Dendritic cell activation is influenced by cyclic mechanical strain when cultured on adhesive substrates

Jamal S. Lewis[1], Natalia Dolgova[1], T.J. Chancellor[2], Abhinav P. Acharya[1], Jerome V. Karpiak[1], Tanmay P. Lele[2], Benjamin G. Keselowsky[1,*]

[1]J. Crayton Pruitt Family Department of Biomedical Engineering,

[2]Department of Chemical Engineering,

University of Florida,

Gainesville, FL 32611 U.S.

* Corresponding Author

Benjamin G. Keselowsky

Tel: (352) 273-5878; Fax: (352) 392-9791; E-mail Address: bgk@ufl.edu

Address: J. Crayton Pruitt Family Department of Biomedical Engineering, PO Box 116131, Gainesville, Florida, 32611-6131, USA

*Abstract
Click here to download Abstract: abstract.docx

Abstract:

Dendritic cells (DCs), key regulators of tolerance and immunity, have been found to reside in mechanically active tissues such as the interior layers of the arterial wall, which experience cyclic radial wall strain due to pulsatile blood flow. Although experimentally difficult to determine in vivo, it is reasonable to postulate DCs experience the mechanical forces in such mechanically active tissues. However, it is currently unknown how DCs respond to cyclic mechanical strain. In order to explore the hypothesis that DCs are responsive to mechanical strain, DCs were cultured in vitro on pre-adsorbed adhesive proteins (e.g., laminin, collagen, fibrinogen) and 1 Hz cyclic strain was applied for various durations and strain magnitudes. It was determined that a strain magnitude of 10% and 24 h duration adversely affected DC viability compared to no-strain controls, but culture on certain adhesive substrates provided modest protection of viability under this harsh strain regime. In contrast, application of 1 h of 1 Hz cyclic 3% strain did not affect DC viability and this strain regime was used for the remaining experiments for quantifying DC activation and T-cell priming capability. Application of 3% strain increased expression of stimulatory (MHC-II) and co-stimulatory molecules (CD86, CD40), and this effect was generally increased by culture on pre-coated adhesive substrates. Interestingly, the cytokine secretion profile of DCs was not significantly affected by strain. Lastly, strained DCs demonstrated increased stimulation of allogeneic T cell proliferation, in a manner that was independent of the adhesive substrate. These observations indicate generation of a DC consistent with what has been described as a semi-mature phenotype. This work begins elucidating a potential role for DCs in tissue environments exposed to cyclic mechanical forces.

1. Introduction:

Vascular homeostasis is maintained through a complex milieu of interacting biochemical and biomechanical factors. Dysregulation of either can lead to pathology. Transmural normal forces due to pulsatile blood flow circumferentially strain arteries, translating to cyclic axial strain of the intimal, medial, and adventitial vessel wall layers [1]. Cyclic mechanical strain effects have been investigated for numerous cell types such as endothelial cells [2-7], fibroblasts [8] and smooth muscle cells [9-13]. For example, physiologic cyclic strain accelerates endothelial cell proliferation [6] while pathologically high cyclic strain induces apoptosis [14]. High cyclic strain also increases endothelial cell permeability and upregulates production of CCL2/MCP-1, a chemokine responsible for monocyte recruitment [7]. *Wilson et al*. demonstrated differences in cyclic strain effects on proliferation depending upon which extracellular matrix (ECM) protein the cells were seeded [15]. These findings suggest that the local biomechanical and adhesive microenvironment has a functional role in modulating cellular responses. Although the role played by endothelial cells, smooth muscle and fibroblasts in mechanical homeostasis of the vasculature has been extensively studied, the contribution of a key immune cell type, tissue-resident dendritic cells (DCs), is yet to be elucidated.

Dendritic cells are critical for both immunity and tolerance and are involved in guiding innate and adaptive immune responses [16,17]. Dendritic cells act as sentinels, constantly patrolling the body and presenting both self and nonself-antigens to lymphocytes, B-cells and T-cells [18-20]. Immature DCs (iDCs), upon activation, up-regulate antigen presenting molecules, co-stimulatory molecules, cytokines and chemokine receptors. Chemokine receptors mediate the migration of DCs to secondary lymphoid tissues where they initiate adaptive immune responses [21]. Notably, functional DCs have been found residing in both human and mouse blood vessels

[22,23]. Moreover, several studies have strongly implicated DC involvement in vascular pathology. For instance, vascular sites of high wall strain (known to be atherosclerosis-prone areas) have elevated numbers of DCs [24]. Furthermore, the number of vascular DCs is elevated in atherosclerotic plaques and advanced atherosclerotic lesions are additionally infiltrated by circulating monocyte-derived DCs [25-27]. While vascular DCs in healthy arterial walls are found in an immature state, the mature DC phenotype is prevalent at the onset of atherosclerosis [28-30]. Histological studies suggest that mature vascular DCs are able to initiate the adaptive immune response by interacting with both $CD4^+$ and $CD8^+$ T cells [26]. It is postulated that within atherosclerotic lesions, an imbalance develops between immunogenic and tolerogenic responses that leads to altered self-antigen presentation by mature DCs [31]. Interestingly, while DCs are not present in the vessel wall of normal veins, they have been detected in vein walls affected by varicosity and thrombophlebitis, and in aortocoronary saphenous vein bypass grafts [32,33]. This suggests that DCs are recruited in response to injury or to a characteristic hemodynamic modulation of the vessels. While it is unclear if mechanical force-modulation of DCs plays a role physiologically, the matter has been unexplored as of yet. If DCs are responsive to cyclic mechanical strain, it would have clear implications in arterial wall homeostasis and local immune cell-driven pathology, to be further explored.

Mechanical stimuli on DC responses should be considered in the context of extracellular matrix composition. Arterial Resident DCs reside in several different zones of the blood vessel, each with a characteristic extracellular matrix composition [34]. In related work, we have found recently that DCs respond differentially to different adhesive environments [35]. Furthermore, DCs from mice with genetic predisposition for autoimmune diabetes (which has been linked to increased atherosclerosis), demonstrate altered immune responses to different ECM proteins

[36]. Integrins, the receptor family which bind extracellular matrix proteins, have been shown to modulate DC functionality and phenotype [37,38]. For numerous cell types, integrin binding to extracellular matrix ligands results in a mechanical coupling between the inside of the cell and its microenvironment [39-41]. While few studies have investigated the role of the cytoskeleton or focal adhesions in DCs, it was recently reported in DCs that the Rho GTPases - RhoA, Rac1, and Cdc42 regulate endocytosis and antigen presentation, demonstrating that mechanotransduction-related signals can regulate DC processes [42,43]. The objective of this study was to quantify DC responses to cyclic mechanical strain and investigate the role of mechanotransduction signals in DC-mediated T-cell priming in context of different ECM adhesive cues. The findings from these studies inform the basic science of DC immunology, and may be relevant to vascular wall biology and vascular tissue engineering [44,45].

