Published in final edited form as:

ACS Appl Mater Interfaces. 2015 March 4; 7(8): 4525–4532. doi:10.1021/acsami.5b00554.

Combined Effects of Substrate Topography and Stiffness on Endothelial Cytokine and Chemokine Secretion

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Abstract

Endothelial physiology is regulated not only by humoral factors but also by mechanical factors such as fluid shear stress and the underlying cellular matrix microenvironment. The purpose of the present study was to examine the effects of matrix topographical cues on the endothelial secretion of cytokines/chemokines *in vitro*. Human endothelial cells were cultured on nanopatterned polymeric substrates with different ratios of ridge to groove widths (1:1, 1:2, and 1:5) and with different stiffnesses (6.7 MPa and 2.5 GPa) in the presence and absence of 1.0 ng/mL TNF- α . The levels of cytokines/chemokines secreted into the conditioned media were analyzed with a multiplexed bead-based sandwich immunoassay. Of the nano-patterns tested, the 1:1 and 1:2 type-patterns were found to induce the greatest degree of endothelial cell elongation and directional alignment. The 1:2 type nanopatterns lowered the secretion of inflammatory cytokines such as IL-1 β , IL-3 and MCP-1, compared to unpatterned substrates. Additionally, of the two polymers tested, it was found that the stiffer substrate resulted in significant decreases in the secretion of IL-3 and MCP-1. These results suggest that substrates with specific extracellular nanotopographical cues or stiffnesses may provide anti-atherogenic effects like those seen with laminar shear stresses by suppressing the endothelial secretion of cytokines and chemokines involved in vascular inflammation and remodeling.

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ASSOCIATED CONTENT

Supporting Information Available: Diagram illustrating the overlapping physiological roles of the secreted cytokine and chemokines analyzed in this study. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Keywords

nanotopography; substrate stiffness; endothelial cells; cytokines; chemokines

INTRODUCTION

As a membranous organ located between the blood stream and the blood vessel wall, the endothelium plays a key role in maintaining vascular health.^{1,2} The structural and functional integrity of the endothelium is important in preventing the leakage of blood proteins and cells out of the blood vessels, and in maintaining the optimal synthesis and secretion of substances involved in vasodilatation, blood coagulation, inflammation and the immune response.^{1,3} Thus, endothelial dysfunction is considered the precursor of various vascular diseases. Cell-cell and cell-extracellular matrix interactions are known to trigger various signal transductions associated with cell fate such as migration, proliferation, death, and differentiation.⁴⁻⁶ The surface topography of underlying vascular components is also an important extracellular cellular environmental factor for endothelial physiology.⁷ Advances in nanofabrication technology make it possible to analyze and control live cell behavior within nanoengineered platforms.⁸⁻¹¹ Recent studies have suggested that nanotopographic cues and substrate rigidities are important factors affecting the morphology and behavior of vascular cell types, including endothelial cells.^{7,12-14}

Cytokines/chemokines are diverse groups of soluble proteins and peptides which serve as communication signals with other cells, as well as with diverse tissues and organs.^{15,16} Vascular endothelial cells are a regulatory target as well as a secreting source of cytokines/chemokines. The major cytokines/chemokines closely associated with endothelial physiology include tumor necrosis factor- α (TNF- α), interferon- γ (IFN- γ), interleukin-4 (IL-4), IL-10, IL-13, IL-1 β , IL-6, IL-3, granulocyte-macrophage colony-stimulating factor (GM-CSF), vascular endothelial growth factor (VEGF), fibroblast growth factor-2 (FGF-2), granulocyte colony-stimulating factor (G-CSF), and monocyte chemoattractant protein-1 (MCP-1). These cytokines have been divided into distinct categories of functions such as immunity, inflammation and thrombosis, and angiogenesis, and many cytokines fall under multiple categories.¹⁷ The analysis of the production of endothelial cytokines/chemokines will help in understanding coordinated endothelial function under specified conditions.

