Supplemental materials

Detailed method descriptions

Cell culture

HUVECs (cat# C-015-5C, lot# 887799, Gibco, NY, USA) were cultured in Endothelial Cell Growth Medium with Growth Supplement (HUVEC medium) (Cell Applications Inc., San Diego, CA, USA) at 37˚C, 5% CO_2 and 95% humidity. The cell culture surfaces were coated with EmbryoMax® 0.1% Gelatin Solution (Merck Millipore, Darmstadt, Germany) for 30 min prior to cell seeding.

Shear stress and flow system

HUVECs were either cultured in condition without flow (termed non-adapted cells) or in 10 dyn flow (termed shear stress adapted cells) condition for 24 h prior to AuNP exposure. It is well-described that endothelial cells adapt their structure during a 24-h exposure to shear stress, characterized by alignment of F-actin stress fibres [1, 2]. We used a 24 h adaptation period because the HUVECs developed F-actin cytoskeleton stress fibres. The adaptation of endothelial cells to shear stress *in vitro* is continuous process as documented in other studies by a gradual change in gene expression profiles and focal adhesion-associated proteins during a 48 h exposure period [3, 4]. The exposure to AuNPs was carried out in either in no-flow (termed static exposed cells) or in flow condition (termed flow exposed cells). The shorter AuNP exposure period was based on previous observations that HUVECs had efficient internalisation during this period.

The flow system was setup with parallel flow-loops each connected to one flow channel (figure 1). The flow system was placed in a cell culture incubator with an external pump. A peristaltic pump (323S

Drive 400 RPM, Watson Marlow Pumps, Falmouth, UK) with a five channel pump head was used (318MC, Watson Marlow). The pump and tubing outside of the incubator was insulated to keep at a constant temperature of 37°C. Low gas permeable PharmaPure® pump tubing (cat# AL242002, Saint-Gobain Performance Plastics, Charny, France) was used together with low gas permeable PharMed® BPT tubing (cat# R6502-26BPT, Saint-Gobain) outside the incubator. For the remaining tubing running inside the incubator (5% CO2 and 95% humidity), high gas permeable platinum-coated Tygon® silicon tubing was used (cat# T3302-13, Saint-Gobain). Polypropylene tube- and Luer-connectors were used for connecting the different tubing and flow-chambers (cat# N004-6005, cat# MLRL004-6005, Watson Marlow Alitea, Stockholm, Sweden). The reservoirs and bubble traps were obtained from DTU Systems Biology (described by Tolker-Nielsen and Sternberg [5]) with a custom made inner diameter of 1.6 mm to fit the flow system. Five ml syringes were used as reservoirs (cat# SS05SE1, Terumo, Elkton, MD, USA) and lids matching the syringe Luer connectors were used. Four days prior to AuNP exposure, $3x10^4$ cells were seeded in each channel to obtain a fully confluent monolayer. Growth medium was exchanged with fresh medium every 24 h. For cells cultured in flow condition, the flow rate was increased every 1.5 h with 2.5 dyn until reaching a final flow rate of 10 dyn. Shear stress adapted cells were cultured in flow for 24 h before exposure to AuNPs. The pump flow rate was measured before each experiment.

Antibody Fragmentation

Monoclonal mouse anti-human CD54 (ICAM-1) IgG1 LEAF™ (low endotoxin and azide-free) purified antibody was obtained from BioLegend® (cat# 322704, lot# B153293, San Diego, CA, USA). To obtain half immunoglobulin G 1 (IgG1) fragments, the CD54 was incubated in 0.1 M phosphate buffer (pH 6) containing 0.15 mM NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA, cat# EDS,

Sigma Aldrich, St Louis, MO, USA) and 10.5 nM and 21 mM 2-mercaptoethylamine (2-MEA, cat# 30078, Sigma-Aldrich) at 37°C for 1½ h and 3 h. The sub 7 kDa compounds were removed by a running the sample twice trough a Zeba Spin Desalting Column (cat# 89882, Thermo Fisher Scientific Inc., Rochester, NY, USA) with a Tris (pH 8) buffer made from ultrapure double distilled water (ddH2O) containing 50 mM Tris (pH 8), 0.15 M NaCl and 5 mM EDTA. The protein recovery yield was measured by absorption at 280 nm by NanoDrop (Thermo Fischer Scientific Inc.). The solution was stored at 4°C and used within a few days.

