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"Optimization of an *in vitro 3D* method for studying modulators of the immunosuppressive tumor microenvironment for enhancing the response to immunotherapy"

Purpose: This study aims to establish physiologically relevant *in vitro* models of immunosuppressive tumor microenvironment (TME) for multiple myeloma (MM) and triple-negative breast cancer (BC) using three-dimensional (3D) multicellular coculture systems incorporating immunosuppressive and inflammatory myeloid cells, including M2-polarized THP-1 macrophages and low-density neutrophils (LDNs) with RPMI-8226 (MM) or MDA-MB-231 cells (BC). These models will provide a platform to investigate the mechanisms by which myeloid cells promote tumor-associated immune suppression and to evaluate strategies that overcome myeloid-driven immunosuppression, thereby enhancing the efficacy of cancer immunotherapies**.**

Methods: Differentiated THP1 into macrophages using phorbol 12-myristate 13-acetate (PMA) were polarized into immunosuppressive-like phenotype (M2) with recombinant human IL4 and IL13 cytokines and cancer cells-derived conditioned media (CC-CM). Primary LDNs from peripheral blood were stimulated with visceral adipose tissue-conditioned media (VAT-CM). Fluorescent labeled myeloid M2-THP1 and LDNs. Activation and M2 markers (CD206, CD163, PD-L1, B7-H4, HLA-DR) were evaluated by flow cytometry on alive cells. Collagen-embedded 3D spheroids from MDA-MB-231 and RPMI-8226 cells were formed in ultra-low-attachment (ULA) 96-round-well-plates and cocultured with different ratios and treatments of myeloid cells. Spheroid size over time and infiltration of myeloid cells was tracked using the Live-Cell Imaging Incucyte SX3.

Findings: Coculture with M2-THP1 macrophages enhanced the growth of 3D spheroids generated from RPMI-8226, with the greatest increase observed when macrophages were exposed to tumor cell-conditioned medium, compared with spheroids cultured alone or with M2-polarized THP1 macrophages in the absence of conditioned medium. Similarly, MDA-MB-231 spheroids exhibited increased size when cocultured with M2-polarized THP1 macrophages, regardless of the presence of conditioned medium, relative to tumor spheroids cultured alone. Notably, coculture with VAT-CM-activated LDNs produced a marked acceleration of MDA-MB-231 spheroid growth over time. This tumor-promoting effect was selective for malignant cells, as VAT-CM-activated LDNs did not alter the growth of spheroids generated from the non-malignant mammary epithelial cell line 184A1.

Conclusion: We were able to replicate an immunosuppressive and inflammatory TME to evaluate tumor growth *in vitro*. Further optimization is fundamental for evaluating the effect of therapeutic modulation of the myeloid phenotype on spheroid growth and response of cellular immunotherapy.