Inflammatory adipocyte-derived extracellular vesicles promote leukocyte attachment to vascular endothelial cells

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Abstract

Background and Aims: Obesity is associated with an increased risk of cardiovascular disease, but the mechanisms involved are not completely understood. In obesity, the adipocyte microenvironment is characterised by both hypoxia and inflammation. Therefore, we sought to determine whether extracellular vesicles (EVs) derived from adipocytes in this setting might be involved in mediating cardiovascular disease, specifically by promoting leukocyte attachment to vascular endothelial cells.

Methods: Mature 3T3-L1 adipocytes were incubated for 24 hours under control, TNF-α (30 ng/mL), hypoxia (1% O_2), or TNF-α+hypoxia (30 ng/mL, 1% O_2) conditions. EVs were isolated by differential ultracentrifugation and analysed by nanoparticle tracking analysis. Primary human umbilical vein endothelial cells (HUVECs) were treated with EVs for 6 hours before being lysed for Western blotting to investigate changes in adhesion molecule production, or for use in leukocyte attachment assays.

Results: EVs from adipocytes treated with TNF-α and TNF-α+hypoxia increased vascular cell adhesion molecule (VCAM-1) production in HUVECs compared to basal level (4.2 ± 0.6 and 3.8 ± 0.3-fold increase, respectively (p<0.05)), an effect that was inhibited by an anti-TNF-α neutralising antibody. Production of other adhesion molecules (E-selectin, P-selectin, platelet endothelial cell adhesion molecule and VE-Cadherin) were unchanged. Pre-incubating HUVECs with TNF-α+hypoxia EVs significantly increased leukocyte attachment compared to basal level (3.0 ± 0.4-fold increase (p<0.05)).

Conclusions: Inflammatory adipocyte EVs induce VCAM-1 production in vascular endothelial cells, accompanied by enhanced leukocyte attachment. Preventing adipocyte derived EV-induced VCAM-1 upregulation may offer a novel therapeutic target in the prevention of obesity-driven cardiovascular disease.

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Introduction

Obesity is a risk factor for the development of cardiovascular disease (CVD)[1] but the complex mechanisms linking the two together are still to be fully elucidated. Obesity combined with metabolic syndrome (an umbrella term encompassing insulin resistance, dyslipidaemia, vascular endothelial dysfunction, hypertension and a hypercoagulable state), further increases the risk of CVD[2, 3]. Whilst a complete understanding of the mechanisms that trigger adipose tissue to become metabolically dysregulated in obesity are not fully understood, evidence suggests that hypoxia and inflammation play a role.

Obese adipose tissue contains localised regions of hypoxia[4-7] thought to arise due to increased cell number and cell size beyond the distance oxygen can diffuse from capillaries. Free fatty acids and cytokines produced by hypoxic adipocytes attract and activate macrophages and in doing so, induce local tissue inflammation[8]. Under such conditions, physiological adipokine production is dysregulated. For example, release of pro-inflammatory tumour necrosis factor (TNF)-α is increased[9] whilst anti-inflammatory adiponectin is decreased[10]. The elevation of TNF-α and decrease of adiponectin in plasma are individually associated with increased leukocyte attachment to vascular endothelial cells via increased production of vascular endothelial cell adhesion molecules[11, 12]. Subsequent phagocytosis of oxidised low-density lipoproteins within vessel walls results in lipid-laden foam cell formation[13] and later, atherosclerotic plaque development.

Like most cells, adipocytes release extracellular vesicles (EVs)[14, 15]. EVs are membrane-bound structures that can convey biological information from a cell of origin to a recipient cell to achieve a target effect. Based on diameter and biogenesis they are categorised into three broad groupings: exosomes (~80-100 nm; released from multivesicular bodies upon plasma membrane fusion), microvesicles (~200-1000 nm; shed directly from the plasma membrane) and apoptotic bodies (~1-5 µm; released as blebs during apoptosis). *In vitro* data show that adipocytes release EVs containing adipokines[16-19]. In obesity, adipocytes reside in a hypoxic and inflammatory environment, therefore the content and function of adipocyte-derived EVs in this environment may be different to adipocyte-derived EVs from lean adipose tissue. Adipocyte EVs circulating in obesity may contribute to vascular endothelial dysfunction which later leads to atherosclerotic plaque formation. We sought to test this hypothesis *in vitro* using a leukocyte attachment assay with vascular endothelial cells isolated from umbilical cords and EVs derived from adipocytes cultured in conditions representative of lean and obese (hypoxic and inflammatory) adipose tissue.

Materials and Methods

Ethical approval for this study was granted by the NHS Health Research Authority (ethics committee reference: 14/NW/1459), and Cardiff Metropolitan University's Research Ethics Committee.

Adipocyte Culture and EV Isolation

3T3-L1 adipocytes were cultured for 14 days as described by Connolly *et al*[16]. For all EV-isolation experiments, serum-containing culture medium was replaced with serum-free medium for 24 hours. During this time, adipocytes were exposed to one of four treatments representative of control (95 % air / 5 % CO_2), inflamed (30 ng/mL TNF-α (13473019, ThermoFisher, USA)), hypoxic (1 % O_2), and inflamed and hypoxic (30 ng/mL TNF-α, 1 % O_2) adipose tissue. Cell media were centrifuged at 4 °C at 1,000 *g* (5 minutes), 15,000 *g* (15 minutes), and finally 100,000 *g* (1 hour) to pellet EVs. EVs were resuspended in 1× PBS and quantified by nanoparticle tracking analysis (NTA; a technique that tracks nanoparticles in suspension using a laser, and based on Brownian motion, determines particle size and concentration). NTA was performed using a NanoSight LM10 with a 642 nm laser (Malvern Instruments Ltd, Malvern, UK), using software version 2.3, screen gain 4 and camera level 10. Five, one-minute videos were recorded per sample with analysis screen gain 10 and detection threshold 4. Temperature ranged from 20 to 23.5 °C.

HUVEC Isolation and Culture

Human umbilical cords were obtained following elective Caesarean sections. Saline was used to flush blood out of the umbilical vein, before one end was clamped. Collagenase type 1A (1 mg/mL; C5894, Sigma) in Medium 199 (M199; 31150, ThermoFisher) at 37 °C, was syringed into the vein until the cord became taut and then the end of the cord was also clamped. After 15 minutes, one clamp was released allowing the vascular endothelial cell suspension to be collected into a Falcon tube. Copious growth medium (M199 containing 10 % (v/v) foetal bovine serum (10500-064, ThermoFisher), human epidermal growth factor (1 ng/mL; 13453029, ThermoFisher), hydrocortisone (1 µg/mL; H0888, Sigma), gentamycin (35 µg/mL; G1272, Sigma) and amphotericin (0.5 µg/mL; A2942, Sigma)) was added to terminate the enzymatic digestion. Cells were centrifuged (300 *g,* 5 minutes, 4 °C), resuspended in growth medium, and plated in 96-well plates for leukocyte attachment assays or 6-well plates for protein assays. Plates were pre-coated with 1 % (w/v) bovine skin gelatin (G9391, Sigma) in 1× PBS. HUVECs were given 2 hours to adhere, before the medium was aspirated and replaced. HUVECs reached confluency within 4-7 days, were never passaged, and were used for experiments within 7 days.

Leukocyte Isolation

Blood (10 mL) was obtained from healthy volunteers by venepuncture and transferred into a universal container (UC) containing Heparin (100 µL, 5,000 I.U/mL,

Wockhardt, India). Dextran (2.5 mL, 6 % (w/v), Sigma) dissolved in 1× balanced salt solution (BSS; 0.13 M NaCl, 2.6 mM KCl, 8.0 mM Na_2HPO_4, 1.83 mM KH_2PO_4, pH 7.4) was added and mixed by a single inversion. Blood was transferred into a fresh UC and allowed to fractionate for 1 hour. The buffy coat layer (~1.5 mL) was transferred into a fresh UC. Cells were collected by centrifugation (300 *g,* 2 minutes, room temperature) and the pellet resuspended in sterile H_2O to burst any contaminating erythrocyte membranes. After 10 seconds the UC was filled with BSS and the leukocytes were pelleted as above. The pellet was resuspended in Krebs-BSA (0.1 % (w/v) bovine serum albumin (BSA) in 1× Krebs (1.2 M NaCl, 0.48 M KCl, 0.12 M KH_2PO_4, 0.12 M $MgSO_4$, 0.13 M $CaCL_2$, 2.5 M HEPES, pH 7.4)) that had been passed through a 0.22 µm pore sterile-filter (Merck Millipore, USA) for sterilisation and remove possible contaminating serum extracellular vesicles. Cells were incubated on ice, in darkness, with CellTrace™ (1:1000; C34851, Invitrogen). After 10 minutes, leukocytes were centrifuged, resuspended in Krebs-BSA and left to settle on ice for 30 minutes. Prior to the attachment assay, the leukocyte suspension was diluted 1:10 with Krebs-BSA pre-warmed to 37 °C.

Leukocyte Attachment Assay

Serum-free M199 (150 µL) containing 1.2 ± 0.4 $\times 10^{10}$ (Control), 2.5 ± 0.4 $\times 10^{10}$ (TNF-α), 8.6 ± 0.8 $\times 10^{10}$ (Hypoxia) and 1.2 ± 0.3 $\times 10^{11}$ (TNF-α & Hypoxia) adipocyte EV/mL (mean ± SEM; N=3, n=9), was added to individual wells of a 96-well plate containing a confluent monolayer of HUVECs. Wells were also used to determine basal leukocyte attachment (no EVs; negative control) or 100 ng/mL TNF-α to increase HUVEC VCAM-1 production (no EVs; positive control). After 6 hours, EVs were removed with three Krebs washes before 150 µL of the fluorescently-labelled leukocyte suspension was added to all wells. Plates were incubated for 30 minutes before non-adherent cells were removed with three Krebs washes. Three images from around the centre of each well were captured using an inverted fluorescence microscope. The percentage of total image area covered by leukocytes was determined using Image J software (1.49v; National Institutes of Health, USA). Incubation timings were based on published protocols[20]. Three separate sets of adipocyte-derived EVs were used in this experiment and each set of EVs was tested on three HUVEC samples (N=3, n=9).

