

Article

Macrophage-Derived Extracellular Particles Conditioned by Vicenin-2 and Advanced Glycation End-Products Reduce TNF- α -Induced ICAM-1 and VCAM-1 Transcript Expression in Human Endothelial Cells

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Abstract: The contribution of macrophage-mediated inflammation to endothelial dysfunction and atherosclerosis has been recognised for several decades. More recent evidence has implicated cell-derived mediators—extracellular particles and vesicles—as central to the intercellular crosstalk that sustains this inflammatory milieu. Extracellular particles (EPs) encompass all structures released by cells into the extracellular space, whereas extracellular vesicles (EVs) represent the subset enclosed by a lipid bilayer membrane and incapable of self-replication. The glycosidic flavonoid Vicenin-2 (V-2) has demonstrated immune-regulatory properties in macrophages; however, its capacity to modulate EP-mediated endothelial responses remains an emerging area of research. This study aimed to determine the effect of V-2 on the release of EPs from differentiated THP-1 macrophages (dTHP-1) stimulated with an advanced glycation end product (AGE; gBSA), and to determine whether these particles alter endothelial cell inflammatory gene expression. Human umbilical vein endothelial cells (HUVECs) were primed by incubating cells with the dTHP-1-derived EPs and subsequently stimulated with tumour necrosis factor- α (TNF- α). The mRNA expression of intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and monocyte chemoattractant protein-1 (MCP-1) was assessed by Reverse Transcription-Polymerase Chain Reaction. EPs derived from V-2-conditioned dTHP-1 cells significantly reduced TNF- α -induced ICAM-1 expression in HUVECs ($p < 0.001$), while EPs from V-2 + gBSA-conditioned cells additionally reduced TNF- α -induced VCAM-1 transcript levels ($p < 0.001$) and produced a non-significant reduction in MCP-1 ($p = 0.16$). These protective effects were EP-mediated and were not recapitulated by direct V-2 treatment at equivalent concentrations. Although further mechanistic investigation is required, these data support a novel role for macrophage-conditioned EPs in mitigating AGE-driven endothelial dysfunction and highlight V-2 as a candidate agent for modulating EP-mediated vascular inflammation in the context of diabetic vasculopathy.

Keywords: Extracellular Particles; Diabetes; Inflammation; Endothelial Inflammation; THP-1; ICAM-1; VCAM-1

1. Introduction

Endothelial dysfunction marks an early, pivotal point in vascular disease, characterised by reduced nitric oxide bioavailability, heightened oxidative stress, and a shift towards a pro-inflammatory endothelial phenotype [1].

One of its defining features is the upregulation of cell adhesion molecules—ICAM-1 and VCAM-1—alongside the chemokine MCP-1, which together drive monocyte recruitment and transendothelial migration. This sets off a vascular inflammatory cascade implicated in atherosclerosis, hypertension, and diabetic vasculopathy [1,2].

Advanced glycation end-products (AGEs) are a diverse family of protein and lipid adducts formed through non-enzymatic glycation of free amino groups, and their levels are markedly increased due to sustained high levels of blood glucose—hyperglycaemia [3]. By attaching to their cognate receptors (receptor for AGE; RAGE) on endothelial cells and monocytes, AGEs switch on downstream transcription factors—primarily via NF- κ B—that drive expression of pro-inflammatory cytokines and adhesion molecules [3,4]. The AGE–RAGE signalling axis has been established as a key contributor to cardiovascular disease through modulating several mechanisms—oxidative stress, inflammatory signalling, and mitochondrial dysfunction within or in the absence of a diabetic state [4]. Glycated bovine serum albumin (gBSA) is a long-standing in vitro tool for modelling this response; gBSA exposure renders endothelial cells more coagulable and more inflammatory [5]. In a recent study, Alanazi et al. demonstrated that conditioned medium obtained from THP-1 macrophages treated with AGE also reproduces the response of elevated inflammatory cytokines and induces barrier disruption in endothelial cells [6].

Extracellular particles (EPs) encompass all structures released by cells into the extracellular space, whereas extracellular vesicles (EVs) represent the subset of these particles that are delimited by a lipid bilayer membrane and are incapable of self-replication. Extracellular vesicles (EVs) are a category of cell-derived vesicles that includes exosomes (30–150 nm) and microvesicles (100–1,000 nm) and are released by almost every cell type, both constitutively and in response to activation [7,8]. Circulating EVs carry proteins, lipids, and non-coding RNAs between cells, making them important vehicles for intercellular communication [7]. Extracellular vesicles (EVs) have emerged as central players in cell-to-cell communication, acting as molecular delivery vehicles that shuttle signals between cells [9]. A growing body of work links EVs to the development of endothelial dysfunction, largely through their capacity to drive inflammatory responses in endothelial cells [10]. This crosstalk between macrophages and endothelial cells is thought to be especially important in diabetes, where it contributes to one of the condition's major vascular complications: atherosclerosis. Within the vasculature, EVs from activated monocytes and macrophages have been shown to deliver pro-inflammatory and procoagulant signals to the endothelium, contributing to endothelial activation, adhesion molecule upregulation, and loss of barrier integrity [11,12]. Wang and colleagues, for instance, found that EVs from LPS-stimulated THP-1 macrophages substantially increased ICAM-1 and VCAM-1 expression in HUVECs through an NF- κ B-dependent pathway [11], while Ismail and colleagues showed that macrophage-derived microvesicles transfer microRNA (miR)-223 to recipient monocytes and drive their differentiation [12].

