Immunomodulatory Hydrogels for Bone Regeneration to Combat Bisphosphonate-Related Osteonecrosis of the Jaw

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INTRODUCTION: Bisphosphonate-related osteonecrosis of the jaw (BRONJ) is a chronic inflammatory disease resulting in bone loss that is caused by long-term use of bisphosphonates. Mesenchymal stromal cells (MSCs) are a promising cell-based therapy for bone regeneration. Biomaterials augment MSC-mediated tissue repair through direct differentiation cues or by promoting trophic factor secretion to recruit endogenous cells and modulate the local immune environment. However, concurrent MSC differentiation and secretome production is difficult to maintain. Upon implantation into harsh microenvironments, MSCs upregulate bioactive factor production in an attempt to modulate the inflammatory environment, yet it is unknown if the controlled delivery of similar biomolecules may allow MSCs to persist longer and undergo osteogenic differentiation toward the osteoblastic phenotype. Here, we aim to use microparticles within hydrogels to locally deliver instructive factors and interrogate MSC differentiation in inflammatory culture conditions relevant to BRONJ.

METHODS: For immunomodulatory (IMF) delivery, unphagocytosable poly(D,L-lactic-co-glycolic-acid) (PLGA) microparticles (MPs) were fabricated using a double emulsion solvent evaporation technique. MPs were loaded into 10% gelatin methacryloyl (GelMA) hydrogels at 0-20 mg/mL to determine their effects on hydrogel mechanical properties. We used a Design of Experiments (DOE) approach to determine the combination of IMFs, specifically IL-10, IL-4, and PGE_2, that maximize osteogenic differentiation and macrophage polarization towards the M2 phenotype. For these experiments, MSC osteogenic differentiation was determined with ALP activity, Alizarin Red (AR) staining, and calcium deposition. Macrophage polarization was determined with flow cytometric analysis for double positive CD206 and Arginase-1 populations. IMF release kinetics from MPs were characterized with ELISA. Lastly, with an eye towards clinical use, we examined the effects of IMF MPs on naïve MSCs when cultured in GelMA under chronic inflammatory conditions (50 pg/mL TNFα). We characterized MSC differentiation potential *via* qPCR and immunomodulatory potential *via* multiplex conditioned media protein analysis and subsequent flow cytometric macrophage polarization. Statistical analysis was performed using a one-way analysis of variance (ANOVA) with a Tukey's multiple comparison *post hoc* test. Data are presented as mean ± SD.

RESULTS: IMF MP density did not affect gel storage modulus or gross appearance (**Fig. 1**), and DOE experiments suggest that 100 ng/mL of both IL-4 and IL-10, with no PGE_2, yield the highest concurrent osteogenic differentiation of MSCs and M2 phenotype (**Fig. 2**). We observed a burst release from the IMF-MPs, with >90% of the IMFs released after 3 days (**Fig. 3**). When we interrogated the response of naïve MSCs to IMF MPs in chronic inflammatory conditions, we found that *Runx2* expression was significantly increased in groups treated with IMF MPs compared to those with unloaded or no MP treatment. Though not significantly different, we also noted the same trend for the late osteogenic marker *Col1a1*, which is meaningful given the lack of osteoinductive stimulus (**Fig. 4**). Furthermore, when MSCs were treated with IMF MPs, we observed an increase in secreted chemotactic factors associated with immune cell recruitment, though no functional changes in macrophage polarization (**Fig 5**). We are currently investigating earlier timepoints to elucidate the dynamics of immune modulation in this system, and preparing for the application of this system to an *in vivo* study in rats.

CONCLUSION: These data indicate that IL-10 and IL-4 loaded into PLGA MPs and delivered in GelMA hydrogels effectively encourage naïve MSC osteogenesis and pro-regenerative M2 macrophage polarization, even in chronic inflammatory conditions. Future studies will examine the efficacy of these constructs *in vivo* in an induced-BRONJ rat model.

