POSTER #

Deep characterization of *in vitro* chronically stimulated T cells via single-cell multiomic analysis.

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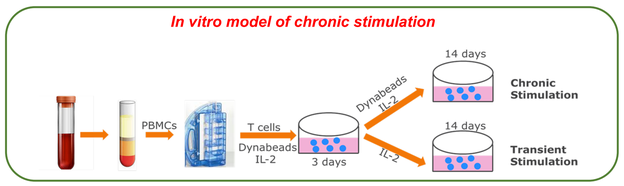
Introduction and Methods

A key step in the clinical production of CAR T cells is the expansion of engineered T cells. To generate enough cells for a therapeutic product, cells must be robustly stimulated, which raises the risk of inducing T-cell exhaustion and reducing therapeutic efficacy. As protocols for T-cell expansion are being developed to optimize CAR T cell yield, function and persistence, fundamental questions about the impact of *in vitro* manipulation on T-cell identity are important to answer. Namely: 1) what types of cells are generated during chronic stimulation? 2) how many unique cell states can be defined during chronic stimulation? We sought to answer these fundamental questions by performing single-cell multiomic analysis to simultaneously measure expression of 38 proteins and 399 genes in human T cells expanded *in vitro*. This approach allowed us to study – with unprecedented depth - how T cells change throughout chronic stimulation. Human PBMCs from three healthy donors were stimulated for 14 days in the presence of CD3/CD28 antibody-coated beads and recombinant human IL-2. This model system was developed to resemble culture conditions that may be used for CAR T cell expansion. Cells were collected at different time points (day 0, 3, 7 and 14) prior to downstream analysis.



AbSeq and Flow Cytometry Panels

CD4	FAS-L (CD178)	CD2	CD54
CD8	CXCR3 (CD183)	CD25	CD62L
PD-1 (CD279)	CXCR5 (CD185)	CD27	CD69
LAG-3 (CD223)	CCR4 (CD194)	CD28	CD7
TIM-3 (CD366)	CCR6 (CD196)	CD30	CD7
GITR (CD357)	CCR7 (CD197)	CD38	CD94
CD25	4-1BB (CD137)	CD39	CD95
CTLA-4 (CD152)	OX40 (CD134)	CD44	CD98
HVEM (CD270)	CD103	CD45RA	
ICOS (CD278)	CD154	CD49a	

38-Plex AbSeq Panel

Marker	Fluorochrome
CD4	BUV805
CD8	BUV395
CD279 (PD-1)	PE-Cy7
CD152 (CTLA-4)	PE
CD223 (LAG-3)	BV480
CD366 (TIM-3)	BV711
CD39	BUV737
CD103	APC
CD45RA	APC-H7
CD62L	FITC
CD95	BV786
CD357 (GITR)	BV421

12-color Flow Cytomety

1. The BD Rhapsody™ Single-Cell Analysis System Sequencing Platform

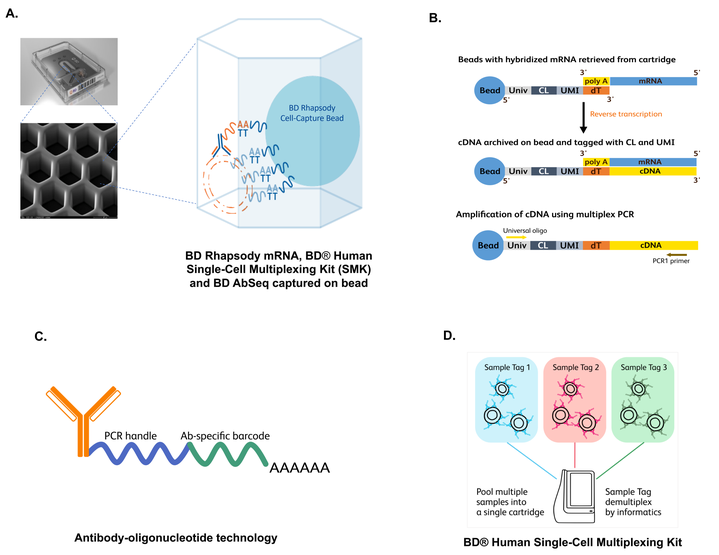


Figure 1. A) Microwell technology is used to partition single cells with BD Rhapsody™ Cell Capture Beads. Upon lysis of the cells, mRNA and oligos from BD SMK and BD AbSeq are captured through hybridization of poly (A) sequences on the oligonucleotides to poly (T) sequences on the beads B) cDNA synthesis is performed on the BD Rhapsody™ beads. Sequences are amplified by using random priming for whole transcriptome amplification (not shown), or highly multiplexed primer panels targeting 300-500 genes of interest. C) Conjugation of oligonucleotides to antibodies can enable readout of protein information from high-throughput sequencing data. D) This technology is used in the BD® Human Single-Cell Multiplexing Kit, in which a universal antibody is conjugated to 1 of 12 oligonucleotides.