Leroyer and colleagues offered some of the clearest evidence for this link when they examined human atherosclerotic plaques directly. They found roughly 200-fold more EVs in plaque tissue than in circulating blood, and macrophage-derived vesicles made up the largest single fraction of those isolated [13]. That finding points to macrophages, acting through their EVs, as a key driver of the endothelial dysfunction seen in atherosclerotic disease.

Mechanistic studies have since begun to elucidate the pathways involved. Mesri and Altieri showed that extracellular vesicles (EVs) isolated from human leukocytes stimulate HUVECs to secrete the proinflammatory mediators IL-6 and MCP-1 [14], while Wang et al. found that microparticles shed by LPS-stimulated THP-1 cells carry IL-1 β along with components of the inflammasome. These vesicles activate endothelial cells and increase adhesion molecule expression through an IL-1 β -dependent pathway [11]. Such vesicles are not merely proinflammatory; they can also be directly cytotoxic. In primary lung endothelial cells, EVs derived from activated THP-1 macrophages induce apoptosis through a caspase-1-dependent mechanism [15]. Collectively, this indicates that EVs secreted by activated monocyte-macrophages damage the endothelium through two processes: sustained local inflammation and compromised vascular integrity through cell death.

Flavonoids have been shown to influence the anti-inflammatory effects of EVs released from mesenchymal stem cells in vitro [16]. Vicenin-2 (V-2) is a glycosidic flavonoid—apigenin 6,8-di-C-glucoside—increasingly recognised for its immunomodulatory properties [17]. Previous work from this group found that V-2 modulates cytokine release and promotes M2-associated marker expression in differentiated THP-1 (dTHP-1) macrophages, partly through upregulation of TET-2 [18]. Furthermore, the macrophage polarisation state has been demonstrated to influence the composition and downstream activity of the EVs it releases [19]. Taken together, this raises the possibility that V-2 exerts its anti-inflammatory effects, at least in part, by altering the release and composition of EVs from dTHP-1 cells.

Populations of extracellular particles and vesicles are commonly isolated by differential ultracentrifugation, as in the present study. This technique alone, however, does not establish vesicular identity, which requires additional morphological and marker-based characterisation. As such characterisation was not undertaken here, the preparations are designated extracellular particles (EPs) throughout. The term extracellular vesicle is reserved for descriptions of published work in which vesicular identity was demonstrated.

Therefore, this present study aimed to explore whether EPs from conditioned dTHP-1 cells modulate TNF- α -induced expression of ICAM-1, VCAM-1 and MCP-1 in HUVECs. Although emerging evidence has started to link EVs from AGE-stimulated macrophages to endothelial dysfunction [20], this remains an emerging area, and to our knowledge no study has examined whether V-2 can modulate this EP-mediated crosstalk.

2. Materials and Methods

2.1. Cell Culture

THP-1 monocytes (ATCC®, TIB-202™) were grown in RPMI 1640 (Gibco, UK) supplemented with 10% heat-inactivated foetal calf serum and 1% each with sodium pyruvate, non-essential amino acids and penicillin–streptomycin (final 100 IU/mL and 100 μ g/mL). Cultures were kept at 37 °C under 5% CO₂ in a humidified incubator and split before density reached 1.5×10^6 cells/mL, equivalent to 80% confluence; only passages 7 to 25 were used. The macrophage-like dTHP-1 phenotype was obtained by exposing cultures for 48 h to 5 ng/mL phorbol-12-myristate-13-acetate (PMA) in the same supplemented medium, after which the medium was replaced, and a further 48 h of rest was allowed before any experiment began. Density at the point of PMA addition was $3\text{--}6 \times 10^5$ cells/mL throughout. Culture and differentiation conditions follow those set out in our own earlier protocol [21].

HUVECs (pooled donor) were obtained from Lonza Ltd (Switzerland) and carried in endothelial basal medium-2 with the manufacturer's supplement set (EGM™-2 BulletKit). Cells were used between passages 3 and 7 in the experiments, and treatment began once monolayers had reached 80–90% confluence.