SDS-PAGE and Coomassie Blue staining

The 2-MEA treated and untreated mouse CD54 antibody were analysed by SDS-PAGE and Coomassie blue staining to check the amount of cleaved antibody. The SDS-PAGE size-separation and stacking gels were prepared with 8% acrylamide (cat# A4058, Sigma-Aldrich), 0.1% SDS (cat# L3771, Sigma-Aldrich) and 0.1% glycerol as described in Simpson (2003) [6]. The loading buffer was modified from Simpson (2003) [6] and prepared without β-mercaptoethanol to ensure that the antibodies were unaffected by the buffer. The gels were run with 100 volt for 2 h with a Tris-glycine-SDS buffer (cat# T7777, Sigma-Aldrich) with PageRuler™ (cat# 26616, Thermo Fisher) protein-ladder. Coomassie blue staining was conducted by staining Coomassie brilliant blue reagent solution (cat# 27813, Sigma-Aldrich) and de-staining of the acrylamide membrane by 10% acetic acid as described in Simpson (2010) [7]. Monochrome images were captured by an ImageQuant LAS 4000 (GE Healthcare Life Sciences, Piscataway, USA) and analysed by ImageJ (v1.47).

AuNP modification

Colloidal spherical AuNPs ø80 nm (coefficient of variation<8% according to manufacturer) suspended in H_2O were obtained from BBI Solutions (cat# EMGC80, BBI Solutions, Cardiff, UK). The intact or cleaved antibodies were conjugated with the AuNPs by adding the intact or cleaved antibodies to the AuNP stock solution at a final concentration of 123 ng protein per µg AuNPs. The solution was mixed overnight at 4°C followed by centrifugation at 2000 x g for 5 min and re-suspension in HUVEC medium (and ddH2O for particle characterisation) just before cell exposure or particle characterisation experiments.

Nanoparticle characterisation

Hydrodynamic size-distribution, stability and precipitation of anti-ICAM-1 AuNPs was compared with unmodified AuNPs re-suspended in ddH2O and supplemented HUVEC medium by Brownian motion video analysis and ultraviolet–visible spectroscopy (UV-Vis). Hydrodynamic size distribution of the AuNPs were analysed by NanoSight (NanoSight LM20, Amesbury, UK) and video analysis software NanoSight Nanoparticle Tracking Analysis (NTA v. 3.0). UV-Vis spectroscopy was performed (UV-2100, Shimadzu, Kyoto, Japan). Analyses were performed at an AuNP concentration of 5 µg/ml.

AuNP exposure

The AuNPs were centrifuged (as in AuNP modification) and re-suspended in HUVEC medium. The AuNP exposure concentration used through-out this work was 5 µg/ml which corresponds to 25.4 µM, $9.67 \cdot 10^8$ particles/ml and a total surface area of 0.1942 cm^2/ml. Growth medium was removed and AuNP containing medium (including a negative control without AuNPs) was added by flushing channels (x 2) and incubated at 37˚C with 5% CO_2 and 95% humidity.

Confocal microscopy

HUVECs were seeded in Ibidi μSlides VI$^{0.4}$ (Ibidi, Germany) multichannel flow chambers (3x10^4 per flow channel) four days prior to AuNP exposure. After 3 h AuNP exposure the HUVECs were washed (x 3) in pre-warmed serum-free HUVEC medium. Cells stained with CellTracker™ Green CMFDA (20 μM) (cat# C7025, Molecular Probes®, Eugene, OR, USA) and Hoechst 33342 (Molecular Probes) dissolved in serum-free HUVEC medium were flushed (x 2) with staining solution followed by 8 min of incubation (37˚C, 5% CO2 and 95% humidity). The cells were subsequently washed with serum-free HUVEC medium (once) and PBS (twice). Cells were fixed in 4% paraformaldehyde (in PBS) for 30 min at room temperature (three times), washed with PBS (twice), followed by two flushings with Ibidi mounting medium (cat# 50001, Ibidi). Images were collected on a Leica SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) using a 100 x magnification Leica NA: 1.42, oil immersion objective (Leica Microsystems). CellTracker Green was imaged at λ 488 and emission was collected at λ 510–550 nm. AuNPs were visualized in reflection mode with a HeNe laser at λ 633 nm laser (collected at λ 631-636 nm). The position of the sample surface was determined (by reflection) and the image background intensity was quantified (5 μm above the surface) by LAS AF Lite (v.1.0.0, Leica Microsystems) using the Stack Profile and Analyse PSF tool. Z-stacks beginning at the sample surface were obtained and subsequent 3D image processing and analysis were done in Volocity® 3D Image Analysis Software (v.6.1.1., Perkin Elmer, Waltham, MA, USA). AuNP uptake analysis was performed as described in Klingberg et al. [8]. The volumes of CellTracker Green signal and AuNP reflection were measured and the AuNPs overlapping the CellTracker Green signal were noted as internalized AuNPs. The ratio between cell volume (CellTracker Green) and AuNP volume was then calculated. For F-actin staining, cells were permeabilised after fixation for 3 min in PBS with 0.1%

