

Development of Robust Evaluation of T Cell Subsets with T cell Activation in a 3D Tumor-PBMC Co-Culture Model Enables Reliable Phenotyping and Cytotoxicity Assessment

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Abstract

Three-dimensional (3D) tumor-immune co-culture systems offer a physiologically relevant platform to evaluate T cell-mediated anti-tumor responses and immunotherapeutic mechanisms of action. In this study, we established a 3D tumor spheroid co-culture model with human PBMCs and implemented anti-CD3/CD28 stimulation under optimized culture conditions to achieve reliable T cell activation within a complex microenvironment. Tumor spheroids were generated under low-adhesion conditions and co-incubated with PBMCs at defined effector-to-target ratios and controlled activator concentrations. Anti-CD3/CD28 antibodies were used to induce strong, polyclonal T cell receptor engagement and to provide a consistent positive control arm for evaluating T cell-engaging therapeutics.

Flow cytometry was employed to assess T cell activation, proliferation, cytotoxicity, and tumor cell viability throughout the co-culture period. Activated PBMCs demonstrated robust upregulation of CD69 and CD25, increased effector cytokine release, and induction of tumor cell apoptosis accompanied by loss of spheroid integrity. CD4⁺ and CD8⁺ T cells displayed distinct patterns of recruitment and effector activity within the 3D model, highlighting functional differences that are not captured in 2D assays.

To enable accurate quantification of CD3⁺ T cells and downstream functional markers, we optimized the activation parameters, spheroid dissociation workflow, and staining conditions to ensure consistent recovery of immune and tumor cell populations from the 3D matrix. This standardized platform provides a reproducible and biologically relevant system for evaluating T cell activation and tumor killing kinetics, supporting preclinical immuno-oncology research, functional profiling, and therapeutic antibody screening. The robust CD3⁺ T cell readout generated under anti-CD3/CD28 stimulation further serves as a reliable reference arm for benchmarking T cell-engaging modalities.

Study Design

Using the previously established PBMC-3D tumor co-culture platform, this study aims to identify an optimal positive control for T cell activation and tumor killing and to develop a reproducible flow cytometry method for surface marker analysis.

Commercial reagents such as ImmunoCult (IMT) and the Miltenyi T Cell Activation Kit induce rapid strong T cell expansion. However, CD3 becomes masked after activation, limiting reliable detection. However, after the T cell activation, the free TCR is important in the 3D co-culture system for immunological synapse formation, chemotaxis, and direct tumor killing (Figure 1).

To address this, we developed a modified activation method using biotin anti-CD3 and purified anti-CD28 antibodies (Biotin-Ab). This approach enables tunable activation while reducing CD3/TCR complex blocking, preserving CD3 detectability. It achieves T cell activation and tumor killing comparable to IMT.

Additionally, surface markers (e.g., CD4, CD8, CD14, CD16, CD44, CD56, PD-1, Tim-3) can be partially degraded during dissociation. An optimized method allows accurate measurement of marker frequencies, although mean fluorescence intensity (MFI) may be slightly reduced compared to non-dissociated cells.

Results: Antibody Saturation for T Cell Activation

A relative antibody binding saturation assay was performed on healthy donor PBMCs using ImmunoCult (IMT), biotin anti-CD3, or PE anti-CD28. Cells were untreated or exposed to serial dilutions of each reagent, followed by staining with anti-CD3-FITC, anti-CD28-PE, or streptavidin-BUV737.

Figure 2. (A) Surface CD3 or CD28 receptor expression curves for IMT show CD3⁺ and CD28⁺ cell percentages normalized on untreated controls. At higher IMT concentrations, CD28 exhibits stronger binding than CD3. **(B)** Receptor binding curves based on gMFI for biotin anti-CD3 and anti-CD28 indicate similar binding affinities at equivalent concentrations.

Higher concentrations promote T cell expansion; but may not be optimal for effective tumor killing in co-culture system.