Effect of Adipocyte EVs on HUVEC Adhesion Molecule Production

Serum-free M199 (1.5 mL) containing 1.2 ± 0.4 $\times 10^{10}$ (Control), 2.5 ± 0.4 $\times 10^{10}$ (TNF-α), 8.6 ± 0.8 $\times 10^{10}$ (Hypoxia) and 1.2 ± 0.3 $\times 10^{11}$ (TNF-α & Hypoxia) adipocyte EV/mL (mean ± SEM, N=3, n=3), was added to individual wells of a 6-well plate of HUVECs for 6 hours. Another well was used for the determination of basal protein production (no EVs; negative control). EVs were removed with three 1× PBS washes before HUVECs were lysed using radioimmunoprecipitation assay buffer (Invitrogen) containing protease inhibitors (Roche, Switzerland). Lysates were centrifuged (12,000 *g,* 10 minutes, 4 °C) and supernatants frozen until required. The protein concentration of each sample was determined using a NanoDrop spectrophotometer

and samples analysed by Western blotting. Lysates were mixed with sample buffer and reducing agent (Invitrogen) and heated (95 °C, 10 minutes). Ten µg of total protein from each sample was loaded onto 4-12 % Bis-Tris gels (Novex™, ThermoFisher) and SDS-PAGE performed (180 V, 1 hour). Resolved proteins were transferred to polyvinylidene difluoride membranes (40 V, 75 minutes). Membranes were blocked using 5 % (w/v) non-fat dried milk in 1× Tris-buffered saline containing 0.05 % Tween-20 (TBST) (1 hour). Primary antibodies directed at proteins of interest (vascular cell adhesion molecule (VCAM-1; ab134047), E-selectin (ab18981), P-selectin (ab59738), platelet endothelial cell adhesion molecule (PECAM; ab28364), vascular endothelial (VE)-cadherin (ab33168), endothelial nitric oxide synthase (eNOS; ab76198) (all Abcam, Cambridge, UK)) were diluted 1:500 in blocking buffer and incubated with membranes overnight. Membranes were then incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (1:1000; 10794347, ThermoFisher) (2 hours). Protein bands developed on enhanced chemiluminescence film (10607665, Fisher Scientific). Membranes were re-probed for β-actin (1:2000; 4970S, Cell Signalling Technology (CST), Netherlands) to confirm equal loading. Band densitometry was performed using Image J. Three sets of adipocyte-derived EVs were used in this experiment with each set tested on one HUVEC sample (N=3, n=3).

Effect of Adipocyte EVs on the Plasma Membrane Expression of HUVEC Adhesion Molecules

HUVECs were treated with 3T3-L1 EVs from each condition (control, TNF-α, hypoxia, TNF-α+hypoxia) as described above, then washed twice and detached from the culture plate using 200 µL 1× PBS. HUVECs were collected by centrifugation (300 *g*, 5 minutes) and then re-suspended in ice-cold FACS Buffer (1% BSA (v/v) in 1× PBS). Flow cytometry was used to assess the surface adhesion molecule profile of HUVECs. Antibodies used for flow cytometric analysis were obtained from Biolegend® (BioLegend, San Diego, CA, USA). They include; phycoerythrin (PE) anti-human CD144, allophycocyanin (APC) anti-human VCAM-1, Alexafluor 647 anti-human PECAM-1, PE anti human E-Selectin, and PECy7 anti-human ICAM-1. Cells were incubated with antibodies for 30 minutes at 4 °C in darkness before being analysed on an FC500 MPL flow cytometer (Beckman Coulter equipped with 488 nm and 633 nm lasers) and data captured on MPX Cytometer List Mode Data Acquisition and Analysis Software (version 2.2). Acquisition was terminated upon recording 10,000 events, and cells gated based on their forward scatter and side scatter characteristics. Fluorescence minus one (FMO) stains were used to set the positive gates for each antibody (See supplemental information and supplementary Figure 3 for gating strategy).Three separate sets of adipocyte- derived EVs were used in this experiment and each set of EVs was tested on one individual HUVEC sample (N=3, n=3). Mean Fluorescence Intensity (MFI) was used to compare surface marker expression between treatments.

TNF-α Neutralisation Assay

A TNF-α neutralising antibody was used to determine whether EVs derived from adipocytes treated with TNF-α, mediated VCAM-1 upregulation in HUVECs. To neutralise any pre-existing TNF-α, HUVECs and adipocyte-derived EVs were incubated separately in serum-free culture medium containing 0, 3, 10, 30, 100 or 300 ng/mL TNF-α neutralising antibody. After 2 hours, HUVEC media were removed and the EV-containing media were added to the corresponding wells. After 6 hours, HUVECs were lysed and VCAM-1 levels assessed by Western blotting, as previously described. Four sets of adipocyte-derived EVs were used in this experiment with each set tested on one HUVEC sample (N=4, n=4).

Analysis of 3T3-L1 Cell and EV Proteins

Adipocyte cell and EV lysates were assessed by Western blotting as previously described. Ten µg of total protein was loaded per lane for cell lysates, and 5 µg of total protein for EV lysates. Primary antibodies directed at fatty acid binding protein-4 (FABP4; 3544S, CST), adiponectin (2789S, CST), peroxisome proliferator-activated receptor gamma (PPARγ; 2443S, CST) and perilipin (9349S, Cell Signalling Technology), were used at a 1:500 dilution, and HRP-conjugated anti-rabbit IgG was used at a 1:1000 dilution. Three sets of adipocyte cell and EV lysates were used (N=3, n=3).

Statistical Analyses

Data are presented as mean or mode ± SEM. A one-way ANOVA with Tucky's Multiple Comparison Test was used to analyse differences. Data were analysed using GraphPad Prism (version 6; GraphPad Software Inc., USA) and *p*-values <0.05 were considered significant.

Results

Effect of Adipocyte EVs on Leukocyte-to-Endothelial Cell Attachment

HUVECs pre-treated with TNFα+hypoxia-derived adipocyte EVs, increased leukocyte attachment to the same extent as the positive control (Figure 1A). These increases were significant when compared to those observed for HUVECs not treated with EVs and HUVECs pre-treated with control adipocyte EVs (Figure 1A). Leukocyte attachment was also greater following treatment with TNFα+hypoxia-derived EVs compared to hypoxia-derived adipocyte EVs. Pre-treating HUVECs with TNF-α-derived adipocyte EVs also increased leukocyte attachment compared to HUVECs not treated with EVs. No other differences were observed in leukocyte attachment to HUVECs after pre-treatment with adipocyte EVs. Using size-exclusion chromatography, we confirm that TNF-α is associated with EVs; there is no free TNF-α (Supplementary Figure 1).

Effect of Adipocyte EVs on HUVEC Protein Production

Following treatment of HUVECs with adipocyte EVs, Western blotting was used to examine the production of proteins involved in leukocyte attachment (Figure 2). VCAM-1 production was increased in HUVECs treated with TNF-α EVs and TNF-α+hypoxia EVs compared to that of untreated HUVECs. No change was observed with control EVs nor hypoxia EVs (Figure 2A). The production of other adhesion proteins including E-selectin (Figure 2B), P-selectin (Figure 2C) and PECAM (Figure 2D) were unaffected by treatment with adipocyte EVs. In addition, no effect on the production levels of the vascular endothelial cell marker proteins VE-Cadherin (Figure 2E) and eNOS (Figure 2F) were identified.

Effect of Adipocyte EVs on the Plasma Membrane Expression of HUVEC Adhesion Molecules

Flow cytometry confirmed the expression of VCAM-1, E-selectin, PECAM-1 and ICAM-1 on HUVEC plasma membranes (Figure 3). However, there were no statistically significant differences in the mean fluorescence intensity for each protein between any of the treatment groups. TNF-α treatment (no EVs) of HUVECs was used as a positive control in all experiments and showed positive expression of adhesion markers, other than for PECAM-1 which showed high expression at baseline and following all treatment conditions.

TNF-α Neutralisation Prevents VCAM-1 Upregulation in HUVEC

To determine if the upregulation of VCAM-1 in HUVECs seen following treatment with TNF-α and TNF-α+hypoxia adipocyte EVs (Figure 2) is due to EV-associated TNF-α, a TNF-α neutralising antibody was used. Compared to untreated HUVECs, TNF-α+hypoxia EVs increased VCAM-1 production (Figure 4). Compared to the level of VCAM-1 production achieved following treatment with TNF-α+hypoxia EVs, co-treatment with 100 ng/mL and 300 ng/mL neutralising antibody completely inhibited VCAM-1 upregulation (Figure 4).

Adipokine Production in Adipocytes and Adipocyte-Derived EVs

No effect on the production of FABP4 in cell lysates due to any treatment was identified (Figure 5A). FABP4 in was increased in EVs lysates of cells treated with TNF-α regardless of normoxia or hypoxia (Figure 5B). Adiponectin production was decreased in cells treated with TNF-α+hypoxia compared to that of control cells, but individually, TNF-α and hypoxia had no effect (Figure 5C). All treatments appeared to decrease adiponectin in EV lysates compared to that of control cells (Figure 5D). PPARγ production in cell lysates decreased in response to hypoxia and TNF-α+hypoxia (Figure 5E). Conversely, hypoxia- and TNF-α+hypoxia-derived EVs contained more PPARγ (Figure 5F). Compared to that of control cells, perilipin production decreased in response to hypoxia and TNF-α+hypoxia (Figure 5G). No differences in the production of perilipin in EVs were identified (Figure 5H).

