

Use of a 50-Marker Mass Cytometry Intracellular Cytokine Staining Panel Reveals Unforeseen Functional Diversity Within Cell Therapy Products and Unmasks Potential Efficacy ‘Saboteurs’

Lauren Tracey¹, Michael J. Cohen¹, Laura Polanco², Erika L. Smith-Mahoney², David King¹, Sarah Adams³, Wilson Wong³, Julian Amirault⁴, Gustavo Mostoslavsky⁴, Amedeo J. Cappione III², Christina Loh¹, Anna C. Belkina^{5*} and Jennifer E. Snyder-Cappione¹

¹Standard BioTools Canada, Markham ON, Departments of ²Virology, Immunology and Microbiology, ³College of Engineering, ⁴Center for Regenerative Medicine, and ⁵Pathology and Laboratory Medicine, and ⁶Flow Cytometry Core Facility, Boston University, Boston, MA, USA

Abstract

Cell-based therapies have proven to be highly effective against certain cancers, yet improvements are needed to extend these successes to a wider variety of malignancies. Single-cell functional assessment of cell therapy products is customarily limited to Th1 cytokines (such as IFN- γ) and the cell survival/proliferative mediator IL-2, overlooking the cellular heterogeneity of CAR T products. This heterogeneity includes diverse antigen-experienced $\alpha\beta$ T cells previously differentiated into distinct lineages (for example, Type 1, 2, 17, Treg) and innate T cell populations (iNKT, MAIT, $\gamma\delta$ T), with many of the latter pre-programmed to exert immunosuppressive and/or cell repair effector functions. This diversity introduces potential “saboteur” cells with off-target effects that may undermine therapeutic efficacy. Surface marker profiling is insufficient for functional characterization, yet traditional flow cytometry lacks resolution for certain intracellular targets, particularly immunosuppressive cytokines. Mass cytometry (CyTOF™ technology) overcomes this technical limitation, providing superior intracellular signal resolution in the context of large panel analyses, a prerequisite of deep functional assessment of single cells. We hypothesized that there are sizeable proportions of saboteur T cells in cell therapy products that exert functions after stimulation that act in direct opposition to the therapeutic intention of the drug. To assess this, we developed a 50-marker CyTOF intracellular cytokine staining (ICS) panel with 24 functional readouts. This enabled clear detection of individual human T cells producing immunosuppressive cytokines such as IL-10, IL-13 and TGF- β , and measured the composite cytokine responses of both T cells in peripheral blood mononuclear cells (PBMC) and cell therapy products. Our data revealed an unforeseen prevalence of T cells that cross dogmatic lineage paradigms when stimulated. For example, after mitogen stimulation, approximately 30% of T cells from both peripheral blood and a cell therapy product produced the TGF- β -inducing cytokine amphiregulin (AREG), often concurrently with IFN- γ within the same cells. In addition, our findings with a Her2-CAR T product show previously unmeasured cytokines are produced in response to specific CAR antigen binding. From these results, we anticipate application of CyTOF ICS technology to cell therapy testing and modification will transform our understanding of these therapies and ultimately enhance their efficacy.

Methods

Cell therapy product generation

iPSC-derived CD4+ (iCD4+) T cells: Induced pluripotent stem cells (iPSCs) were differentiated to CD4+/CD8+ double positive T cell progenitors through directed differentiation over 40 days of culture. Stimulation of the TCR in a Notch-ligand free context allowed differentiation to the CD4 single positive lineage. iCD4+ T cells were then expanded through anti-CD3/CD28 stimulation in the presence of IL-2.

Her2 CAR T cells: Human primary T cells (CD3+) were purified using a negative selection kit from leukocyte reduction cones of a healthy donor. Her2 CARs were introduced via lentiviral transduction. Expression of the CAR was quantified using V5-tag immunostaining and quantified by flow cytometry. Engineered T cells were at sub-cultured for 8 days prior to the overnight culture described below (no Her2 antigen exposure) to assess functional activity upon CAR T antigen encounter. For CAR T stimulated samples, Her2 antigen coated wells were prepared prior to CAR T cell addition.