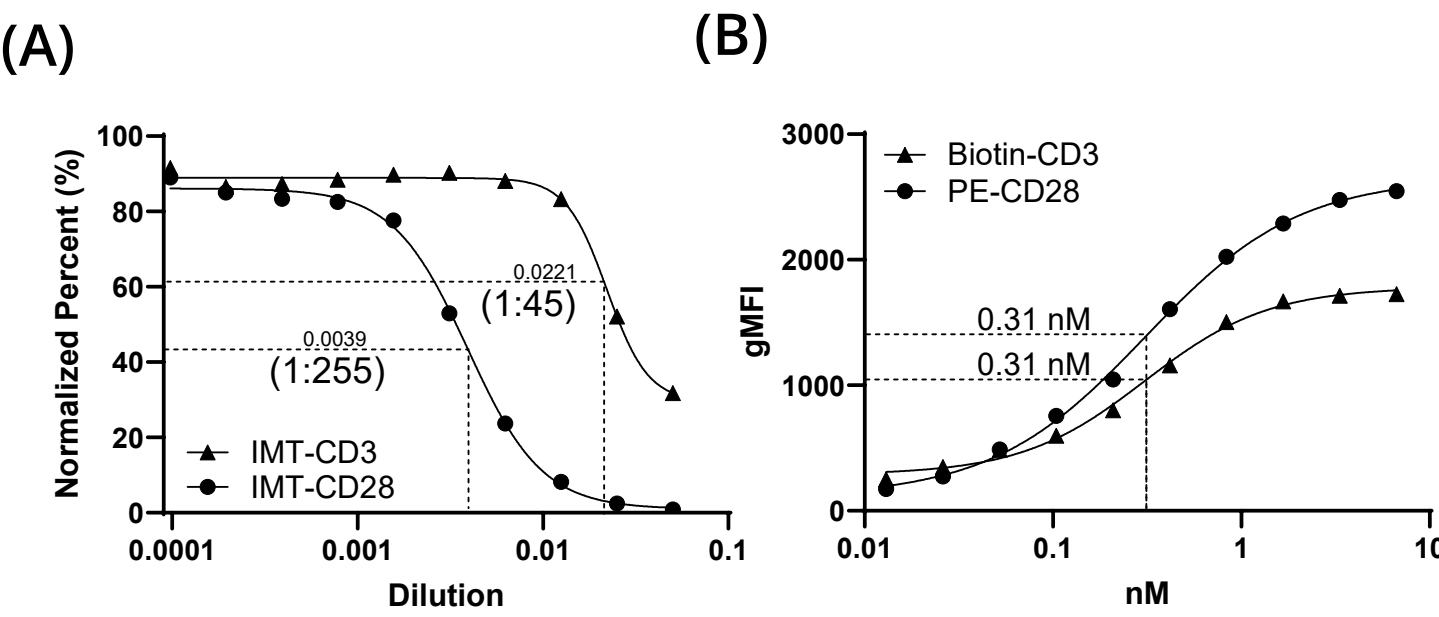


Figure 2. Relative Receptor Binding Assay

Results: T Cell Activation

Different concentrations of IMT and Biotin-Ab were tested with different PBMC numbers in TumorGraft3D media or cRPMI (RPMI-1640, 10% FBS, 1% Pen/Strep) using an optimized PBMC-3D tumor co-culture setup. To reduce FBS-related variability, TumorGraft3D medium was optimized with a serum replacement. The optimal concentrations of IMT (1:150 dilution) and Biotin-Ab was confirmed. PBMCs were co-cultured with TumorGraft3D model-0064 (E:T 10:1) using IMT (1:150), Biotin-Ab, or no treatment in TumorGraft3D3D medium (Day 4). Viable CD45⁺ and EpCAM⁺ populations, and expression of CD4⁺, CD8⁺, and CD25⁺ were analyzed.