The purpose of the present study is to study the effects of nanotopography and substrate stiffness on the endothelial secretion of major cytokines/chemokines *in vitro*. Endothelial cells were cultured on substrates of varying nanotopographical dimensions, and comprised of polymers of two different moduli, NOA76 (6.7 MPa) and NOA86 (2.5 GPa). Because cell physiology can be affected not only by physical micro-environmental factors but also by inflammatory agonists, experiments were performed under both basal conditions and more inflammatory conditions stimulated by TNF- α . This inflammatory cytokine was chosen for this purpose because it is an multipotent cytokine that plays a major role in the regulation of cell survival, proliferation, differentiation and death.¹⁸

Analysis of the conditioned media was performed using a multiplexed immunoassay that allows simultaneous analysis of many cytokines/chemokines using a small volume of serum samples.^{19,20} The results of this study suggest that endothelial secretion of cytokines/chemokines is affected by substrate topography and stiffness, and some of the effects, ie., decreased MCP-1 secretion, are similar to those seen in endothelial cells exposed to laminar shear stress (LSS) compared to non-uniform shear stress.²¹ Such information would be useful in understanding the effects of extracellular environmental factors on vascular physiology associated with innate immunity, inflammation, thrombosis, and angiogenesis.

EXPERIMENTAL SECTION

Fabrication and characterization of nanopatterned substrates

The topographic nano-patterns were fabricated on flexible polyethylene terephthalate (PET) films using two different poly(urethane acrylate) (PUA)-based polymers (NOA76 and NOA86, Norland Optical Adhesives, Cranbury, NJ, USA), via ultraviolet (UV)-assisted capillary force lithography as previously described (Figure 1A).^{12,22} A small amount of polymer was drop-dispensed onto a glass coverslip (No.1, 25mm, Fisher Scientific, Pittsburgh, PA, USA), and a PUA nanopattern mold on PET film was placed onto the polymer drop. The polymer spontaneously filled the cavity of the mold through capillary action. After curing by exposure to UV light ($\lambda = 250 \sim 400$ nm, dose = 100 mJ/cm²) for 30 s through the transparent backplane, the mold was peeled off from the substrate, leaving a nanopatterned polymer on the the glass coverslip. The fabricated nanopatterns consisted of an array of parallel ridges with a constant ridge width (550 nm) and depth (600 nm), and variable groove widths (550 nm, 1.10 μ m, and 2.75 μ m), as verified by measurement with a scanning electron microscope (S-4300 FESEM, Hitachi high tech, Tokyo, Japan). The ratios of ridge width to groove width (1:1, 1:2, and 1:5) were used in designating the nanopatterns in the present study. The stiffnesses of NOA76 (6.7 MPa) and NOA86 (2.5 GPa) were commercially characterized by the polymers' manufacturer.

Surface wettability of polymer substrates

A goniometer (First Ten Angstroms FTA200, Portsmouth, VA, USA) was used to take contact angle measurements of a water droplet on substrates comprised of unpatterned and patterned NOA76 and NOA86. Approximately 10 μ L of water was carefully pipetted onto the samples, and images were taken five seconds after the droplet was first placed on the samples. For patterned substrates, the water contact angle parallel and orthogonal to the direction of the nanopatterning were measured, with the angles defined as θ_y and θ_x , respectively. The settings of the camera were set at 75% contrast and 43% brightness. Water contact angles were quantified using the goniometer's software package.

Cell culture on the nanopatterned substrates

EA.hy926 human endothelial cells (American Type Culture Collection, Manassas, VA, USA) were cultured on 100-mm tissue culture dishes (BD Biosciences, San Jose, CA, USA) at 37°C and 5% CO₂ in Dulbecco's modified Eagle's medium (Gibco BRL, Grand Island, NY, USA) with 10% fetal bovine serum (Gibco BRL,) and antibiotics (100 U/mL penicillin, 100 μ g/mL streptomycin, 0.25 μ g/mL amphotericin B). Nanopatterned substrates were

attached to a bottomless culture plate (SPL, Pocheon, Gyeonggi-Do, Korea) with the nanopattern facing up. The plate was washed with PBS, sterilized with UV on a clean bench, and used in cell culture without any additional surface treatments. Cells were seeded onto the nanopatterned substrates and cultured in growth media without phenol red for 24 h. Some groups were then treated with 1.0 ng/mL TNF- α for 12 h. The conditioned cell culture media were collected and stored for further analysis.

Cell morphology analysis

Cell orientation angles were quantified by the analysis of microscopic cell images using ImageJ software (National Institutes of Health, Bethesda, Maryland, USA). The cell orientation angle represents the angle between the longer axis of the approximated boundary of individual cell and the nanoridges' direction (0°). The measured angle was from -90 to +90°. Data were presented as the % distribution of cell orientation angles.