Effect of Inflammatory and Hypoxic Stimuli on Adipocyte EV Yield and Size.

Compared to control adipocytes, treatment with TNF-α, hypoxia and TNF-α+hypoxia all increased the number of EVs produced per cell (Figure 6A). Treatment with TNF-α, hypoxia, or TNF-α+hypoxia all decreased EV size compared to control adipocytes (Figure 6B).

Discussion

Increased adiposity has long been recognised as a risk factor for cardiovascular disease development[1]. In obesity, adipose tissue inflammation is associated with vascular inflammation[21] and one of the primary mediators of this process is the inflammatory cytokine, TNF-α. Whilst TNF-α is secreted by adipocytes themselves, levels in obese adipose tissue are predominantly raised by activated macrophages[22]. TNF-α desensitizes adipocytes to insulin[23], but distally and in terms of effects on vascular endothelial cells, plasma TNF-α levels positively correlate with adhesion molecule production, disrupted eNOS activity and oxidative stress[24]. We are the first to show that EVs derived from adipocytes residing within a hypoxic and inflammatory, TNF-α-containing environment (modelling the pathophysiological inflammatory nature of obese adipose tissue *in vivo*), are implicated in the onset of vascular disease by increasing vascular endothelial cell adhesion molecule production and thereby promoting leukocyte attachment. In addition, we show that inflammatory and hypoxic stimuli affect the content of adipocyte-derived EVs, as well as their yield and size.

Leukocyte-to-endothelial cell attachment was increased following treatment of HUVECs with TNF-α and TNF-α+hypoxia derived adipocyte EVs when compared to untreated HUVECs. However, when compared to the level of leukocyte attachment following treatment of HUVECs with control EVs, attachment was only increased following treatment with TNF-α+hypoxia EVs. This suggests that a factor(s) conferred by hypoxia, can elicit a functional effect on vascular endothelial cells that exacerbates leukocyte adhesion in the presence of inflammation, and this is worthy of future investigation. The data do however, indicate that inflammatory EVs, regardless of whether they were produced under normoxic or hypoxic conditions, prime endothelial cells for subsequent leukocyte attachment.

Whilst flow cytometry confirmed the expression of the adhesion molecules VCAM-1, E-selectin, PECAM-1 and ICAM-1 on the surface of HUVEC plasma membranes, no differences between EV treatment groups were observed. Although not significant VCAM-1 surface expression did, however, show a trend to increase in cells treated with TNF-α and TNF-α+hypoxia derived adipocyte EVs and mirrored the increase in VCAM-1 detected by Western blotting. We hypothesize that in order to detect subtle changes in surface marker expression between treatment groups will likely require a very high number of HUVEC/experimental replicates and it is acknowledged further studies will be required in order to investigate differences in surface expression.

The fact that inflammatory adipocyte EVs increase HUVEC VCAM-1 production, does however, offer a mechanism through which leukocyte-to-endothelial cell attachment is achieved. TNFR1 receptors have been shown to induce VCAM-1 mRNA and protein via a pathway mediated by NF-κB but not ERK, p38MAPK or JNK kinase[25]. Future experiments aim to determine if the TNF-α delivered to HUVECs by

3T3-L1 EVs activates TNFR1 receptors and mediates VCAM-1 upregulation via a similar mechanism. As TNF-α neutralisation prevents VCAM-1 upregulation, we hypothesise that this would also prevent the increase in leukocyte-to-endothelial cell attachment following incubation of HUVEC with TNF-α and TNF-α+hypoxia derived adipocyte EVs. *In vivo*, VCAM-1 is involved in the firm attachment of leukocytes to endothelial cells prior to their transmigration though the vessel wall[26, 27], and whilst enhanced VCAM-1 production accompanied by leukocyte attachment is known to contribute to the progression of atherosclerosis[28, 29], we can now suggest that this is at least partly mediated by EVs derived from hypoxic and inflamed adipocytes.

In this study, EVs were not processed further to remove possible contaminating "free" TNF-α. However, we do provide evidence that TNF- α co-elutes with CD63 and FABP-4 following size exclusion chromatography of EV samples and that no "free" TNF-α elutes in later fractions. This indicates that TNF- α within EV samples is EV-associated (Supplementary Figure 1). In addition, it is important to note that the leukocytes for the leukocyte-to-endothelial attachment assay were isolated from heparin-anticoagulated blood. Heparin may activate platelets[30] causing them to expose P-selectin. P-selectin can consequentially bind to P-selectin glycoprotein on leukocytes, and this may have affected leucocyte attachment[31]. However, this effect would be equal across all experimental conditions, and as such, the results observed in this study can be attributed to 3T3-L1 EVs. It is also important to note that once isolated from blood, the leukocytes were resuspended in 0.22 µm sterile-filtered Krebs-BSA. As such, there is a chance that bovine serum EVs smaller than 0.22 µm were present in this buffer and that they too could have affected leukocyte adhesion. Likewise, this effect would have been equal across all conditions and consequentially the levels of leukocyte attachment observed across all experimental conditions would remain in proportion to each other.

Our results add to the growing body of literature confirming that adipocytes not only release EVs [15, 16, 32], but that the information conveyed through their content has a functional effect on other cell types. As such, we investigated the effect of inflammatory and hypoxic stimuli on the production of several proteins (FABP4, adiponectin, PPARɣ and perilipin) linked to cardiovascular disease in both adipocyte cell and EV lysates. We hypothesise that differences in the content of these proteins within EVs derived from inflammatory and/or hypoxic adipocytes may confer mechanistic influences on leukocyte attachment to vascular endothelial cells. Future studies will seek to identify and elucidate such mechanisms.

Plasma levels of FABP4, a fatty-acid chaperone protein[33], have been shown to increase in obesity[34] and are associated with vascular endothelial cell dysfunction[35]. We observed no change in the FABP4 content of adipocyte cell lysates in response to inflammatory or hypoxic stimuli. This result supports a previous study whereby FABP4 production in adipocyte cell lysates was also shown not to change in response to hypoxia[36]. What is interesting, however, is that the authors of this study

did identify an increased level of FABP4 in adipocyte culture media in response to hypoxia. In our study, hypoxia did not increase the FABP4 content of EVs suggesting that perhaps FABP4 is released by adipocytes in an EV-independent mechanism in this setting. However, we did observe an increase in FABP4 in the lysates of EVs derived from TNF-α treated adipocytes (regardless of normoxia or hypoxia). As such, future experiments will aim to elucidate the influence of FABP4 within inflamed adipocyte-derived EVs on vascular endothelial dysfunction.

Adiponectin is abundant within the circulation of healthy people and exerts regulatory insulin-sensitising and anti-inflammatory effects[37,38]. Plasma adiponectin levels negatively correlate with degree of obesity[39] and therefore also correlate with increased obesity-related co-morbidities including insulin resistance, type II diabetes and cardiovascular disease[40]. We found that a combined inflammatory and hypoxic stimulus decreased adiponectin in adipocyte cell lysates. EV-associated adiponectin also appeared to decrease compared to control in response to all treatments, but particularly hypoxia. Using the same adipocyte cell model, Chen *et al.,* showed that hypoxia prevents soluble adiponectin secretion[40], complementing our finding. Our data add to this by suggesting that at least a proportion of this reduction in adiponectin secretion under hypoxic conditions is because it is not being packaged into EVs. Reduced adiponectin within adipocyte EVs may contribute to the decrease in plasma adiponectin during obesity and thereby may contribute to the development of cardiovascular disease.

PPARɣ is a nuclear receptor regulating the transcription of genes central to fatty acid and energy metabolism[41]. We found that hypoxia decreased PPARɣ production in adipocyte cell lysates. Down-regulation of intracellular PPARɣ in response to hypoxia is well documented[16, 42] and is mediated by inhibition of its transcription by hypoxia inducible factor-1 (HIF-1α). Interestingly, in our study we also observed a simultaneous increase of PPARɣ within the lysates of EVs derived from hypoxic adipocytes, confirming that adipocyte EVs are selectively packaged depending on environmental cues. We hypothesize that HIF-1α may also be implicated in the selective packaging of PPARɣ into EVs destined for release, as a further means to reduce intracellular PPARɣ levels. Overall, this finding highlights that EVs do not necessarily possess the same content as their cell-of-origin, but rather that their content reflects the cell-of-origin's current state.

Perilipin is a protein localised to the surface of lipid droplets and is involved in lipolysis[43]. In our study, hypoxia decreased perilipin production within adipocyte cell lysates when normalised to total protein. Wang *et al.,*[44] previously showed no difference in total perilipin production per individual adipocyte sampled from lean and obese adipose tissue but when normalised to total protein or fat cell surface area, perilipin levels were also significantly lower in obese samples. We propose that hypoxia restricts perilipin production in adipose tissue in obesity *in vivo*. In addition, whilst perilipin levels are increased in the circulation in obese mice and humans[15]

this may be because it is released in to the circulation from adipocytes as a free protein rather than being associated with EVs. This hypothesis fits with our results as we identified no difference in the perilipin content of adipocyte EVs in response to inflammatory and hypoxic stimuli.

