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The effect of cyclic mechanical strain on activation of dendritic cells cultured on adhesive substrates

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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 costimulatory 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.

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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], fibroblasts [3] and smooth muscle cells [4]. For example, physiologic cyclic strain accelerates endothelial cell proliferation [5] while pathologically high cyclic strain induces apoptosis [6]. 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 [8]. 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 [9,10]. Dendritic cells act as sentinels, constantly patrolling the body and presenting both self and nonself-antigens to lymphocytes, B-cells and T-cells [11]. Immature DCs (iDCs), upon activation, upregulate antigen-presenting molecules, costimulatory molecules, cytokines and chemokine receptors. Chemokine receptors mediate the migration of DCs to secondary lymphoid tissues where they initiate adaptive

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immune responses [12]. Notably, functional DCs have been found residing in both human and mouse blood vessels [13]. 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 [14]. Furthermore, the number of vascular DCs is elevated in atherosclerotic plaques and advanced atherosclerotic lesions are additionally infiltrated by circulating monocyte-derived DCs [15,16]. While vascular DCs in healthy arterial walls are found in an immature state, the mature DC phenotype is prevalent at the onset of atherosclerosis [17]. Histological studies suggest that mature vascular DCs are able to initiate the adaptive immune response by interacting with both $CD4^+$ and $CD8^+$ T cells [16]. 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 [18]. 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 [19,20]. 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 [21]. In related work, we have found recently that DCs respond differentially to different adhesive environments [22]. 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 [23]. Integrins, the receptor family which bind extracellular matrix proteins, have been shown to modulate DC functionality and phenotype [24]. For numerous cell types, integrin binding to extracellular matrix ligands results in a mechanical coupling between the inside of the cell and its microenvironment [25–27]. 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. 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.

2. Methods and materials

2.1. Generation of murine bone marrow-derived DCs

Dendritic Cells were generated from bone marrow obtained from 8 to 12 week old, female, C57BL6/j mice in accordance with animal care guidelines approved by University of Florida using a modified 10 day protocol [28,29]. 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 25 g 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 200× g for 7 min. The red blood cells (RBCs) were removed by lysing with ACK lysis buffer (Lonza, Walkersville, MD) followed by centrifugation at 270× 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 (1×10^6 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 °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 °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-annexin V 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 °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 300× 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 300× 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 °C. 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 °C, cells were centrifuged at 300× 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.