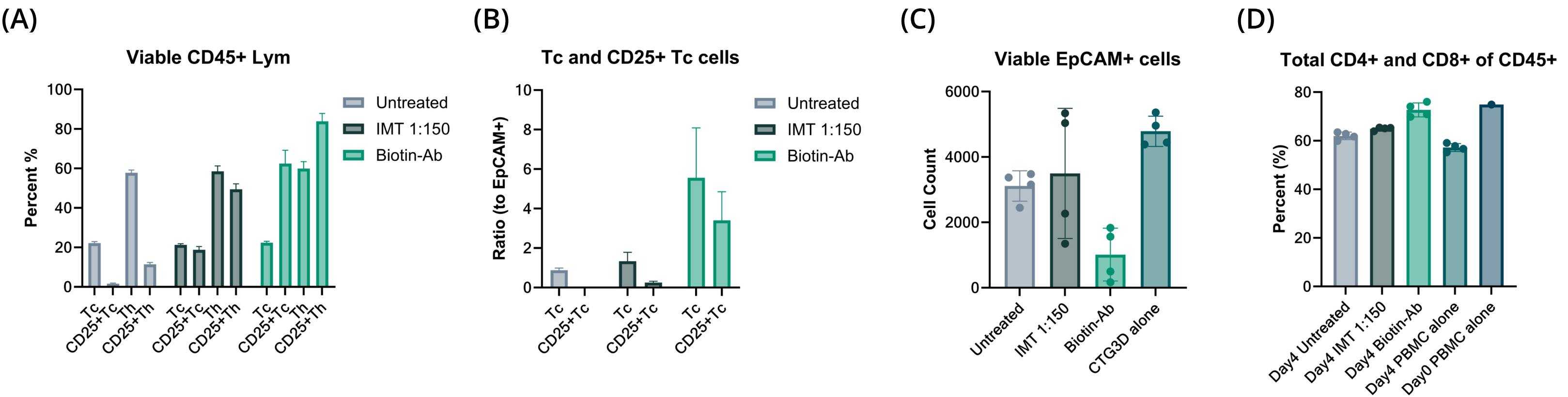


Figure 3. (A) CD8⁺ (Tc) and CD4⁺ (Th) cells were gated from CD45⁺ lymphocytes excluding non-T cells (CD14⁺, CD16⁺, CD19⁺, CD33⁺ and CD56⁺). Frequencies of Tc and Th cells were similar across groups, but activation increased with IMT and Biotin-Ab, with stronger effects from Biotin-Ab. **(B)** Ratios of viable CD8⁺ T cells to tumor cells and **(C)** reduced tumor cell numbers indicate more effective killing with Biotin-Ab. **(D)** Total T cell levels remained comparable to untreated controls. These results suggest both optimal IMT (1:150) and Biotin-Ab-activated PBMCs provide an effective positive control compatible with the optimized method.

Results: Dissociation for Marker Preservation & Accurate T Cell Deletion

The CD3 may be partially or completely blocked following IMT or T cell engager treatment, therefore, accurate detection of T cells requires clear CD4⁺ and CD8⁺ readouts. Furthermore, exclusion of BMNK (non-T cell) populations using additional markers such as CD14, CD16, CD19, CD33, and CD56 improves the accuracy of the indirect total T cells detection. In contrast, the Biotin-Ab method provides a direct detection of total T cells with streptavidin staining following T cell activation.

To enable reliable indirect T cell measurement for IMT or T cell engager applications, we optimized dissociation and staining conditions to preserve enzyme-sensitive surface markers. PBMC-TumorGraft3D model-2687 co-cultures were untreated or treated with optimal IMT, with PBMC-only controls in 0.1% DMSO, and analyzed at Day 4. Following optimized method, highly enzyme-sensitive markers (CD4, CD14, CD16, CD56) were accurately detected.

