Novel workflow for characterizing T-cell functional heterogeneity in response to checkpoint inhibitors

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INTRODUCTION

Since the approval of the first checkpoint inhibitor, ipilimumab (α-CTLA4), there continues to be enthusiasm for leveraging the immune system to eradicate solid tumors. However, unknowns about the cellular mechanisms that cause some patients to durably respond, while others relapse, continue to stymie the field. Efforts to drive field consensus around the cell subset(s) responsible for the MoA of immunotherapies have been bottlenecked by limitations of current technologies. Single-cell RNA-seq has enabled discoveries of transcriptionally defined cell subsets, but understanding their function requires inference from transcription. On the other hand, bulk readouts with plate-based assays are biased by pre-selection of subsets with specific markers, rely on surrogate markers for cell-extrinsic functions (e.g., cytotoxicity), and can dilute the cytotoxic impact of key subsets. (**Figure 1**).

To address these limitations, we leveraged a novel technology that converges live, cellular functions with transcriptomics at the resolution of single cells or single-cell interactions. This technology captures tens of thousands of individual cells or defined combinations of groups of cells in bio-compatible hydrogel compartments, CellCage™ enclosures (CCEs), enabling longitudinal imaging over multiple days. Throughout this period, cells are characterized via bright-field or immunofluorescence, enabling assessment of cytotoxicity, changes in cell morphology, surface receptor expression, and cytokine secretion. Cells are then lysed within compartments to generate barcoded libraries for mRNA sequencing to generate matched datasets across imaging and transcriptomics. Here, we leveraged this technology to develop a novel assay to enable longitudinal, unbiased functional characterization of individual T cells mapped to transcriptomics.

CHALLENGES DEFINING AND CHARACTERIZING T-CELL SUBSETS RESPONSIVE TO IMMUNOTHERAPIES

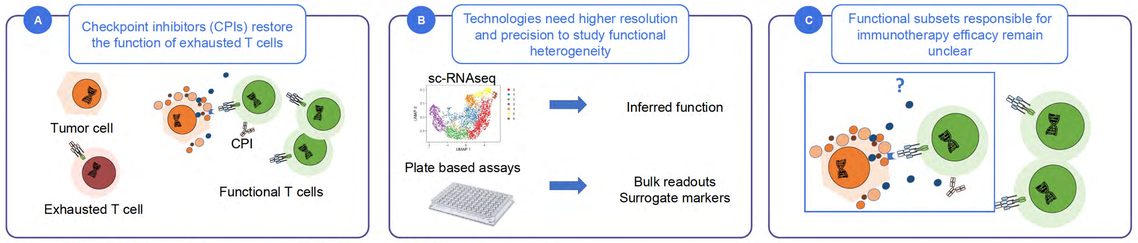

Figure 1: T-cell subsets driving efficacy of checkpoint inhibitors remain unclear
(A) Checkpoint inhibitors improve the capability of exhausted T-cells to kill tumor cells. **(B)** Current technologies are limited in their ability to assess functional heterogeneity. Single-cell sequencing requires function to be inferenced from transcriptional data, and plate-based assays potentially obscure functional subsets due to bulk readouts and / or utilization of imprecise surrogate markers. **(C)** Our goal is to characterize the functional heterogeneity amongst CD8⁺ T cells and identify potential subsets responsible for driving tumor cell killing in response to immunotherapy treatment.