2.2. dTHP-1 Treatment and Collection of Conditioned Medium

Differentiated THP-1 cells were cultured by seeding in 75 cm² flasks at a density of 6×10^5 cells/mL. V-2 was added to a final 80nM 1 h ahead of glycated BSA (gBSA, 500 μ g/mL) treatment, and the cells were then left for a further 24 h. Four treatment arms were run: untreated control, V-2 alone, gBSA alone, and V-2 + gBSA. The concentration of 80nM V-2 was selected on the basis of previous work from this laboratory in the same dTHP-1 model, in which this concentration was sufficient to modify cytokine secretion and induce M2-associated marker expression [18]. Following incubation, conditioned medium was collected for further processing as per Section 2.3. EP isolation medium was prepared using Foetal Bovine Serum (FBS) pre-cleared of endogenous EPs by ultracentrifugation at $100,000 \times g$ for 16 h. It is acknowledged that ultracentrifugation-based depletion does not remove bovine vesicles completely, and that residual material may therefore be present in all arms including controls.

2.3. Isolation of Extracellular Particles (EPs)

Differential ultracentrifugation, as employed in the present study, remains a mainstay for isolating extracellular material; the technique nonetheless co-isolates vesicles alongside non-vesicular particles, protein aggregates and lipoproteins [22]. Current consensus guidance from the International Society of Extracellular Vesicles (ISEV) therefore requires that vesicular identity be demonstrated before a preparation is assigned to the EV category. The recommended evidence comprises transmission electron microscopy, immunoblotting for transmembrane and cytosolic vesicle markers (such as CD63, CD81, TSG101 or Alix), and immunoblotting for a negative marker of cellular contamination (such as calnexin) [23]. As this characterisation was not undertaken, the preparations described here are designated extracellular particles (EPs), a term reflecting the isolation procedure rather than confirmed vesicular identity. Accordingly, no claim is made that the biological effects reported below are mediated by extracellular vesicles specifically, and the active species within these preparations remains undefined.

EPs were isolated from conditioned medium by sequential differential ultracentrifugation: $300 \times g$ for 10 min (cell debris), $2,000 \times g$ for 20 min (apoptotic bodies), $10,000 \times g$ for 30 min (large vesicles), and $100,000 \times g$ for 70 min (EPs). The final EP pellet was resuspended in phosphate-buffered saline (PBS) and stored at -80 °C until use. EP concentration (particles/mL) and size distribution were determined by nanoparticle tracking analysis (NTA)

using the NanoSight NS300 system with NTA v3.1 software (Malvern Panalytical). All samples were measured in triplicate at 25 °C.

2.4. HUVEC Treatment with Extracellular Particles and TNF- α Stimulation

HUVECs were seeded in 12-well plates in complete EGM-2 as described in Section 2.1 and grown to 80–90% confluence before treatment. EP preparations were thawed on ice immediately before use, having undergone a single freeze–thaw cycle since isolation. For each condition, 100 μ L of EP suspension in PBS—derived from the conditioned medium of the corresponding dTHP-1 treatment group (control, V-2, gBSA or V-2 + gBSA)—was added to each well and incubated for 16 h at 37 °C, then stimulated with 100 ng/mL TNF- α for 4 h before isolation of RNA. Vehicle control wells received an equal volume (100 μ L) of PBS.

2.5. RNA Extraction and Quantitative RT-PCR

Total RNA was extracted from HUVECs using TRIzol reagent (Invitrogen, Thermo Fisher Scientific) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesised from 500 ng of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Thermo Fisher Scientific). Quantitative real-time PCR was carried out on an Applied Biosystems (7500 Real-Time PCR System) using TaqMan® assays with the following validated primer/probe sets (Thermo Fisher Scientific): ICAM-1 (Hs00164932_m1), VCAM-1 (Hs01003372_m1) and MCP-1/CCL2 (Hs00234140_m1). The housekeeping gene GAPDH (Hs02758991_g1) was used for normalisation. Relative gene expression was calculated using the $2^{-\Delta\Delta C_t}$ method and expressed as fold-change relative to untreated control.

2.6. Statistical Analysis

All data are expressed as mean $\pm$ standard deviation (SD). Statistical comparisons between groups were performed by one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple comparisons, using GraphPad Prism Version 9. Differences were considered statistically significant at $p < 0.05$. All experiments were conducted in triplicate or quadruplicate as stated; significance levels are denoted as follows: ** $p < 0.01$; *** $p < 0.001$; NS = not significant.

3. Results

3.1. V-2 Amplifies gBSA-Induced Particle Release from dTHP-1 Macrophages

To establish whether V-2 modulates extracellular particle biogenesis under AGE-driven inflammatory conditions, dTHP-1 macrophages were pre-treated with 80nM V-2 prior to stimulation with the AGE compound gBSA, and the EP released by cells were quantified. V-2 treatment alone did not significantly alter basal EP release relative to untreated control ($p = 0.98$; **Figure 1**). By contrast, stimulation with gBSA alone significantly increased EP release by 1.9-fold compared to control ($p < 0.01$; **Figure 1**). Pre-treatment with V-2 prior to gBSA stimulation amplified this response further, producing a 3.7-fold increase in EP output relative to control ($p < 0.001$, **Figure 1**)—demonstrating an amplified effect between V-2 and AGE-induced stimulation on particle biogenesis in dTHP-1 macrophages.