2. Methods and Materials

2.1 Generation of murine bone marrow-derived DCs

Dendritic Cells were generated from bone marrow obtained from 8 – 12 week old, female, C57BL6/j mice in accordance with animal care guidelines approved by University of Florida using a modified 10 day protocol [46,47]. For DC culture, mice were euthanized by CO_2 asphyxiation followed by cervical dislocation and tibias and femurs were harvested for isolating marrow cells. The marrow cells were obtained by flushing the shaft of the bones with a 25g needle using RPMI medium (MP Biomedicals, OH, USA) containing 1% fetal bovine serum (Lonza, Walkersville, MD) and 1% penicillin-streptomycin (Hyclone) and mixed to make a homogenous suspension. The suspension was then strained using 70 μm cell strainers (Becton

Dickinson, NJ, USA) and cells were collected at 200x g for 7 min. The red blood cells (RBCs) were removed by lysing with ACK lysis buffer (Lonza, Walkersville, MD) followed by centrifugation at 270x g for 5 min to recover leukocytes. Leukocytes were then re-suspended in DMEM/F-12 with L-glutamine (Cellgro, Herndon, VA), 10% fetal bovine serum, 1% sodium pyruvate (Lonza, Walkersville, MD), 1% non-essential amino acids (Lonza, Walkersville, MD), 1% penicillin-streptomycin (Hyclone) and 20 ng/ml GM-CSF (R&D systems, MN, USA) (DC media) and plate on tissue culture flasks for 2 d in order to remove adherent cells. At 2 d the floating cells were transferred to low attachment plates and cultured in fresh DC media for expansion of DC precursor cells. At 7 d, cells were transferred to tissue culture plates to allow for DC adhesion and proliferation. At 10 d, cells were lifted with 5 mM solution of Na2EDTA (Fisher Scientific) in phosphate buffer saline (PBS) (Hyclone) and used for all the experiments. Purity (CD11c+ > 90%) and immaturity (major histocompatibility complex (MHC-II+) < 6% and CD86+ < 6%) were verified by flow cytometry.

2.2 Protein coating and application of mechanical strain

Extracellular matrix proteins were coated onto 6-well Bioflex plates (Flexcell, Hillsborough, NC) by overnight incubation of 20 μg/ml protein solution in PBS. For these single-component coating conditions, substrates are expected to be fully saturated with respect to protein surface densities. The wells were then washed with PBS supplemented with calcium and magnesium to remove excess protein. Immature DCs were seeded (1 $X10^6$ cells/well) on the following protein-coated substrates: human plasma-derived fibronectin (FN) (BD Bioscience), Engelbreth–Holm–Swarm mouse tumor-derived laminin (LN) (BD Bioscience), bovine dermis-derived collagen type I (Col) (BD Bioscience), human plasma-derived vitronectin (VN) (BD

Bioscience) and bovine plasma fibrinogen (FG) (Mp Biomedicals). Non-coated Bioflex wells were included as a reference (no coat control), where serum proteins are expected to adsorb out of the culture media during the time of cell seeding. Additionally included was a 10% serum overnight coating (Ser) group, along with a bovine serum albumin (BSA) (Fisher Bioreagents) group as reference substrates. Species-specific protein sequence homologies, as compared to murine, are as follows: COL – 89%, FG – 81%; determined by HomoloGene, an online resource made available through the National Center for Biotechnology Information. DCs were cultured on each substrate for 24 h, and then exposed to 3% or 10% equi-axial (radial) cyclic strain with a frequency of 1 Hz for 1 h, followed by 23 h without mechanical strain, or 24 h of strain using the Flexcell-4000 system (Flexcell, Hillsborough, NC). Cyclic strain was applied by deformation of the plate through regulated air vacuum supplied to the bottom of the plate causing the membrane to stretch. No strain protein-coated wells were included as a control.

2.3 Flow Cytometry and Cytokine Analysis

DC activation was quantified by measuring cell surface marker levels by flow cytometry. Monoclonal antibodies (mAbs) specific for mouse CD40 (clone HL40-3, IgG1, κ λ2), CD83 (clone 16-10A1, IgG2, κ), CD86 (clone GL1, IgG2a, κ), I-A/I-E (clone M5/114.15.2, IgG2b, κ), were purchased from BD Biosciences. Briefly, DCs were lifted by incubating with 5 mM Na2EDTA solution in PBS at 37 ^{o}C for 20 min. Dendritic cells were then washed with 1% fetal bovine serum in PBS and incubated with antibodies against CD16/CD32 (clone 2.4G2, IgG2b, κ) (BD Pharmingen, CA) for 10 min at 4 ^{o}C to block Fcγ receptors on DCs. The cells were then incubated for 40 min on ice in 100 µl of PBS with 1 µg of relevant mAbs and then washed twice with cold PBS. Viability was evaluated by staining with APC-annexinV and

7-amino-actinomycin D (7AAD; BD Pharmingen, CA) according to the manufacturer's instructions. Appropriate isotypes were used for each antibody as negative controls. Data acquisition was performed using FACSCalibur cytometer (Becton Dickinson, NJ). More than 10,000 events were acquired for each sample and data analysis was performed using FCS Express version 3 (De Novo Software, Los Angeles, CA).

Cell culture supernatants were collected after cell cultures on the various protein-coated substrates, centrifuged to remove any cell debris and stored at -20 ^{o}C until analysis. Sandwich enzyme-linked immunosorbent assays (ELISA) were performed to detect IL-12, IL-10, IL-1β in culture supernatants using commercial ELISA kits (Becton Dickinson, NJ) according to manufacturer's directions.

2.4 Isolation of T-cells and Mixed Lymphocyte Reaction

T-cells were isolated from spleen of 8-week-old BALB/cbyj mice by negative selection using MACS separation system (Miltenyi Biotec). Single cell suspensions were prepared by mincing the spleen through a 70 μm cell strainer. The effluent was centrifuged for 10 min at 300x g. This suspension was strained to remove debris and the remaining single cells were counted using a hemocytometer. The cells were then spun down at 300x g for 10 min and the pellet was re-suspended in 40 μl of cold MACS buffer (0.5% BSA and 2 mM EDTA in PBS) per 10^7 cells. Negative selection of Pan T-cells was performed. A biotin-labeled antibody cocktail against CD11b, CD11c, CD19, CD45R (B220), CD49b (DX5), CD105, Anti-MHC class II, and Ter-119 (Miltenyi Biotec) was added (10 μl per 10^7 cells) and incubated for 10 min at 4 ºC. A buffer volume of 30 μl and 20 μl anti-biotin microbeads (Miltenyi Biotec) were added to the mixture per 10^7 cells. After 15 min incubation at 4 ºC, cells were centrifuged at 300x g for 10

min and re-suspended in 500 μl of buffer per 10^8 cells. The LS magnetic column (Miltenyi Biotec) was pre-washed with 5 ml of buffer solution. Cell suspension was added to the column and the effluent comprised of Pan T-cells was collected. The column was then washed thrice with buffer solution and the effluents were mixed. The T-cells were pelleted and used in mixed lymphocyte reaction (MLR) assays. More specifically, T-cells purified from spleen of BALB/cbyj mice were co-cultured with stretched or non-stretched DCs in 96-well U-bottom tissue culture-treated plates at a 6:1 T-cell to DC ratio for 96 h. At 92 h, cultures were pulsed with 10 μM bromodeoxyuridine (BrdU; Beckton Dickinson). Non-adherent cells were then collected and immuno-fluorescently stained using mAbs against mouse CD3 and BrdU according to manufacturer's (BD Pharmingen) specifications. Flow cytometry was then used to quantify T-cell proliferation for the different conditions. To verify T-cell proliferation potential, control T-cells were stimulated with a phorbol 12-myristate 13-acetate (PMA)/ ionomycin mixture for 48 h.

2.5 Statistical analysis

Statistical analyses were performed using general linear nested model ANOVA. Based on the overall ANOVA p value ($p \leq 0.05$), a post-hoc assessment using Tukey test was subsequently performed to make pair-wise comparisons. Differences were considered significant when $p \leq 0.05$ using Systat (Version 12, Systat Software, Inc., San Jose, CA).

