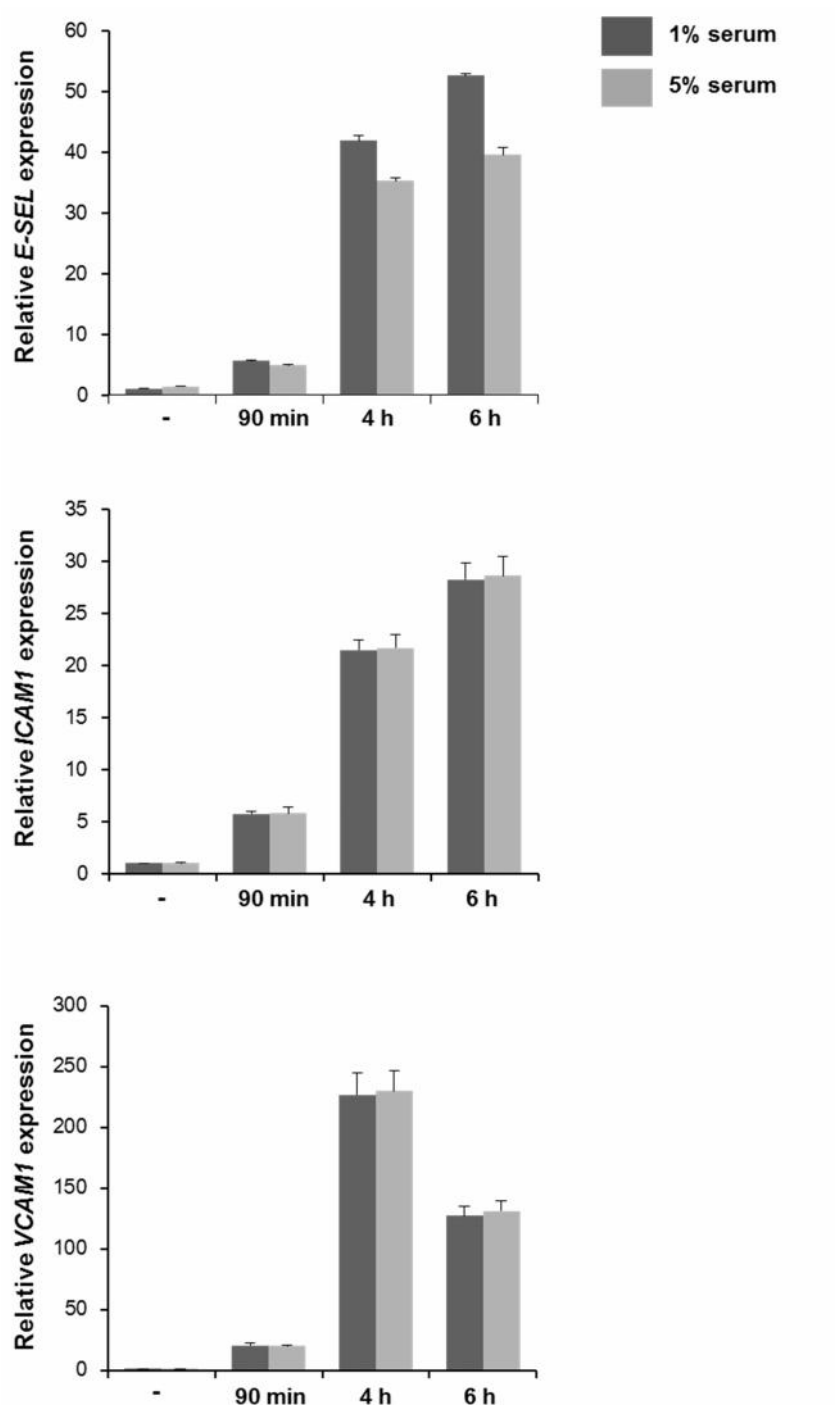
Supplementary Figure S1. The effect of SEVs on HDMEC proliferation. HDMECs were treated with various concentrations of SEVs for 6 h in 1% serum-containing basal medium. The cell viability was determined using the CCK-8 activity assay. The data are expressed as the mean $\pm$ SD of three independent experiments (*, $P < 0.05$ compared to vehicle control). Veh, HBS-treated.