3.2. Direct V-2 Treatment Differentially Modulates TNF- α -Induced Adhesion Molecule and Chemokine Transcript Levels in HUVECs

In order to delineate the direct effects of V-2 as a potential anti-inflammatory agent on endothelial cells compared to the EP-mediated effects, HUVECs were pre-treated with 80nM V-2 and subsequently stimulated with the proinflammatory cytokine TNF- α . TNF- α is known to induce the expression of adhesion molecules ICAM-1 and VCAM-1, which in turn facilitate the binding of monocytes to the endothelial layer [10,20]. In line with previous findings, TNF- α significantly induced ICAM-1 and VCAM-1 expression ($p < 0.001$; **Figure 2a,b**). Interestingly, V-2 co-treatment significantly augmented TNF- α -induced ICAM-1 upregulation ($p < 0.001$; **Figure 2a**) but did not significantly alter TNF- α -induced VCAM-1 expression ($p = 0.98$; **Figure 2b**). At the concentration used here, V-2 applied directly to endothelial cells does not reduce the TNF- α -induced ICAM-1 and VCAM-1 expression; in the case of ICAM-1, it acts to upregulate transcript expression. A distinct expression pattern was observed for the endothelial

cell-derived chemokine, MCP-1. V-2 treatment alone significantly induced MCP-1 transcript levels, whereas in the presence of TNF- α , V-2 significantly attenuated MCP-1 expression relative to TNF- α alone ($p < 0.001$; **Figure 2c**).

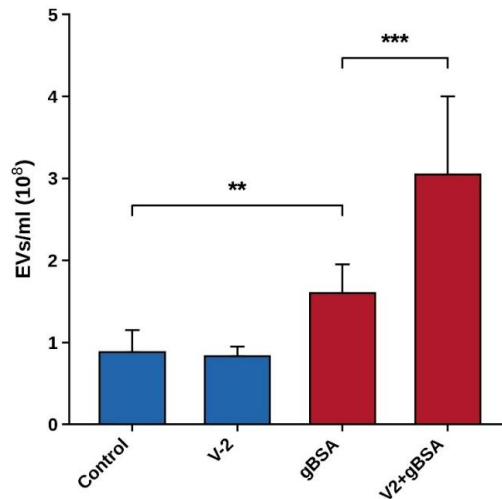


Figure 1. Effect of V-2 on gBSA-induced extracellular particle release from dTHP-1 macrophages. Differentiated THP-1 cells were pre-treated with 80nM V-2 for 1 h, then stimulated with 500 μ g/mL gBSA for 24 h. Graph showing EP count ($\times 10^8$ particles/mL) across four treatment groups: Control, V-2, gBSA, and V-2 + gBSA.

Note: ** $p < 0.01$, *** $p < 0.001$; $n = 3$, mean $\pm$ SD.

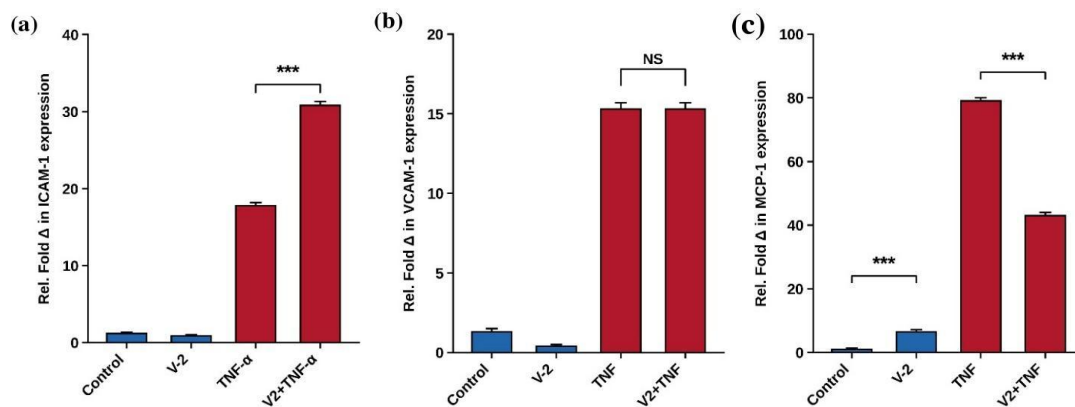


Figure 2. Direct effect of V-2 on TNF- α -induced inflammatory gene expression in HUVECs. HUVECs were pre-treated with 80nM V-2 for 1 h, then stimulated with 100 ng/mL TNF- α for 4 h. Relative mRNA expression of (a) ICAM-1, (b) VCAM-1 and (c) MCP-1, quantified by TaqMan® RT-PCR and expressed as fold change relative to untreated control ($2^{-\Delta\Delta C_t}$ method).