Cytochemistry

Cells were rinsed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min. The fixed cells were washed with PBS, and permeabilized with 0.1% Triton X-100 in PBS for 5 min. The cells were stained with Alexa Fluor Phalloidin (Invitrogen, Carlsbad, CA, USA) at 5 units/mL for 20 min to detect F-actin. After rinsing the cells 5 times with PBS, cells were stained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma, St. Louis, MO, USA) at 1 μ g/mL for 10 min to detect nuclei. The cells were air dried and then mounted in a permanent mountant ProLong®Gold reagent (Invitrogen) under a coverglass and examined with a confocal microscope (LSM 5 PASCAL, Carl Zeiss, Oberkochen, Germany).

Multiplexed immunoassay of cytokine/chemokines

The conditioned media from the EA.hy926 cell culture were subjected to a multiplexed capture sandwich immunoassay using MILLIPLEX™ MAP (Multi-Analyte Profiling) Human Cytokine/Chemokine Magnetic Bead Panel (kit No. HCYTOMAG-60 K, Millipore Corporation, Billerica, MA, USA), as previously described.²⁰ This panel enabled simultaneous detection of IFN- γ , IL-4, IL-10, IL-13, IL-1 β , IL-6, IL-3, GM-CSF, VEGF, FGF-2, G-CSF, and MCP-1. Either standard solutions in a fresh medium or samples of conditioned medium in triplicate were incubated with the pre-mixed capture antibody-coupled bead sets in 96 well plates, at 4 °C overnight. The beads were washed and incubated with biotinylated secondary antibodies for 1 h at room temperature. Streptavidin-phycoerythrin was added and incubated for additional 30 min at room temperature. After the beads were washed and suspended in sheath fluid, the levels of cytokines/chemokines were quantified using a Luminex 100™ (Luminex, Austin, TX, USA). Data were reported as the median fluorescence intensity. The concentrations of cytokines/chemokines were estimated using standard curves constructed with high purity recombinant cytokines/chemokines (0.64 ~ 10,000 pg/mL).

Statistical analysis

Data are presented as the means $\pm$ SEM of the experiments. Significant differences among the groups were determined using one-way ANOVA with a Tukey post-hoc test, where $p < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Fabrication of nanopatterned substrates of varying topography and stiffnesses

Nanopatterned substrates were fabricated from UV-curable polymers by utilizing capillary force lithography (Figure 1A). This technique allows for the simple and reproducible fabrication of a variety of molded nanopatterned substrates with excellent pattern fidelity regardless of the polymer used, and this was confirmed using SEM and AFM (Figure 1B). It is well known that cell reorganization, motility, and adhesion is greatly affected by substrate surface wettability.²³⁻²⁶ Therefore, to ensure that changes in endothelial cell morphology and cytokine/chemokine secretion would be due to differences in substrate stiffnesses and topographies, rather than surface chemistries, surface wettability measurements on both unpatterned and patterned substrates were taken, and the results indicate that there were no significant differences due to polymer composition (Figure 2). The specific nanopattern dimensions and structures used for this study were based on the native extracellular matrix (ECM) structure of vascular tissue,²⁷ thus providing a biomimetic platform for studying the effects of the ECM on endothelial cell morphology and activity. As changes in the structure and stiffness of vascular ECM can occur as a result of disease,^{28,29} our platform could be used in future studies that utilize disease-in-a-dish models to better understand how these pathologies can affect cell behavior, and how this leads to the disease progression and complications seen in patients.

Effect of nanotopography on endothelial cell morphology

Cells have the ability to sense the local topography of the extracellular matrix structure containing nano- and micro-scale ridges, grooves and pillars with variable local densities in order to control their own morphology, movement and fate.^{4,30,31} It is therefore hypothesized that the topographic feature of the extracellular basement may play a role in the regulation of endothelial physiology. This concept has been experimentally supported using nanopatterned substrates that mimic topographic features encountered in the native extracellular basement.^{5,7,14} Cells were cultured for 24 h on various nanopatterned substrates with different groove widths, and their morphological changes were observed. Cells showed an altered morphology dependent on not only the presence of nanopatterning, but also on the ratio of ridge and groove dimensions of these patterns (Figure 3). Specifically, cells grown on 1:5 type nanopatterned substrates exhibited the typical cobblestone morphology similar to cells on unpatterned substrates and on conventional tissue culture plastics. In contrast, cells grown on nanopatterned substrates of 1:1 and 1:2 ridge:groove dimensions were much more elongated and aligned in parallel with the fabricated nanopatterns, regardless of substrate stiffness. To further examine the cell morphology change at the subcellular level, the cells cultured on unpatterned and 1:1 patterned substrates and were stained for F-actin and nuclei using fluorescent phalloidin and DAPI, respectively. The stained cells were then observed using a confocal fluorescent

microscope (Figure 4). As expected, the underlying nanotopographical cues of the substrates changed the subcellular organization of F-actin to a more directional pattern.

