Effects of Gravitational Mechanical Unloading in Endothelial Cells: Association between Caveolins, Inflammation and Adhesion Molecules

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Mechanical forces including gravity affect endothelial cell (ECs) function, and have been implicated in vascular disease as well as physiologic changes associated with low gravity environments. The goal of this study was to investigate the impact of gravitational mechanical unloading on ECs phenotype as determined by patterns of gene expression. Human umbilical vascular endothelial cells were exposed to 1-gravity environment or mechanical unloading (MU) for 24 hours, with or without periods of mechanical loading (ML). MU led to a significant decrease in gene expression of several adhesion molecules and pro-inflammatory cytokines. On the contrary, eNOS, Caveolin-1 and -2 expression were significantly increased with MU. There was a decrease in the length and width of the cells with MU. Addition of ML during the MU period was sufficient to reverse the changes triggered by MU. Our results suggest that gravitational loading could dramatically affect vascular endothelial cell function.

Alterations in endothelial function has been involved in the pathogenesis of cardiovascular diseases on Earth through angiogenesis, vascular remodeling and atherosclerosis. Changes in endothelial hemo-
tasis have also been associated with post-spaceflight orthostatic intolerance. Hence understanding functioning of the vascular endothelium becomes critical for different diseases pathogenesis. The healthy endothelium maintains a critical relationship with the outside environment through the blood and hemodynamic forces. This relationship is governed by a well-studied frictional force: shear stress. Regulation of vascular endothelial cells (ECs) responses to shear stress involve a complex cascades of gene responses with different temporal profiles and shear stress impacts ECs morphology, function and gene expression, the latter taking place transcriptionally and/or post-transcriptionally. In fact, DNA microarray analysis has demonstrated that 3% of all genes responded to shear stress. If ECs express 20,000 genes, then approximately 600 would be responsive to shear stress. If ECs express 20,000 genes, then approximately 600 would be responsive to shear stress.

The cytoskeleton is at the center of the ECs responses to shear stress. The cells respond to rapid laminar flow by becoming spindle-shaped and aligned with their long axis parallel to the direction of blood flow. This is accompanied by cytoskeletal reorganization with actin filaments rearranged into bundles of stress fibers and aligned in the direction of the shear stress. When flow is turbulent or stagnant, the cells become rounder in shape and do not have a uniform orientation. Hence, the cytoskeleton is critical in sensing mechanical forces, with a change in their morphology leading to changes in inflammation and atherogenesis.

Although shear stress has been studied extensively, mechanotransduction, that is the mechanisms by which cells convert mechanical stimulus into chemical activity are not fully understood. It is thought that sensing of mechanical forces and shear stress signal transduction through the cytoskeleton take place, at least partially through caveolae. The caveolae are membrane microdomains measuring approximately 50–10 nm in length that are visible as flask-shaped invaginations below the surface of cells, containing many signaling molecules. In response to increased flow, calcium gradients develop close to caveolae and propagate through the entire cell in the form of a calcium wave. The calcium increase close to caveolae causes the caveolae to rapidly liberate the nitric oxide (NO) synthase eNOS into the cytoplasm, where it catalyzes the production of NO. Caveolins have also...
been reported to be involved in the early phases of atherosclerosis\textsuperscript{22} with absence of caveolin-1 in mice characterized by impaired blood-flow-dependent vascular remodeling and vasodilator responses\textsuperscript{19}.

In-vitro techniques used to study mechanotransduction have included fluid flow (shear stress), four-point bending, substrate stretch, as well as gravity force, vibration, magnetic fields, atomic forces and shockwaves\textsuperscript{24}. The effects of gravitational forces on mechanotransduction in ECs responses have been the matter of only a few investigations and remain largely unknown. It is well known that astronauts experience cardiovascular deconditioning during spaceflight manifested among others as orthostatic intolerance, and some investigators have suggested that the nitric oxide system is involved in these changes\textsuperscript{1}. At the physiological level, we demonstrated changes in the cardiovascular system with simulated microgravity characterized by alterations in sympathetic function, the renin-angiotensin system and electrolyte excretion\textsuperscript{25–27}. Overall, understanding the effects of gravitational mechanical forces is important as mechanical unloading (MU) of cells has been shown to alter cell cytoskeleton\textsuperscript{28}, affect caveolae\textsuperscript{29} and eNOS\textsuperscript{30,31}. If shear stress can affect the cytoskeleton, cell function and expression, can gravitational mechanical forces do the same? Since caveolins are gravity-sensing elements and eNOS is tightly related to inflammation and cell-cell interaction including adhesion\textsuperscript{32}, could microgravity have the potential to alter processes such as inflammation and cell-to-cell interaction?