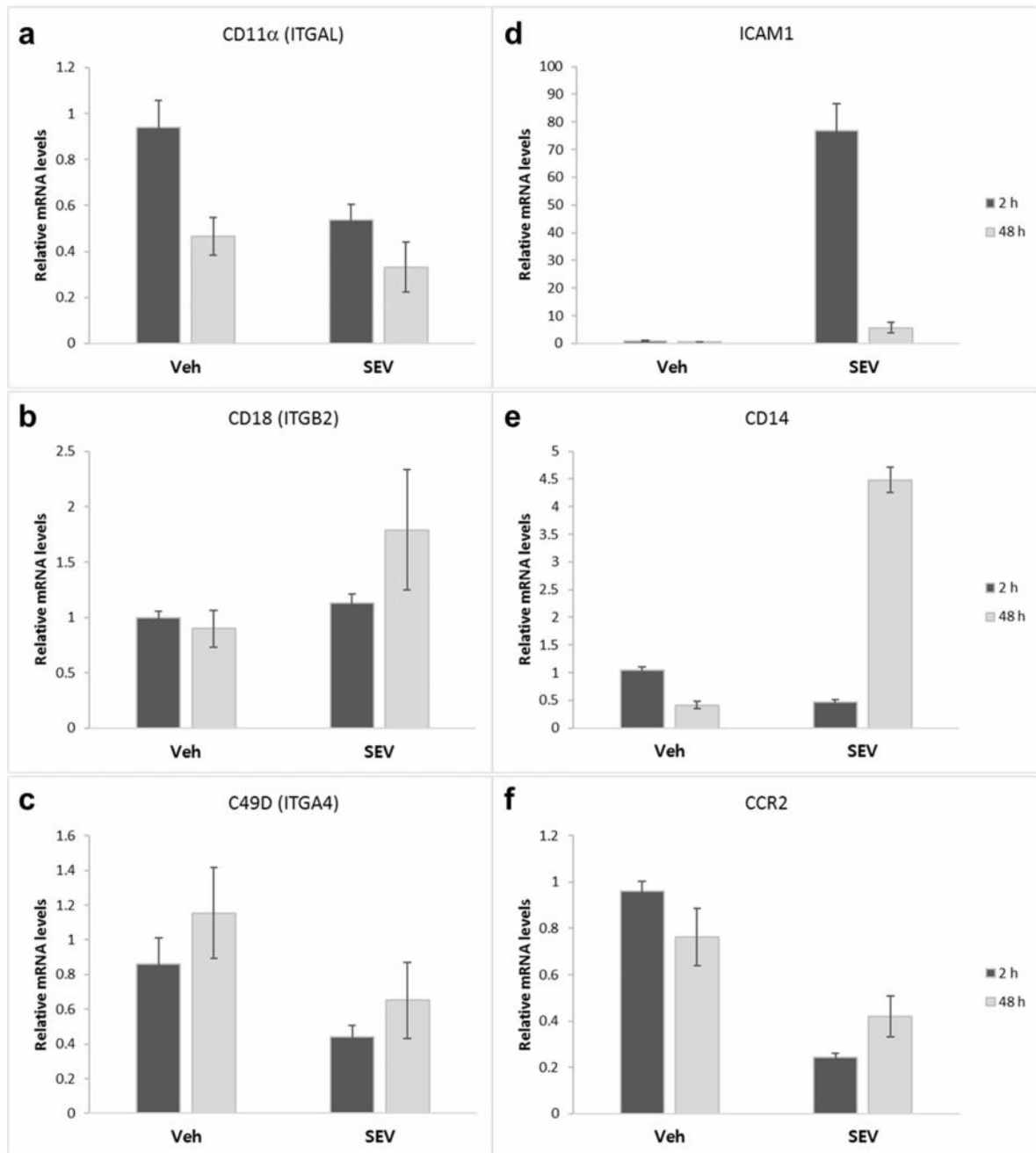
Supplementary Figure S2. The effect of SEVs on tube formation and migration of human endothelial cells. HDMECs (a) or HUVECs (b) were treated with VEGF (10 ng/ml) or various concentrations of SEVs in 1% serum-containing basal medium. The representative images for tube formation were taken at 16 h post-treatment under phase-contrast microscopy. Magnification, $\times 200$. (c) HDMECs pretreated with SEVs for 6 h in 1% serum-containing basal medium were plated in the Boyden chamber and migrated for 6 h. The migrated cells were stained with hematoxylin and eosin and counted per area. The data are expressed as the mean $\pm$ SD of three independent experiments. -, HBS-treated. Magnification, $\times 200$.