The observation that changes to endothelial cell morphology were dependent on the ratio of nanopatterned grooves and ridges indicates that these cells, like other cell types, are sensitive to the nanoscale organization and composition of the underlying ECM. While the molecular mechanisms governing these processes remain elusive, several possibilities have arisen. It is suggested that nanotopographical cues are sensed by cells through the formation of focal adhesions that then activate downstream signaling pathways, and several studies by our group have supported this.^{12,22,32,33} For instance, the increased cell-surface contact guidance of nanopatterned substrates can result in directed focal adhesion formation, as well as promote actin polymerization and protrusion in the direction parallel to grooves. Kim et al. addressed the issue of how topographical pattern density affects this process, observing that fibroblasts established focal adhesions localized to ridge contact points and extended lamellipodia preferentially along more densely spaced features, while focal adhesions were more randomly distributed in cells cultured on sparsely-spaced ridges, similar to those cultured on flat surfaces.²² Therefore, topographical pattern density plays a significant role in defining cell shape and orientation throughout the adhesion process. Our findings in this study appear to confirm this, as the morphology of cells cultured on the 1:5 type nanopatterned substrates are indistinguishable from those that were cultured on unpatterned substrates, suggesting that this lower density pattern was insufficient to impart the necessary cell-substrate interactions to significantly alter cell behavior.

Interestingly, the observed elongated cell and cytoskeletal morphology here is similar to that observed of endothelial cell cultures that have been exposed to laminar shear stresses (LSS).^{34,35} A unique feature of the vascular environment in which these endothelial cells reside is the continuous flow of blood, which generates shear stress. Fluid shear stress regulates a variety of vascular functions and endothelial gene expression depending on its pattern and magnitude.³⁶⁻³⁸ The straight regions of the arteries that experience pulsatile LSS are usually protected from atherosclerotic lesion formation, while the arterial branch points, curvatures, and bifurcations are more prone to atherogenesis due to the disturbed blood flow generating oscillatory shear stresses.^{39,40} The anti-atherogenic effects of pulsatile LSS and steady LSS have been attributed to enhanced endothelial nitric oxide (NO) production,^{41,42} inhibited monocyte adhesion to endothelial cells,^{43,44} inhibited endothelial apoptosis,⁴⁵⁻⁴⁷ and enhanced wound healing.^{48,49} Although LSS and nanotopographical cues are mutually unrelated physical factors, they both appear to alter subcellular distribution of F-actin to a more directionally aligned pattern. This may be due to the fact that the nanopatterned substrates that induced the greatest amount of cellular elongation and alignment featured topography similar to that found in the subendothelial extracellular matrix of native vascular tissue.^{27,50,51} However, because of the observation that both factors appear to induce similar morphological changes, it was hypothesized that these two different physical factors may regulate endothelial secretion of cytokines/and chemokines in a similar manner.

Effect of nanopatterned substrates on endothelial secretion of cytokines/chemokines

Cytokines/chemokines secreted from the endothelium are mediators of complex interactions between endothelial cells and blood cells including immunocompetent leukocytes.⁵² They also have an autocrine function and regulate the interaction of endothelial cells with cellular microenvironments such as neighboring cells, extracellular matrix, and interstitial fluids.^{4,53} Thus, endothelial secretion of cytokines/chemokines could affect major vascular events involved in immunity, inflammation, hemostasis, and angiogenesis.¹⁷ Because defining specific function of each cytokine and chemokine is a very challenging task and because of their various effects on vascular physiology, we attempted to divide them into three overlapping physiological roles: immunity, inflammation/thrombosis, and angiogenesis, based on the work by Mantovani et al. (Supplementary Figure 1).¹⁷ This approach allows us to interpret the changes of various cytokines/chemokines in an integrative way.