In terms of specific EV character, our group has previously shown that EVs isolated from adipocytes express the vesicular proteins CD9, CD63, Alix, tumour susceptibility gene (TSG101)[16], and now show that they also exhibit characteristic cup-shape morphology by electron microscopy (Supplementary Figure 1). Here we show the yield and size of adipocyte-derived EVs can be modulated independently by external stimuli. TNF-α, hypoxia and TNF-α+hypoxia increased the yield of EVs/cell whilst decreasing their size. These physical changes may themselves also confer distinct functional effects. However, it is important to note that the size data presented is based solely on raw NTA values; no refractive index[45] nor mathematical modelling[46] was applied.

In summary, we provide evidence that adipocytes residing in a hypoxic and inflammatory environment produce EVs capable of inducing VCAM-1 production in vascular endothelial cells, and that this effect promotes leukocyte attachment. We also show that both inflammatory and hypoxic stimuli not only influence the adipokine content of adipocytes and their EVs, but also effect EV yield and size. Future studies will further explore the functional impact inflammatory and hypoxic adipocyte EVs have on cardiovascular disease. Indeed, the functional effects of adipocyte EVs in the setting of obesity, are likely to be not solely limited to vascular endothelial cells. We anticipate that a better understanding of the mechanisms though which adipocyte EVs negatively impact the vascular endothelium will open the door for the development of novel therapies for preventing and treating obesity-driven cardiovascular disease.

Conflict of Interest

The authors declare no conflict of interest.

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Author contributions

Rebecca M. Wadey performed the experiments. Katherine D. Connolly performed pilot experiments and manuscript editing. Donna Mathew and Gareth Walters performed the flow cytometry experiments. D. Aled Rees and Philip E. James were the Principal Investigators leading the project.

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Figure Legends

Figure 1 – Leukocyte attachment to HUVECs pre-treated with and without adipocyte-derived EVs. (A) Ratio of percentage area covered by leukocytes to a confluent monolayer of HUVECs pre-treated with (C) control, (T) TNF-α, (H) hypoxia, and (TH) TNF-α+hypoxia-derived adipocyte EVs relative to that of (B; Basal) HUVECs not treated with EVs (negative control). Direct treatment with TNF-α acted as a positive control (mean ± SEM; N=3, n=9; *** p<0.001). (B) Representative fluorescent images used to determine the percentage area of HUVECs covered by leukocytes. White dots are leukocytes stained with CellTrace™. Scale bar = 200 μm.

Figure 2 – Expression of adhesion and endothelial marker proteins in HUVECs following treatment with adipocyte EVs. Densitometry graphs and representative Western blots showing the ratio of (A) VCAM-1 (110 kDa), (B) E-Selectin (80 kDa), (C) P-Selectin (140 kDa), (D) PECAM (130 kDa), (E) VE-Cadherin (115 kDa) and (F) eNOS (140 kDa) expression in HUVECs following treatment with (C) control, (T) TNF-α, (H) hypoxia and (TH) TNF-α+hypoxia derived adipocyte EVs, relative to that of (B; basal) untreated HUVECs (mean ± SEM; N=3, n=3; *** p<0.001). β-actin confirms equal loading (bottom blots). Ten μg total protein per lane.

Figure 3 - Surface expression of HUVEC adhesion proteins following treatment with adipocyte EVs. Mean fluorescence intensity graphs showing the ratio of (A) VCAM-1 (-APC), (B) E-Selectin (-PE), (C) PECAM-1 (-AlexoFluor) and (D) ICAM-1 (-PE/Cy7) expressed on the plasma membrane of HUVECs following treatment with (C) control, (T) TNF-α, (H) hypoxia and (TH) TNF-α+hypoxia derived adipocyte EVs, relative to that of (B; basal) untreated HUVECs. Direct treatment with TNF-α was used as a positive control (mean ± SEM; N=3, n=3 for all except ICAM-1 where N=2, n=2).

Figure 4 – TNF-α neutralisation inhibits endothelial VCAM upregulation following treatment with TNF-α+hypoxia adipocyte EVs. (A) Western blotting densitometry of VCAM-1 expression in control HUVEC lysates, and lysates of HUVECs treated with TNF-α+hypoxia EVs (denoted by +) in the presence of 0, 3, 10, 30, 100 and 300 ng/ml TNF-α neutralising antibody (mean ± SEM; N = 4, n = 4; ** $p<0.01$). (B) Representative VCAM-1 (110 kDa; top) and β-actin (40 kDa; bottom) blots. Twenty µg total protein per lane.

Figure 5. FABP4, adiponectin, PPARγ and perilipin expression in adipocyte and adipocyte-derived EV lysates. Densitometry graphs and representative Western blots for FABP4 and adiponectin in cell and EV lysates following (C) control, (T) TNF-α, (H) hypoxia and (TH) TNF-α+hypoxia treatment of adipocytes. FABP4 expression in (A) cell lysates (mean ± SEM; N=6, n=6) and (B) EV lysates (mean ± SEM; N=4, n=4) (15 kDa). Adiponectin expression in (C) cell lysates (mean ± SEM; N=5, n=5; * $p<0.05$) and (D) EV lysates (mean ± SEM; N=2, n=2) (30 kDa). PPARγ expression in (E) cell lysates (mean ± SEM; N=6, n=6; *** $p<0.001$) and (F) EV lysates (mean ± SEM; N=3, n=3; ** $p<0.01$) (53 and 57 kDa). Perilipin expression in (G) cell lysates (mean ± SEM; N=5, n=5; *** $p<0.001$) and (H) EV lysates (mean ± SEM; N=3, n=3) (56 kDa). Twenty µg total protein per lane of cell lysates, and 5 µg total protein per lane of EV lysates.

Figure 6. Effect of inflammatory and hypoxic stimuli on adipocyte EV yield and size. The effect of (C) control, (T) TNF-α, (H) hypoxia and (TH) TNF-α+hypoxia treatments on (A) EV yield per cell (mean ± SEM; n=3, n=9; *** $p<0.001$) and (B) EV size (mode ± SEM; n=3, n=9; *** $p<0.001$).

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Key Words

Adipocyte, adipokine, obesity, endothelial dysfunction, vesicles.

Materials and Methods

Size Exclusion Chromatography

EVs from 3T3-L1 adipocytes treated with 30 ng/mL TNF-α for 24-hours were collected by differential ultracentrifugation; cell medium was centrifuged at 4 °C at 1,000 *g* (5 minutes), 15,000 *g* (15 minutes), and finally 100,000 *g* (1 hour) to pellet EVs. EVs were re-suspended in 1.0 mL of 0.22 µm sterile-filtered 1X PBS which was then loaded onto a size-exclusion chromatography column (Exo-spin™ midi columns; Cell Guidance Systems; EX04-20). Thirty 0.5 mL 1X PBS fractions were collected and then Nanoparticle Tracking Analysis, Nanodrop protein concentration and Western blotting assays performed. Nanoparticle Tracking Analysis and Western Blotting were performed as described in the manuscript. The primary antibodies FABP4 (3544S, CST), CD63 (sc-15363, SantaCruz) and TNF-α (6945; CST) were used at a 1:500 dilution. (N=1, n=1). Protein concentration was determined using a NanoDrop 1000 Spectrophotometer (Thermo Scientific).

Transmission Electron Microscopy

3T3-L1 EVs were resuspended in 1X 0.22 µm-filtered BPS and then fixed with an equal volume of 4 % (v/v) paraformaldehyde and kept in at 4°C until processing for Transmission Electron Microscopy (TEM). Briefly, EVs (10 µl) were adsorbed onto glow discharged carbon formvar 200 mesh copper grids for 2 minutes, Grids were then blotted using filter paper, stained for 10 seconds with 2& (w/v) uranyl acetate before surplus stain was removed and grids were air dried. Grids were imaged using a FEI Tecnai 12 TEM at 120 kV fitted with a Gatan OneView CMOS camera. (N=1, n=1).

Flow Cytometry Gating Strategy

Acquisition was terminated upon recording 10,000 events and events gated based on their forward scatter and side scatter characteristics, as we have described in detail previously for HUVEC [1].

Results

Size Exclusion Chromatography

A peak in particle concentration is observed between fractions 6-8 (Supplementary Figure 1A). Protein content shows low levels across the 30 fractions (Supplementary Figure 1B). The protein content is highest in fractions 7 and 8. TNF-α appears in fractions 6-8 which also contain CD63 and FABP4 and does not appear in later fractions where soluble protein would elute (Supplementary Figure 1C). This provides evidence that a small level of TNF-α is strongly associated with EVs. This also confirms that the majority of soluble TNF-α added to the initial cell culture is removed during washing/centrifugation steps