Triton-X-100 and blocked for unspecific binding for 30 min in PBS with 1% bovine serum albumin (BSA) (Sigma Aldrich) and stained for 30 min in PBS with 1% BSA and (1:100) rhodamin phalloidin (Molecular Probes). Cells were washed with PBS and mounted with Ibidi mounting medium. Rhodamin phalloidin was exited at λ 543 nm and emission was collected at λ 551–606 nm and Hoechst was excited at λ 406 nm and emission was collected at λ 428–464 nm.

Flow cytometry

Samples were analysed on a flow cytometer BD Accuri™ C6 Flow Cytometer (Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA). For TNF response (dilution series) and full antibody versus antibody fragment comparison HUVECs were seeded ($1x10^5$ cells per well) on to Nunc™ 12 well plates (Nunc - Thermo Fisher Scientific., Roskilde, Denmark) one day prior to AuNP-exposure. For particle uptake and ICAM-1 and PECAM-1 expression experiments HUVECs were seeded as for shear stress experiments. AuNP-exposure was ended by washing (x 3) in HEPES buffered saline solution (Lonza, Walkersville, MD, USA) followed by 3-5 min incubation with Accutase™ Cell Detachment Solution (cat# 561527, BD). Cell detachment was terminated by addition of cold HUVEC medium and samples were kept on ice until they were analysed or stained with antibodies. Antibody staining was initiated by centrifuging the HUVECs for 5 min at 2000 x g. The cells were re-suspending and incubated for 20 min in staining buffer (cat# 554656, BD) with antibodies against ICAM-1 (25µl/ml, PE mouse anti-human CD54, cat# 555511, lot# 70073, BD) and PECAM-1 (25µl/ml, FITC mouse anti-human CD31, cat# 555445, lot# 29583, BD). Compensation for fluorescent overlap was performed on anti-mouse Ig, κ/negative control compensation particles set (cat# 552843, BD). A gate that included the entire cell population was established and 10^4 events were recorded for static samples while fewer cell events were recorded within this gate (on fast collection to avoid sedimentation of large cells).

TNF activation and ICAM-1 cell surface expression

The HUVECs were seeded ($1x10^5$ cells per well) on to Nunc™ 12 well plates (Nunc, Denmark). To determine the concentration of TNF needed for optimal activation of HUVECs, the cellular surface-expression of ICAM-1 was measured by flow cytometry after 24 h of TNF activation. This exposure period was chosen because it has been shown that the ICAM-1 expression reaches a plateau in endothelial cells at 24 h exposure to TNF [9, 10]. We observed that the ICAM-1 surface expression reached a plateau at concentrations between 10 and 100 ng/ml (figure S1). Neither SSC-A nor FSC-A was significantly changed by TNF activation (figure S2).

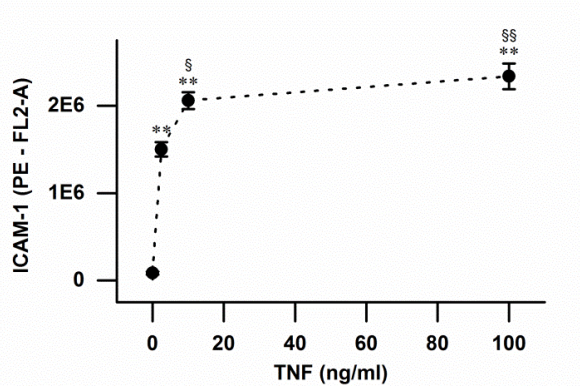

Figure S1 – Flow cytometry analysis of intercellular adhesion molecule 1 (ICAM-1) surface expression

Error bars represent SEMs for 4 (and 3 for untreated control cells) independent experiments, $P^{**} < 0.001$ compared to untreated control cells, $P^{§} < 0.05$ and $P^{§§} < 0.001$ compared to 2.5 ng/ml TNF-activated cells.

