Compressive Load Influences the Immunomodulatory Profile of MSC Spheroids

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INTRODUCTION: Cell-based treatments are an alternative approach for rejuvenating injured tissues and are currently being studied for their potential to treat bone defects under mechanical load. Mesenchymal stromal cells (MSCs) are commonly used due to their innate ability for multipotent differentiation and bioactive secretome that modulates the local inflammatory environment and recruits host cells to the implantation site for repair. Cell-based therapies are often placed in defects under mechanical load. However, the response of the cells under a dynamic physical microenvironment (*i.e.*, compressive mechanical loading) has not been well characterized. Mechanical loading impacts MSC behavior, from cell proliferation to directing cell lineage and modulating the secretome. While the importance of compressive load to direct MSC differentiation has been reported, there is limited knowledge on the application of physical compressive load and its immunomodulatory effects on MSC spheroids. Previous studies report elevated levels of cytokines when MSCs are mechanically stimulated *via* hydrostatic and pneumatic pressure compared to unstimulated MSCs. Monodispersed MSCs secrete more vascular endothelial growth factor (VEGF), a potent proangiogenic factor, when stimulated by pneumatic driven mechanical load. However, these studies have only focused on monodispersed MSCs and introduced mechanical forces *via* hydrostatic or pneumatic pressure. Such models fail to capture extrinsic physical, mechanical forces that the body undergoes. MSC spheroids present numerous advantages over monodispersed MSCs including improved survival and increased secretion of many trophic factors. Nonetheless, the effects of compressive mechanical loading remain largely unknown on MSC spheroids. We hypothesized that cyclic compressive load and loading regime influence the immunomodulatory potential of MSC spheroids, evidenced by changes in cytokine production and gene expression.

METHODS: Human bone marrow-derived MSC spheroids consisting of 8,000 cells were formed using forced aggregation for 48 h prior to entrapment in gels. Alginate gels were made with 2% w/v RGD (Arginine-Glycine-Aspartic Acid)-modified MVG alginate and crosslinked with 200 mM $CaCl_2$ and 10 mM $BaCl_2$. Gels possessed a shear modulus of 11 kPa, as measured by rheometry. MSC spheroids were encapsulated in alginate hydrogels at 5 x 10^6 cells/mL. Spheroid-loaded alginate gels were allowed to reach swelling equilibrium for 24 h before moving to 1 mL of complete alpha-MEM in the MechanoCulture TX bioreactor (CellScale) for 3 days. The loading regime was changed for load magnitude (5 or 10 kPa) and duration (30 or 240 s) with the overall time maintained for the cycle. Mechanical loads were chosen to interrogate the influence of load amplitude on MSC spheroid behavior and were based on the maximum load the gels could sustain without fracturing. Gels cultured in static conditions served as the control. Culture media was refreshed 24 h before collecting as conditioned media. Multiplex cytokine arrays were used to characterize the immunomodulatory cytokine production from MSC spheroids entrapped in alginate hydrogels. The mechanism regulating the secretory potential of MSC spheroids was also examined using the ROCK inhibitor Y-27632. Statistical analysis was performed using GraphPad Prism 9 software. Data are presented as means ± standard deviation. Statistical significance was assessed by one-way ANOVA, where *p*-values <0.05 were considered statistically significant. Significance is denoted by alphabetical letterings, with different letters denoting statistical significance between groups.

RESULTS: Compressive loads of 5 kPa and 10 kPa and hold times of 30 s and 250 s were chosen to generate three loading regimes – 5 kPa load with 30 s hold (L5H30), 10 kPa with 30 s hold (L10H30), and 10 kPa with 250 s hold (L10H250) (**Fig. 1A**). Secretion of pro- and anti-inflammatory cytokines was dependent on compressive load magnitude and duration, with a general shift towards pro-inflammatory and away from the anti-inflammatory profile with the application of compressive loading (**Fig. 1B**). We measured changes in gene expression of *CYR61* and *CTGF*, two downstream targets of the Hippo pathway, to determine the effect of compressive loading on mechanosensing. With the addition of a ROCK inhibitor (Y-27632), expression of these genes decreased compared to both static and compressive loaded gels without the inhibitor for the L10H30 condition (**Fig. 1C**).

DISCUSSION: These data demonstrate that the secretome of MSC spheroids entrapped in alginate gels is influenced by the magnitude of compressive loading. When exposed to three different loading regimes (different load magnitudes and duration), the immunomodulatory secretome of the spheroids in the alginate gels was altered. The application of compressive load shifted the secretome to an upregulation of pro-inflammatory factors and a downregulation of anti-inflammatory factors. We next examined how the responsiveness of MSC spheroids with cyclic compressive load was influenced by cellular mechanisms. Treatment of spheroids with Y-27632 resulted in reduced expression of *CYR61* and *CTGF* compared to samples that were mechanically loaded without the inhibitor. These data emphasize the importance of cyclic mechanical load on the immunomodulatory potential of MSC spheroids. Taken together, this study reveals the influence of the compressive loading regime on the immunomodulatory potential of MSC spheroids and emphasizes the importance of considering cyclic compressive load in current therapeutic approaches.

SIGNIFICANCE/CLINICAL RELEVANCE: The impact of compressive mechanical load on MSC spheroids remains largely unknown. Unlike other methods of load application, our approach utilizes physical compressive mechanical load to interrogate the behavior of cells. This work provides new insight into characterizing the response of cellularized materials when transplanted into tissues under mechanical load.

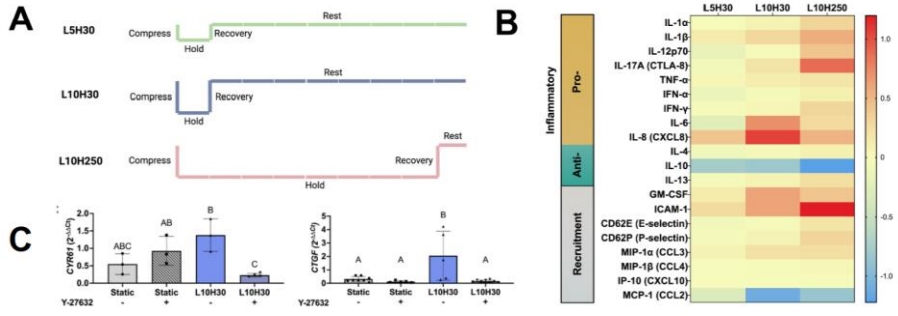

Figure 1. (A) Bioreactor loading regimes (L5H30, L10H30, L10H250) with varyling load (L) magnitudes (*i.e.*, 5 or 10 kPa) and hold (H) durations (*i.e.*, 30 or 250 s). **(B)** Heat map shows the results of a quantitative 20-plex Luminex assay with reactivity for pro- and anti-inflammatory human analytes. Data are normalized to static culture controls. Scale bar represents fold changes in cytokine/chemokine content where 0 represents no change (yellow), positive values are an upregulation (red) and negative are down regulation (blue). **(C)**. Gene expression related to mechanosensing is altered with Y-27632. Different letters denote statistical differences. Data are mean ± SD (n = 3).