In this report, we investigate the effects of gravitational MU on primary ECs. The goals are to assess whether cell morphology is involved in functions such as eNOS regulation, inflammation and adhesion. To assess this, primary vascular endothelial cells (human umbilical vein endothelial cell- HUVECs) were placed under MU and mechanical loading (ML) conditions after which gene expression profiles and the cytoskeleton were studied. We hypothesized that MU would lead to changes in the morphology of the cells and their gene expression, which could be reversed by ML.

**Results**

**Effects of mechanical unloading on adhesion molecules and inflammation.** To assess if exposure of endothelial cells to simulated MU has a significant impact on expression of adhesion molecules or inflammatory angiogenic factors, we used quantitative RT-PCR and flow cytometry to measure the levels of gene expression and presence at the cell surface respectively. We found that 24 hours of MU leads to a significant decrease in the expression of adhesion molecules ICAM-1, VCAM-1 and E-Selectin as well as inflammatory mediators IL-6 and TNF-\(\alpha\) (Figure 1a). This response remained similar after 48 hours of exposure to MU (Figure 1a). Reflecting changes in gene expression, the presence on the cell surface of ICAM-1, VCAM-1 and E-Selectin were also significantly decreased by MU at 24 and 48 hours (Figure 1b).

**Effects of gravitational mechanical unloading on eNOS and caveolins.** In order to investigate a possible pathway involved in the regulation of gene expression of inflammatory and adhesion molecules in ECs, we studied endothelial NOS (eNOS), caveolin-1 and caveolin-2 expression levels. There was an increase in eNOS, Caveolin-1, Caveolin-2 gene expression after 24 h of MU (Figure 2a). This was also coupled with a significant increase in nitrite concentration in the media (Figure 2b).

**Inhibition of NOS activity does not reverse the effects of mechanical unloading.** To assess if an increase of NOS activity could underlie the changes in expression levels of adhesion molecules and inflammatory factors triggered by MU, we treated the HUVECs with the selective inhibitor of NO synthesis L-NAME. Interestingly, the inhibition of NOS activity did not reverse the changes associated with MU (Figure 3), suggesting that NO production is not responsible for the decrease of adhesion molecules and pro-inflammatory gene expression in this model. Compare to MU alone, the addition of L-NAME to MU led to a decrease in eNOS and Cav-1 (although not a complete return to the levels seen with control) and an increase in Cav-2.

**Mechanical loading reverses the changes mediated by mechanical unloading in endothelial cells.** To further investigate the causes of the molecular changes triggered by MU, we studied the effect of short reloading periods by applying mechanical loading (ML) to the ECs during the period of MU. Strikingly, three short periods of ML during 24 hours of MU were sufficient to completely reverse the changes seen in adhesion molecules, inflammatory mediators, eNOS and caveolins (Figure 4).

**Cytoskeletal changes associated with MU.** MU significantly affected the morphology of HUVECs. There was a decrease in the length and
width of the cells with MU (Figure 5a). There appeared to be disorganization of the F-actin network with clustering of the fibers around the nucleus. Addition of L-NAME did not return the shape of the cells to baseline. However, paralleling changes in gene expression, addition of ML in the MU environment tended to reverse the shape of the cells towards 1 G conditions.

Using immunofluorescent labeling, we found that caveolin-1 are less associated to the plasma membrane and adopt a perinuclear localization under MU compared to the 1 G conditions (Figure 5b). Interestingly, the translocation of Cav-1 from caveolae to the Golgi apparatus has been shown to be responsible for an increase of eNOS activity in vascular ECs. The exact localization of Cav-1 under MU and a direct link with the increase of eNOS activity need to be established. However, the disruption of the actin cytoskeletal organization by MU could impair the translocation of Cav-1 to the caveolae, and together with the increase of eNOS expression, could contribute to the increase of NOS activity. Addition of L-NAME during the MU exposure had no effect on Caveolin-1 distribution, which remains perinuclear, however ML seemed to change towards 1 G conditions.

The results of the immunofluorescent labeling of VCAM-1 (Figure 5c) and E-selectin (Figure 5d) are consistent with the analysis by qRT-PCR and flow cytometry (Figure 1) and show a decrease of their presence at the cell surface under MU condition. Interestingly, the process tended to go back to 1 G conditions with the addition of ML.