TEM Electron Microscopy

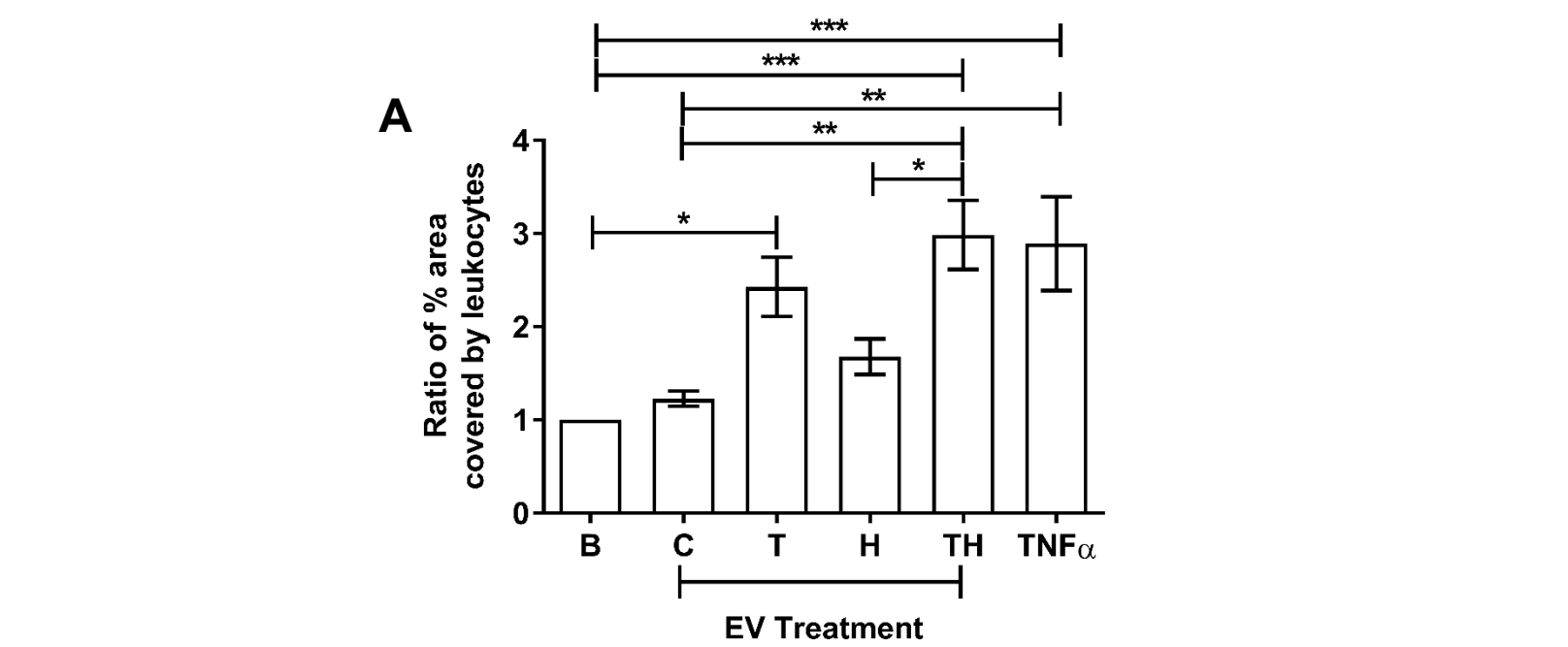
3T3-L1 EVs display typical cup-shape morphology (Supplementary Figure 2).

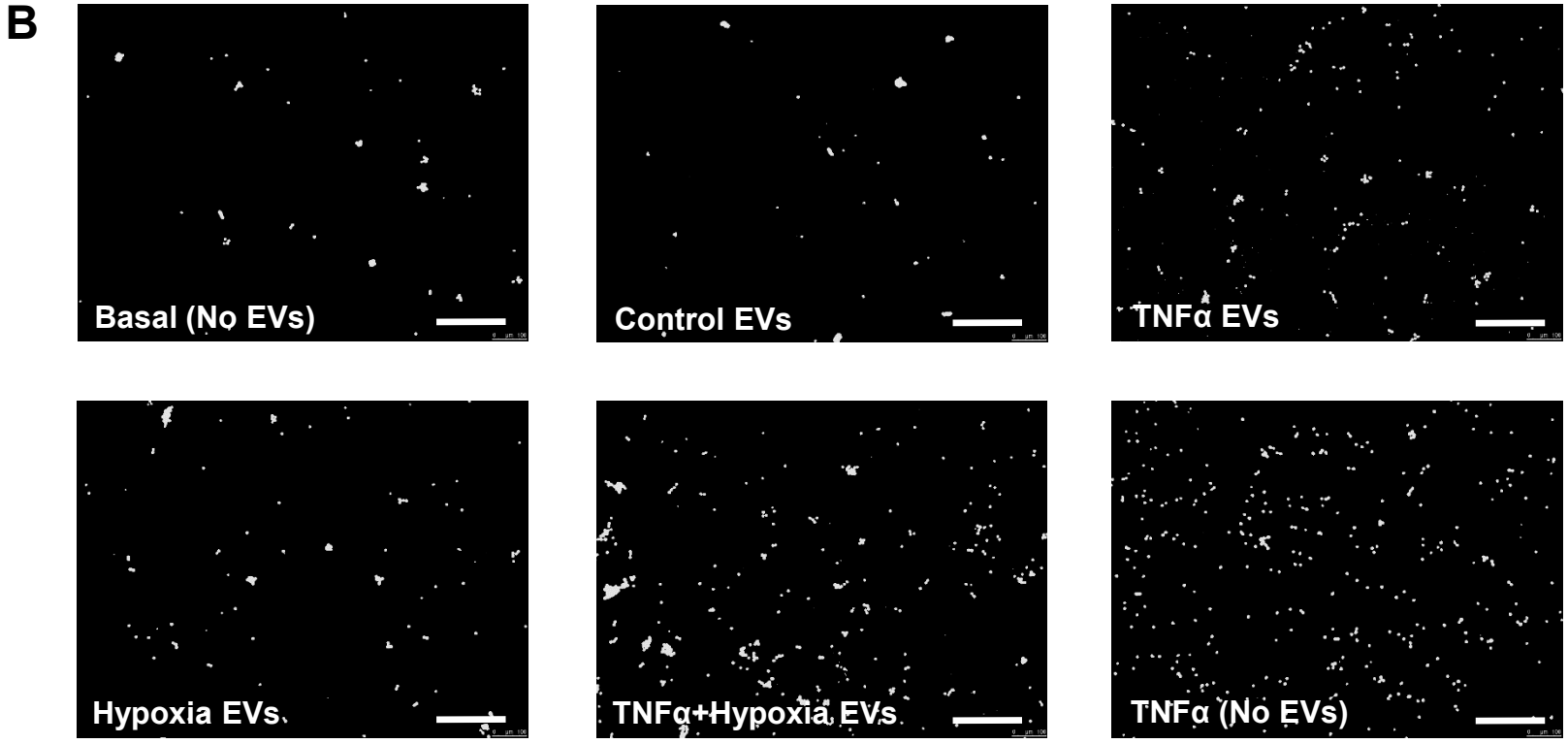
Flow Cytometry Gating Strategy

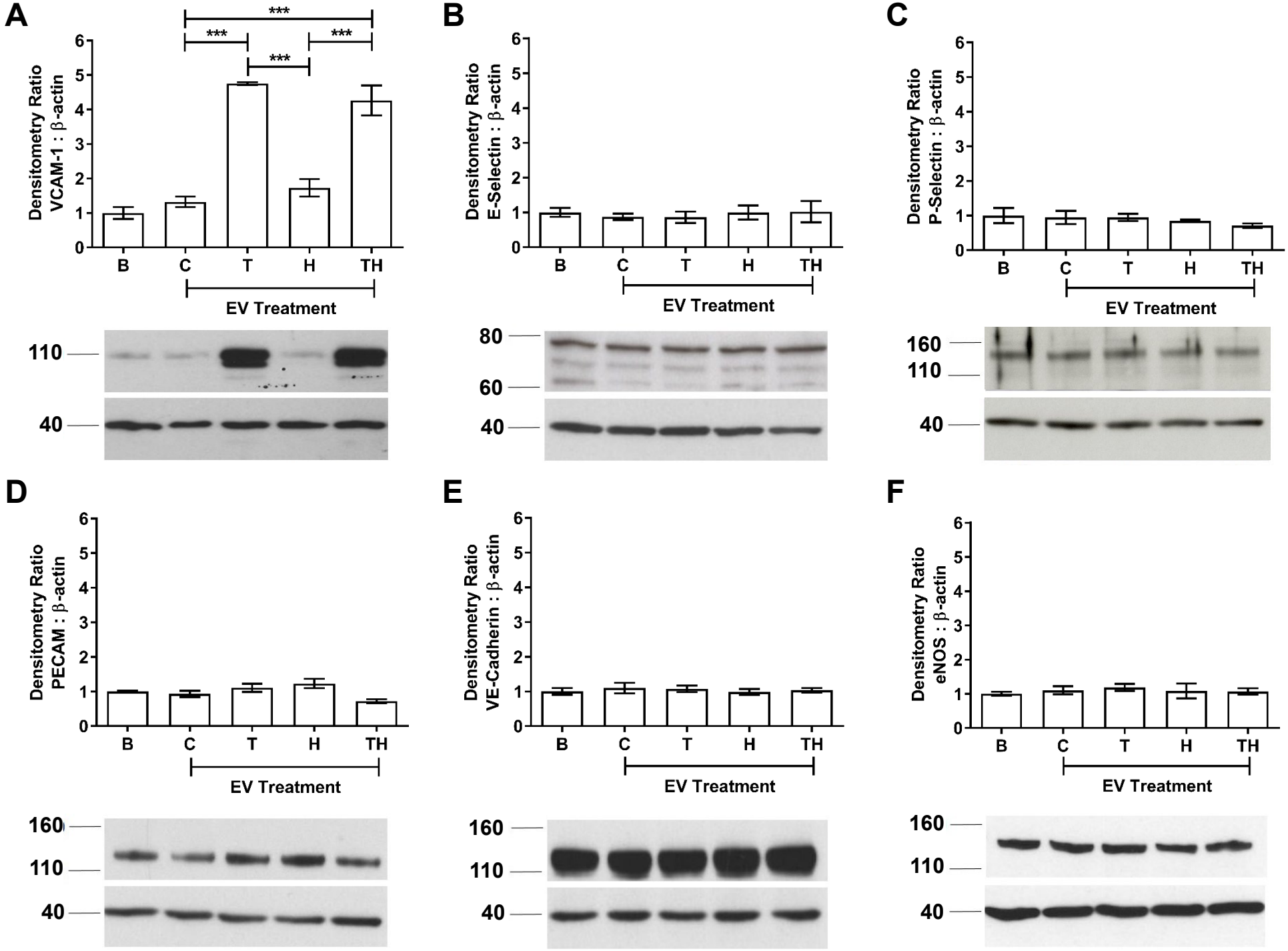
Unstained HUVEC and single positive CD144-PE fluorescence (using FL-2 channel) or single positive adhesion marker (using FL-4 channel) were analysed to ensure compensation. Double stained CD144 (PE) +ve and VCAM-1 (APC) +ve was used to confirm HUVEC phenotype (Supplementary Figure 3). Cytometric gating for cell adhesion molecule profile. A FSC versus SSC region was set to exclude cell aggregates and debris. Representative dot plots showing fluorescence-minus-one for unstained cells, CD144+ cells, VCAM-1 + cells, and CD144+ and VCAM-1 + cells.

