Cell killing dynamics and heterogeneity in engineered T-cells measured in live CellCage enclosures

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QUESTIONS?

ABSTRACT

Predicting CAR-T clinical efficacy remains a central challenge to the cell therapy field. Existing potency assays generally rely on bulk endpoints and may fail to capture cellular heterogeneity and dynamic interactions governing in vivo efficacy. We developed a technology to reveal CAR-T functional heterogeneity at single-cell resolution. Using CellCage Enclosures™, we encapsulated thousands of CAR-T cells at single-cell density with NALM-6, HeLa, and A549 targets across 1:10 and 25:1 E:T ratios in one experiment, enabling parallel cytotoxicity assessment under diverse conditions. Studies with AIC100, a preclinical ICAM1 CAR-T product, revealed striking heterogeneity: individual CAR-T cells exhibited killing at 55% compared to 80% with multiple cells working together. Optimal activity required both CD4+ and CD8+ CAR-T cells within the same microenvironment, suggesting critical cooperative mechanisms, which were product-specific, and impactful to clinical efficacy. A second CD19-targeting CAR-T product against NALM6 targets showed individual cells could kill effectively (50-60%), though cytotoxicity still improved to more than 80% with multiple effectors—but at different E-T ratios. Interestingly, CD8+ CAR-T killing was enhanced ~30% by non-transduced CD4+ T-cells lacking the CAR construct entirely, demonstrating that therapeutic potency can be modulated by cell-extrinsic factors independent of antigen recognition or cytokine secretion. This approach profiles thousands of cellular interactions per experiment, revealing that CAR-T activity emerges from complex cell-intrinsic properties and microenvironment factors unpredictable from bulk measurements. Each product exhibits unique cellular dynamics requiring individual optimization. The CellCage platform enables identification of potent CAR-T subpopulations and quantification of functional heterogeneity, providing a path toward predictive potency assays and rational optimization of cellular therapeutics.

CELLANOME TECHNOLOGY

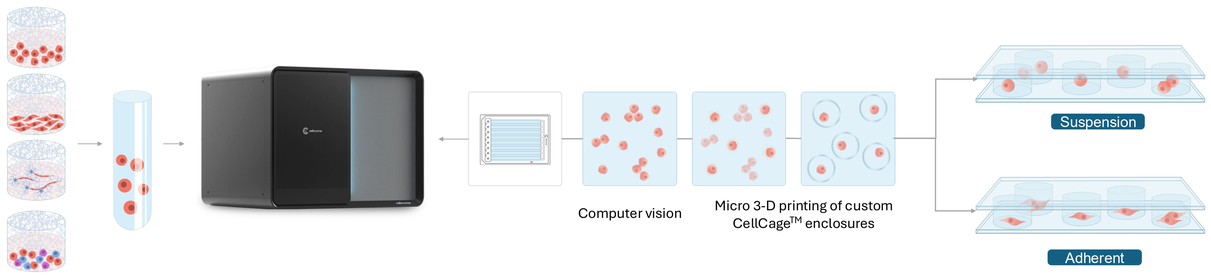
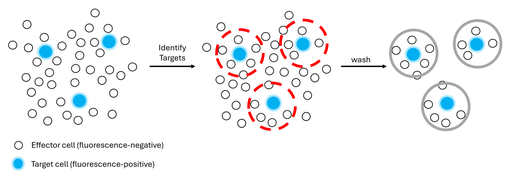


Fig 1A. Schematic of workflow for novel Cellanome technology enabling the measurement of multiple phenotypic and functional assays from the same cells in CellCage™ enclosures. Cells in suspension are mixed with hydrogel precursor and loaded on an 8-lane flow cell. Positions of cells are identified and CellCage™ enclosures are generated around cells with light-guided polymerization in an automated fashion. CellCage™ enclosures are permeable to reagents enabling long-term culturing and a variety of imaging-based, longitudinal assays to be performed on the same cells (e.g., small molecules, immunofluorescent antibodies). Cells can be lysed within CellCage™ enclosures to generate barcoded cDNA for downstream library prep and sequencing off the instrument.