**Hindlimb suspension: Effects on caveolins.** Consistent with our *in vitro* cellular model, simulation of MU by hindlimb suspension led to a significant increase in eNOS and Caveolin-1 and -2 expression in mouse aortas (Figure 6). IL-6 also significantly decreased. Furthermore, there was a significant decrease in E-Selectin expression. However, expression of other inflammatory or adhesion molecules did not decrease significantly with hindlimb suspension and ICAM-1 expression was significantly increased.

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**Figure 2 | Gene Expression of Endothelial Nitric Oxide Synthase, Caveolins and Nitrite concentration with Mechanical Unloading.** (a) Quantitative analysis by qRT-PCR of gene expression of cultured Human Umbilical Vein Endothelial Cells (HUVEC) placed in Random Positioning Machine (RPM) simulating Mechanical Unloading (MU) vs. ground controls (1 G) after 24 hours. (b) Measure of the Nitrite Concentration in the Media of HUVECs under 1 G or MU conditions. Results are representative of five separate cell donors. Bars represent mean ± SD. (n = 5, * p ≤ 0.05; with two-tailed Student’s t test against control samples).

**Figure 3 | Gene Expression with Mechanical Unloading and Treatment with L-NAME.** Quantitative analysis by qRT-PCR of gene expression in cultured Human Umbilical Vein Endothelial Cells (HUVEC) subjected to 24 hours of ground control conditions (1 G) or Mechanical Unloading (MU) or MU with L-N^3^-Nitroarginine methyl ester (L-N) treatment. (a) Expressions of adhesion molecules (ICAM-1, VCAM-1, E-selectin), Cytokines (IL-6 and TNF-α) decrease significantly when exposed to MU and MU + L-N with no difference between MU and MU + L-N samples. (b) eNOS, CAV-1 and CAV-2 expression increases significantly upon exposure to MU and MU + L-N but the addition of L-N decrease significantly the increase of eNOS and CAV-1 gene expression induced by MU alone. Bars represent mean ± SD (n = 5, * p ≤ 0.05 with two-tailed Student’s t test against control samples, NS non significant).
**Figure 4 | Gene Expression with Mechanical Unloading and Mechanical Loading.** Quantitative analysis by qRT-PCR of gene expression in cultured Human Umbilical Vein Endothelial Cells (HUVEC) subjected to 24 hours of ground control conditions (1 G) or Mechanical Unloading (MU) or MU with 3 periods of 30 minutes of Mechanical Loading (MU + ML). (a) Expressions of adhesion molecules (ICAM-1, VCAM-1, E-selectin), cytokines (IL-6 and TNF-α) decrease significantly when exposed to MU but were not significantly decreased by the MU + ML conditions. (b) eNOS, CAV-1 and CAV-2 expression increases significantly upon exposure to MU but this effect was significantly reverted by the addition of ML. Bars represent mean ± SD (n = 5, * p ≤ 0.05 with two-tailed Student’s t test against control samples, NS non significant).

**Figure 5 | Immunofluorescence of Mechanically Unloaded cells with and without L-NAME or Mechanical Loading.** Labeling of cytoskeletal F-Actin, nuclei, Caveolin-1, VCAM-1 and E-selectin antibodies. (a) Both cell dimensions width and length significantly decrease under Mechanical Unloading (MU) conditions with and without L-Name (L-N), more so when cells are both mechanically unloaded and treated with L-N. However, cell dimensions seems to be reversed or remains about the same as 1 G control upon exposure to MU with 3 periods of 30 minutes of Mechanical Loading (ML). (b) Caveolin-1 adopt a perinuclear localization upon exposing HUVECs to MU and MU + L-N compared to control but the effect seems to be reverted under MU + ML conditions. (c and d) Surface presence of VCAM-1 and E-selectin tends to decrease upon exposing HUVECs to MU and MU + L-N, more so when cells are both mechanically unloaded and treated with L-N. Nonetheless, the effect tends to be reverted under MU + ML conditions. Bars represent mean ± SD (n = 5, * p ≤ 0.05, ** p ≤ 0.01 with two-tailed Student’s t test against control samples). Scale bars: 50 μm.
Discussion

In this study, we demonstrated that MU changes the morphology of cells and leads to a decrease in inflammatory gene expression (i.e. IL-6, TNF-α), adhesion molecule gene expression and cell-surface expression (i.e. ICAM-1, VCAM-1, E-Selectin) as well as an increase in eNOS, nitrite concentration and an increase in Caveolin-1 and Caveolin-2 expression. We also demonstrated reversal of the morphology and gene expression towards baseline with three short periods of mechanical loading. Since alterations in gene expression were accompanied by changes in the cytoskeleton and both cell morphology and gene expression returned to baseline with the addition of hypergravity, our findings suggest that ECs gene expression and cytoarchitecture are tightly linked to gravitational mechanical forces, with a possible role for the caveolins[35,36].