Cell culture conditions, mass cytometry sample preparation, and data analysis

iCD4+ T cells and **Her2 CAR T cells** were cultured overnight with either media alone or PMA + Ionomycin/Her2 CAR T antigen, respectively, in the presence of brefeldin A and monensin (added 1-2 hours after stimulation at 5 μ g/mL final concentration each). At the end of the culture period, cells were washed, counted and stained with surface and cytoplasmic antibodies according to the Maxpar™ Cell Staining with Fresh Fix User Guide (the 50-marker panel markers are listed in **Figure 1**). **iCD4 cells:** note that these cells were stained with a reduced panel excluding non-T cell lineage markers, V γ 9, CD107a, and IL-3. Samples were acquired on either a CyTOF Helios™ of XT system and normalized with EQ™ Six Element Calibration Beads.

Data analysis was conducted using OMIQ to perform expert gating of the datasets for intracellular cytokine expression. OMIQ cloud analysis platform was utilized for computational studies. Multidimensional data from live singlet events were projected into opt-SNE space and clustered using PhenoGraph. Signal intensity was visualized as dotplot heatmap overlays on opt-SNE projections. PhenoGraph cluster median intensities were hierarchically clustered using R. Collected data was visualized into 2D space using opt-SNE and clustered via PhenoGraph from expression of both surface and intracellular markers.

Funding:

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Reference:

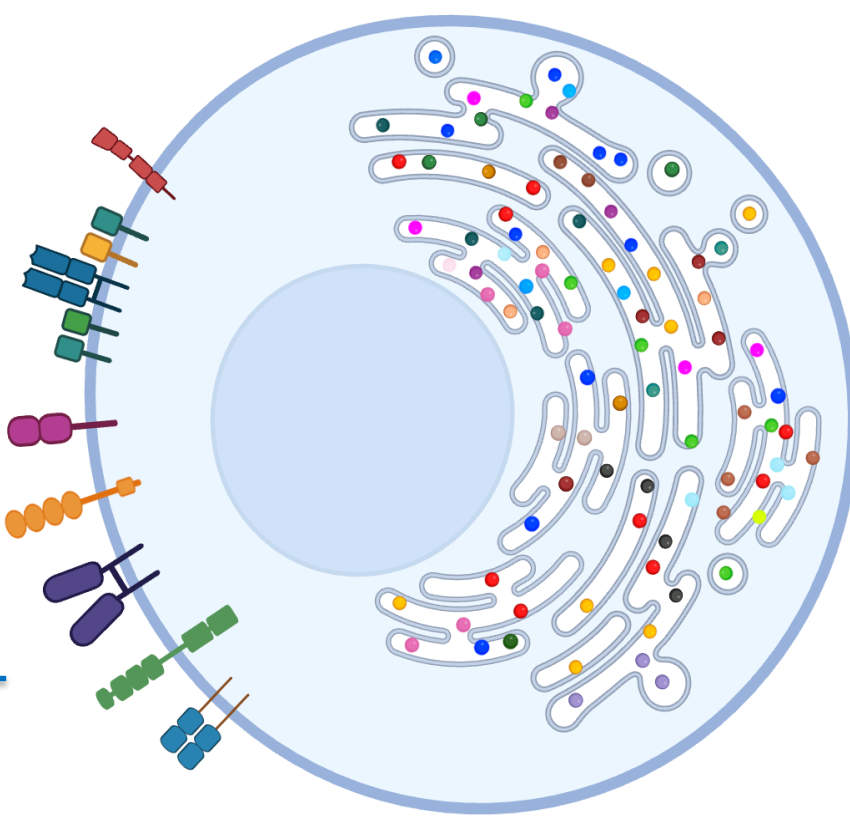
iPSC study



Our 50-parameter panel

26 Surface Antigens

Lineage
CD3 CD161
CD4 V α 7.2
CD8 V α 24
CD127 V β 11
CD25 V δ 1
CD16 V δ 2
CCR6 V γ 9
CD14
IgD
CD19
Differentiation/activation/exhaustion
CD38 CD27
CD57 CD28
CD56 PD-1
CD45RO TIGIT
CD45RA