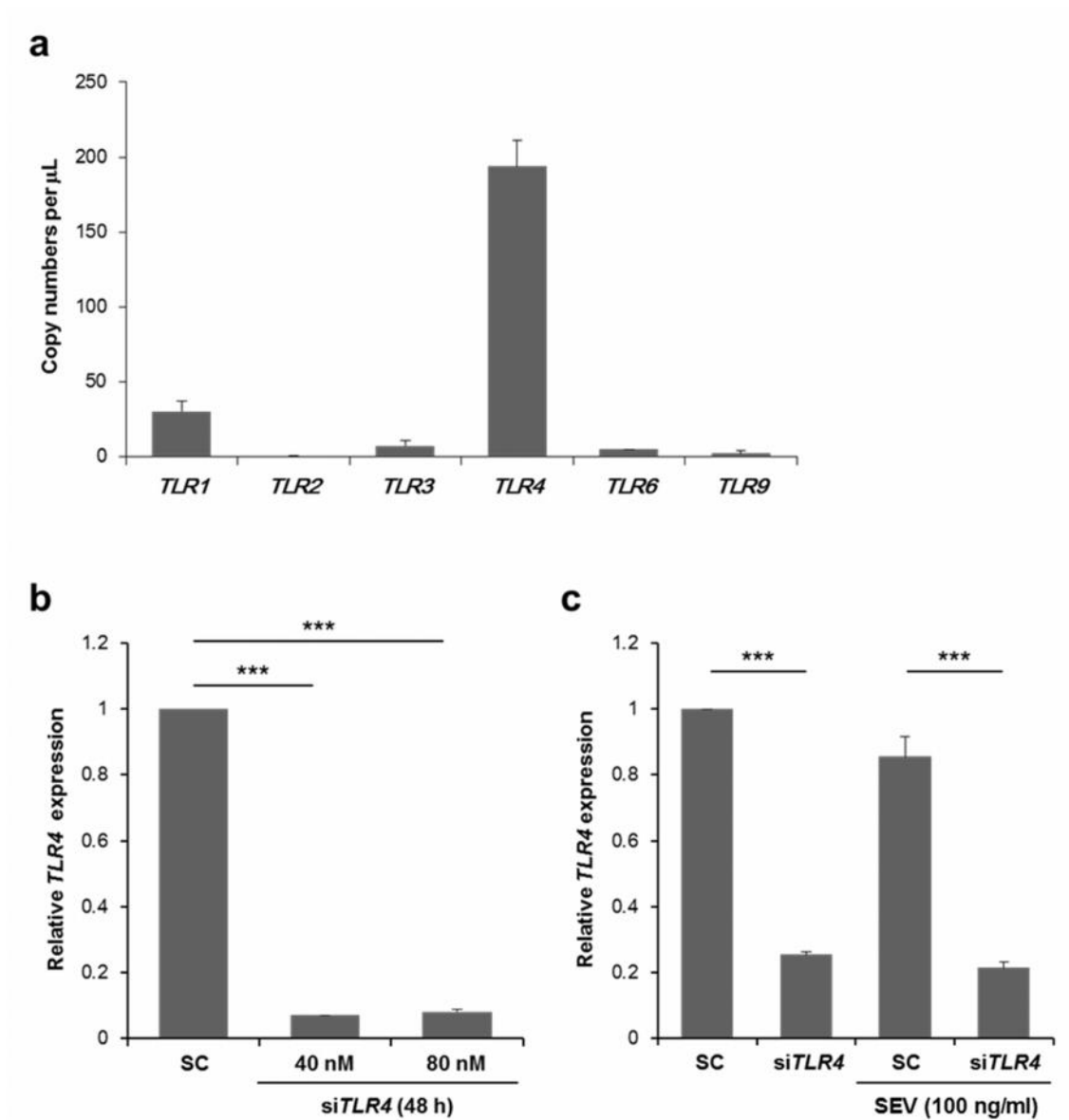
Supplementary Figure S3. The expression patterns of adhesion molecules under different media conditions. HDMECs were cultured in 1% or 5% serum-containing media and treated with the indicated concentrations of SEVs for 6 h. The mRNA levels of *E-Selectin*, *ICAM1* and *VCAM1* were measured by qRT-PCR and normalized to *GAPDH* levels. TNF- α was used as a positive control. -, HBS-treated.