The present study demonstrates that microgravity alters the cytoskeleton of ECs. It appears that the actin filaments become disorganized and redistributed within the cells, with as a possible consequence an inability to transport molecules to the outside of the cells, such as the caveolins to the caveolae at the plasma membrane. Cells are known to stabilize their structure and shape by means of interconnected network of cytoskeletal components including microfilaments, microtubules, and intermediate filaments. In vitro, when subjected to shear stress in a flow-loading device, net cellular movement happens orienting them in the direction of the shear stress5, a phenomenon accompanied by cytoskeletal reorganization with actin filaments rearranged into bundles of stress fibers and aligned in the direction of the shear stress10-12.

In microgravity, cytoarchitectural alterations of different endothelial cell lines have been reported to occur. Buravkova et al demonstrated changes in the cytoskeleton of HUVECs within 1–2 hours of simulated microgravity with actin filament thinning and their redistribution to cell borders (with the central area becoming practically free of actin fibers while there was formation of continuous F-actin band at the intercellular contact area)37. Siamwala demonstrated that 2 hours of simulated microgravity in EAhy926 ECs lead to actin rearrangements and increase in nitric oxide production38. Versari et al also showed that HUVECs in microgravity for 96 hours had disorganization of the actin cytoskeleton and a decrease in the total amount of actin39. Infanger et al also showed that EA.hy926 cells in microgravity had altered cytoskeletal components and the formation of cell aggregates in the monolayer after 12 hours. Carlsson et al also demonstrated disorganization of the actin fibers with clustering of the fibers around the nucleus up to 96 hr in simulated microgravity, then disappearing after 144 hours40. Grimm et al41 showed that EA.hy926 cells in microgravity formed tubular structures that actually had an increased amount of actin that covered the entire surface of the tubular structure.

It appears that expression and localization of key integrins that mediate EC attachment and spreading, such as the vitronectin and fibronectin receptors, could likely be involved in the changes seen in the cytoskeleton. For example, integrins have been shown to be decreased during parabolic flight in HUVECs[42]. The expression of fibronectin has been found to be increased with simulated microgravity in EA.hy.92643. Tube-shaped structures of EA.hy.926 forming after 2 weeks of simulated microgravity produced more fibronectin. Although not demonstrated in endothelial cells to our knowledge, Rho GAP expression has been found to be increased two fold with spaceflight in rat osteoblasts, suggesting that microgravity suppress Rho signals regulating actin filament rearrangement44. Since the cytoskeleton plays an important role in shear-stress-induced NO production and ICAM-1 gene expression by ECs with shear stress[15,16], it could then be expected that MU also alters expression of eNOS, inflammatory and adhesion molecules.

We demonstrated in this study a decrease in IL-6 and TNF-α expression with MU, which was reversed by hypergravity. This was coupled with a decrease in adhesion molecules ICAM-1, VCAM-1 and E-Selectin, also reversed with hypergravity. Investigators have reported different effects of mechanical unloading or microgravity in the literature. For example, IL-6 was found to be increased45, decreased46, and unchanged47 in separate studies, which may be related to different conditions. IL-8 was also found to be increased48 and unchanged49 in separate studies. Buravkova et al found that 12-24 hours of simulated microgravity increased ICAM-1 and VCAM-1 when subjected to shear stress[15,16] but not VCAM-1 or E-Selectin- in fact microgravity had opposite effects on ICAM-1 compared to VCAM-1 and E-Selectin[51].

In this study, we found that mechanical unloading led to an increase in Caveolin-1 and -2 gene expression. This correlated with an increase in eNOS expression and nitrite concentration. Our results are overall consistent with the forming body of knowledge on the effects of simulated microgravity on endothelial cells. For example, Spisni et al.46 found that caveolin-1 protein expression was increased with 24 hours of microgravity. With regards to NO production, two studies showed an increase in eNOS protein levels and increased NO production at 48 hours and 72 hours respectively[10,31]. However, Spisni et al.49 found no change in eNOS or iNOS protein levels yet an increase in NO production at 24 hours. Ulbrich et al.45 actually found decreased eNOS gene expression at 24 hours. Finally, Wang et al.50 found increased iNOS protein levels.