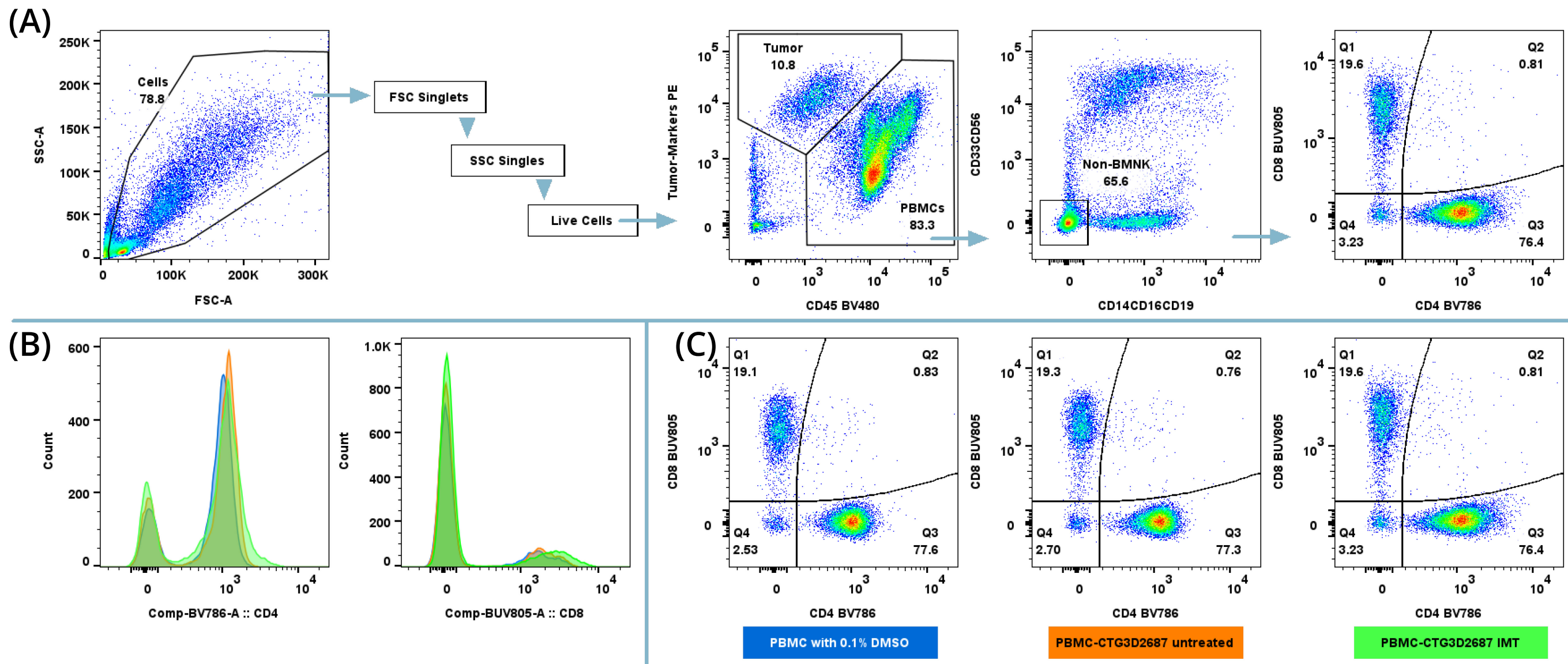


Figure 4. (A) Gating strategy for tumor cells (EpCAM, HLA-ABC), CD45⁺ PBMCs, non-T cell exclusion (CD14, CD16, CD19, CD33 and CD56), and CD4 vs CD8 T cells. **(B)** Overlapped CD4⁺ and CD8⁺ histograms. The increased gMFI of CD4⁺ and CD8⁺ with IMT group indicates higher activation than other groups. **(C)** CD4/CD8 plots showed clear positive and negative separation across groups, despite reduced MFI compared to cells without dissociation (data not shown).

Additional markers (CD25, CD44, CD69, PD-1, TIM-3) were also reliably detected (reduced MFI) with optimized method cross experiments. CD44 was observed highly enzyme-sensitive, while others were moderately affected. These markers also support accurate total T cell readouts in anti-CD3-activated TBMNK panels.