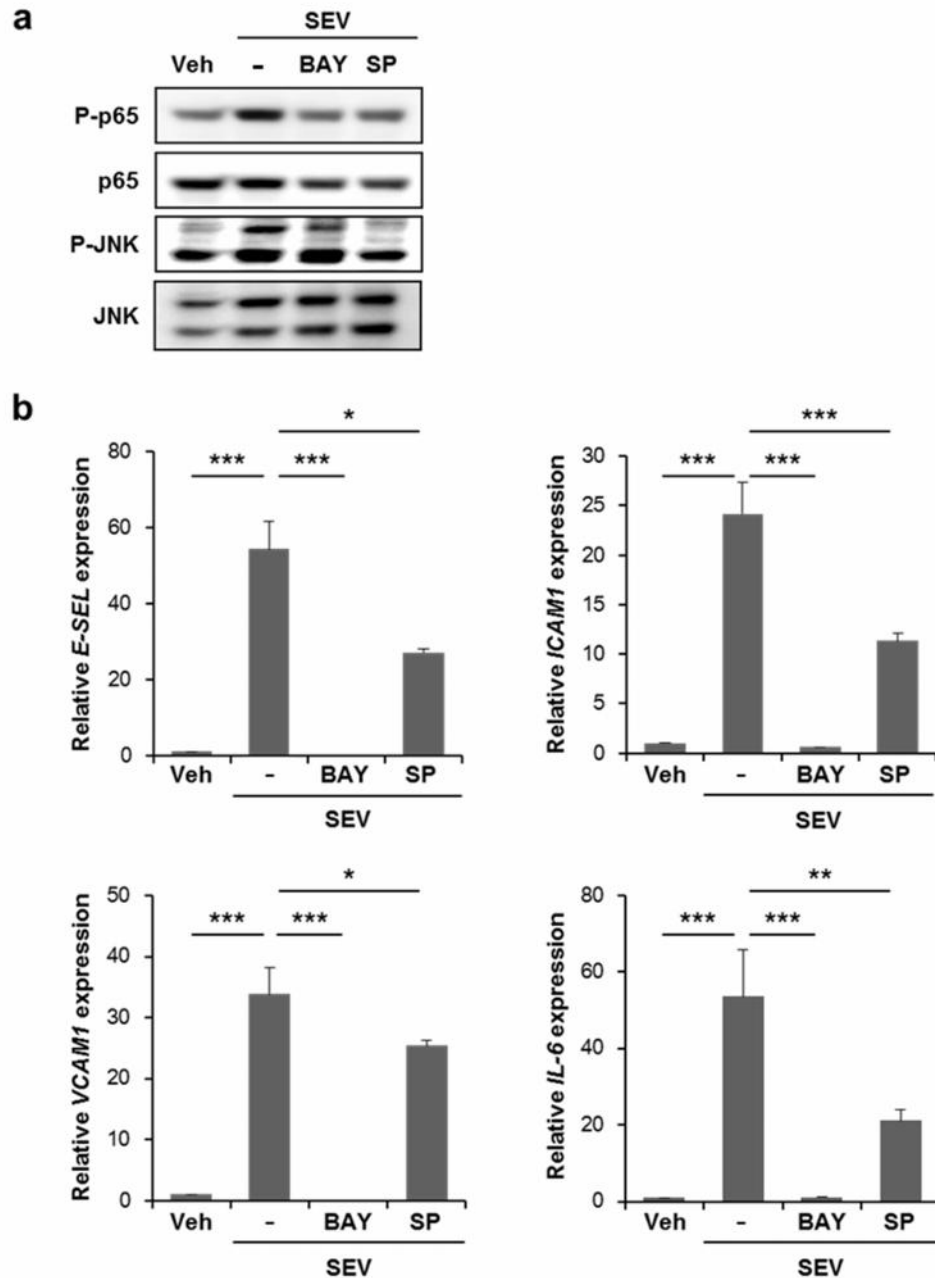
Supplementary Figure S4. The expression patterns of adhesion molecules during SEV treatment. HDMECs were treated with SEVs (100 ng/ml) for various times in 1% or 5% serum-containing media. The mRNA levels of adhesion molecules were analyzed by qRT-PCR and normalized to *GAPDH* levels. -, HBS-treated.



