IL-1β Does Not Exacerbate Cell Death in a Large-Scale Tissue-Engineered Intervertebral Disc

Matthew Fainor[1,2], Alexandra J. Baranyai[1], Robert L. Mauck[1,2], Harvey E. Smith[1,2], Sarah E. Gullbrand[1,2]
[1]University of Pennsylvania, Philadelphia, PA; [2]Corporal Michael J. Crescenz VA Medical Center, Philadelphia, PA
Disclosures: MF – Matthew.Fainor@pennmedicine.upenn.edu (N), AJB (N), RLM (5), HES (N), SEG (6)

INTRODUCTION: Much work has been completed assessing how mesenchymal stromal cells (MSCs) respond to the challenges inherent to large-scale (>4mm diameter) tissue engineering, including the presence of stark oxygen [1-2] and glucose gradients [2-3]. However, the transition of tissue-engineered intervertebral discs from nutrient-rich in vitro cultures to low nutrient in vivo environments leads to changes in cell morphology, decreases in cell viability, and alterations to extracellular matrix post-implantation [4-5]. It's unclear how the interplay of nutrient deprivation, inflammatory molecules, and immune cell infiltration contribute, both independently and synergistically, to MSC death and tissue remodeling. Here, we hypothesized that inflammation would exacerbate cell death and matrix loss in a large tissue-engineered intervertebral disc made from MSCs when deployed in conjunction with oxygen and glucose deprivation.

METHODS: Mesenchymal stromal cells (MSCs) were obtained from the bone marrow of six skeletally-mature goats and previously screened using a chondrogenic pellet assay [6]. Endplate-modified Disc-like Angle Ply Structures (eDAPS) (16 mm diameter x 9 mm height) were then fabricated and seeded with the P3 MSCs from the most chondrogenic goat donor, as previously described [7]. Tissue-engineered discs were matured under normoxic conditions (21% oxygen) in high-glucose (4.5g/L) chondrogenic media supplemented with TGF-β3 (10ng/mL) under constant mechanical agitation for 10 weeks. After 10 weeks of culture, eDAPS were subject to either in vitro or in vivo challenges. During the in vitro challenge, eDAPS were cultured in hypoxia (2% oxygen) using low-glucose (1g/L) chondrogenic media supplemented with IL-1β (50ng/mL), which was refreshed every 3 days, for up to 2 weeks. At 0, 1, and 2 weeks eDAPS were removed from hypoxic culture. Half of each eDAPS (n=3-4) was utilized for quantification of DNA, glycosaminoglycan (GAG), and collagen content in the NP and AF regions. The NP and AF analogs from the second half of each eDAPS were separated for live/dead staining and MTT quantification, respectively (n=3-4). Additional full eDAPS were taken for histological staining at each time point (n=3). Additional full eDAPS (n=3) were processed for paraffin histology and stained with alcian blue, picrosirius red, and hematoxylin/eosin. Sections also underwent immunofluorescence staining for human collagens I, II, and X, as well as human chondroitin sulfate and rat CD68. During the in vivo challenge, eDAPS were implanted in the subcutaneous space of 8-week-old athymic rats, as approved by IACUC. After 0 and 5 weeks of subcutaneous implantation, eDAPS were processed as described above (n=3).

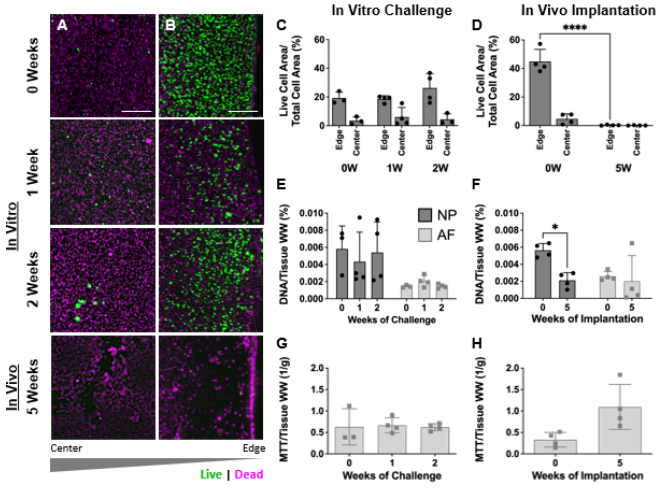

Figure 1: Cell Viability. Live (green)/Dead (pink) staining at the (A) center and (B) edge of eDAPS' NP-analog cross sections at 1 and 2 weeks following in vitro challenge and 5 weeks following in vivo subcutaneous implantation (scale = 300 μm). Quantification of (C-D) NP live cell area/total cell area, (E-F) DNA content, and (G-H) AF metabolism measured via MTT for in vitro and in vivo challenges, respectively.