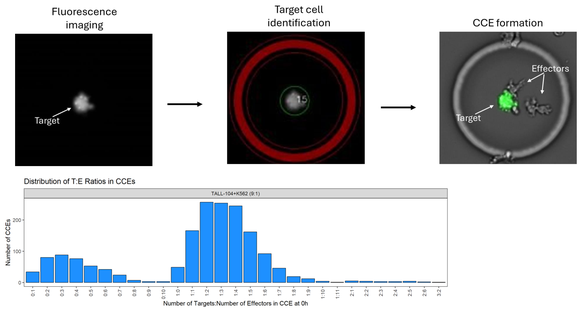
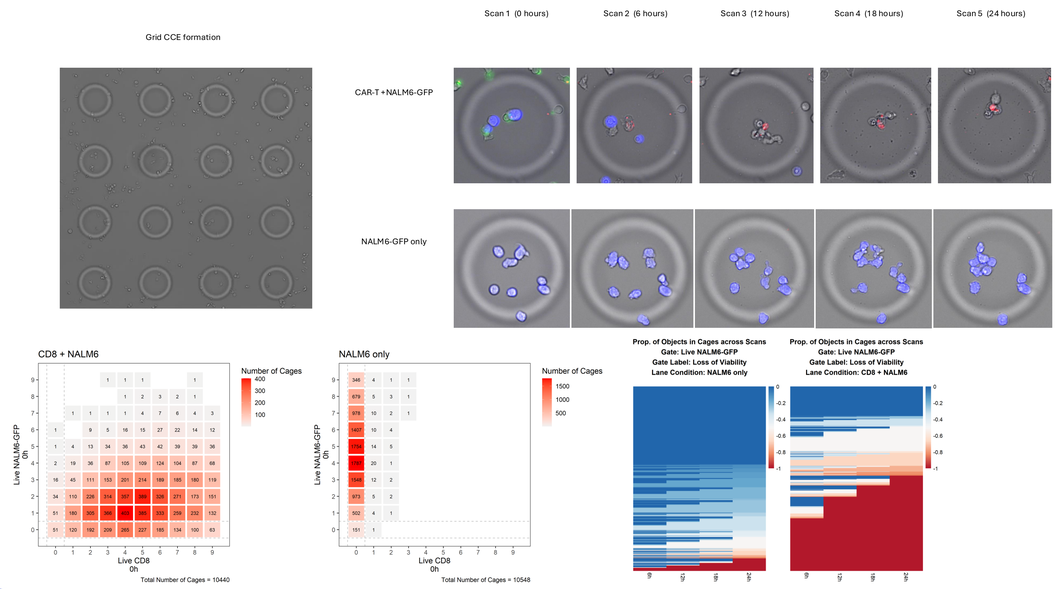
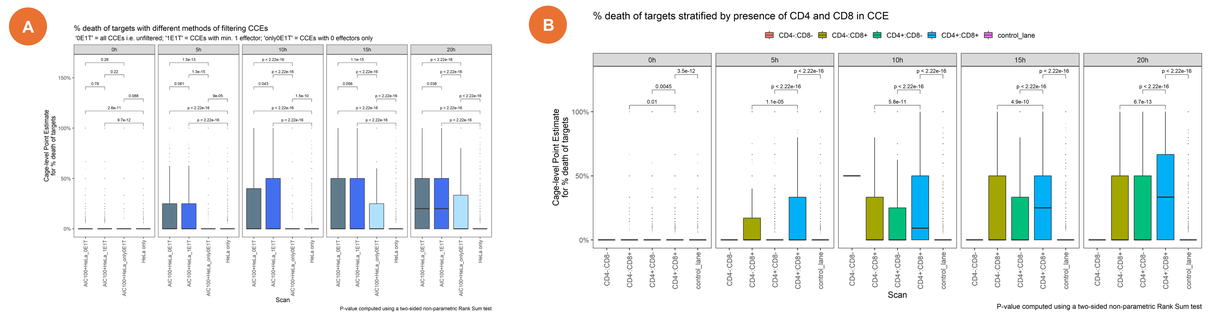


Figure 1B. Schematic of multicell caging process. Using fluorescent markers to generate a range of effector to target ratios and enable a range of killing dynamics to be observed in a single experiment.