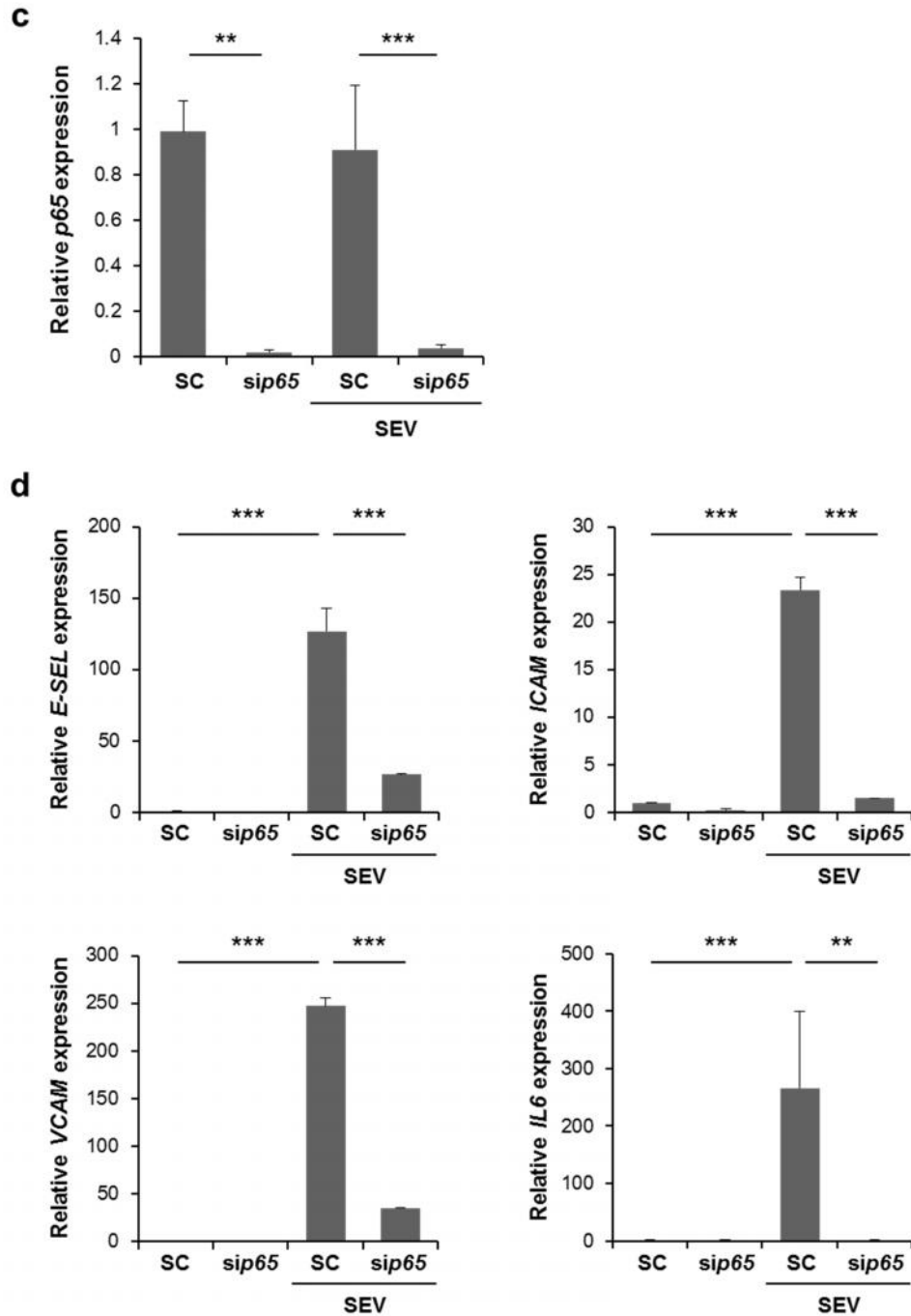
Supplementary Figure S5. The mRNA expression levels of receptors for cell adhesion molecules and differentiation markers for macrophage lineage after SEV treatment in THP-1 cells. Human monocytic THP-1 cells were treated with SEVs (1 μ g/ml) for 2 h or 48 h in 10% serum-containing media. The mRNA levels of receptors for adhesion molecules (a-c) and differentiation markers for macrophage lineage (d-f) were analyzed by qRT-PCR using six samples from two biological replicates and normalized to *GAPDH* levels. The mRNA levels of the SEV-treated group at 2 h and Veh- or SEV-treated groups at 48 h were relatively compared to those of Veh-treated group at 2 h. Veh, HBS-treated.