3. Results

3.1 Limited influence of substrate on mechanically strained dendritic cell viability

Dendritic cell viability was assessed by flow cytometry at time, t=24 h, following initiating exposure, at t=0, to 3% or 10% cyclic 1 Hz strain for both 1 h and 24 h strain durations (**Figure 1**). Application of 3% strain for 1 h did not induce elevated levels of apoptosis (**Figure 1a**) or necrosis (**Figure 1b**), in comparison to the no strain controls for each adhesive substrate, and there was no effect comparing across adhesive substrates (Col = collagen type 1; LN = laminin; FG = fibrinogen). On the other hand, 24 h of 3% strain resulted in a significant increase in the percent of apoptotic cells and necrotic cells for all substrates, also with no effect when comparing across substrates. Camptothecin, a standard apoptotic agent [48], was used as a positive control for apoptosis. Strain of 10% for 1 h did not induce apoptosis that significantly differed from that of the no strain control condition, while application of 10% strain for 24 h induced elevated apoptosis levels, again with no significant effect of the substrate observed for either duration with the exception of the 24 h 10% strain FG condition, which demonstrated a protective effect from apoptosis compared to the uncoated control (**Figure 1c**). Strain at 10% for 1 h yielded increased necrosis on the no coat, Col and LN substrates when compared to the no strain control (**Figure 1d**). The FG substrate, on the other hand, was not different from the no strain control. Application of 10% strain for 24 h further increased DC necrosis on the adhesive substrates, this time with the exception of the Col substrate which protected from necrosis rising beyond the level of the 1 h 10% strain Col condition. For the conditions examined, DC viability was adversely affected in proportion to the magnitude and duration of cyclic mechanical strain, with indication that there is potential for modulation by the adhesive substrate culture condition employed. In light of these findings, in order to maintain DC viability, the 1 h 3% strain regime was selected for further analysis and the influence of adhesive substrates on DC responses to strain was further investigated.

3.2 Application of strain induces DC activation

The next question explored was whether the application of mechanical strain would modify DC maturation state in an adhesive substrate-dependent manner. Maturation state was evaluated based on expression of major histocompatibility complex class II (MHC-II), and co-stimulatory molecules – CD40 and CD86. These surface molecules are typically upregulated as a DC becomes mature, following antigen recognition. They interlink with their cognate receptors on the surfaces of T cells, thereby stimulating clonal expansion of specific T cell populations. These stimulatory and costimulatory molecules, along with DC-produced cytokines, modulate lymphocytes to aid in clearance of pathogenic agents, infected cells and tumors [16]. As a result of applying 1 h of 3% cyclic strain at 1 Hz, expression of MHC-II was increased compared to no strain controls for all substrates (**Figure 2a**). This effect was largely independent of adhesive substrate, with the exception of the Col substrate, which, in conjunction with applied strain directed slightly higher MHC-II expression than the strained, no coat control. Similarly, CD40 expression increased in response to strain for all substrate conditions compared to no strain controls. Comparing across substrates, strain applied to DCs on the FG substrate increased CD40 levels the most, higher than the strained, no coat control (**Figure 2b**). In contrast to the no strain MHC-II and CD40 expression levels, which were insensitive to substrate, increased CD86 expression was evident on the no strain Col, LN and FG substrate groups compared to the no coat, no strain control (**Figure 2c**). Note that in the present work, the solid substrates (Bioflex plates) being coated with adhesive proteins, are different in nature from the tissue culture-treated polystyrene used in prior work [35,36] reporting substrate-dependent differences in stimulatory and co-stimulatory molecules, which can give rise to surface-dependent protein adsorption and

presentation. Application of strain further increased dendritic cell CD86 expression on the Col and FG substrates in comparison to the no strain controls, but not on the LN substrate on which was no difference from the no strain control. Lipopolysaccharide (LPS), an endotoxin derived from the cell wall of gram-negative bacteria which promotes an inflammatory response in immune cells [49], was added to DCs as a positive control group and LPS exposure significantly increased MHC-II, CD40 and CD86 levels in comparison to all conditions.

The effects of strain and adhesive substrate on DC cytokine secretion of IL-10, IL-12 and IL-1β were also quantified. Treatment with LPS as a positive control for inflammatory cytokine secretion was included. Recognition of pathogen or tissue damage associated material by relevant DC receptors often triggers signaling pathways that induce expression and secretion of cytokines, soluble mediators that can act in an autocrine or paracrine manner to influence immune cell behavior. Mature DCs characteristically express and secrete pro-inflammatory cytokines such as IL-12, whilst immature or suppressive DCs have been characterized to secrete anti-inflammatory cytokines, for example, IL-10 [16,17]. Interestingly, strain did not substantially increase the secretion of the pro-inflammatory cytokine IL-12 for the substrates tested compared to the no strain controls, except for the FG group (**Figure 3a**), which was only a minor increase. Further, strain failed to influence DC secretion of both the IL-10 (**Figure 3b**) and IL-1β (**Figure 3c**) cytokines, with no effect of substrate evident. Positive controls for secretion of the anti-inflammatory cytokine IL-10, and the pro-inflammatory cytokine IL-1β were bovine serum albumin (BSA) and LPS, respectively. Both of these controls induced significantly higher levels of cytokine secretion in comparison to all other conditions for their relevant cytokine tests. These results, when considered in conjunction with the increased surface expression of MHC-II,

CD40 and CD86, suggest a semi-mature DC phenotype, as described by *Lutz et al*. [50], as a result of the application of strain.

3.3 Strain-activated DCs stimulate downstream T-cell proliferation

We investigated the ability of strained DCs cultured on adhesive substrates to initiate adaptive immunity responses in vitro. Specifically, following culture on adhesive substrates and application of 3% cyclic strain for 1 h, C57Bl6 mouse bone marrow-derived DCs were co-localized with allogeneic, BALB/cbyj T-cells in a mixed lymphocyte reaction. Poly I:C-treated DCs were included as a positive control for T-cell stimulation capacity. All conditions where cyclic strain was applied stimulated T-cell proliferation to levels significantly higher than their respective no strain control (**Figure 4**), for all adhesive substrates (Col, LN, and FG-prepared surfaces, and no coat control). In general, this increased T-cell stimulatory capacity corresponds well with the increased expression of stimulatory and costimulatory molecules on strained DCs. However, DCs cultured on Col- and FG surfaces had higher levels of stimulatory molecules than LN substrates and the no coat control, but this did not translate into superior T-cell stimulation. This suggests a potential role for the involvement of co-stimulation molecules other than the ones investigated, for example, perhaps CD83.

3.4 Results for other adhesive substrates (BSA, FN, Ser, VN) investigated

In addition to the adhesive substrates reported above (Col, LN and FG), pre-coating the culture wells with the adhesive substrates BSA, FN, Ser and VN (BSA = bovine serum albumin; FN = fibronectin; Ser = 10% serum; VN = vitronectin) were also investigated in conjunction with cyclic mechanical strain. In general, the results were consistent with those for the Col, LN

and FG substrates and were not included in the figures to reduce redundancy and improve clarity. The findings for the BSA, FN, Ser, and VN substrates are summarized here. In terms of apoptosis and necrosis, the BSA, FN, and VN substrates yielded similar effects to strain as the other substrates, with the exception of the Ser substrate for the condition of 10% strain applied for 24 h, which protected DCs from necrosis, similar to the Col substrate (**data not shown**). Expression of MHCII and CD86 when DCs were cultured on the BSA, FN, Ser, and VN substrates followed the same trend as the Col, LN and FG substrates, namely that the substrates alone increased the level of CD86 expression, and the application of strain induced higher expression of both MHCII and CD86 for all substrates. Lastly, allogeneic T cell proliferation was upregulated by DCs exposed to strain cultured on the BSA, FN, Ser, and VN substrates, in a substrate-independent manner, again correlating well with stimulatory and costimulatory molecule expression (**data not shown**).