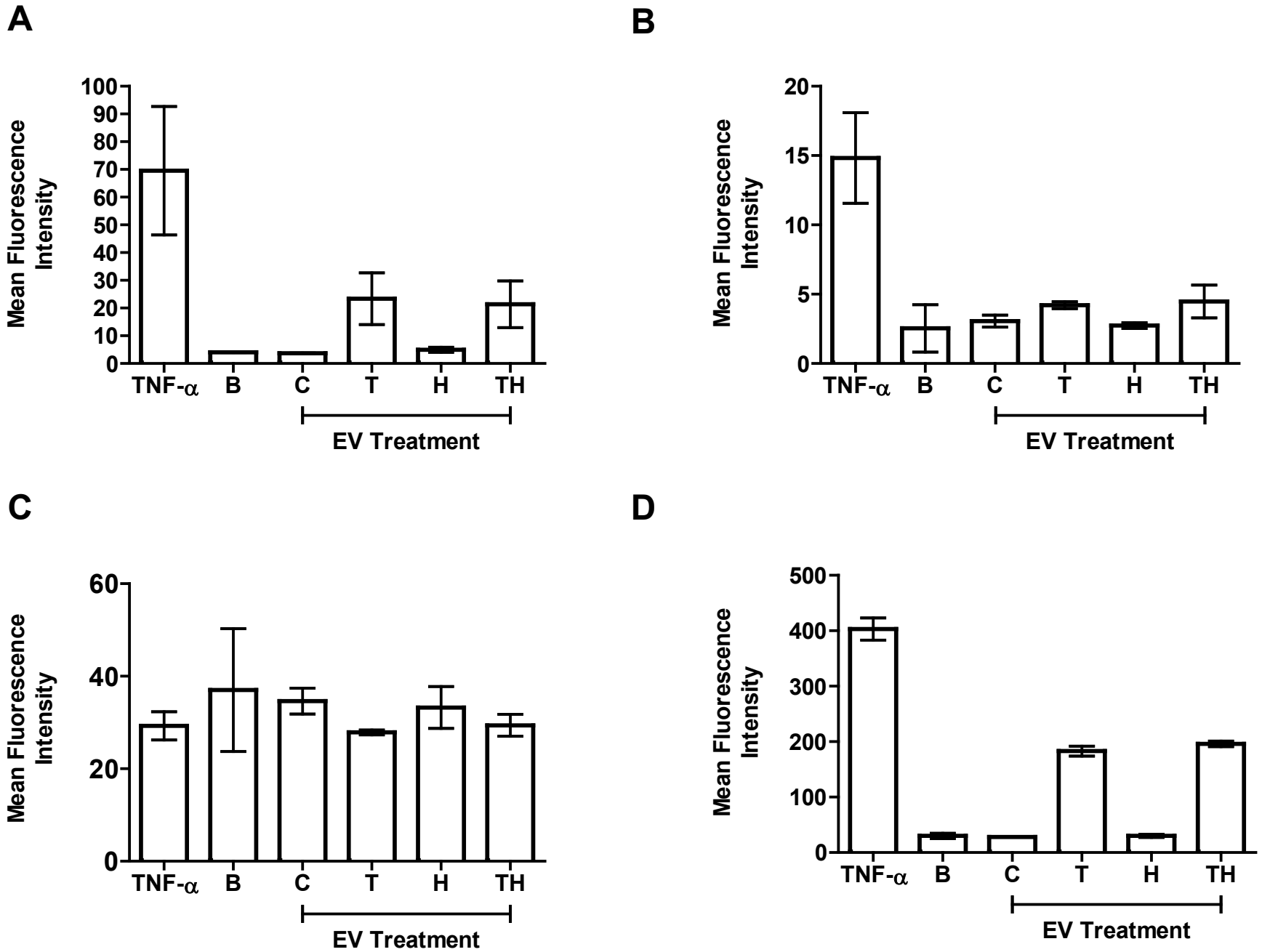
References

Burnley-Hall N, Willis G, Davis J, Rees DA, James PE. Nitrite-derived nitric oxide reduces hypoxia-inducible factor 1α-mediated extracellular vesicle production by endothelial cells. *Nitric Oxide* 2017;63:1-12.

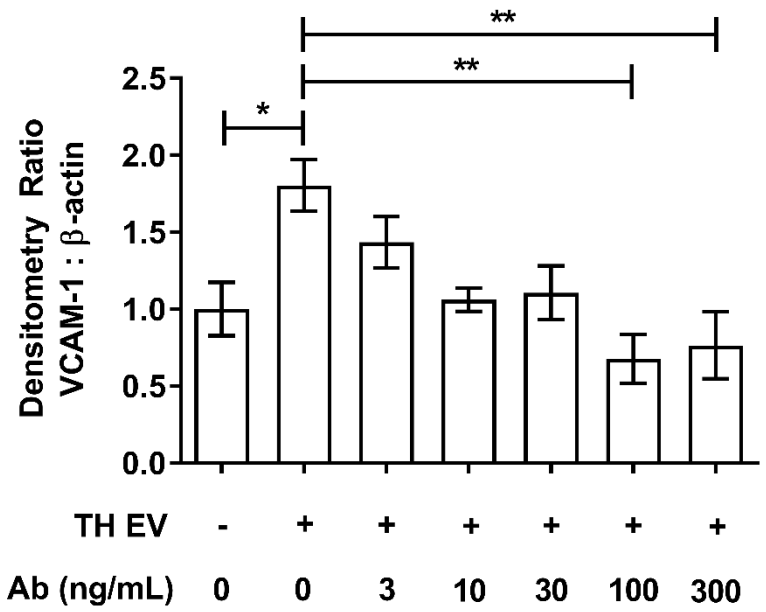

A
***
***
**
**
*
*
Ratio of % area covered by leukocytes
0
1
2
3
4
B
C
T
H
TH
TNFα
EV Treatment


B
Basal (No EVs)
Control EVs
TNFα EVs
Hypoxia EVs
TNFα+Hypoxia EVs
TNFα (No EVs)

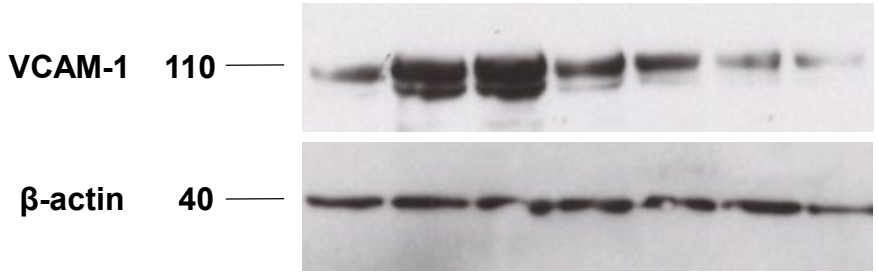

A
B
C
D
E
F
Densitometry Ratio
VCAM-1 : β-actin
E-Selectin : β-actin
P-Selectin : β-actin
PECAM : β-actin
VE-Cadherin : β-actin
eNOS : β-actin
***
B
C
T
H
TH
EV Treatment
110
40
80
60
160