A NEW APPROACH TO THE ANALYSIS OF CELL KILLING DATA



DETERMINATION OF CONDITIONS THAT MEDIATE EFFECTIVE CELL KILLING



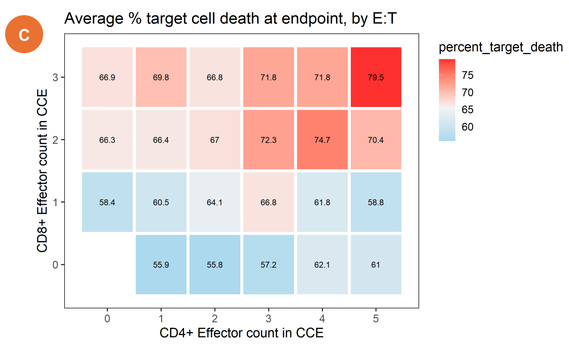


Fig 4. Correlation of CCEs with high and low cell killing with AIC100 cell type and number elucidates conditions needed for effective killing. (A) The distribution of target cytotoxicity among CCEs with different filtering conditions within the same lane. These distributions show that the presence of effectors in CCEs leads to a statistically significant increase in target cytotoxicity, (B) The distribution of target cytotoxicity is dependent on the composition of the effectors encapsulated in the same CCE. In later timepoints, we observe a statistically significant difference in target cytotoxicity among CCEs in the presence of CD4+ and/or CD8+ AIC100 effectors. CCEs that contain both CD8+ and CD4+ cells are associated with the highest average target cytotoxicity, compared to CCEs with only CD4+ or only CD8+ AIC100 cells. This suggests that CD8+ and CD4+ T-cells can effect greater cytotoxicity when encapsulated together in close proximity.

C) Average target cytotoxicity varies by the specific numbers of different effector cell profiles, defined by surface positivity for CD4 and CD8. CCEs have increasing target cytotoxicity with increasing numbers of both CD4+ effectors and CD8+ effectors, independently and together. This dependence is stronger with CD8+ effector count in the CCE, and aligns with our observation in (B).