4. Discussion

Efforts to elucidate mechanical properties of cells in general [51,52], and in particular, the role of mechanical stimuli on cells in vascular homeostasis have been extensive [53,54]. However, the potential for immune cells to respond to physical forces such as transmural forces found in blood vessels has not yet been considered. If immune cells are indeed responsive to mechanical strain, it would have potentially broad implications in vascular biology because the immune system has been linked to vascular genesis, growth and repair [55-58]. Dendritic cells are professional antigen-presenting cells that are pivotal in regulating immune responses, particularly T-cell stimulation [59]. Moreover, DCs have been implicated in vascular pathologies including aortic aneurysm and atherosclerosis [22,60,61]. For instance, activated DCs have been

shown to accumulate in atherosclerotic lesions in the aortic intima at the onset and throughout development of this disease whereas, in contrast, in the absence of disease, the resident DCs found in the intima of normal human aortae display an immature phenotype [62,63]. Dendritic cells are thought to regulate inflammation in atherosclerotic lesions through generation of pro- or anti-inflammatory factors, in addition to controlling plasma cholesterol levels [64]. In conjunction, it has been reported that atherosclerotic lesion sites occur where the vasculature is under increased strain [24]. This suggests potential for mechanical factors to be involved in shaping the inflammatory responses of DCs in the intima of vasculature and thereby playing a role in vascular homeostasis. Understanding the effect of mechanical stimuli on DCs in vascular homeostasis is nuanced by the fact that if mechanical forces are transmitted to tissue resident immune cells in vivo, it is likely through integrin-ligand coupling, and it has been demonstrated that DC maturation state can also be influenced by adhesive cues from the extracellular matrix [35,36]. Furthermore, extracellular matrices may be remodeled due to the cyclic strain in the vessel wall [65,66]. The present work begins to explore dendritic cell immune responses to radial cyclic strain when cultured on ECM proteins found in the blood vessel intima.

To investigate the effect of mechanical stimulus on dendritic cells, it is pertinent to establish strain regimes in which viability is either directly impacted, or unaffected. In context, a number of studies have demonstrated mechanical stretch may induce vascular cell death via the activation of cell apoptotic mechanisms [67]. For example, *Wernig et al.* showed that smooth muscle cells subjected to mechanical strain undergo apoptosis via β-1 integrin signaling pathway [68]. It has been speculated that DC apoptosis in the vascular wall may be relevant to vascular pathology and remodeling [69-71]. Our investigations revealed that DC apoptosis and necrosis were dependent on stretching strain magnitude and duration time, independent of adhesive

substrate. Application of 10% strain on DCs resulted in high cell death, particularly through necrotic pathways, indicating disruption of the plasma membrane and perhaps rupture of the underlying cytoskeletal framework of the cell [72]. Dendritic cells subjected to a long period of strain, underwent cell death through necrosis as well as apoptosis, for both strain magnitudes investigated. It has also been shown that over time mechanical stretching of various cell types may result in detachment from ECM substrate and cell shape deformation [73,74], which can trigger apoptotic pathways [73-76].

To initiate adaptive immunity, DCs must first be activated by pathogen or damage associated stimuli. Activation state is determined by major histocompatibility complex (MHC) and co-stimulatory molecules expression levels as well as the cytokine-secreting profile. We investigated the surface expression of stimulatory molecules (MHC-II) and costimulatory molecules (CD86 and CD40) following application of 3% strain for 1 h, when DCs were cultured on various ECM proteins. Our results show that mechanical loading of DCs induced increased expression of all the stimulatory/costimulatory molecules assessed. In general, this increased expression was independent of adhesive cues provided by ECM proteins. However, it should be noted that different protein substrates did modulate expression of CD86 differentially without any mechanical input. Similarly, *Acharya et al*. found that CD80 expression increased on DCs cultured on various protein substrates [35]. This is understandable, as these two costimulatory molecules are share similar expression pathways [77]. Interestingly, the DC cytokine profile was not coupled to MHC-II, CD86 and CD40 expression. Mechanical cyclic strain, by and large, failed to significantly alter the cytokine secretion profile of DCs for the cytokines analyzed. Recently, *Lutz et al.* characterized this type of DC phenotype, distinguished by high expression of MHC-II and costimulatory molecules but low secretion of pro-inflammatory cytokines, as

having a ‘semi-mature’ phenotype [50]. It is proposed that this class of DCs is induced by a number of agents including lactobacilli from the gut flora [78], apoptotic cells [79] and TNF-α [80,81]. Our results suggest that mechanical strain could be added to the list of inducers of ‘semi-mature’ DCs.

Lutz and co-workers further elaborated that the level of T-cell stimulation by this subset of DCs is still relatively high despite the loss of the third signaling mechanism. Our observations are consistent with these findings and conclude that DCs subjected to cyclic mechanical strain have the capacity to significantly increase proliferation of T-cells from an allogeneic source. *Lieshout et al.* explained that this stimulation may be heavily dependent on signaling networks initiated by costimulatory molecules on the surface of DCs (e.g. CD40, CD86) [82]. Moreover, it is suggested that this proliferative response is of a tolerogenic mode inducing IL-10^{+}, CD4^{+}, regulatory T-cells [50,80,81]. While this possibility was not investigated in this study, it suggests a role for strain-induced, ‘semi-mature’ DCs in the maintenance of vascular homeostasis under normal conditions.

5. Conclusion

The effect of cyclic mechanical strain on dendritic cell phenotype and function in the context of ECM protein adhesives cues was investigated. We demonstrated that application of mechanical strain to dendritic cells can influence viability, phenotype and stimulatory capacity. Additionally, adhesive cues provided by different ECM proteins can exert influence over DC phenotype and functionality in concert with mechanical stimulus. In general, we can conclude that application of mechanical strain and ECM protein to DCs induces a ‘semi-mature’ phenotype with increased expression of stimulatory molecules but insignificant secretion of

inflammatory cytokines. These DCs are capable of increased stimulation of T cells from an allogeneic source. However, further investigation is required to determine whether these stimulated T-cells are of an inflammatory or regulatory nature, and further investigation is required to elucidate the role of the 'semi-mature' DC type in vascular homeostasis.

6. Acknowledgment

This work was supported in part by National Science Foundation grants, CMMI-0927918 and CMMI-0954302.