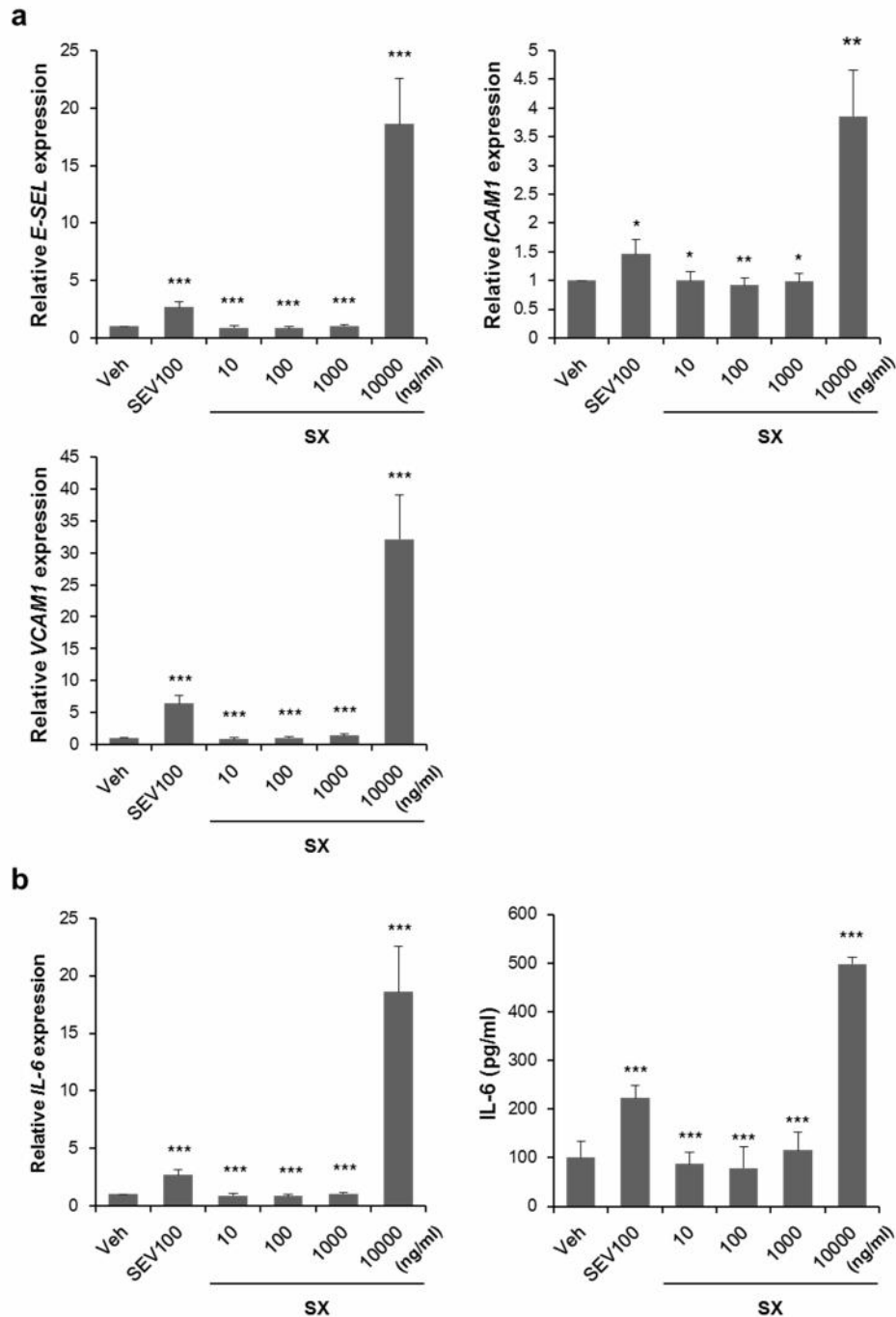
Supplementary Figure S6. The expression levels of *TLR4* in HDMECs. (a) The endogenous transcript levels of *TLR* family members in HDMECs were analyzed by ddPCR. (b) HDMECs were transfected with 40 or 80 nM *TLR4* siRNAs for 48 h. The knockdown of *TLR4* was confirmed by qRT-PCR. The data are expressed as the mean $\pm$ SD of three independent experiments (***, $P < 0.001$). (c) HDMECs were transfected with *TLR4* siRNAs for 48 h and then treated with SEVs (100 ng/mL) for another 6 h. *TLR4* mRNA levels were analyzed by qRT-PCR. The data are expressed as the mean $\pm$ SD of three independent experiments (***, $P < 0.001$).



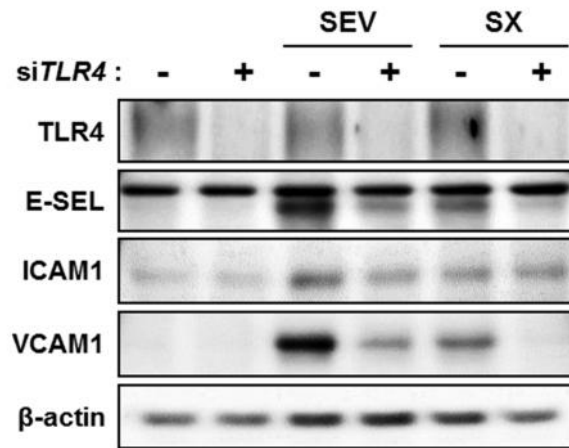
Supplementary Figure S7. The effects of signaling inhibitors or p65 siRNAs on the expression of adhesion molecules and IL-6. (a, b) HDMECs were pretreated with 5 μ M BAY 11-7082 or 10 μ M SP600125 for 1 h and then incubated with SEV (100 ng/ml) for an additional 6 h. (a) The total of phosphorylated p65 (p65 vs. P-p65) and JNK (JNK vs. P-JNK) protein levels were analyzed by western blotting. (b) The mRNA levels of adhesion molecules and IL-6 were analyzed by qRT-PCR. Veh, HBS-treated; -, DMSO-treated; BAY, BAY 11-7082; SP, SP600125.