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Results: Established Tumor Models

We established over 125 ex vivo TumorGraft3D models across multiple tumor types for PBMC-tumor co-culture assays, demonstrating robust viability and well-defined tumor readouts. Tumor dump markers (EpCAM, EGFR and E-Cadherin), HLA-ABC or CD44 are used to ensure accurate tumor cell detection for evaluation of tumor cell killing.

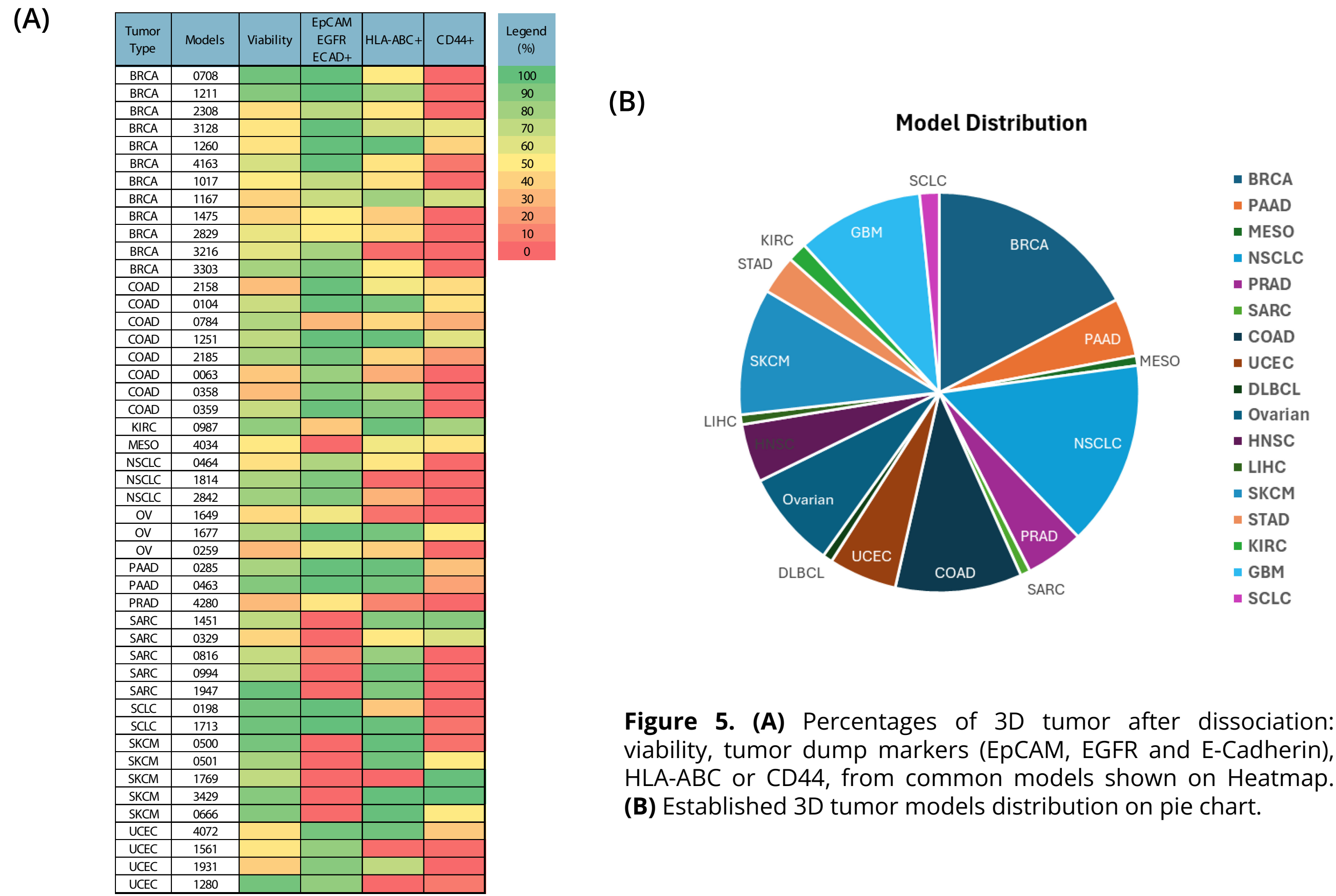


Figure 5. (A) Percentages of 3D tumor after dissociation: viability, tumor dump markers (EpCAM, EGFR and E-Cadherin), HLA-ABC or CD44, from common models shown on Heatmap. **(B)** Established 3D tumor models distribution on pie chart.