Figures

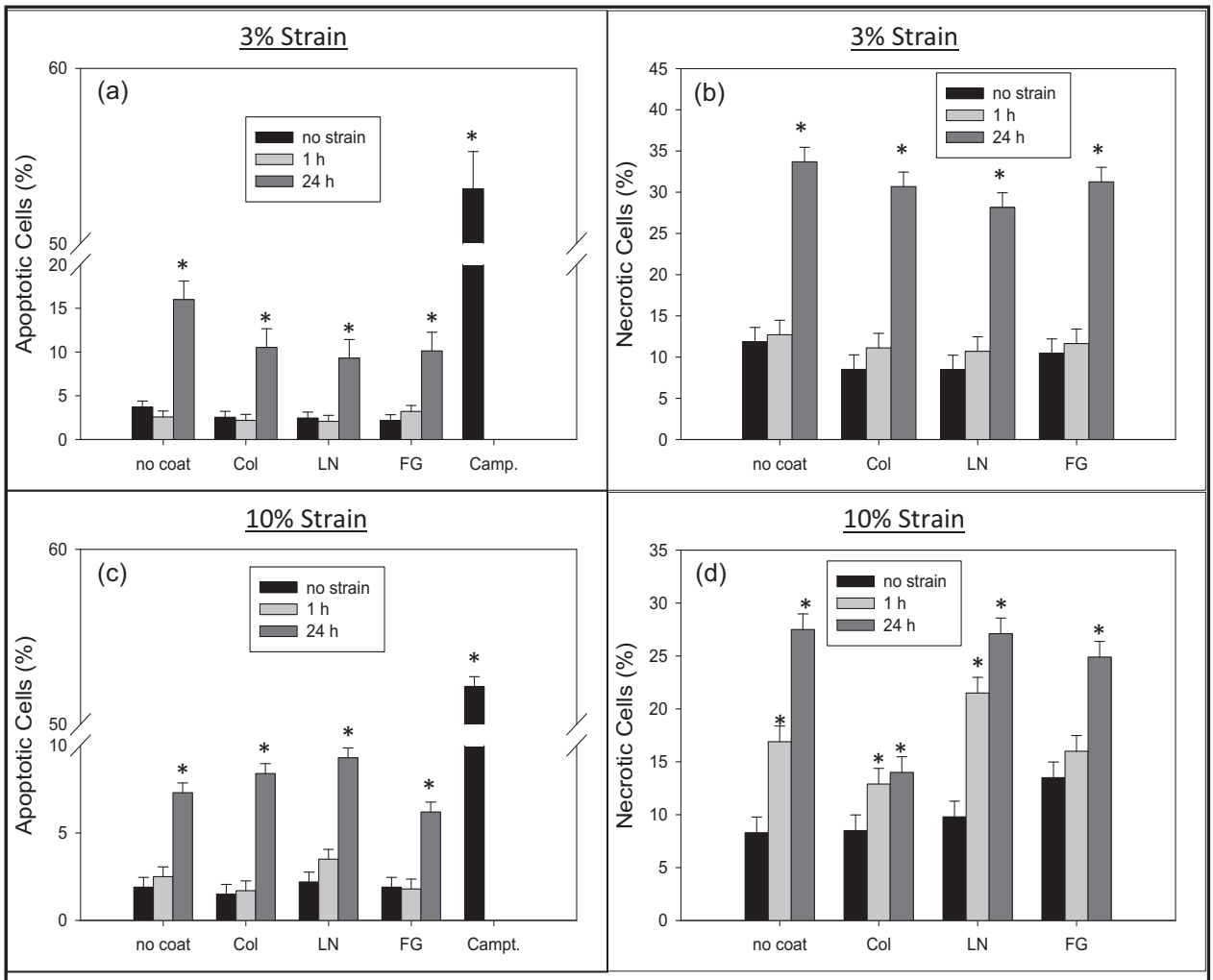

Figure 1. The length of exposure and magnitude of cyclical strain on dendritic cells impacts viability, for all adhesive substrates. Dendritic cells were seeded (1 x 10^6cells/well) on protein-coated substrates as well as a non-coated reference surface. Dendritic cells were exposed to either 3% (a, b) or 10% (c, d) cyclic strain with a frequency of 1 Hz for 1hr or 24 hrs using the Flexcell-4000 system. Viability was then quantified by flow cytometry using Annexin V and 7AAD as markers of apoptosis and necrosis respectively. Camptothecin-treated dendritic cells (5μM for 3h) were used as a positive control for apoptosis. An overall ANOVA was performed on all the treatments shown. Tukey's pair-wise significant differences from control condition (no strain) for each substrate is denoted by the * symbol (p value < 0.05).

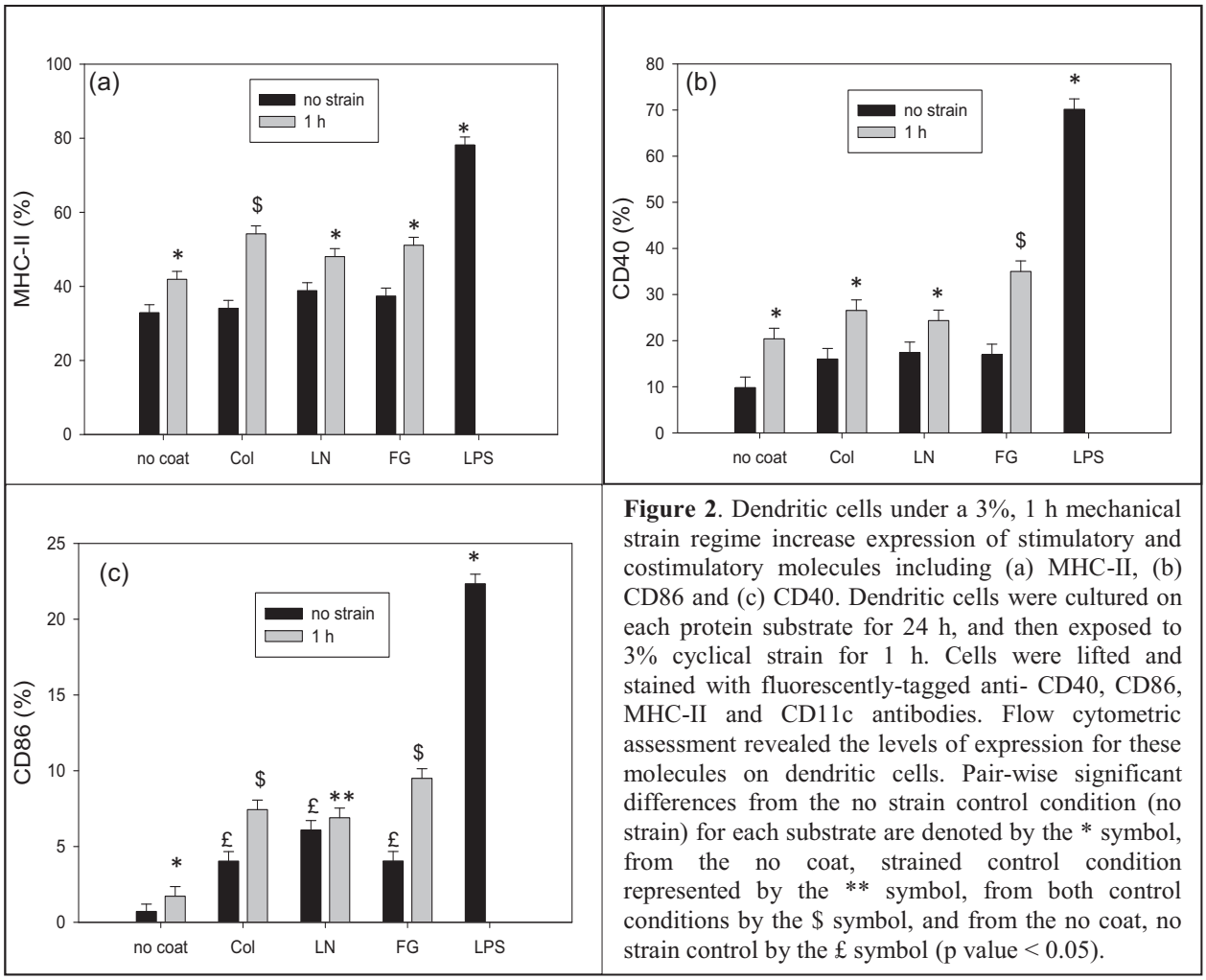

Figure 2. Dendritic cells under a 3%, 1 h mechanical strain regime increase expression of stimulatory and costimulatory molecules including (a) MHC-II, (b) CD86 and (c) CD40. Dendritic cells were cultured on each protein substrate for 24 h, and then exposed to 3% cyclical strain for 1 h. Cells were lifted and stained with fluorescently-tagged anti- CD40, CD86, MHC-II and CD11c antibodies. Flow cytometric assessment revealed the levels of expression for these molecules on dendritic cells. Pair-wise significant differences from the no strain control condition (no strain) for each substrate are denoted by the * symbol, from the no coat, strained control condition represented by the ** symbol, from both control conditions by the $ symbol, and from the no coat, no strain control by the £ symbol (p value < 0.05).