Note: *** $p < 0.001$, NS = not significant; $n = 4$, mean $\pm$ SD.

3.3. Particles from V-2-Conditioned dTHP-1 Cells Reduce TNF- α -Induced ICAM-1 Transcript Expression in HUVECs

It is noteworthy that because preparations were dosed by volume (100 μ L) rather than by particle number or protein mass, and particle output varied up to 3.7-fold across donor-cell conditions (Section 3.1), the following comparisons confound the composition of the delivered material with its amount. This also applies to the data reported in Sections 3.4 and 3.5.

To investigate whether the anti-inflammatory properties of V-2 are transmitted to the endothelial cells via the released particles, HUVECs were incubated with EPs isolated from conditioned dTHP-1 cells and subsequently stimulated with TNF- α . TNF- α treatment significantly increased ICAM-1 transcript levels in the HUVECs (44.5 ± 0.17 -fold; $p < 0.001$). Pre-incubation with EPs from V-2-conditioned dTHP-1 cells [EP(V-2)] significantly reduced

TNF- α -induced ICAM-1 transcript levels (EP(V-2) + TNF- α : 37.3 ± 0.02 -fold; $p < 0.001$; **Figure 3**). Likewise, EPs from V-2 + gBSA-conditioned cells also significantly reduced ICAM-1 expression (EP(V-2 + gBSA) + TNF- α : 41.8 ± 0.24 -fold; $p < 0.001$; **Figure 3**). EPs from control and gBSA-conditioned cells did not significantly alter TNF- α -induced ICAM-1 expression.

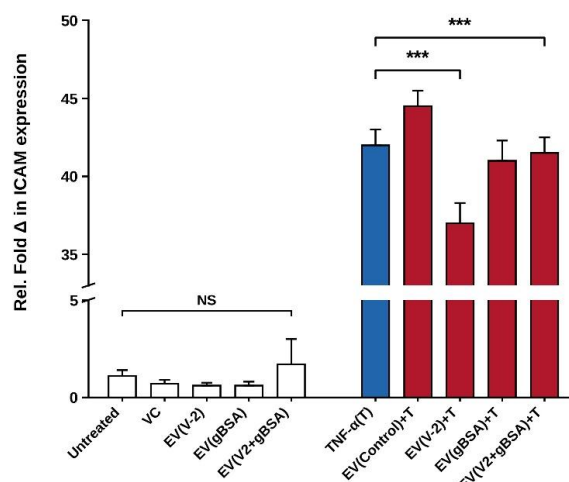


Figure 3. Effect of dTHP-1-derived extracellular particles on TNF- α (T)-induced ICAM-1 mRNA expression in HUVECs. Differentiated THP-1 cells were pre-treated with 80nM V-2 for 1 h, then stimulated with 500 μ g/mL gBSA for 24 h. HUVECs were incubated with 100 μ L of vehicle control (PBS) or particle suspension for 16 h, then stimulated with 100 ng/mL TNF- α for 4 h. Graph showing relative ICAM-1 mRNA expression, quantified by RT-PCR.

Note: *** $p < 0.001$, NS = not significant; $n = 4$, mean $\pm$ SD.

3.4. Particles from V-2 + gBSA-Conditioned dTHP-1 Cells Reduce TNF- α -Induced VCAM-1 Transcript Expression in HUVECs

Particles isolated from dTHP-1 cells treated with the combination of V-2 and gBSA produced a statistically significant reduction in TNF- α -induced VCAM-1 expression in HUVECs (TNF- α : 72.6 ± 0.16 -fold vs. EP(V-2 + gBSA) + TNF- α : 70.0 ± 0.16 -fold; $p < 0.001$; **Figure 4**). Particles from the other treatment conditions—control, V-2 alone, and gBSA alone—did not significantly alter TNF- α -induced VCAM-1 expression.

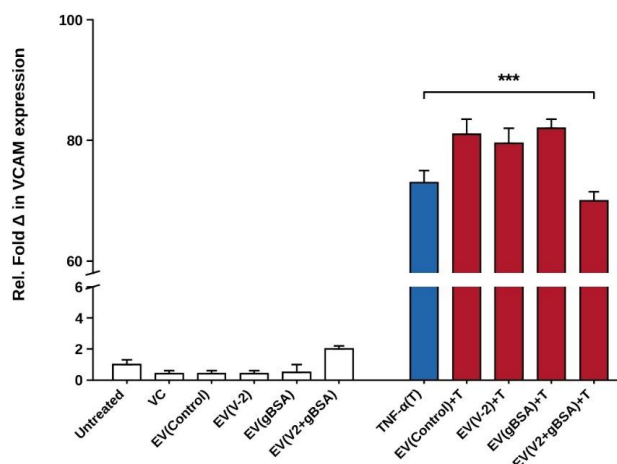


Figure 4. Effect of dTHP-1-derived EPs on TNF- α (T)-induced VCAM-1 mRNA expression in HUVECs. Differentiated THP-1 cells were pre-treated with 80nM V-2 for 1 h, then stimulated with 500 μ g/mL gBSA for 24 h. HUVECs were incubated with 100 μ L of vehicle control (PBS) or EP suspension for 16 h, then stimulated with TNF- α for 4 h. Graph showing relative VCAM-1 mRNA expression, quantified by RT-PCR.