CELLANOME TECHNOLOGY

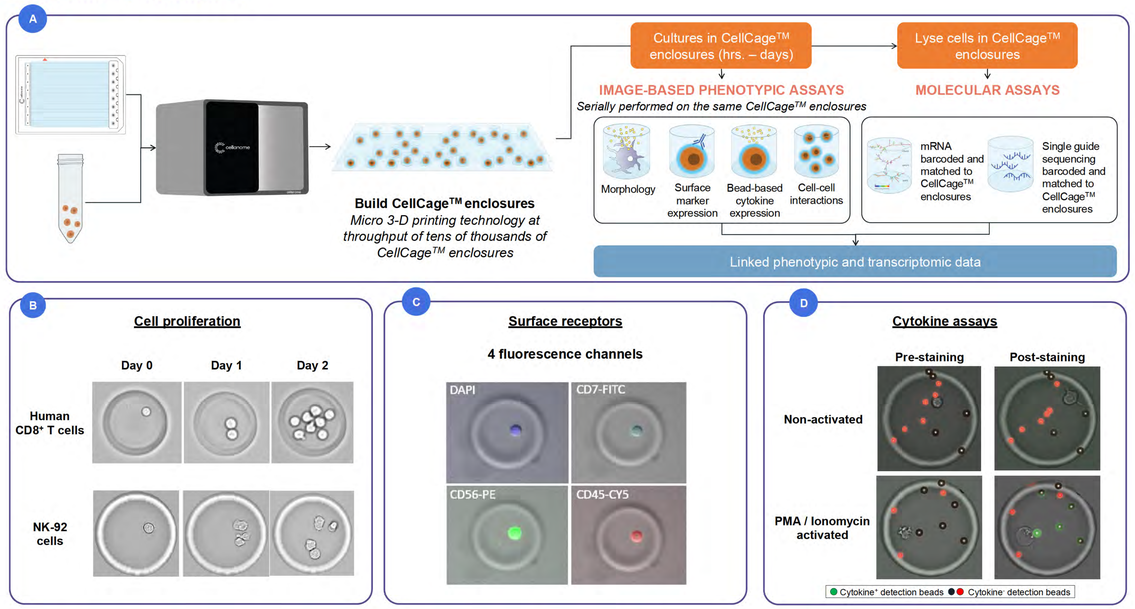

Figure 2: Cellanome's novel Micro-3D printing technology enables multiple phenotypic and functional assays to be performed on the same cells in CellCage™ enclosures at high throughput
(A) Schematic of workflow for novel Cellanome technology enabling the measurement of multiple phenotypic and functional assays from the same cells in CellCage™ enclosures. Tens of thousands of suspension or adherent cells are mixed with hydrogel precursor and loaded on an 8-lane flow cell. Positions of cells are identified and CellCage™ enclosures are generated around cells with light-guided polymerization in an automated fashion. Bio-compatible CellCage™ enclosures can be formed around single cells, multiple cells, or cells with objects (e.g., cytokine beads). CellCage™ enclosures are permeable to reagents enabling long-term culturing and a variety of imaging-based, longitudinal phenotypic and functional assays to be performed on the same cells (e.g., small molecules, immunofluorescent antibodies). Cells can be lysed within CellCage™ enclosures to generate positionally barcoded cDNA for downstream library prep and sequencing off the instrument. **(B)** Brightfield imaging of activated human T cells and NK-92 cells on days 0, 1, and 2 of culture in CellCage™ enclosures. **(C)** Fluorescent imaging of NK92 cells in CellCage™ enclosures following staining with α-CD7-FITC, α-CD56-PE, α-CD45-CY5 and DAPI. The same individual cell was imaged across 4 channels with Cellanome's technology. **(D)** Activated NK-92 cells (PMA/Ionomycin) and cytokine detection beads. Beads were co-encapsulated in CellCage™ enclosures and stained with IFNγ antibodies prior to imaging.