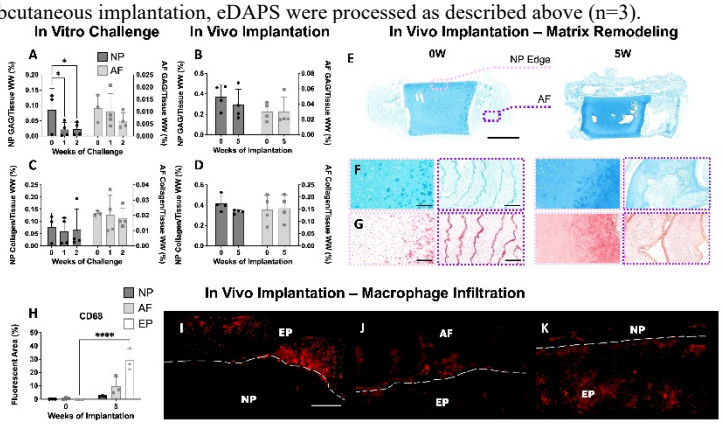

Figure 2: Extracellular Matrix Changes. (A-B) GAG and (C-D) collagen content for eDAPS subjected to in vitro and in vivo challenges, respectively. Histologic staining of eDAPS subjected to in vivo subcutaneous implantation at 0 and 5 weeks using (E-F) alcian blue and (G) picrosirius red of the NP-analog (left inset) and AF-analog (right inset) [scale=3mm (E), 500μm (F-G)]. Immunofluorescence (IF) (H) quantification of CD68 positive macrophages (red) in the (I) NP-analog, (J) AF-analog, and (K) endplate (EP)-analog (scale=400μm).

RESULTS: After 10 weeks of in vitro preculture, the vast majority of goat MSCs were dead in the center of the constructs (Figure 1A) but were alive at the constructs' edge (Figure 1B). After two weeks of in vitro challenge, cell viability in the NP was unchanged (Figure 1C,E), whereas 5 weeks of in vivo subcutaneous implantation killed every MSC in the NP-analog (Figure 1D,F). Following in vitro challenge, MSC metabolism and DNA content in the AF was unchanged (Figure 1E,G), whereas overall cell metabolism in the AF of the in vivo implantation group increased from 0 to 5 weeks (Figure 1H). Glycosaminoglycan content decreased significantly in the NP of in vitro-challenged eDAPS after 1 week but was unchanged in the NP and AF of in vivo-challenged eDAPS (Figure 2A-B). Hydroxyproline (collagen) content was stable over time in all compartments of both experimental groups (Figure 2C-D). Both alcian blue and picrosirius red stained more intensely after 5 weeks of subcutaneous implantation, particularly in the NP-analog (Figure 2E-G). Collagen I, collagen X, and chondroitin sulfate all decreased significantly in quantity by 5 weeks, indicating that extracellular matrix proteins of all kinds, while present in higher quantities, are non-functional and fragmented. Finally, CD68 positive macrophages were found in the DAPS NP-, AF-, and EP-analogs after 5 weeks in the subcutaneous space (Figure 2H-K).

DISCUSSION: Surprisingly, there was no change in goat MSC survival in a low oxygen, low glucose, IL-1β-induced inflammatory environment over the course of two weeks in vitro, which may indicate that the cells unfit to survive these conditions already perished during the preculture period. Both in vitro- and in vivo-challenged eDAPS lost functional GAG content over time, indicating that even if cells are not dying, they are struggling to maintain ECM integrity, likely in response to upregulated MMPs from IL-1β stimulation [8-9]. Although T cell deficient athymic rats are a useful model to study an in vivo challenge, it is clear that local macrophages responded to the presence of these xenogenic implants. When hMSCs were delivered intraarticularly to the knees of T and B cell deficient SCID mice, their number was reduced by 87% one month following transplantation [10], supporting the theory that local macrophages likely contributed to cell death across the NP-analog in this study. The eDAPS provide a system to test the performance of cell therapies in 3D hypoxic, hypoglycemic environments, and this data provides a critical baseline against which to compare the future performance of eDAPS seeded with native disc cells, as well as eDAPS seeded with CRISPR-modified MSCs [11] with edits targeting cell proliferation, senescence, metabolism, and fibrocartilaginous matrix production, in order to better understand what mechanisms enable cells to thrive in inflammatory/low glucose/low oxygen environments.

SIGNIFICANCE: Characterization of MSC response to inflammation in low glucose and low oxygen environments will allow us to better understand what phenotypes allow cells to thrive under duress in order to engineer more successful cell therapies for large-scale tissue engineering interventions.

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