Figure 6 | Gene expression in Aortic Endothelial Cells from Hindlimb suspension mouse model. Mice were used as animal model in a hind limb suspension experiment. The tails of the animals were suspended to simulate physical inactivity and mechanical unloading. (a) eNOS, CAV-1, CAV-2 expression increased in aortic endothelial cells of suspended animal (HL) compare to controls (CL) as seen in the mechanically unloaded HUVECs. (b) IL-6 and E-Selectin significantly decreased with simulated physical inactivity. Bars represent mean ± SD. (n = 4, * p ≤ 0.05; with two-tailed Student’s t test against control samples).
at 24 hours. Recently, Shi et al reported that HUVECs exposed to 24 hours of simulated microgravity using a 2-D clinostat had an enhanced eNOS activity and promoted angiogenesis51.

It is likely that the changes seen in inflammation, adhesion molecules expression, eNOS and caveolins are mediated through a change in the cytoskeleton since the cytoskeleton is altered by MU and returns toward baseline with ML, along with gene expression. This study has important implications for spaceflight. It is well known that astronauts suffer from orthostatic intolerance (OI) upon returning to a gravity environment after spaceflight. It has been suggested that the nitric oxide system may be involved4. Our findings support a role of increased NO production in the pathophysiology of post-spaceflight OI but more importantly, suggest that this variable may be improved by the addition of artificial gravity since 3 periods of 30 minutes of hypergravity in this study reversed the changes seen in microgravity. In addition to the implications of our findings to OI, it is interesting to note that the changes seen would be considered “anti-inflammatory” and possibly protective for early atherogenesis (Figure 7). This opens the way to explore new therapies to treat cardiovascular diseases by manipulation of cell phenotype using gravitational forces. Although the significance of the findings with regards to an overall “dysregulation” of the cells compared to a true change towards an anti-inflammatory phenotype need to be further assessed, our findings would suggest that microgravity may have a beneficial impact on the health of blood vessels. It has been of important interest to know that astronauts are more at risk of immune dysfunction52–54. The changes in the vascular ECs could therefore be involved in the overall changes in the immune system seen with spaceflight. Our findings support the fact that the cells respond to altered gravitational environments, which could have an important impact on organ physiology.

In summary, gravitational mechanical unloading affects inflammatory and adhesion molecules expression in ECs towards an “anti-inflammatory” phenotype. This is coupled with an increase in eNOS and caveolin gene expression. In view of the changes seen in the

![Diagram](https://www.nature.com/scientificreports)

**Figure 7 | Proposed Model for the Effects of Microgravity on Endothelial Cells.** Proposed model by which microgravity leads to changes in inflammation, adhesion molecules, caveolins and eNOS. Those changes are thought to be mediated through a change in the cytoskeleton.

| Table 1 | Human and mouse primers |
|---|---|---|
| **Human Primers** | | |
| Primer | Forward Primer 5’-3’ | Reverse Primer 5’-3’ |
| eNOS3 | GAGACTTCCGAATCTGGAAACAG | GCTCGGTGATCTCCACCGT |
| CAV1 | CGACCTTAAACACCTCCCAAG | TAAATGGCCCGAGTGGTGG |
| CAV2 | ATGCCTCTTCTTGAATCAGC | CTTGACAAATGGACTATT |
| ICAM1 | GTGGTAGCAGCGCGGATC | GCCTTAGTATCGGTTTCA |
| VCAM1 | AATGGGAATCTACAGCACCT | ATACCGGTATCTCTAAAA |
| E-sel | ACCCTCCAGGAAAGCTATGACT | CAGACCCACACATGTGATT |
| IL-6 | GCTGAAAAGAGATGGATGCTT | GCTTTGTCCTCAGAATCT |
| TNFa | TCAGATCATCTTCTCGAACC | ATCTTCAGCTCCACCC |
| **Mouse Primers** | | |
| Primer | Forward Primer 5’-3’ | Reverse Primer 5’-3’ |
| eNOS3 | TCAGGCTATCACGAGTGGTCCC | ATAGGCGGCGCATGAC |
| CAV1 | ACAGGGCAACATATCTCAAAGC | ATCTTAGACGAACAGC |
| CAV2 | CCCCTGTGAGCTTCATGCG | ATCTCCATATTCTGAC |
| ICAM1 | GGCCTGCTGTTTGAGAACAT | ATACAGGCTTAGTGGG |
| VCAM1 | GCCCCTAATACGCGAAGG-T | ATGTTCAACCGGATCTG |
| E-sel | CAAATCTGAAAACATTACCCAG | CGAGTCTTCTCTCATG |
| IL-6 | TCTATACACTTCTCAGCGA | GAATGCGCATGACAAAT |
| TNFa | CCCTTCACTGACATCCCATCTT | GCTACGAGCCTGAG |