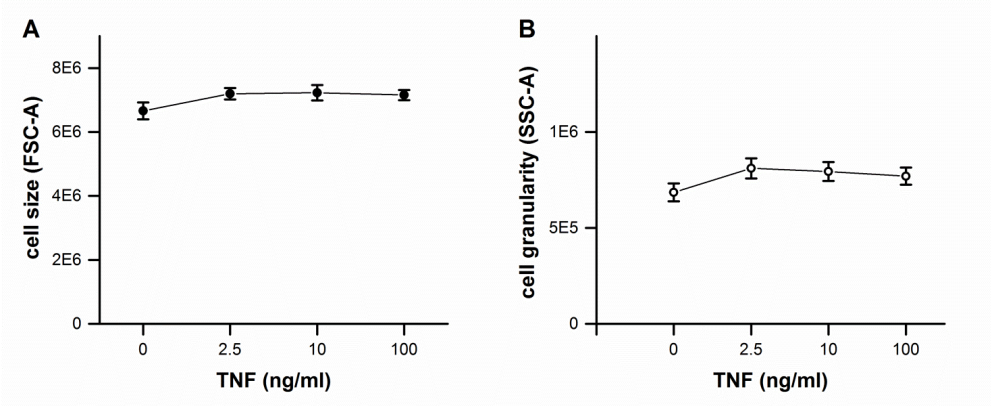

Figure S2 – Cell size and granularity with increasing TNF concentration asses by flow cytometry.

Panel A shows the cell size (FSC-A) plotted against tumour necrosis factor (TNF) concentration. Panel B shows cell granularity (SSC-A) plotted against TNF concentration. Error bars represent SEM of 4 experiments.

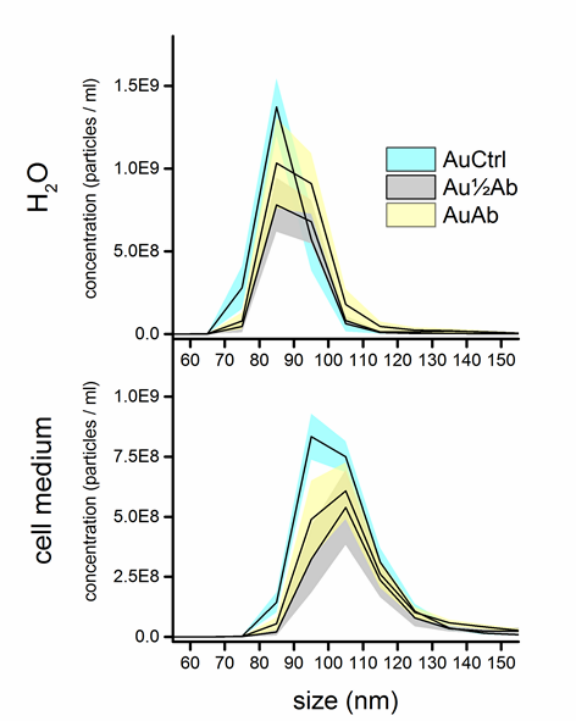

Figure S3 – Hydrodynamic size distribution histograms

Averaged size distribution histograms of unmodified AuNPs (AuCtrl) compared with AuNPs conjugated with cleaved (Au½Ab) and intact (AuAb) re-suspended in H_2O (top graph) and serum containing HUVEC medium (bottom graph). The shading represents SEM of 20 measurements on 4 different days (15 measurements on 3 different days for Au½Ab).

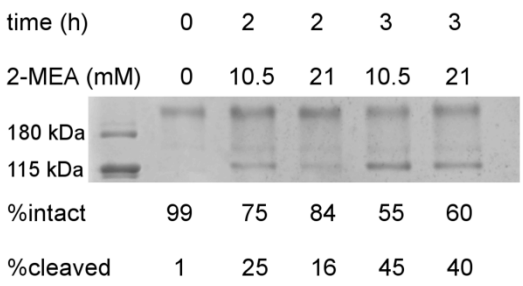

Figure S4 – SDS-PAGE gel of 2-mercaptoethylamine (2-MEA) digested mouse anti human-ICAM-1 antibodies

The quantitated size indicated with protein ladder is not directly comparable with the antibody or antibody-fragment size due to the lack of β-mercaptoethanol in the sample loading-buffer.

