Analyzing Immune Response to Engineered Hydrogels by Hierarchical Clustering of Inflammatory Cell Subsets

Marc A. Fernandez-Yague[1], Lauren A. Hymel[1], Edward A. Botchwey[1,] and Andrés J. García[1].
[1]Georgia Institute of Technology, Atlanta, GA, USA.

Introduction: Synthetic hydrogels have great potential to enable a wide range of novel immunotherapies[1]**.** In the present study, we explored the progression of inflammation around degradable PEG hydrogels presenting adhesive peptides (RGD) and angiogenic growth factor (VEGF) using SPADE clustering algorithms to analyze high-dimensional flow cytometry data paired with intravital imaging and cytokine secretory profiling. The results shed light on the importance of macrophages functions in wound healing progression influenced by adhesive engineered hydrogels and demonstrate the advantages of unbiased high-dimensional analysis methods over traditional methods that identify previously excluded populations sensitive to biomaterial adhesive cues.

Methods: PEG4-MAL 4.5% w/v functionalized with 1mM of RGD peptide (GRGDSPC) or RDG scrambled peptide control (GRDGSPC) and crosslinked with the cysteine-flanked peptide VPM (GCRDVPMSMRGGDRCG) in 0.5M MES buffer, pH 5.5. The VEGF concentration was 10µg/mL. Male C57BL/6J mice or B6.129P-Cx3cr1tm1Litt/J mice (CX3CR1GFP/+) mice (8-12 weeks) were used for dorsal skinfold window chamber (DSWC) studies. Animals were euthanized for flow cytometry and single-cell protein analysis at 1, 3, 7, or 14 dpi. Subsequent analysis was using bi-plot gating, UMAP, X-shift, and SPADE.

Results: We performed real-time migration analyses of cells on multiple areas around engineered hydrogels implanted in a dermal wound (DSWC) model. Using 3D image processing software we found an increase, the mean and maximum velocity of CX3CR1+ cells within <30 µm of the surface of RGD-presenting hydrogels. However, flow cytometry results did not reveal clear differences between the two hydrogel formulations in terms of recruited immune cell clusters. Whereas temporal shifts in myeloid populations' heterogeneity and recruitment frequency were observed for RGD- and RDG-presenting hydrogels, the analyses based on traditional gating strategy did not reveal more complex, functionally relevant subpopulations associated with the adhesive ligand presentation. In contrast, in-depth cytokine profiling of single macrophages from RGD- and RDG-presenting hydrogels revealed clear differences in the immune response to RGD-presenting hydrogels (Fig. 1). RGD presentation recruited preferentially macrophages with modulated polyfunctionality. Although these underlying differences in the immune response are easily overlooked in flow cytometry cell frequency analysis, these RGD-dependent macrophage secretions may significantly alter autocrine and paracrine signaling within the microenvironment crucial to the wound healing process. In addition, using an unbiased clustering algorithm, SPADE, we determined that a rare macrophage subpopulation that accumulated preferentially on RGD- compared to RDG-presenting gels.

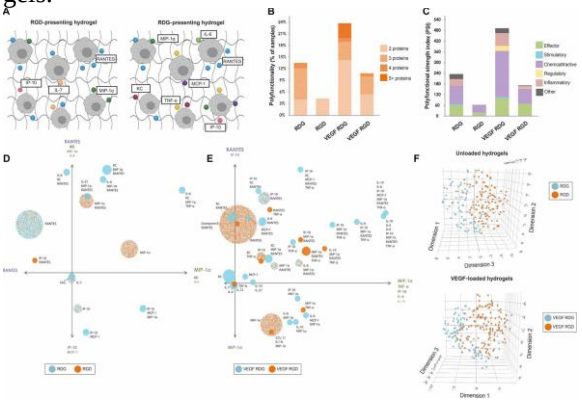

Fig. 1. RGD-presenting hydrogels stimulate specific macrophage cytokine signatures. MerTK+CD64+ macrophages were FACS-sorted from RGD- or RDG-functionalized hydrogels with or without VEGF at 7 dpi. (A) Schematic representation of cytokine. (B) Polyfunctionality or heterogeneity of single macrophages. (C) Polyfunctional strength index (PSI) of macrophages from each treatment group. (D and E) Polyfunctional activation topology PCA plot representing each polyfunctional group. (F) 3D t-SNE plots of single macrophages .

Conclusions: Single-cell proteomics revealed previously unreported adhesion-dependent functional heterogeneity in immune populations defined as relatively homogeneous by traditional surface markers. We expect that this advanced, in-depth temporal study of biomaterial immune response will inform the future development of biomaterial-based strategies for immunomodulation applications.

References: [1] E. A. Phelps . Adv. Mater. 24, 64–70 (2012).

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