(c, d) HDMECs were transfected with scrambled control (SC) or *p65* siRNAs for 48 h and then treated with SEV (100 ng/ml) for 6 h. The mRNA levels of *p65* (c), adhesion molecules, and IL-6 (d) were determined by qRT-PCR. The data in (b), (c), and (d) are expressed as the mean $\pm$ SD of three independent experiments (*, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$).



Supplementary Figure S8. The effects of *S. aureus* extracts at various protein concentrations on the expression of adhesion molecules and IL-6. HDMECs were treated with SEVs (100 ng/ml) or various concentrations of *S. aureus* derived extracts (SXs) for 6 h. (a) The mRNA levels for adhesion molecules were analyzed by qRT-PCR and normalized to *GAPDH* levels. (b) The mRNA (left) and protein (right) levels of IL-6 were analyzed by qRT-PCR and ELISA, respectively. The data are expressed as the mean $\pm$ SD of three independent experiments (*, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$ compared to vehicle control). Veh, HBS-treated.



Supplementary Figure S9. TLR4-dependent regulation of adhesion molecules by SEVs and SXs. HDMECs were transfected with scrambled control (SC) or TLR4 siRNAs (siTLR4) for 48 h and then treated with 100 ng/ml SEVs or SXs for another 6 h. The protein expression of TLR4 and adhesion molecules was assessed by western blotting. β -actin was used as a loading control.