Note: *** $p < 0.001$, NS = not significant; $n = 4$, mean $\pm$ SD.

3.5. Particle-Associated Effects on TNF- α -Induced MCP-1 Transcript Expression Did Not Demonstrate Statistical Significance

Following HUVEC incubation with particles from conditioned dTHP-1 cells and subsequent TNF- α stimulation, a trend toward reduced MCP-1 transcript levels was observed with EPs from V-2 + gBSA-conditioned cells; however, this did not reach statistical significance ($p = 0.16$; **Figure 5**). No significant differences in TNF- α -induced MCP-1 expression were observed with EPs from any other treatment group.

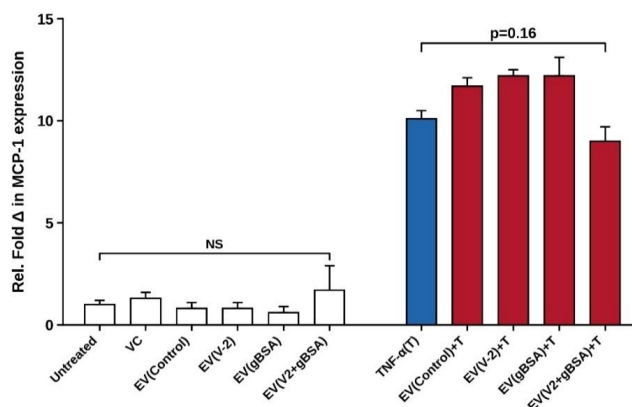


Figure 5. Effect of dTHP-1-derived EPs on TNF- α (T)-induced MCP-1 mRNA expression in HUVECs. Differentiated THP-1 cells were pre-treated with 80nM V-2 for 1 h, then stimulated with 500 μ g/mL gBSA for 24 h. HUVECs were incubated with 100 μ L of vehicle control (PBS) or EP suspension for 16 h, then stimulated with TNF- α for 4 h. Graph showing relative MCP-1 mRNA expression, quantified by RT-PCR.

Note: NS = not significant; n = 4, mean $\pm$ SD.

4. Discussion

Our current study aimed to assess whether the glycosidic flavonoid, V-2, in addition to its regulatory effects on pro-inflammatory cytokines and M2-like polarisation of dTHP-1 cells, affects extracellular particle-mediated crosstalk between macrophages and endothelial cells within a pro-diabetic milieu. The majority of previous studies have utilised LPS to activate macrophages. However, because AGEs play a pivotal role in diabetic vascular disease [24], we employed gBSA in our study. Our results show that there is a significant increase in the quantity of EPs released from dTHP-1 cells when exposed to gBSA alone, and an even greater increase in the presence of V-2. Our results indicate that co-stimulation of V-2 and gBSA demonstrated an amplified effect on particle biogenesis in dTHP-1 macrophages. However, an important caveat should be considered while interpreting this experiment. Namely, particle yield is expressed per millilitre of conditioned medium rather than per viable donor cell; if gBSA alters dTHP-1 adherence, proliferation or survival, an apparent change in release could partly reflect a change in cell number.

Next, we investigated whether direct treatment of HUVECs with V-2 alters the transcript levels of pro-inflammatory markers ICAM-1, VCAM-1 and the chemokine MCP-1. Our results indicate that direct treatment of HUVECs with V-2 did not significantly attenuate TNF- α induced ICAM-1 and VCAM-1 transcript levels. In the case of ICAM-1, the response was unexpected, with a significant increase in ICAM-1 transcript levels observed upon co-stimulation with V-2 and TNF- α . This contrasts starkly with the findings of Kang et al. [25], who reported that V-2 reduced ICAM-1, VCAM-1, IL-6 and TNF- α in HUVECs. The most probable explanation for this discrepancy is the concentration employed in each of the studies. Kang et al. used 20 μ M, which was 250-fold higher than the concentration employed here. To contextualise this, the reported pharmacology of the compound has shown that the anti-inflammatory IC_{50} values for V-2 in macrophage systems lie in the low micromolar range, at 6.8 μ M for suppression of TNF- α transcript levels and 5.2 μ M for nitric oxide production [26]. At 80 nM, the concentration used in the present study is roughly two orders of magnitude below that range. The absence of a direct protective effect on the endothelium is therefore the expected result.