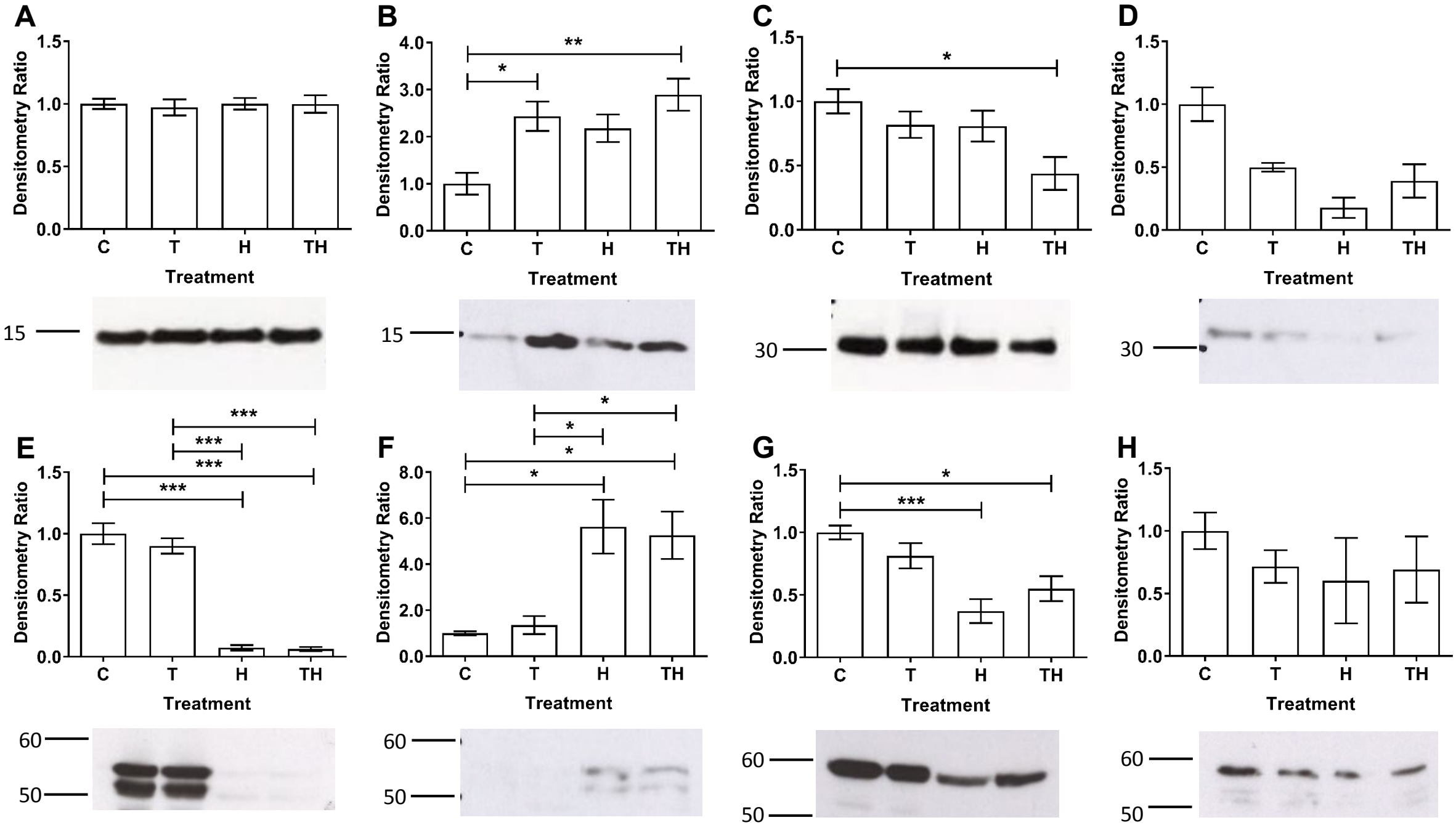

A
Mean Fluorescence Intensity
100
90
80
70
60
50
40
30
20
10
0
TNF-α
B
C
T
H
TH
EV Treatment
B
Mean Fluorescence Intensity
20
15
10
5
0
TNF-α
B
C
T
H
TH
EV Treatment
C
Mean Fluorescence Intensity
60
40
20
0
TNF-α
B
C
T
H
TH
EV Treatment
D
Mean Fluorescence Intensity
500
400
300
200
100
0
TNF-α
B
C
T
H
TH
EV Treatment

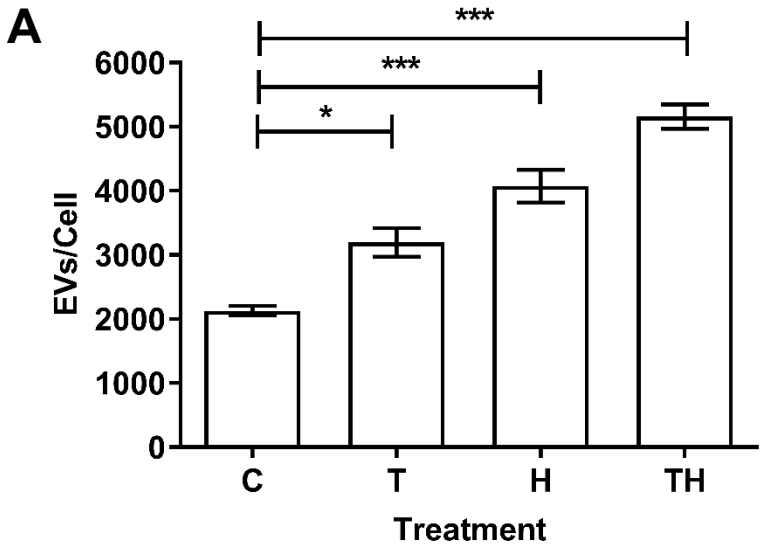
A

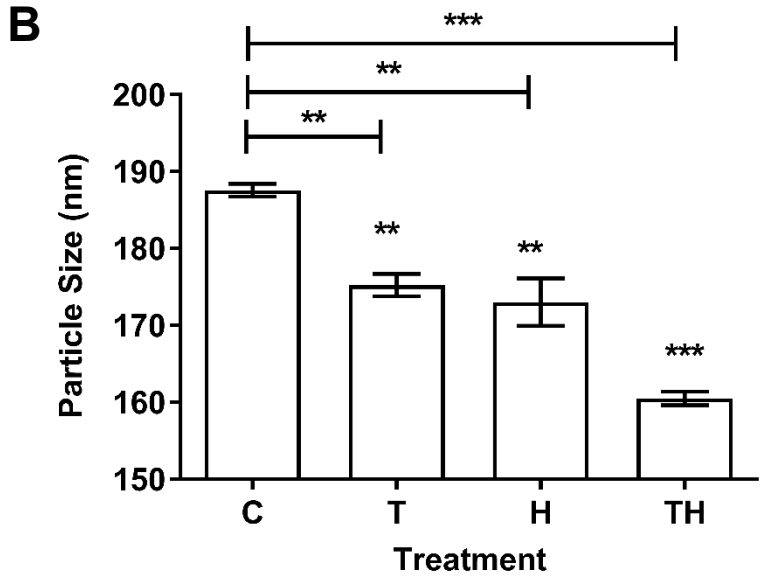

**
**
*
2.5
2.0
1.5
1.0
0.5
0.0
Densitometry Ratio
VCAM-1 : β-actin
TH EV - + + + + + +
Ab (ng/mL) 0 0 3 10 30 100 300

B


VCAM-1 110
β-actin 40


A
B
C
D
E
F
G
H
Densitometry Ratio
Treatment
C
T
H
TH
15
30
60
50


A
***
***
*
6000
5000
4000
3000
2000
1000
0
EVs/Cell
C
T
H
TH
Treatment


B
***
**
**
200
190
180
170
160
150
Particle Size (nm)
**
**
***
C
T
H
TH
Treatment

1

‘Inflammatory’ and ‘Inflammatory & Hypoxic’ adipocytes release TNF-α-containing extracellular vesicles (EVs)

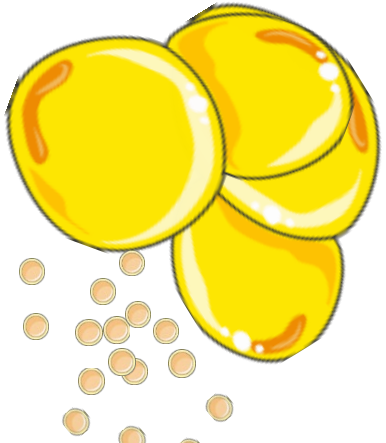


Image Key:

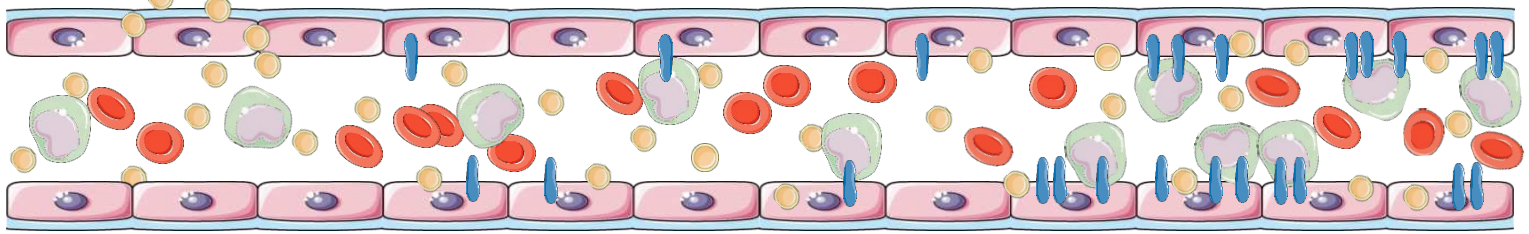
- Adipocytes
- Adipocyte EVs
- Leukocytes
- Endothelial Cells
- Erythrocytes
- VCAM-1 protein