PROFILING THE KILLING ACTIVITY OF CLINICAL GRADE CAR-T CELLS PRODUCTS

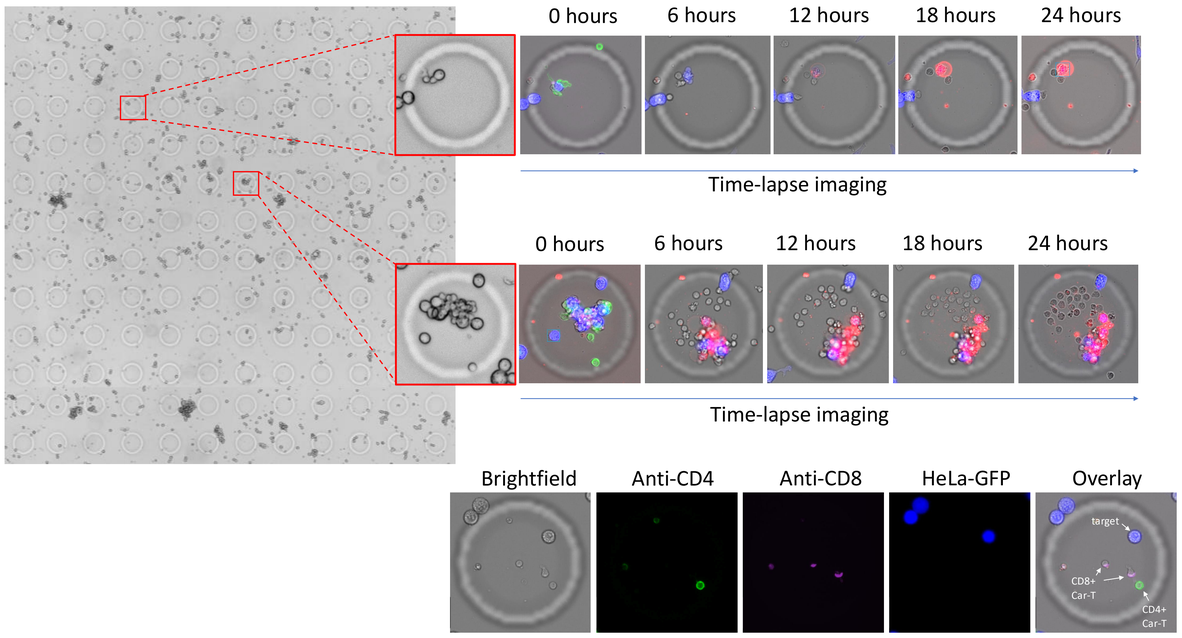


Fig 2. Identification of T-cell types and longitudinal monitoring cell killing activity using AIC100 CAR T-cells. AIC100 cells, labeled with anti-CD4 (green) and anti-CD8 (violet) antibodies were co-encapsulated in CellCage™ enclosures with HeLa-GFP target cells (blue). Cell killing, as indicated by Annexin V staining (red), was monitored for each CellCage™ enclosures via time-lapse imaging over 24 hours.

DISCUSSION AND FUTURE DIRECTIONS

In this study we established an experimental workflow that can be leveraged by cell therapy developers / researchers to deeply understand the products and engineering strategies they are developing. By creating high-resolution, multi-modal data that can maximize information yielded from product or patient samples, researchers and developers can conserve precious samples and extend discovery efforts. Cellanome's technology provides data on cell behavior with multi-modal measurements across surface receptors, cytokine secretions, and cytotoxic capabilities from single cells and multi-cell interactions. Because they are generated from the same cell, this platform yields matched datasets, removing the need to infer relationships across molecular properties from cells collected across different samples, experiments, and technologies. The observation that a combination of CD4+ and CD8+ AIC100 CAR T-cells increased cytotoxicity suggests that individual CAR T effectors are not sufficient for optimal activity. Effectors and targets were analyzed based on stochastic distribution, enabling the survey of a wide range of E:T ratios within a single lane. A greater proportion of HeLa-GFP cell death was observed in the lane with AIC100 CAR T effectors compared to the HeLa-GFP only negative control lane. CCEs that encapsulated both CD8+ and CD4+ effectors had the highest proportion and rate of target cell death. Further analysis of these CCEs showed that the increasing the number of CD8+ effectors alone increased target cell death while increasing CD4+ effectors alone did not, suggesting that CD8+ CAR T-cells played a more direct role in target cell cytotoxicity. Interestingly, increasing the number of both CD8+ and CD4+ led to the greatest amount of target cell death, suggesting that the coordinate activities of both T-cell types were required for efficient cell killing. Being able to make these observations underscores the capabilities of the Cellanome platform in not only executing cytotoxicity assays but also enabling the examination of conditions required for effective cell killing. These results underscore the value to the CAR-T field of being continuously vigilant in comprehensively characterizing CAR-T products both pre- and post-patient infusion across modalities, coupled with patient response data. Our next steps are to leverage Cellanome's platform to comprehensively profile AIC100 cells from patient blood, evaluate what cellular and molecular predictors correlate with better or worse patient responses, and compare them to the properties of functional subsets identified in vitro.