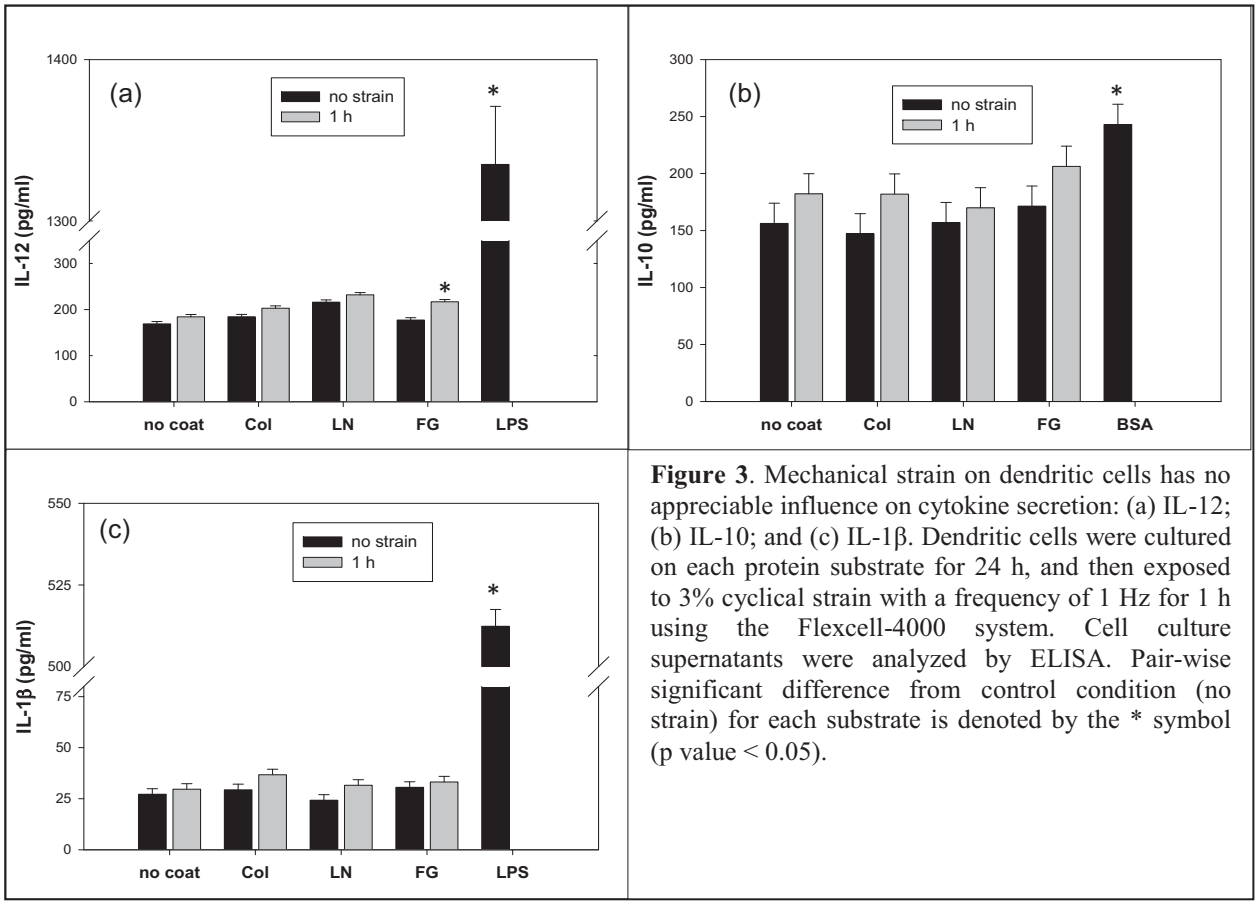

Figure 3. Mechanical strain on dendritic cells has no appreciable influence on cytokine secretion: (a) IL-12; (b) IL-10; and (c) IL-1β. Dendritic cells were cultured on each protein substrate for 24 h, and then exposed to 3% cyclical strain with a frequency of 1 Hz for 1 h using the Flexcell-4000 system. Cell culture supernatants were analyzed by ELISA. Pair-wise significant difference from control condition (no strain) for each substrate is denoted by the * symbol (p value < 0.05).

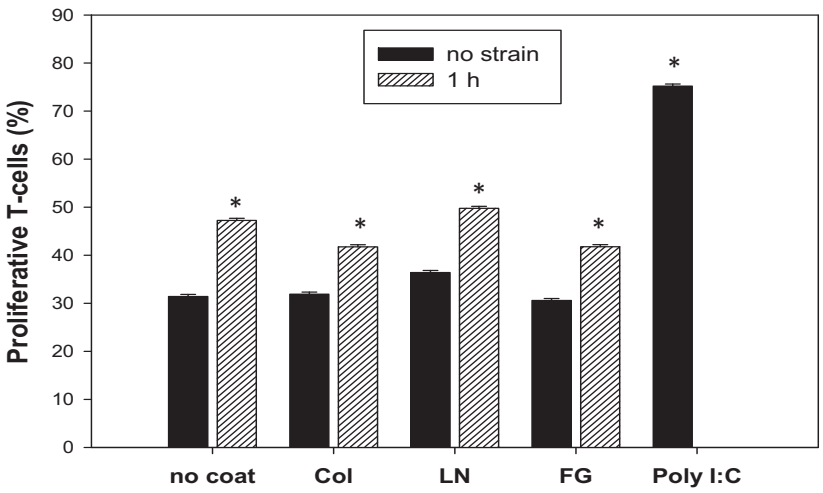

Figure 4. Mechanically-strained dendritic cells stimulate allogeneic T-cell proliferation in a substrate-independent manner. T-cells were isolated from Balb/c mouse spleen by negative selection and coupled with dendritic cells previously stretched on various protein and control substrates. T-cell proliferative response following a 4 d mixed lymphocyte reaction was determined as measuring BrdU incorporation by flow cytometry. Poly I:C-stimulated dendritic cells (12.5µg/ml) were included as a positive control. Data shown represent the mean proliferation indices ± SE (n = 3). Pair-wise significant difference from proliferative effect of non-strained dendritic cells for each condition (as determined by ANOVA and Tukey's Significance Test) is denoted by the * symbol (p value < 0.05).

7. References

[1] VanEpps J, Vorp DA. Mechanopathobiology of atherogenesis: A review. Journal of Surgical Research 2007;142(1):202-17.

[2] Pradhan S, Sumpio B. Molecular and biological effects of hemodynamics on vascular cells. Frontiers in Bioscience 2004;9:3276-85.

[3] Garcia-Cardena G, Gimbrone MAJ. Biomechanical modulation of endothelial phenotype: implications for health and disease. Handbook of experimental pharmacology 2006;(176 Pt 2):79-95.

[4] Kaunas R, Usami S, Chien S. Regulation of stretch-induced JNK activation by stress fiber orientation. Cellular Signalling 2006;18(11):1924-31.

[5] Lehoux S, Castier Y, Tedgui A. Molecular mechanisms of the vascular responses to haemodynamic forces. Journal of Internal Medicine 2006;259(4):381-92.

[6] Nishimura K, Li W, Hoshino Y, Kadohama T, Asada H, Ohgi S, et al. Role of AKT in cyclic strain-induced endothelial cell proliferation and survival. American Journal of Physiology-Cell Physiology 2006;290(3):C812-C821.

[7] Wung BS, Cheng JJ, Chao YJ, Lin J, Shyy YJ, Wang DL. Cyclical strain increases monocyte chemotactic protein-1 secretion in human endothelial cells. American Journal of Physiology 1996;270(4 PART 2):H1462-H1468.

[8] Nishimura K, Blume P, Ohgi S, Sumpio BE. Effect of different frequencies of tensile strain on human dermal fibroblast proliferation and survival. Wound Repair and Regeneration 2007;15(5):646-56.

[9] Kakisis JD, Pradhan S, Cordova A, Liapis CD, Sumpio BE. The role of STAT-3 in the mediation of smooth muscle cell response to cyclic strain. International Journal of Biochemistry & Cell Biology 2005;37(7):1396-406.