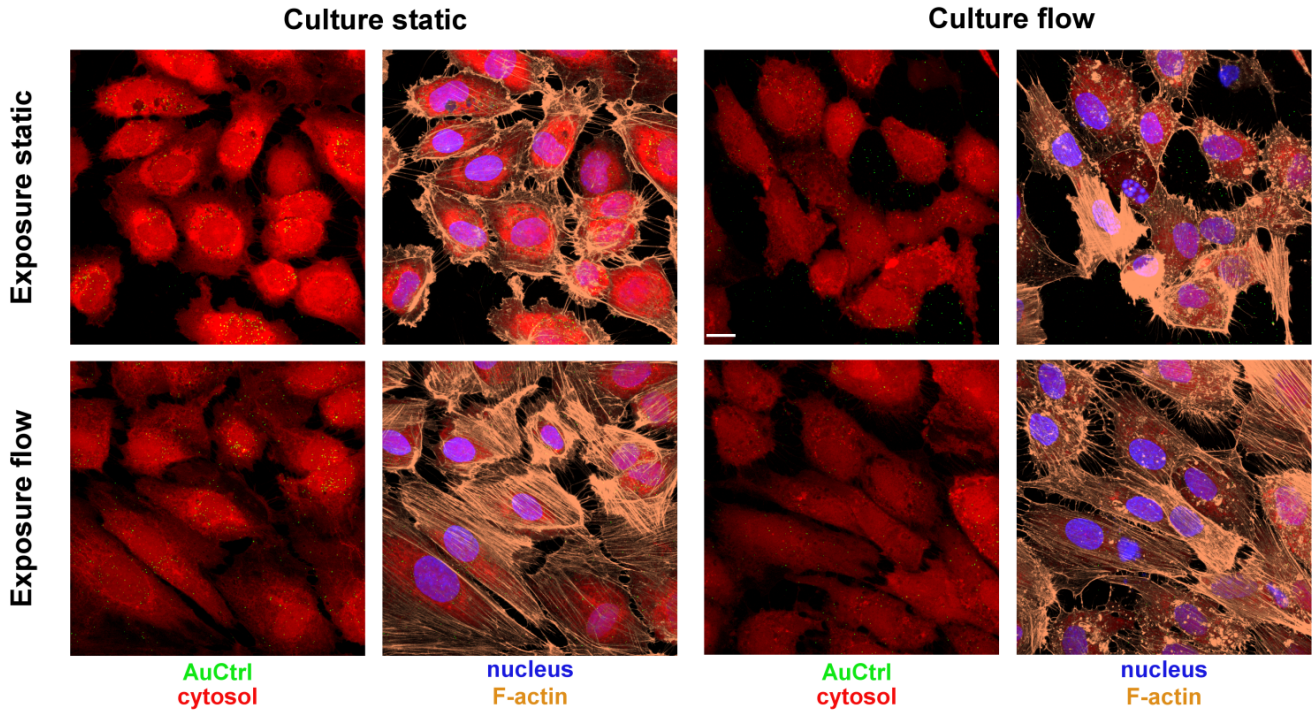

Figure S5 – Effects of 10 dyn shear-stress on F-actin organization in HUVECs

The cells were cultured static or adapted to shear stress for 24 h in flow (10 dyn) prior to exposure to unmodified AuNPs (AuCtrl) (5µg/ml for 3 h) in either static or flow conditions. Extended focus images of representative image-stacks were captured with AuCtrl visualised by reflection (green), the cytosol by CellTracker (red), the nucleus by Hoechst (blue), and F-actin by rhodamin phalloidin (coral) (n = 5). Scale bar = 14 µm, scaled for all images.