Direct exposure of HUVECs to V-2 at the concentration tested failed to reduce TNF- α -driven ICAM-1 or VCAM-1

transcript levels. On this basis, attention turned to the alternative possibility that particles released by conditioned dTHP-1 cells, rather than the flavonoid itself, account for any endothelial effect. Preparations derived from cells exposed to V-2 in isolation produced a significant reduction in TNF- α -driven ICAM-1 mRNA expression, while those obtained from the V-2/gBSA condition suppressed both ICAM-1 and VCAM-1 relative to TNF- α alone. There was also an observed reduction in TNF- α -induced MCP-1 expression, although the effect was not statistically significant. Overall, our data provide additional evidence for the anti-inflammatory effects of EPs derived from activated THP-1 macrophages on inflammatory markers expressed in HUVECs.

At this juncture, it is noteworthy that the disparity between these two sets of observations is the central finding of this study; direct V-2 exposure of HUVECs at 80nM either had no effect on, or augmented, TNF- α -induced adhesion molecule expression, while particle preparations from V-2-conditioned donor cells reduced it. The effect, therefore, cannot be attributed to carry-over of free compound into the particle preparation. This indicates that conditioning the donor macrophages changes the inflammatory profile of the HUVECs.

Our findings in this current study indicate that EPs obtained from dTHP-1 cells conditioned with V-2 and gBSA attenuated TNF- α -induced inflammatory marker transcript levels in the HUVEC model. Since V-2 applied directly to HUVECs did not reduce the endothelial response to TNF- α , and did not significantly alter basal particle release, the effect is putatively attributable to the particles rather than to the compound acting directly.

In a well-designed study that validates the phenomenon we observed in our current work, Ji et al. demonstrated that exosomes secreted by AGE-stimulated macrophages inhibit proliferation, migration and tube formation of HUVECs and induce inflammation through activation of the miR-22-5p/FOXP1 axis [20]. Their study not only confirms that AGE-conditioned macrophage exosomes are genuinely bioactive on endothelial cells, but it also points to a specific miRNA mechanism that could be tested against the EP preparations used in our current study. While the observed effects in their study are attributable to the exosome subpopulation of the macrophage-derived extracellular particles, it is conceivable that the active species in our extracellular particles preparation may overlap with the results reported by Ji et al. Notwithstanding, further characterisation is warranted to confirm this. It is also notable that Ji et al. included a non-glycated BSA control, distinguishing an AGE-specific effect from a protein-load effect. The present study did not include such a control, and this should be regarded as a design limitation: we cannot fully exclude the plausibility that some part of the response we attribute to gBSA reflects exposure to a high concentration of protein rather than to glycation specifically.

In an earlier study by Wang et al., they showed that dTHP-1-derived EVs are internalised by HUVECs, and that EVs from LPS-activated THP-1 cells upregulate ICAM-1 and VCAM-1 through an NF- κ B-dependent mechanism [11]. More recent studies further support this observation; for example, monocyte microvesicles generated in response to *Treponema pallidum* antigen drive HUVEC adhesion molecule expression via ERK1/2–NF- κ B signalling [27], and EVs from high-glucose-treated THP-1 cells impair HUVEC function by delivering miR-142-5p, again converging on NLRP3/NF- κ B signalling [28]. Taken together, this makes NF- κ B modulation a reasonable working hypothesis for how V-2-conditioned particles may act on HUVECs.

Furthermore, the cargo composition of these particles should also be considered. Ismail et al. [12] observed that dTHP-1-derived EVs drive differentiation of THP-1 cells and primary monocytes and identified part of this effect to be attributed to miR-223 contained within the EVs. The microRNA miR-223 has since been implicated as a functionally important EV cargo well beyond monocyte differentiation; with roles in kidney injury, hepatic lipid metabolism, and arthritis, as reviewed by Jiao and colleagues [29]. Therefore, examining the functional cargo composition of V-2-conditioned EPs is relevant to understanding mechanisms underlying their anti-inflammatory activity.

Vesicle biogenesis and cargo sorting are regulated in a context-specific manner, with donor-cell metabolic state and signalling environment determining which molecules are packaged for export [30]. Our prior demonstration that V-2 drives dTHP-1 cells towards an M2-like phenotype, combined with independent confirmation of this polarising effect [31] and with evidence that polarisation state governs the composition of macrophage-derived vesicles [19], provides a coherent framework in which the present observations would be expected. Zhang et al. showed that vesicles released from M1-polarised THP-1 macrophages impaired adipocyte insulin signalling whereas vesicles from M2-polarised macrophages improved glucose uptake [32], and this has since been corroborated mechanistically by work showing that miR-690-rich exosomes from M2 macrophages increase insulin sensitivity in obese mice, an effect lost when miRNA content is depleted [33]. Whether V-2 conditioning might protect against metabolic

dysfunction driven by macrophage-derived particles and vesicles through a similar route is a distinct question from the one addressed here, and one we note only as a separate future direction. Exploring this would be worthwhile given the pace at which researchers are identifying diagnostic and therapeutic roles for extracellular vesicles in metabolic disease, as recently reviewed by Pavlović and colleagues [34].