[10] Julien MA, Haller CA, Wang P, Wen J, Chaikof EL. Mechanical strain induces a persistent upregulation of Syndecan-1 expression in smooth muscle cells. Journal of Cellular Physiology 2007;211(1):167-73.

[11] Julien MA, Wang P, Haller CA, Wen J, Chaikof EL. Mechanical strain regulates syndecan-4 expression and shedding in smooth muscle cells through differential activation of MAP kinase signaling pathways. American Journal of Physiology-Cell Physiology 2007;292(1):C517-C525.

[12] Li L, Chaikof EL. Mechanical stress regulates syndecan-4 expression and redistribution in vascular smooth muscle cells. Arteriosclerosis Thrombosis and Vascular Biology 2002;22(1):61-8.

[13] Li C, Xu Q. Mechanical stress-initiated signal transduction in vascular smooth muscle cells in vitro and in vivo. Cellular Signalling 2007;19(5):881-91.

[14] Birukov KG, Jacobson JR, Flores AA, Ye SQ, Birukova AA, Verin AD, et al. Magnitude-dependent regulation of pulmonary endothelial cell barrier function by cyclic stretch. American Journal of Physiology-Lung Cellular and Molecular Physiology 2003;285(4):L785-L797.

[15] Wilson E, Sudhir K, Ives HE. Mechanical Strain of Rat Vascular Smooth-Muscle Cells Is Sensed by Specific Extracellular Matrix/Integrin Interactions. Journal of Clinical Investigation 1995;96(5):2364-72.

[16] Medzhitov R, Janeway CA. Innate immune recognition and control of adaptive immune responses. Seminars in Immunology 1998;10(5):351-3.

[17] Matzinger P. An innate sense of danger. 2002.

[18] Lambrecht BN, Salomon B, Klatzmann D, Pauwels RA. Dendritic cells are required for the development of chronic eosinophilic airway inflammation in response to inhaled antigen in sensitized mice. Journal of Immunology 1998;160(8):4090-7.

[19] Fernandez NC, Lozier A, Flament C, Ricciardi-Castagnoli P, Bellet D, Perricaudet M, et al. Dendritic cells directly trigger NK cell functions: A cross-talk relevant in innate antitumor immune responses in vivo. Journal of Leukocyte Biology 1998;79.

[20] Moser M. Balancing life and death. Nature Immunology 2004;5(6):559-60.

[21] Rot A, von Andrian UH. Chemokines in innate and adaptive host defense: Basic chemokinese grammar for immune cells. Annual Review of Immunology 2004;22:891-928.

[22] Cheong C, Choi JH. Dendritic cells and regulatory T cells in atherosclerosis. Molecules and Cells 2012;34(4):341-7.

[23] Hansson GK, Hermansson A. The immune system in atherosclerosis. Nature Immunology 2011;12(3):204-12.

[24] Lord RSA, Bobryshev YV. Clustering of dendritic cells in athero-prone areas of the aorta. Atherosclerosis 1999;146(1):197-8.

[25] Bobryshev YV, Lord RSA. S-100 Positive Cells in Human Arterial Intima and in Atherosclerotic Lesions. Cardiovascular Research 1995;29(5):689-96.

[26] Bobryshev YV, Lord RSA. Mapping of vascular dendritic cells in atherosclerotic arteries suggests their involvement in local immune-inflammatory reactions. Cardiovascular Research 1998;37(3):799-810.

[27] Bobryshev YV, Cherian SM, Inder SJ, Lord RSA. Neovascular expression of VE-cadherin in human atherosclerotic arteries and its relation to intimal inflammation. Cardiovascular Research 1999;43(4):1003-17.

[28] Bobryshev YV, Lord RSA. Ultrastructural Recognition of Cells with Dendritic Cell Morphology in Human Aortic Intima - Contacting Interactions of Vascular Dendritic Cells in Athero-Resistant

and Athero-Prone Areas of the Normal Aorta. Archives of Histology and Cytology 1995;58(3):307-22.

[29] Bobryshev YV, Lord RSA. Langhans cells of human arterial intima: Uniform by stellate appearance but different by nature. Tissue & Cell 1996;28(2):177-94.

[30] Waltner-Romen M, Falkensammer G, Rabl W, Wick G. A previously unrecognized site of local accumulation of mononuclear cells: the vascular-associated lymphoid tissue. Journal of Histochemistry & Cytochemistry 1998;46(12):1347-50.

[31] Taleb S, Tedgui A, Mallat Z. Regulatory T-cell immunity and its relevance to atherosclerosis. Journal of Internal Medicine 2008;263(5):489-99.

[32] Cherian SM, Bobryshev YV, Inder SJ, Lord RSA, Ashwell KWS. Dendritic cells in venous pathologies. Angiology 1999;50(5):393-402.

[33] Cherian SM, Bobryshev YV, Inder SJ, Lord RSA, Reddi KH, Farnsworth E, et al. Involvement of dendritic cells in long-term aortocoronary saphenous vein bypass graft failure. Cardiovascular Surgery 1999;7(5):508-18.

[34] Raines EW. The extracellular matrix can regulate vascular cell migration, proliferation, and survival: relationships to vascular disease. International Journal of Experimental Pathology 2000;81(3):173-82.

[35] Acharya AP, Dolgova NV, Clare-Salzler MJ, Keselowsky BG. Adhesive substrate-modulation of adaptive immune responses. Biomaterials 2008;29(36):4736-50.

[36] Acharya AP, Dolgova NV, Xia CQ, Clare-Salzler MJ, Keselowsky BG. Adhesive substrates modulate the activation and stimulatory capacity of non-obese diabetic mouse-derived dendritic cells. Acta Biomaterialia 2011;7(1):180-92.

[37] Rogers TH, Babensee JE. The role of integrins in the recognition and response of dendritic cells to biomaterials. Biomaterials 2011;32(5):1270-9.

[38] Andersen CA, Handley M, Pollara G, Ridley AJ, Katz DR, Chain BM. beta 1-integrins determine the dendritic morphology which enhances DC-SIGN-mediated particle capture by dendritic cells. International Immunology 2006;18(8):1295-303.

[39] Romer LH, Birukov KG, Garcia JGN. Focal adhesions - Paradigm for a signaling nexus. Circulation Research 2006;98(5):606-16.

[40] Keselowsky BG, Garcia AJ. Quantitative methods for analysis of integrin binding and focal adhesion formation on biomaterial surfaces. Biomaterials 2005;26(4):413-8.

[41] Lele TP, Thodeti CK, Ingber DE. Force meets chemistry: Analysis of mechanochemical conversion in focal adhesions using fluorescence recovery after photobleaching. Journal of Cellular Biochemistry 2006;97(6):1175-83.

[42] Tourkova IL, Shurin GV, Wei S, Shurin MR. Small Rho GTPases mediate tumor-induced inhibition of endocytic activity of dendritic cells. Journal of Immunology 2007;178(12):7787-93.

[43] Shurin GV, Tourkova IL, Chatta GS, Schmidt G, Wei S, Djeu JY, et al. Small rho GTPases regulate antigen presentation in dendritic cells. Journal of Immunology 2005;174(6):3394-400.

[44] Matzelle MM, Babensee JE. Humoral immune responses to model antigen co-delivered with biomaterials used in tissue engineering. Biomaterials 2004;25(2):295-304.

[45] Babensee JE, Anderson JM, McIntire LV, Mikos AG. Host response to tissue engineered devices. Advanced Drug Delivery Reviews 1998;33(1-2):111-39.

[46] Lewis JS, Zaveri TD, Crooks CP, Keselowsky BG. Microparticle surface modifications targeting dendritic cells for non-activating applications. Biomaterials 2012;33(29):7221-32.