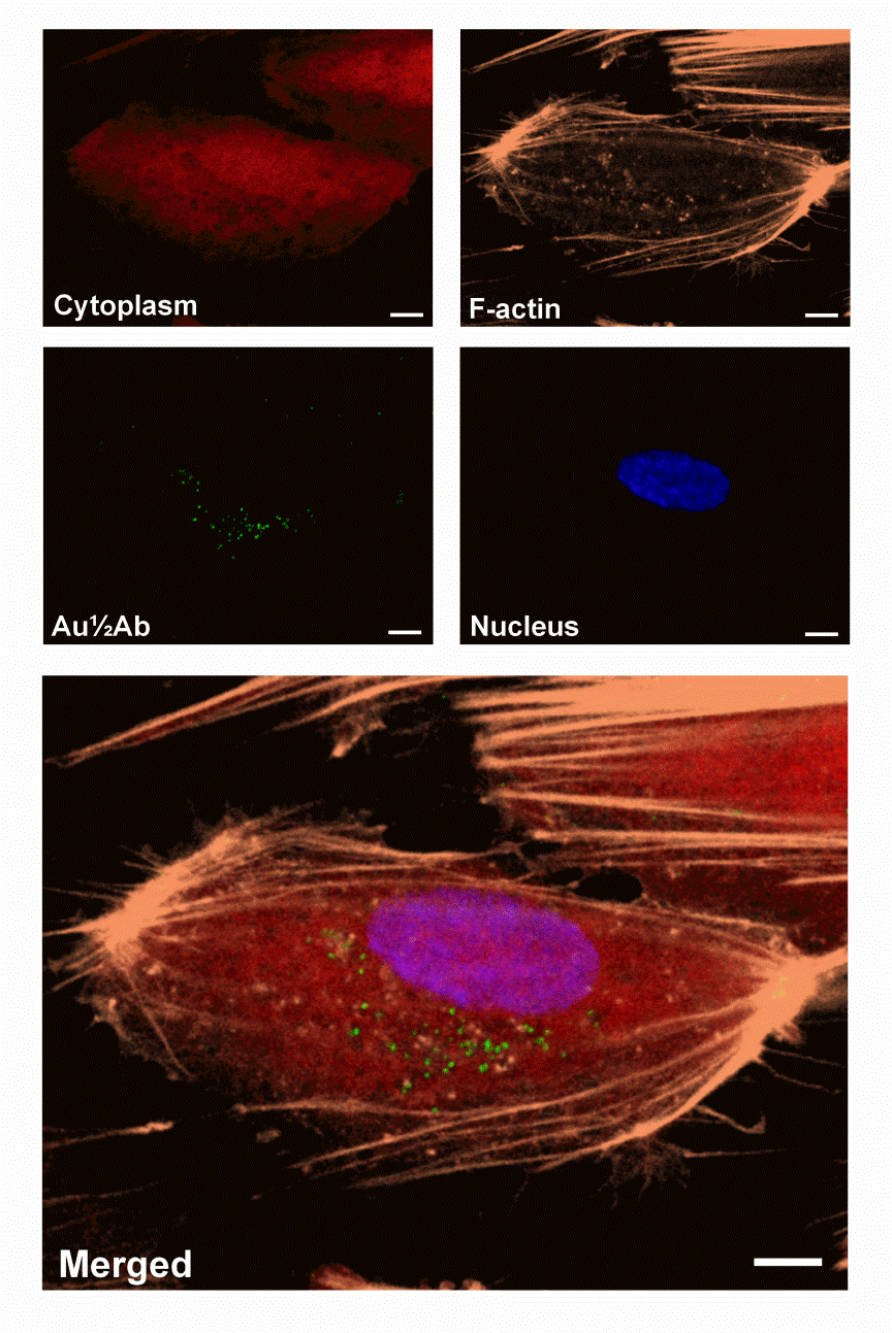

Figure S6 – Selected confocal microscopy z-plane image of static TNF activated HUVECs and internalised anti-ICAM AuNPs.

Representative z-plane of cells were visualised with CellTracker (red), rhodamin phalloidin (sandy brown), AuNPs by reflection (green), and Hoechst (blue). Scale bar = 6 μm.