SIGNIFICANCE/CLINICAL RELEVANCE: These data represent key findings that will facilitate the reversal of the chronic bone loss and inflammation characteristic of BRONJ. This work has the potential to provide a strategy to regenerate bone tissue in patients with BRONJ.

IMAGES AND TABLES:

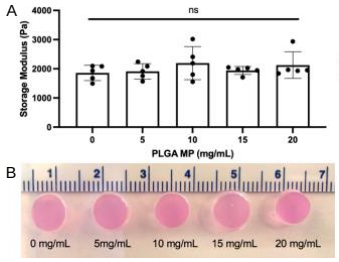

Figure 1. Microparticles do not affect hydrogel mechanical properties. **(A)** Shear storage moduli and **(B)** gross morphology of 10% GelMA hydrogels loaded with 0-20 mg/mL MPs (n=4-5).

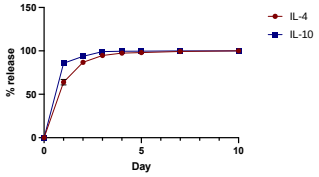

Figure 3. MPs exhibit burst release of encapsulated IMFs. Percentage release of IL-4 and IL-10 over 10 days of IMF-MPs as measured by ELISA (n=4).

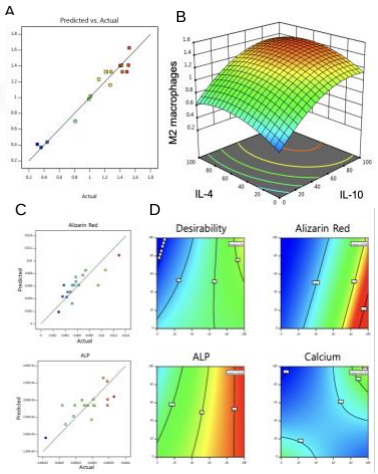

Figure 2. Treatment with 100 ng/mL of IL-4 and IL-10 induces the greatest M2 macrophage polarization (A, B) and MSC osteogenesis (C, D). Model Fit of M2 polarization **(A)** and Alizarin Red (AR) and ALP **(C)** as visualized by plotting predicted versus actual output values. The effect of IL-4 (y-axes) and IL-10 (x-axes) on M2 polarization **(B)** MSC osteogenic potential **(D)**. "Desirability" maps combine maximized outputs with IL-4 and IL-10 concentrations.

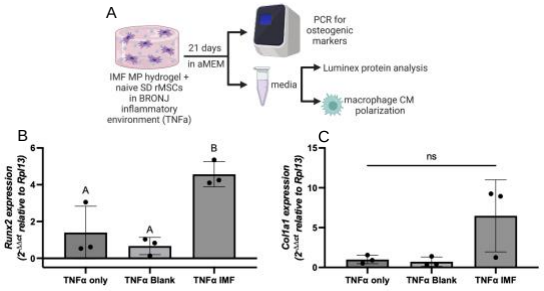

Figure 4. Osteogenesis is upregulated in naïve MSCs cultured for 21 days in GelMA loaded with IMF MPs treated with 50 pg/mL TNFα. **(A)** Schematic of workflow. *Runx2* **(B)** and *Col1a1* **(C)** expression as osteogenic differentiation markers. (n=3).

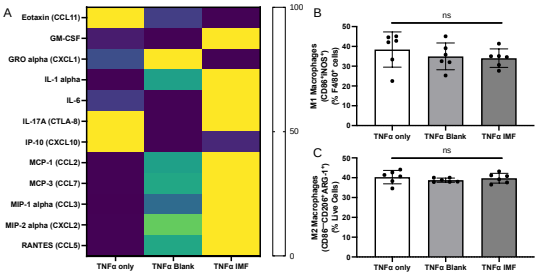

Figure 5. **(A)** Heatmap of secreted chemotactic proteins *via* Luminex analysis. Data are normalized on a scale of 0-100%, where the lowest absolute value per row is "0" and the highest is "100". Flow cytometric analysis of IC-21 macrophages treated with media from **(A)** to evaluate M1 **(B)** and M2 **(C)** polarization. (n=6).