[47] Acharya AP, Clare-Salzler MJ, Keselowsky BG. A high-throughput microparticle microarray platform for dendritic cell-targeting vaccines. Biomaterials 2009;30(25):4168-77.

[48] Legarza K, Yang LX. New molecular mechanisms of action of camptothecin-type drugs. Anticancer Research 2006;26(5A):3301-5.

[49] Alexander C, Rietschel ET. Bacterial lipopolysaccharides and innate immunity. Journal of Endotoxin Research 2001;7(3):167-202.

[50] Lutz MB, Schuler G. Immature, semi-mature and fully mature dendritic cells: which signals induce tolerance or immunity? Trends in Immunology 2002;23(9):445-9.

[51] Matsumoto T, Nagayama K. Tensile properties of vascular smooth muscle cells: Bridging vascular and cellular biomechanics. Journal of Biomechanics 2012;45(5):745-55.

[52] Straehla JP, Limpoco F, Dolgova NV, Keselowsky BG, Sawyer W, Perry SS. Nanomechanical Probes of Single Corneal Epithelial Cells: Shear Stress and Elastic Modulus. Tribology Letters 2010;38(2):107-13.

[53] Dunn AC, Zaveri TD, Keselowsky BG, Sawyer W. Macroscopic friction coefficient measurements on living endothelial cells. Tribology Letters 2007;27(2):233-8.

[54] Humphrey J. Vascular adaptation and mechanical homeostasis at tissue, cellular, and sub-cellular levels. Cell Biochemistry and Biophysics 2008;50(2):53-78.

[55] Ross R. Mechanisms of disease - Atherosclerosis - An inflammatory disease. New England Journal of Medicine 1999;340(2):115-26.

[56] Guzik TJ, Hoch NE, Brown KA, McCann LA, Rahman A, Dikalov S, et al. Role of the T cell in the genesis of angiotensin II-induced hypertension and vascular dysfunction. Journal of Experimental Medicine 2007;204(10):2449-60.

[57] Hoefer I, Schoneveld A, Grundmann S, van Keulen K, Bot P, Timmers L, et al. Arteriogenesis requires leukocytic co-signaling of TLR2 and TLR4. Circulation 2007;116(16):42.

[58] Herold J, Tillmanns H, Xing Z, Strasser RH, Braun-Dullaeus RC. Isolation and transduction of monocytes: promising vehicles for therapeutic arteriogenesis. Langenbecks Archives of Surgery 2006;391(2):72-82.

[59] Banchereau J, Steinman RM. Dendritic cells and the control of immunity. Nature 1998;392(6673):245-52.

[60] Manthey HD, Zernecke A. Dendritic cells in atherosclerosis: Functions in immune regulation and beyond. Thrombosis and Haemostasis 2011;106(5):772-8.

[61] Bobryshev YV, Lord RSA, Parsson H. Immunophenotypic analysis of the aortic aneurysm wall suggests that vascular dendritic cells are involved in immune responses. Cardiovascular Surgery 1998;6(3):240-9.

[62] Bobryshev YV, Lord RSA. Ultrastructural Recognition of Cells with Dendritic Cell Morphology in Human Aortic Intima - Contacting Interactions of Vascular Dendritic Cells in Athero-Resistant and Athero-Prone Areas of the Normal Aorta. Archives of Histology and Cytology 1995;58(3):307-22.

[63] Bobryshev YV, Lord RSA. Langhans cells of human arterial intima: Uniform by stellate appearance but different by nature. Tissue & Cell 1996;28(2):177-94.

[64] Gautier EL, Huby T, Saint-Charles F, Ouzilleau B, Pirault J, Deswaerte V, et al. Conventional Dendritic Cells at the Crossroads Between Immunity and Cholesterol Homeostasis in Atherosclerosis. Circulation 2009;119(17):2367-U151.

[65] O'Callaghan CJ, Williams B. Mechanical strain-induced extracellular matrix production by human vascular smooth muscle cells - Role of TGF-beta(1). Hypertension 2000;36(3):319-24.

[66] Kolpakov V, Rekhter MD, Gordon D, Wang WH, Kulik TJ. Effect of Mechanical Forces on Growth and Matrix Protein-Synthesis in the In-Vitro Pulmonary-Artery - Analysis of the Role of Individual Cell-Types. Circulation Research 1995;77(4):823-31.

[67] Wernig F, Xu QB. Mechanical stress-induced apoptosis in the cardiovascular system. Progress in Biophysics & Molecular Biology 2002;78(2-3):105-37.

[68] Wernig F, Mayr M, Xu QB. Mechanical stretch-induced apoptosis in smooth muscle cells is mediated by beta(1)-integrin signaling pathways. Hypertension 2003;41(4):903-11.

[69] Lee Y, Gustafsson AB. Role of apoptosis in cardiovascular disease. Apoptosis 2009;14(4):536-48.

[70] Fisher SA, Langille BL, Srivastava D. Apoptosis during cardiovascular development. Circulation Research 2000;87(10):856-64.

[71] Paulson KE, Zhu SN, Chen M, Nurmohamed S, Jongstra-Bilen J, Cybulsky MI. Resident Intimal Dendritic Cells Accumulate Lipid and Contribute to the Initiation of Atherosclerosis. Circulation Research 2010;106(2):383-90.

[72] Danial NN, Korsmeyer SJ. Cell death: Critical control points. Cell 2004;116(2):205-19.

[73] Levkau B, Herren B, Koyama H, Ross R, Raines EW. Caspase-mediated cleavage of focal adhesion kinase pp125(FAK) and disassembly of focal adhesions in human endothelial cell apoptosis. Journal of Experimental Medicine 1998;187(4):579-86.

[74] Meredith JE, Fazeli B, Schwartz MA. The Extracellular-Matrix As A Cell-Survival Factor. Molecular Biology of the Cell 1993;4(9):953-61.

[75] Liu S, Ruan Y, Tang D, Li Y, Goldman J, Zhong L. A possible role of initial cell death due to mechanical stretch in the regulation of subsequent cell proliferation in experimental vein grafts. Biomechanics and Modeling in Mechanobiology 2002;1(1):17-27.

[76] Moore MM, Goldman J, Patel AR, Chien S, Liu SQ. Role of tensile stress and strain in the induction of cell death in experimental vein grafts. Journal of Biomechanics 2001;34(3):289-97.

[77] Lenschow DJ, Walunas TL, Bluestone JA. CD28/B7 system of T cell costimulation. Annual Review of Immunology 1996;14:233-58.

[78] Christensen HR, Frokiaer H, Pestka JJ. Lactobacilli differentially modulate expression of cytokines and maturation surface markers in murine dendritic cells. Journal of Immunology 2002;168(1):171-8.

[79] MacPherson G, Huang FP, Platt N, Wykes M, Major J, Powell T, et al. Dendritic cells constitutively transport apoptotic intestinal epithelial cells to T cell areas of mesenteric lymph nodes. Immunology 1999;98(suppl. 1):15.

[80] Voigylander C, Rossner S, Cierpka E, Theiner G, Wiethe C, Menges M, et al. Dendritic cells matured with TNF can be further activated in vitro and after subcutaneous injection in vivo which converts their tolerogenicity into immunogenicity. Journal of Immunotherapy 2006;29(4):407-15.

[81] Menges M, Rossner S, Voigtlander C, Schindler H, Kukutsch NA, Bogdan C, et al. Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. Journal of Experimental Medicine 2002;195(1):15-21.

[82] van Lieshout AWT, Barrera P, Smeets RL, Pesman GJ, van Riel PLCM, van den Berg WB, et al. Inhibition of TNF alpha during maturation of dendritic cells results in the development of semi-mature cells: a potential mechanism for the beneficial effects of TNF alpha blockade in rheumatoid arthritis. Annals of the Rheumatic Diseases 2005;64(3):408-14.