ESTABLISHING POC FOR MULTIPLE FUNCTIONAL ASSAYS ON SAME CELLS ENCAPSULATED IN CELLCAGE™ ENCLOSURES

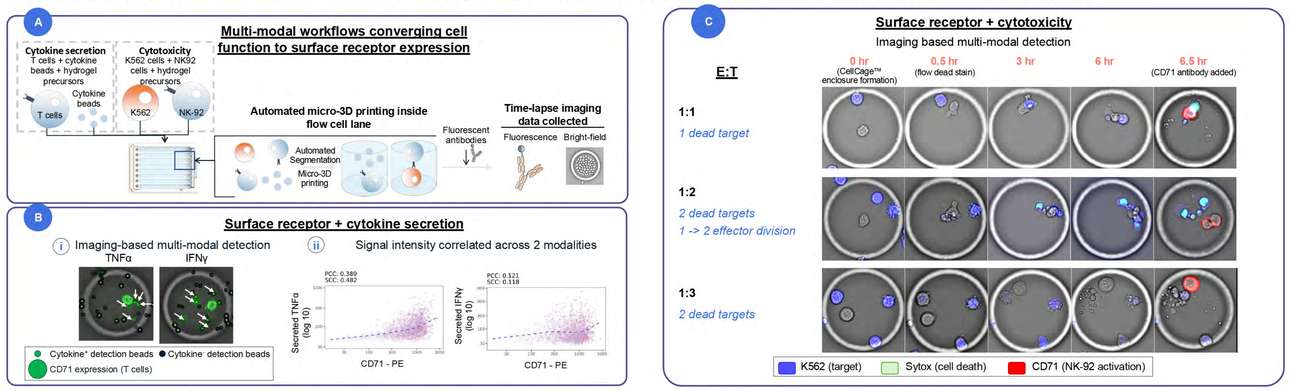

Figure 3: Converging multi-modal measurements to single cells or single cell-cell interactions with Cellanome's technology
(A) Schematic of workflow for multi-modal assays on Cellanome Technology. POC was established for assays converging cytokine or cytotoxic capabilities to surface markers. For cytokine secretion assays, PBMCs were activated with α-CD3 / α-CD28 in flask, mixed with cytokine detection beads and hydrogel precursor, and loaded onto Cellanome flow cell. For cytotoxicity assays, NK-92 Effector cells and K562 Target cells were mixed with hydrogel precursors and loaded onto flow cells. For both these assays, cells were detected and CellCage™ enclosures formed around cells alone, or with proximal cytokine beads. Media containing reagents that label cells or beads, including α-CD71 (lymphocyte activation), α-IFNγ / α-TNFα (cytokine beads), and / or Sytox (cell death for cytotoxicity assays), were perfused onto the flow cell. Subsequently, time-lapse images of cells within CellCage™ enclosures were collected for multi-modal evaluation. **(B)** Converging cytokine secretion to activation markers on individual cells. **i)** Representative images of brightfield and fluorescence imaging data generated to collect multi-modal data (secreted cytokines, surface receptors). **ii)** Signal intensity of cytokine beads and surface receptor expression can be quantified and linked to perform pairwise analysis across modalities, with each individual dot representing an individual CellCage™ enclosure. **(C)** Demonstrating longitudinal assessment of cell killing capabilities converged to activation markers by individual cells. Representative longitudinal images of individual Effector cells (NK-92) co-encapsulated with one more Target cells (K562). Representative images of brightfield and fluorescence imaging data to monitor cell activation (CD71 expression on NK-92), viability (both NK-92 and K562), and proliferation (both NK-92 and K562). This data is coupled with the evaluation of K562 cell death for direct evaluation of killing by individual NK-92 cells in a manner that enables evaluation of kinetics, validation of NK-92 viability, and NK-92 proliferation to ensure increased killing in a single CellCage™ enclosure is due to killing, as opposed to increased numbers of NK-92 cells from proliferation.