The effects of nanotopographic cues on endothelial secretion of cytokines/chemokines were examined in the absence and presence of TNF- α , and it was observed that the nanopatterns indeed had an effect on the endothelial secretion of cytokines/chemokines (Figures 5-7). In the absence of TNF- α , the 1:2 type nanopattern in particular appeared to significantly down-regulate the secretory levels of IL-1 β , IL-4, IL-3 and MCP-1, which are cytokines and chemokines that play significant roles in inflammation and angiogenesis. Interestingly, fluid shear stress has been known to control vascular inflammation and angiogenesis.⁵⁴⁻⁵⁶ Previous studies reported that the secretion of inflammatory cytokines/chemokines from HUVECs was reduced by exposure to LSS.²¹ Thus, the results of this study suggest that certain topographies, such as the 1:2 nanopatterns, can attenuate vascular inflammation or thrombosis in a similar manner as LSS, because both of these physical factors suppressed the levels of cytokines/chemokines involved in inflammation/thrombosis.

TNF- α treatment itself increased the levels of IL-6, GM-CSF, G-CSF and MCP-1 in cells cultured on un-patterned substrates. The nanopatterns had no significant effect on cytokine/chemokine secretion under TNF- α -treated conditions. These results indicate that the “anti-inflammatory” effects of substratum topography may not be robust enough to overcome inflammation processes triggered by strong inflammatory agonists such as TNF- α . However, we speculate that substratum topography, in combination with other physical factors and stimuli such as LSS, may play a role in the prevention of inflammation under basal conditions.

Effect of substrate stiffness on endothelial secretion of cytokines/chemokines

The influence of substrate stiffness on cytokine/chemokine secretion from cultured endothelial cells was also investigated (Figures 5-7). It was found that cells on the stiffer substrate (2.5 GPa), when nanotopography was held constant, secreted significantly lower levels of IL-3, IL-13, IL-4 and MCP-1 compared to cells cultured on the softer substrate (6.7 MPa). Additionally, the decreased secretion of IL-13, IL-4 and MCP-1 in relation to stiffness was somewhat mitigated by the presence of TNF- α , with a significant decrease in secretion levels only seen when comparing the unpatterned groups. However, the decrease in IL-3 secretion from cells cultured on the stiffer substrate was maintained even with TNF- α present.

It has been suggested that the mechanical properties of the extracellular matrix play a significant role in regulating endothelial cell health and function.⁵⁷ Indeed, in atherosclerotic or calcified tissues in which the tissue modulus is greater than in healthy vasculature, it has been found that the presence of inflammatory cytokines, such as IL-3 and MCP-1, is increased.⁵⁸⁻⁶⁰ While the moduli of the polymers used in this study are greater than that of healthy subendothelial matrix (2.5 ± 1.9 kPa) and of even calcified tissues (1.47 MPa),^{61,62} the results still provide additional insights into the effects of substrate stiffness on endothelial cell behavior. In this case, it was found that inflammatory cytokine/chemokine secretion levels decreased as the matrix modulus increased, suggesting that while some degree of matrix stiffness is detrimental to vascular health, it appears that very stiff substrates may in fact impart a small anti-inflammatory effect similar to what was observed with the nanopatterned topography.

CONCLUSIONS

In this work, human endothelial cells were cultured on nanopatterned substrates with varying topographical feature dimensions and substrate stiffnesses, and the effects of such cues on cytokine/chemokine secretion from the cells was investigated. In the context of vascular health, the study of endothelial cell-regulated inflammatory responses is crucial, as these are responsible for the majority of detrimental tissue pathologies due to disease and stent implantation.^{63,64} It was found that nanotopographical cues composed of aligned grooves and ridges not only induced cultured cells to take on an elongated morphology, but also caused a decrease in the secretion of inflammatory cytokines. These effects were found to be similar to those observed with endothelial cells subjected to laminar shear stresses. Similar decreases in cytokine secretion were observed on stiffer nanopatterned substrates. These results suggest that topographical and mechanical environmental cues are able to contribute to the maintenance of vascular health by inhibiting inflammatory and thrombogenic events, and by promoting endothelial repair. In conclusion, this study showed that there are important extracellular physical factors influencing vascular remodeling and pathophysiology aside from laminar shear stresses, and that endothelial cell behavior can be modulated by local topography and underlying matrix stiffnesses. An understanding of this relationship would be useful in predicting the effects of extracellular environmental factors on vascular physiology associated with innate immunity, inflammation, thrombosis, and angiogenesis. Furthermore, by utilizing other nanopatternable materials with tunable stiffnesses that can accurately model healthy and diseased vascular tissue,⁶⁵ future work can be done to explore the interplay of these factors in a disease-in-a-dish or tissue engineering application. Additionally, this knowledge could then assist in the design of next-generation vascular implants, such as stents, so that complications from restenosis or undesired thrombosis can be avoided.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

D. H. Kim thanks the Department of Bioengineering at the University of Washington for support from the new faculty startup fund. D. H. Kim is also supported by an American Heart Association Scientist Development Grant (13SDG14560076). This work was also supported by the Mid-Career Researcher Program (2011-0014527) through a National Research Foundation grant funded by the Ministry of Education, Science and Technology, Republic of Korea.