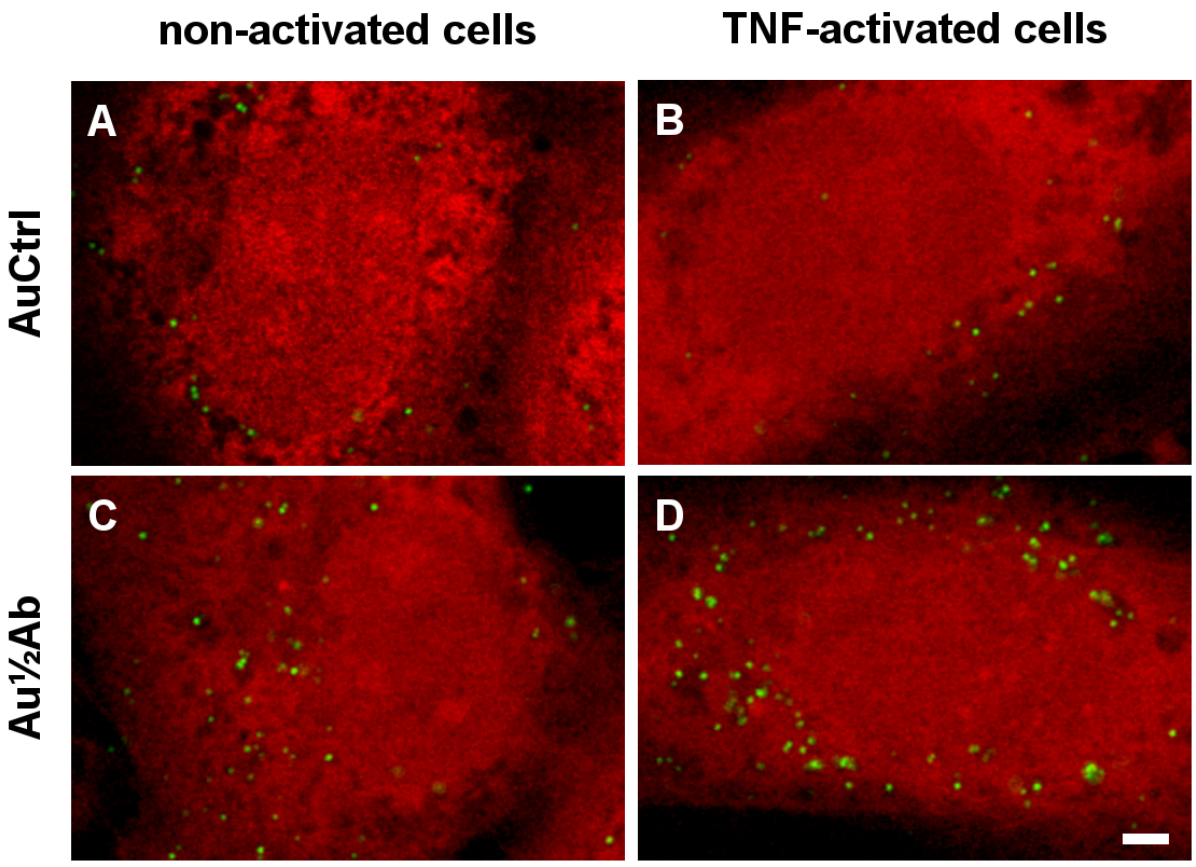

Figure S7 – Internalised AuNPs localised within vesicular structures in HUVECs in static exposure conditions

Representative z-planes of (A and C) non-activated HUVECs and (B and D) TNF-activated HUVECs (10 ng/ml, 24 h) either (A and B) exposed to unmodified AuNPs (AuCtrl) or (C and D) exposed to anti-ICAM-1 AuNPs (Au½Ab). Cells exposed to AuNPs were exposed for 3 h at a concentration of 5 µg/ml. Cells were stained by Cell Tracker (red) and AuNPs imaged by laser reflection (green). Scale bar = 3 µm.