Limitations

This study has a number of limitations that should be considered when interpreting the findings. The most significant is the absence of complete characterisation of the isolated preparations to confirm EV identity in line with ISEV guidelines. We have accordingly adopted operational nomenclature throughout, referring to extracellular particles (EPs) rather than extracellular vesicles (EVs). This limitation propagates: it means the effects we report cannot be definitively assigned to a defined biological entity, and it constrains comparison with the published literature, most of which reports characterised vesicle preparations.

While the data presented herein are consistent with extracellular particle-mediated, rather than direct, modulation of HUVEC inflammatory signalling, the mechanistic pathways modulated by V-2- and gBSA-conditioned EPs were not directly investigated. However, the NF- κ B hypothesis proposed above—based on previous studies [26,27] remains a plausible mechanism. To confirm the putative involvement of the NF- κ B pathway, immunoblotting for phospho-p65 should be undertaken in the cell system employed here, namely HUVECs. Additionally, confirmatory work using pathway-specific inhibitors or reporter assays would be required to establish causality.

Another important limitation of this study is that the effects of Vivenin-2-conditioned EPs on endothelial inflammatory marker production were solely determined at the RNA transcript level. Since the amounts of RNA transcripts can sometimes not accurately reflect the amount of proteins being produced—due to post-transcriptional regulation of RNA and post-translational modifications of protein—it is necessary to perform additional protein-level analysis such as ELISA and Immunoblotting to determine if the observed differences in gene expression are also reflected in differences in protein production. Additionally, no functional readout, such as monocyte adhesion or transendothelial electrical resistance was performed. This current study, therefore, establishes a transcript-level association only at this stage. A further limitation is that the particle preparations were applied by fixed volume and were not normalised to particle number or protein content, so the doses delivered across treatment groups are not directly comparable. Together with the absence of a non-glycated BSA control noted above, this constrains the inferences that can be drawn about the specificity of the gBSA effect.

It is also noteworthy that the magnitude of several of the statistically significant effects is small. The reduction in VCAM-1 transcript associated with EP(V-2 + gBSA) corresponds to approximately 3.6% of the TNF- α -induced level, and the corresponding ICAM-1 reductions are approximately 16% and 6%. Whether changes of this size have any consequence for endothelial function is unknown, and the statistical significance of these differences should not be read as an indication of biological importance. This consideration reinforces the need for protein-level and functional validation. We further note that the TNF- α concentration used (100 ng/mL) is at the upper end of the range typically applied to HUVECs and produces a near-maximal stimulus; a submaximal stimulus would provide greater dynamic range in which to detect modulation.

Although uptake of dTHP-1-derived vesicles by HUVECs has been shown previously [11], we did not perform labelling experiments to confirm it in our own system, and an effect exerted at the endothelial cell surface rather than intracellularly cannot be excluded.

Finally, all experiments were conducted utilising only one type of endothelial cell (a pooled population of human umbilical vein endothelial cells), and only one donor cell line of macrophage-like cells (PMA-differentiated THP-1 cells). Although human umbilical vein endothelial cells are commonly used to model endothelial function—and represent a well-characterised system—they likely do not accurately model the functional properties of disease-relevant microvascular or arterial endothelia in diabetic vasculopathy. Likewise, THP-1 cells—like most other monocytic/macrophagic cell lines—exhibit different characteristics compared to primary human monocyte-derived macrophages. Therefore, extending this work to primary human macrophages, disease-relevant endothelial cell types, and eventually an *in vivo* model would greatly enhance the translational significance of these findings.

5. Conclusions

This study demonstrates that vicienin-2 (V-2) modulates the quantity of extracellular particles released by AGE-stimulated dTHP-1 macrophages, and extracellular particles obtained from V-2-conditioned macrophages are associated with reduced TNF- α -induced ICAM-1 and VCAM-1 transcript expression in HUVECs. These findings extend the existing literature on macrophage-derived particles in vascular inflammation by identifying V-2 as a novel modulator of EP biogenesis and function under AGE-driven inflammatory conditions—a context of direct relevance to diabetic vasculopathy. Future work should prioritise effective characterisation of the particles along with mechanistic validation of the putative NF- κ B-dependent pathway, and extension of this model to primary human macrophages/disease-relevant endothelial cell types, and in vivo systems.

Author Contributions

Conceptualization, K.M. and N.H.; methodology, N.H.; investigation, N.H.; resources, K.M., N.H., A.A. and Y.K.A.H.; data curation, N.H.; writing—original draft preparation, N.H.; writing—review and editing, N.H.; supervision, K.M.; project administration, N.H. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest

The authors declare no conflicts of interest.

AI Use Statement

The authors declare that no artificial intelligence (AI) tools were used in the preparation (data and analysis) of this manuscript.

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