2

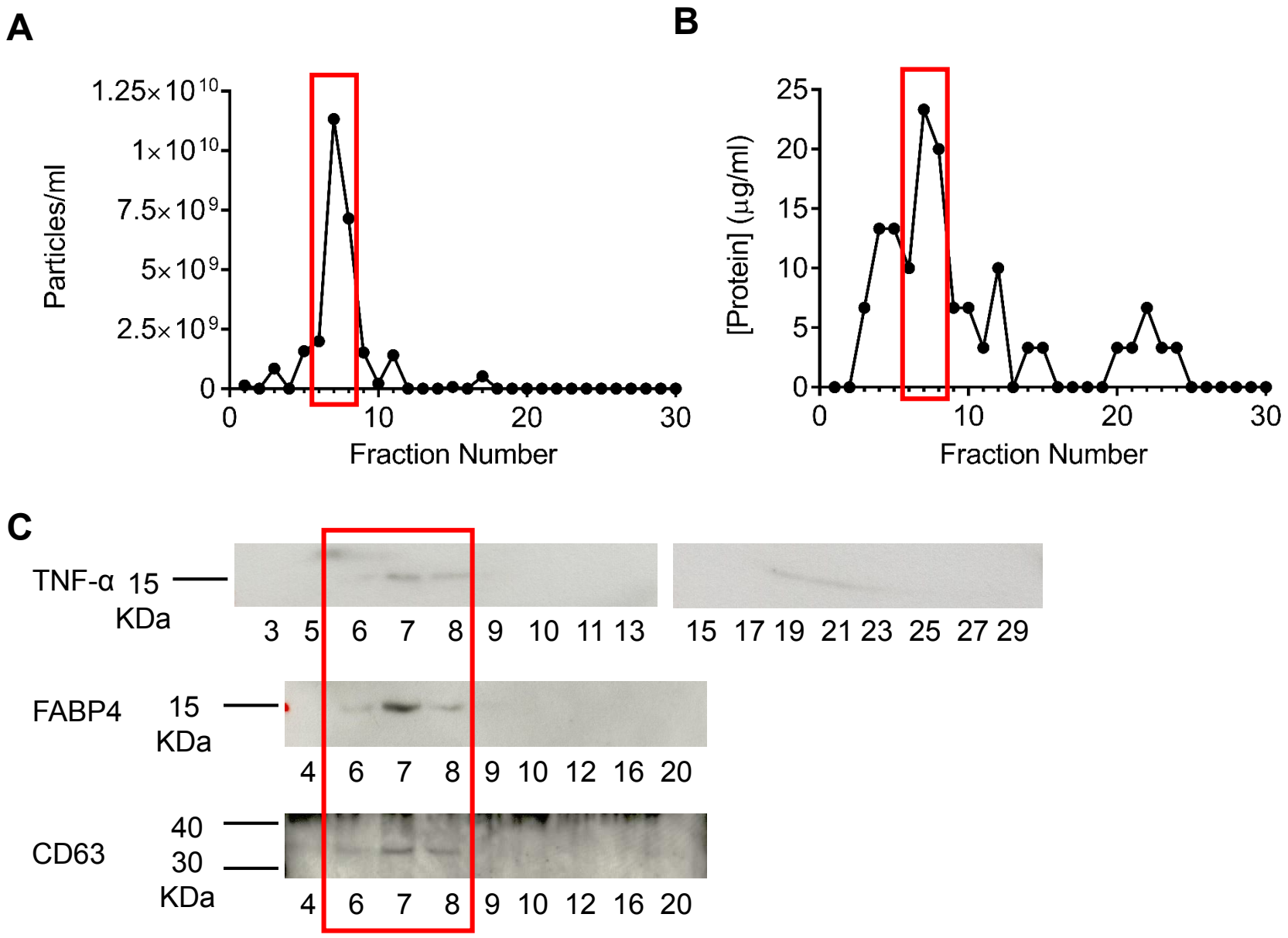
Following entry of these EVs into the circulation, there is an increase in vascular endothelial cell VCAM-1 production

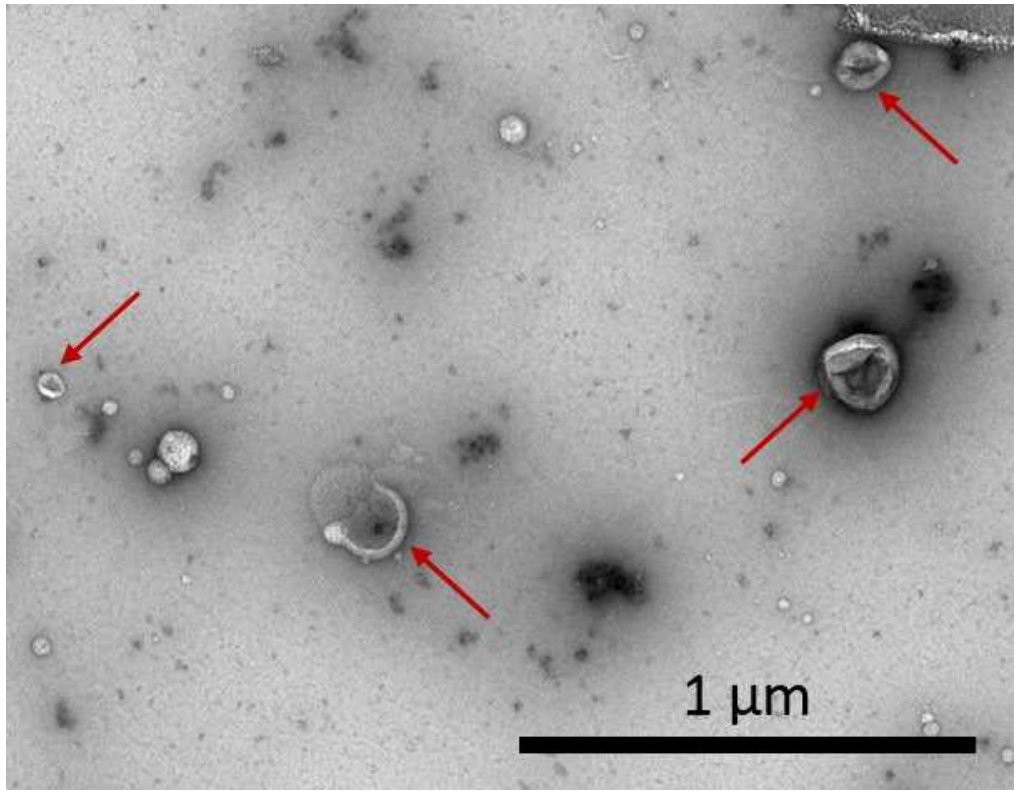
3

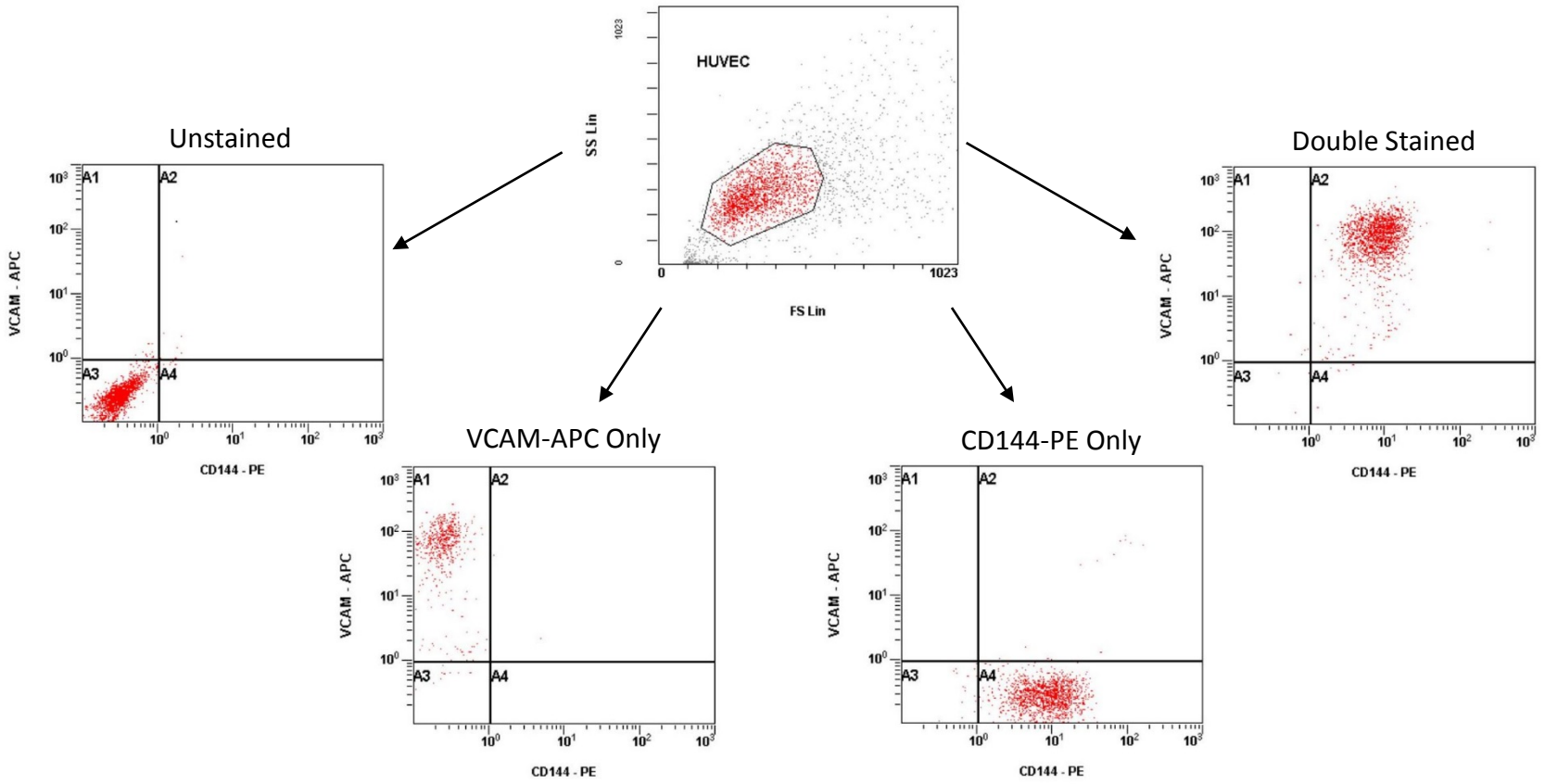
This is followed by increased leukocyte attachment



N.b. Image not to scale.


A
Particles/ml
1.25×10^10
1×10^10
7.5×10^9
5×10^9
2.5×10^9
0
0
10
20
30
Fraction Number
B
[Protein] (μg/ml)
25
20
15
10
5
0
0
10
20
30
Fraction Number
C
TNF-α 15 KDa
3 5 6 7 8 9 10 11 13
15 17 19 21 23 25 27 29
FABP4 15 KDa
4 6 7 8 9 10 12 16 20
CD63 40 30 KDa
4 6 7 8 9 10 12 16 20


1 µm


HUVEC
SS Lin
FS Lin
1023
0
Unstained
Double Stained
VCAM-APC Only
CD144-PE Only
VCAM - APC
CD144 - PE
A1
A2
A3
A4