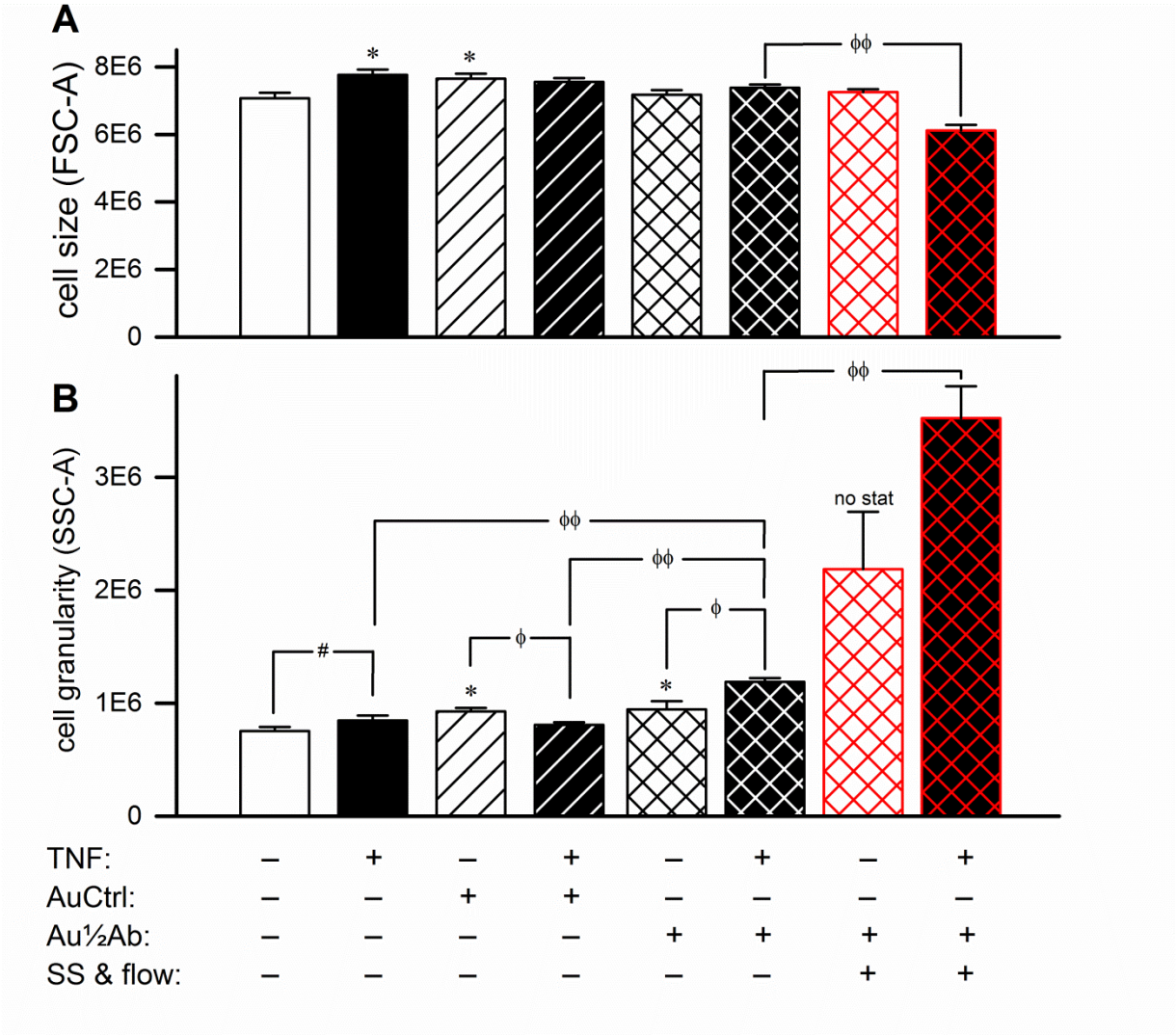

Figure S8 – Flow cytometry forward- and side-scatter data of HUVECs exposed to gold nanoparticles (AuNPs) in static condition or in shear stress & flow (SS & flow) conditions

Cells were exposed for 3 h to 5 µg/ml of unmodified AuNPs (AuCtrl) or anti-ICAM-1-AuNPs (Au½Ab). Cells were incubated with or without TNF (24 h, 10 ng/ml) prior to AuNP-exposure. Cells were either cultured and exposed in static conditions or cultured in shear stress for 24 h followed by exposure to AuNPs in flow conditions (SS & flow). Panel A shows cell size represented by forward-scatter (FSC-A). Panel B shows cell total reflection represented by side-scatter (SSC-A). *P < 0.05, **P < 0.001 compared to unexposed non-activated cells. $^{\phi}$P < 0.05, $^{\phi\phi}$P < 0.01, $^{\#}$P = 0.09 compared as indicated by the bracket lines. Error bars represent SEM of 5 (SS & flow-exposure = 3) independent experiments.

Description of files for videos

File S1 – 3D confocal images of non-activated HUVECs exposed to unmodified 80 nm gold nanoparticles (AuNPs) (5 μg/ml for 3 h) in static condition. The cytosol was stained by CellTracker (red) and the AuNPs were visualised by light reflection (green). The grid units are 14.13 μm.

File S2 – 3D confocal images of 24 h shear stress adapted and non-activated HUVECs exposed to unmodified 80 nm gold nanoparticles (AuNPs) (5 μg/ml for 3 h) in flow condition. The cytosol was stained by CellTracker (red) and the AuNPs were visualised by light reflection (green). The grid units are 14.13 μm.

File S3 – 3D confocal images of TNF-activated (10 ng/ml for 24 h) HUVECs exposed to 80 nm gold nanoparticles (AuNPs) conjugated with cleaved anti-ICAM-1 antibodies (5 μg/ml for 3 h) in static condition. The cytosol was stained by CellTracker (red), the nucleus by Hoechst (blue), and the AuNPs were visualised by light reflection (green). The grid units are 14.13 μm.

File S4 – 3D confocal images of TNF-activated (10 ng/ml for 24 h) and shear stress adapted (10 dyn for 24 h) HUVECs exposed to 80 nm gold nanoparticles (AuNPs) conjugated with cleaved anti-ICAM-1 antibodies (5 μg/ml for 3 h) in flow exposure condition (10 dyn). The cytosol was stained by CellTracker (red), the nucleus by Hoechst (blue), and the AuNPs were visualised by light reflection (green). The grid units are 14.13 μm.

Supplement Material Reference List

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