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cytoskeleton and the reversal of both gene expression and morphology with exposure to ML, it is highly likely that this “anti-inflammatory” phenotype is mediated through changes in the cytoskeleton. Our findings further support a role for caveolae as gravity-sensing elements and demonstrate plasticity of changes with gravitational mechanical forces.

Methods

Cell culture. Human Umbilical Vascular Endothelial Cells (HUVECs; HUVECs Clonetics, Lonza) were cultured at 37°C/5% CO2 in EBM-2 Endothelial Cell Basal Medium-2 supplemented with 2% fetal bovine serum (FBS), 0.2% (v/v) Gentamicin Sulfate and Amphotericin-B, 0.2% (v/v) Heparin, 0.2% (v/v) Hydrocortisone, 0.2% 57,58. The research protocol was reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the Veterans Affairs Medical Center San Francisco. Three months old FBV/bN mice were used. Hindlimbs of 8 mice were lifted approximately 0.5–1 cm off the floor and beared no weight. The tail was cleaned with alcohol and sprayed with small hook was then applied to the tail length-wise. Filament tape was used to wrap a Bio-Rad’s 3Q Single-Cell Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA). Gene expression level were quantified using the primers listed in Table 1 and were normalized to cyclinH (CPII) internal standard. Five independent biological samples were used.

Fluorescence microscopy. Confluent HUVECs were trypsinized and plated on Nuncl Lab Tek Chamber Slides (Fisher Scientific Pittsburg, PA USA). Immediately after the experiments, the cells were fixed with 3.7% Formaldehyde for 30 minutes, then blocked and permeabilized in PBS with 1%BSA and 0.2% Triton X-100 overnight at 4°C. Cells were then incubated with primary antibodies for 1 hour at room temperature. Rabbit polyclonal anti-human-E-selectin (20 μg/mL, Abcam, Cambridge, MA USA) or Rabbit polyclonal anti-human-Caveolin-1 (dilution 1: 250, Novus Biologicals, Littleton, CO, USA) were used in combination with Hoescht dye (2 μg/mL) and Rhodamine Phalloidin (2 μM) (Invitrogen, Eugene, OR USA). After 3 washes in PBS, cells were incubated with secondary antibody Alexa Fluor 488 Goat Anti Rabbit IgG (H+L) (1:1000, Invitrogen, OR USA) during 1 hour at room temperature. After 3 washes in PBS, cells were mounted using Permount histological mounting medium (Fisher Scientific, Pittsburgh, PA) and imaged using a Zeiss Axioscope Fluorescent Microscope (Carl Zeiss, Germany) camera and a (Hamamatsu Corporation, Bridge-water, NJ USA) fluorescent microscope.

Flow Cytometry. Upon completion of the experiments, cells were washed 2 times with PBS. Cells were detached from the 25 cm² flasks using 5 mL EDTA in PBS containing Ca²⁺/Mg²⁺, resuspended in ice-cold PBS with 2% FBS and filtered with a 40 μm cell strainer. Cells were blocked in PBS with 2% FBS containing 10% antibody host serum and 1% IgG/Fc Block (Sigma, St. Louis, MO USA). Cells were washed with PBS 2 times and then incubated with monoclonal mouse anti-human FITC-Conjugated ICAM-1/CD54 (dilution 1:100), Monoclonal mouse Anti-human Phyceroerythrin-Conjugated VCAM-1/CD106 (dilution 1:100, R&D Systems, Minneapolis, MN USA) and monoclonal mouse anti-human PE-Cy5-Conjugated E-Selectin/CD62E (dilution 1:100, BD Biosciences, San Diego, CA USA). Cells were resuspended in 200 μL of PBS with 2% FBS and analyzed using a LSR II flow cytometer (BD Biosciences) and FlowJo software (Tree Star).

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Author contributions

S.M.G., M.J. and M.H.F. designed the experimental protocols and interpreted the

results. J.A.Z., S.M.G., M.J. and M.H.F. performed the experiments. S.M.G. primarily

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Additional information

Competing financial interests: The authors declare no competing financial interests.

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