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Figure. 1. Fabrication and characterization of nanopatterned substrates. (A) Schematic illustration of fabricating nanopatterned polymer *via* capillary force lithography. (B) SEM images of 550nm 1:1, 1:2, 1:5 nanopatterned PUA substrates and an AFM image of a 1:1 nanopatterned substrate.

Figure 2. Analysis of substrate wettability as a function of polymer composition and topography. (A) Representative images of an approximately 10 μ L drop of water on substrates comprised of polymers with moduli of 6.7 MPa (NOA76) and 2.5 GPa (NOA86). For patterned substrates, the water contact angle parallel and orthogonal to the direction of the nanotopography were measured, with the angles defined as θ_y and θ_x , respectively (inset). (B) Measurement of water contact angles reveals no significant difference imparted by polymer composition, regardless of nanotopography. Data are presented as the means $\pm$ SEM ($n=5$), * $p < 0.05$.

Figure. 3. Effects of substrate nanotopography and substrate stiffness on endothelial cell morphology. EA.Hy926 cells were cultured on unpatterned or nanopatterned substrates with moduli of (A) 6.7 MPa or (B) 2.5 GPa for 24 h. The fabricated nanopatterns consisted of an array of parallel ridges with different ratios of ridge width to groove width (1:1; 1:2, and 1:5). Representative microscopic cell images and the distribution of cell orientation angles are shown. The cell orientation angle represents the angle between the longer axis of the approximated boundary of individual cell and nanopatterning. Alignment of nanoscale ridges are indicated with white arrows.

Figure. 4.

Representative confocal microscopic images illustrating the effects of nanopatterning on cytoskeletal organization of cultured endothelial cells. Cells were plated on (A) unpatterned or (B) 1:1 nanopatterned 6.7 MPa substrates and cultured for 24 h. Cells were subjected to fluorocytochemical detection of F-actin (red) and nuclei (blue) using fluorescent phalloidin and DAPI, respectively. Alignment of nanopatterned ridges is indicated with white arrows.

Figure. 5. Effects of nanopatterning on the endothelial secretion of cytokines/chemokines mainly associated with immunity on substrates with stiffnesses of (A) 6.7 MPa and (B) 2.5 GPa. EA.Hy926 cells were cultured on polymeric substrates for 36h, with some groups treated TNF- α . Collected conditioned media was used to determine the levels of cytokines/chemokines by a multiplexed capture sandwich immunoassay. Data are presented as the means $\pm$ SEM (n=3). Statistical differences between nanopatterns, TNF- α treatment and stiffnesses are denoted with a, b, and c, respectively ($p < 0.05$).

Figure. 6. Effects of nanopatterning and substrate stiffness on the endothelial secretion of cytokines/chemokines mainly associated with inflammation and thrombosis on substrates with stiffnesses of (A) 6.7 MPa and (B) 2.5 GPa. EA.Hy926 cells were cultured on polymeric substrates for 36h, with some groups treated TNF- α . Collected conditioned media was used to determine the levels of cytokines/chemokines by a multiplexed capture sandwich immunoassay. Data are presented as the means $\pm$ SEM (n=3). Statistical differences between nanopatterns, TNF- α treatment and stiffnesses are denoted with a, b, and c, respectively ($p < 0.05$).

Figure. 7. Effects of nanopatterning and substrate stiffness on the endothelial secretion of cytokines/chemokines mainly associated with angiogenesis on substrates with stiffnesses of (A) 6.7 MPa and (B) 2.5 GPa. EA.Hy926 cells were cultured on polymeric substrates for 36h, with some groups treated TNF- α . Collected conditioned media was used to determine the levels of cytokines/chemokines by a multiplexed capture sandwich immunoassay. Data are presented as the means $\pm$ SEM (n=3). Statistical differences between nanotopographies, TNF- α treatment and stiffnesses are denoted with a, b, and c, respectively ($p < 0.05$).