Results: Cytokine Quantification via Luminex Platform

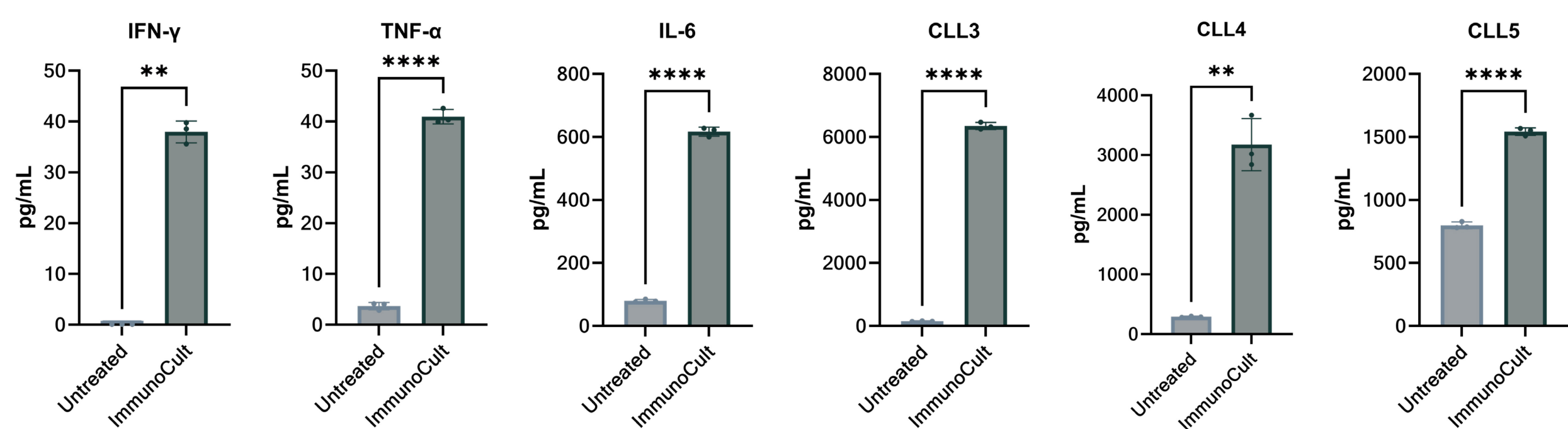


Figure 6. Cytokine analysis (IFN-γ, TNF-α, IL-6, CCL3, CCL4, CCL5) of Day 4 PBMC-3D tumor co-culture supernatants. ImmunoCult treatment induced T cell activation and tumor-associated effector activity, shown by increased IFN-γ, TNF-α, and chemokines (CCL3, CCL4, CCL5). (GraphPad Prism unpaired t-test).

Summary

This study establishes optimized culture conditions, Tumor dissociation, and staining protocols for accurate flow cytometry analysis. Using anti-CD3/CD28 stimulation and refined methods, we achieved reliable T cell activation and quantification of tumor killing, with upregulation of activation markers (CD69, CD25) and distinct CD4⁺ and CD8⁺ recruitment in the 3D system. This platform enables precise measurement of T cell activation, proliferation, cytokine release, and tumor cell death, and serves as a standardized benchmark for preclinical immuno-oncology and T cell engager screening. Baseline phenotyping will be expanded to additional PBMC donors, and combined with Day 4 readouts, will further strengthen evaluation of test agents in the PBMC-3D tumor co-culture platform. Additionally, the multiplex platform allows flexible customization of selective cytokine analysis panels.