CONFIGURING CELLANOME WORKFLOW FOR UNBIASED CHARACTERIZATION OF MOUSE PRIMARY LYMPHOCYTES

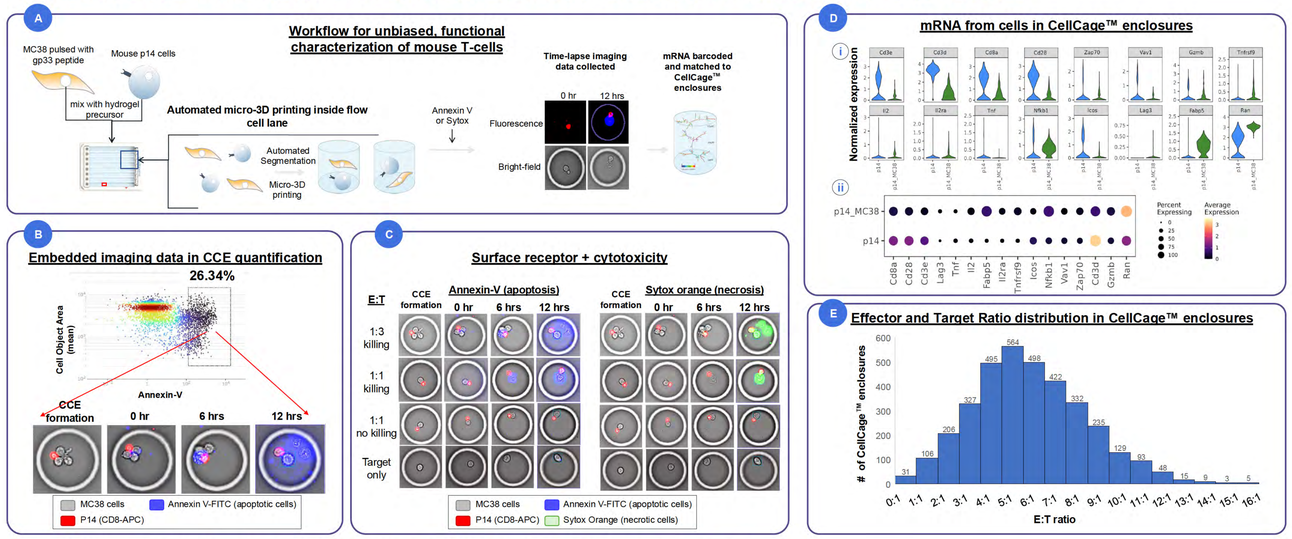

Figure 4: Preliminary data characterizing heterogeneity in cytotoxic capabilities by mouse primary T-cells
(A) Schematic showing how workflow can be adapted for primary mouse T cells and adherent target cells. MC38 cells were treated with IFNγ (10ng / ml) and gp33-41 peptide for 24 hours. α-CD3/α-CD28 activated P14 CD8⁺ T cells and MC38 cells were encapsulated on the Cellanome flow cell and media containing Annexin-V-FITC and Sytox Orange were diffused through permeable CellCage™ enclosures. After imaging data was collected, cells were processed for sequencing off-instrument as described in Figure 2a **(B)** Quantification of CellCage™ enclosures containing positive Annexin-V signal. Each dot represents the average signal and size of cells within an individual CellCage™ enclosure and are embedded with longitudinal imaging data. Representative images illustrate CellCage™ enclosures with 3 Targets and 1 viable Effector. **(C)** Representative CellCage™ enclosures with P14 cells across E:T ratios, killing kinetics, and potentially, cell-intrinsic capability to kill target cells. **(D)** POC that cells in individual CellCage™ enclosures can be barcoded to map mRNA data to imaging-based functional analysis. Violin plot **i)** and Dot plot **ii)** of T-cell activation genes from P14 and P14:MC38 cocultures. **(E)** Representative distribution of E:T ratios detected within CellCage™ enclosures (~3-4K). This representative plot showcases co-encapsulation of a single Target with multiple Effectors 3 hours after initial CellCage™ enclosure formation. Workflow can be configured to encapsulate a single Effector with multiple Targets.

DISCUSSIONS AND FUTURE DIRECTIONS

For this study, we leveraged Cellanome's novel technology to establish a cytotoxicity workflow that evaluates dimensions of cell killing inaccessible with other technologies at high throughput. Longitudinal evaluation of cell killing at the resolution of single CD8⁺ T cells will enable novel insights to be gleaned around killing kinetics, the number of cells that can be killed by single effector cells, and the durability of CD8⁺ T cell killing. Our next steps are to leverage this workflow to functionally characterize single tumor-infiltrating T cells from tumor-bearing mice treated with checkpoint inhibitors. By combining imaging-based characterization with transcriptomics, we aim to discover novel functional subsets and generate hypotheses around mechanisms governing the anti-tumor efficacy of checkpoint inhibitors. These workflows can enable the discovery of novel targets that could amplify the efficacy, safety, and durability of new immunotherapies.

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