

**FIGURE 9**

Effect of soluble stimuli on macrophage polarization in well-defined, bioprinted 3D cultures. Effect of polarization stimuli on macrophage polarization was determined for RAW 264.7 cells and primary BMMs (C57BL/6) in 3D culture (1.1 kPa PEG-peptide hydrogels with RGD, GFOGER, YIGSR, and HA). (A) CD86 and (B) MHCII were used as M1 markers. (C) CD206 and (D) EGR2 were used as M2 markers. All data are normalized to the UT control for the respective cell type. (E) Cell area and (F) circularity were quantified for all the cell types across different conditions. (G) Representative images of BMMs with nuclei (blue) and F-actin (red) qualitatively show morphology of BMMs across all the treatment groups. Statistics were performed using Tukey's post-hoc test with one-way ANOVA. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Scale bar: 100 μm .

promote more M2-like phenotypes (He et al., 2018; Bu et al., 2022; Ciciuendez Maroto et al., 2024). Others also have reported that RAW 264.7 cells in PEG-based hydrogels increase baseline expression of both M1 and M2 markers in 3D culture in both compliant and stiff cell-degradable PEG-peptide hydrogels relative to 2D culture on TCP (Kim et al., 2019). Future work is needed to address how stiffness impacts the phenotype in such well-defined 3D cultures alongside synergistic effects of stiffness and external stimuli.

Based on previous literature as noted above (Cha et al., 2017; Kim et al., 2019; Lee and Ki, 2020), we speculate that higher base level expression of inflammatory markers prior to stimulation may hinder subsequent polarization with external stimuli when compared to the untreated control. There may also be less availability of receptors in 3D culture for binding to applied ligands or nonspecific interactions between the hydrogel components and cytokines that impact ligand-receptor binding. Note, the diffusion of cytokines through the full hydrogel thickness is expected to occur within an hour, based on the literature and assuming one-dimensional Fickian diffusion ($t_d = L^2/D$, where the length (L) is 0.055 cm and the diffusion coefficient (D) of the proteins through a PEG-hydrogel is estimated to be $\sim 1E-6$ cm²/s) (Weber et al., 2009). In this context, we do not expect cytokine diffusion to be limiting the macrophage response in these studies. Macrophages may need higher concentrations of cytokines and longer incubation times, or repeated exposures to see pronounced responses to stimuli. Opportunities for future studies include probing tissue-specific responses within such culture systems for their validation and application. Overall, our results establish the relevance of a bioprinted synthetic ECM for well-defined 3D culture of both immortalized and primary macrophages and provide insights into differences in their function in 3D culture toward informing cell type selection in both fundamental and applied studies, from biological mechanisms to biomaterials designs.

4 Conclusion

Our findings demonstrate that macrophages from different cell lines display marked differences in phenotypical and morphological profile, phagocytosis function, and repolarization with alternative stimuli. Both immortalized and primary cells vary in polarization based on the tissue origin and mouse strain. Macrophages are less sensitive to stimuli in 3D hydrogel culture and have notably different morphologies than on TCP. While limited to the specific cell lines and markers addressed here, through this analysis, we aim to provide researchers with an important baseline for selecting a macrophage cell line or primary cells for biomaterial applications. Our results further showcase bioprinted well-defined 3D cultures for studies of immortalized or primary macrophages in bioactive hydrogel-based synthetic ECMs with high throughput; future work with such systems has the potential to bridge an important gap between *in vitro* and *in vivo* systems for probing tissue-relevant responses of macrophages and other innate immune cells. Here, we focused on cell-by-cell analysis to understand the phenotype and function of the cell. In future work, phenotypic characterization of selected cell lines or primary cells of interest in a specific application should also include gene expression and functional differences in cytokine release profiles to fully elucidate differences between these macrophage research tools.

Data availability statement

The raw data supporting the conclusion of this article will be made available by the authors, without undue reservation.

Ethics statement

Primary BMMs and PMs were isolated from healthy BALB/c and C57BL/6 mice (6–12 weeks old, Jackson Laboratories, Bar Harbor, Maine, USA), respectively, following the approved Institutional Animal Care and Use Committee (IACUC) protocol at the University of Delaware. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JG: Writing–original draft, Writing–review and editing, Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Supervision, Visualization. KB: Conceptualization, Data curation, Formal Analysis, Investigation, Methodology, Project administration, Visualization, Writing–original draft, Writing–review and editing. MT-R: Conceptualization, Formal Analysis, Investigation, Writing–original draft, Writing–review and editing. BJ: Conceptualization, Formal Analysis, Investigation, Writing–original draft, Writing–review and editing. NG: Formal Analysis, Investigation, Writing–original draft, Writing–review and editing. AK: Writing–original draft, Writing–review and editing, Conceptualization, Funding acquisition, Project administration, Resources, Supervision. CF: Writing–original draft, Writing–review and editing, Conceptualization, Funding acquisition, Project administration, Resources, Supervision.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

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