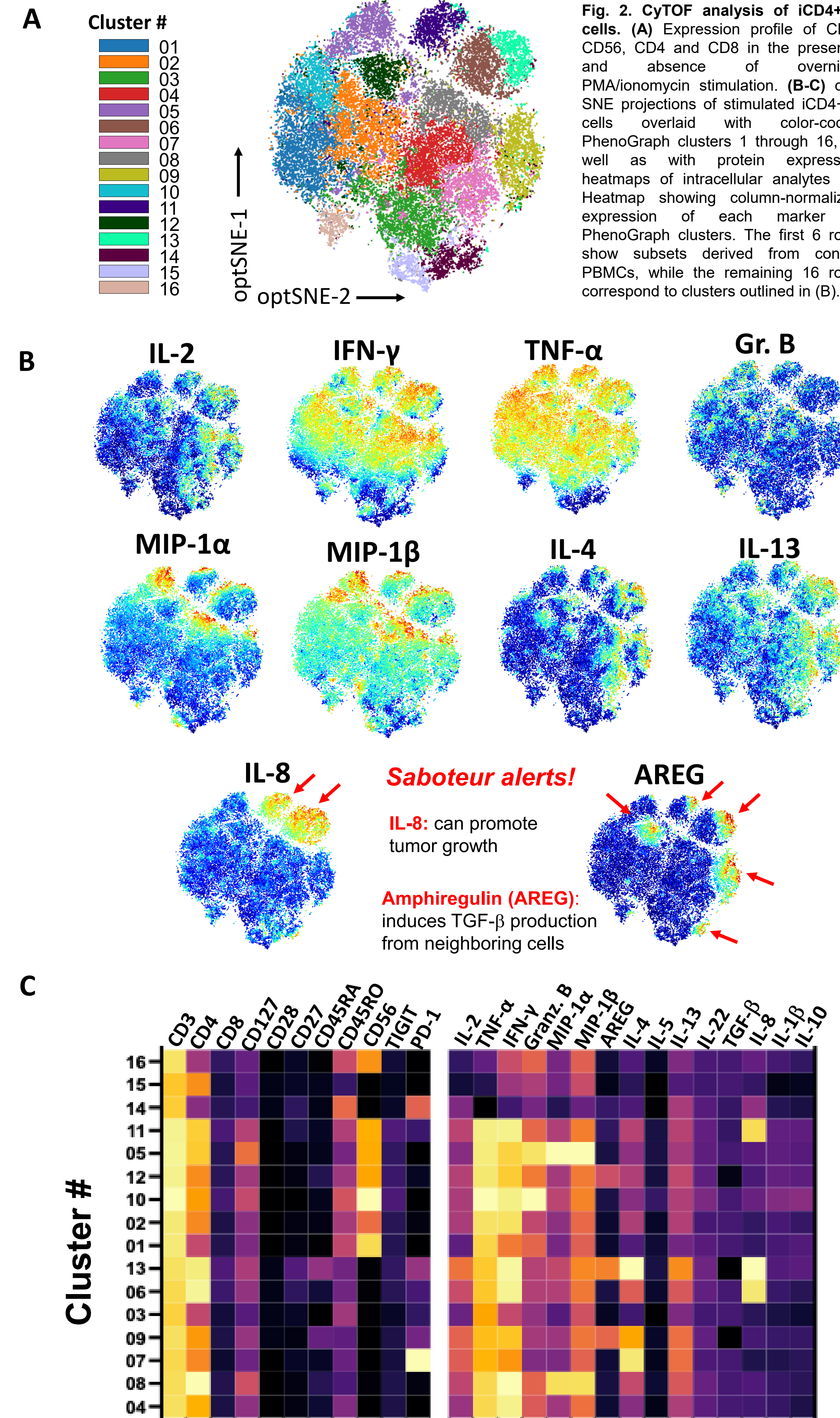


24 Intracellular (Functional) Readouts

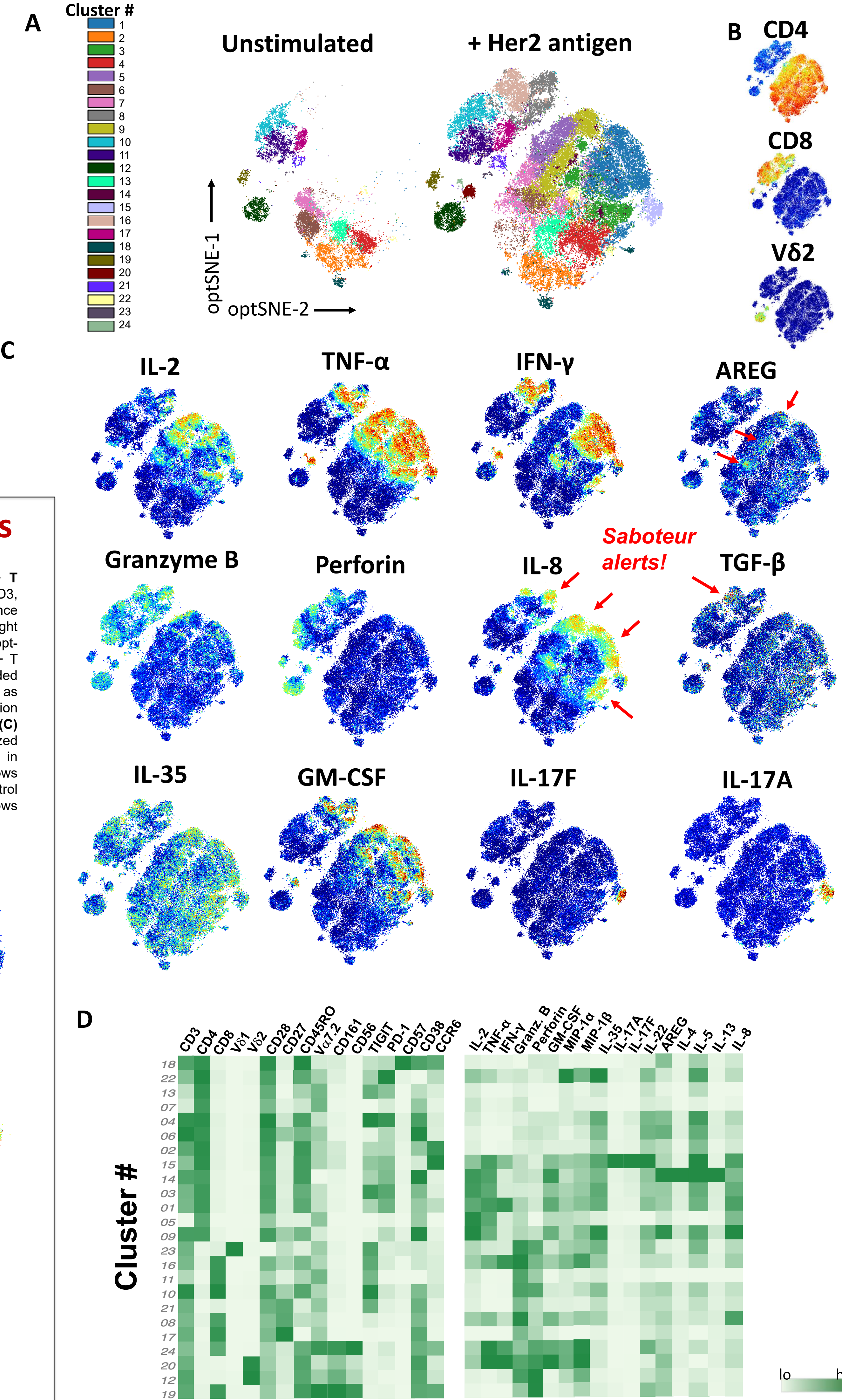
Type1
IFN- γ
TNF- α
MIP1- α
MIP1- β
Type2
IL-4
IL-5
IL-13
Treg/cell repair
TGF- β
IL-10
Amphiregulin
IL-8
Cytotoxicity
CD107A
Granzyme B
Perforin
Type9/Type17
IL-9
IL-17A
IL-21
IL-22
IL-35
IL-17F
Other
IL-2
GM-CSF
IL-3
IL-1 β

Figure 1. Optimization of a 50-marker CyTOF panel for deep intracellular functional profiling. (Left) 26 surface targets were included for cell surface marker characterization. (Right) 24 intracellular functional analytes were selected to profile the breadth of immune functional diversity. Surface staining permits fractionation of cells into distinct compartments based on historical CD markers and markers of lineage segmentation (e.g., differentiation, maturation and activation status). When measured in combination with functional readouts with single-cell resolution, human cell therapy product proteomic ‘fingerprinting’ is now achievable.

iCD4+ T product: not just ‘Th1’, many AREG+ cells



Her2 CAR T product: includes IL-8+ (pro-tumor?) T cells



Key conclusions

- This is the first look, to our knowledge, of the **cross-functional single-cell proteomic heterogeneity of T cells** within a cell therapy product in response to CAR engagement.
- Vast functional diversity is present within cell therapy products** (even within an iPSC-derived preparation!), with substantial proportions of the cells producing cytokines that likely act to sabotage the efficacy of the drug.
- Selective enhancement/purification of T cells within products with the precise functional profiles desired could dramatically improve CAR T efficacy.

Interested in applying this technology to your product? contact Jennifer Snyder-Cappione (cappione@bu.edu)