2. Concordance Between AbSeq and flow Cytometry

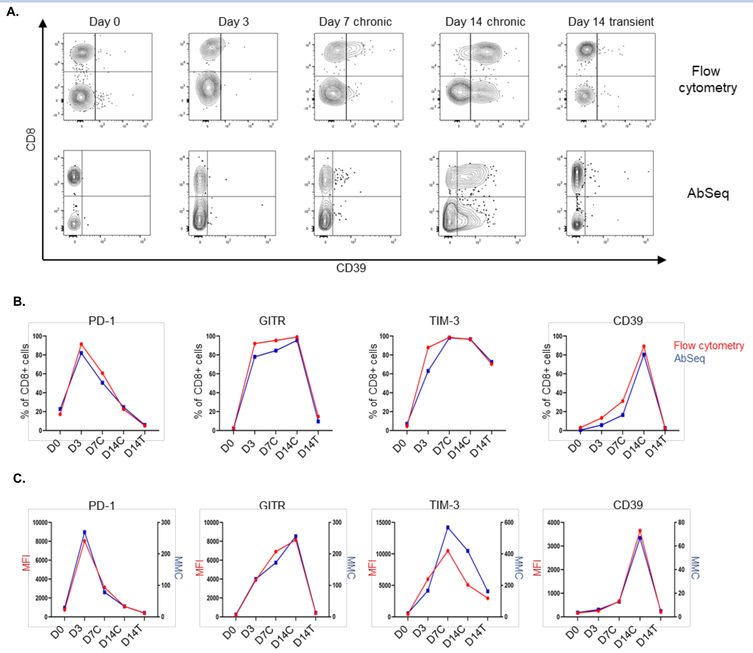


Figure 2. **A)** Representative analysis of CD39 kinetic expression within CD8⁺ and CD8⁻ T cells B) Equivalent quantitation of CD8+ subsets expressing several activation markers was observed at each time point. **C)** Changes in Median Fluorescence Intensity (MFI; left y axis) and Median Molecular Count (MMC; right y axis) measured via flow cytometry and AbSeq.

3. Deep profiling of resting CD8⁺ T cells

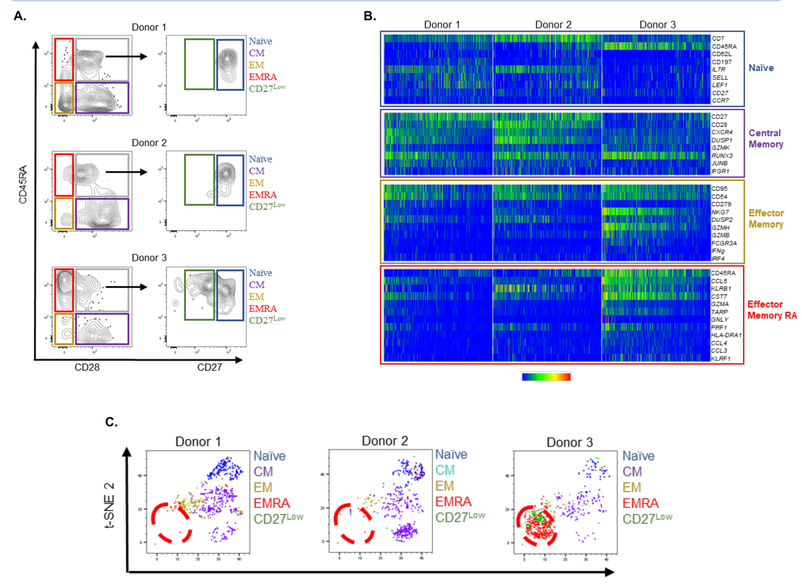


Figure 3. Refined characterization of resting CD8⁺ cells via simultaneous protein and gene expression analysis. A) Gating strategy used to identify CD8⁺ maturational subsets based on measurement of CD45RA, CD28 and CD27 expression via AbSeq. A unique population of CD45RA⁺CD28⁺CD27^{low} cells (CD27^{low}, green box) was detected in donor 3. (B) Single-cell heatmap displays the expression of selected proteins and genes (italic) differentially expressed in each T-cell subset across the three donors (fold change ≥2, q≤0.05). (C) t-SNE visualization of the CD8+ T-cell subsets showing different cell clusters with a naïve, CM, EM, EMRA and CD27^{low} phenotype across the three donors.

3. Identification of common activation signatures

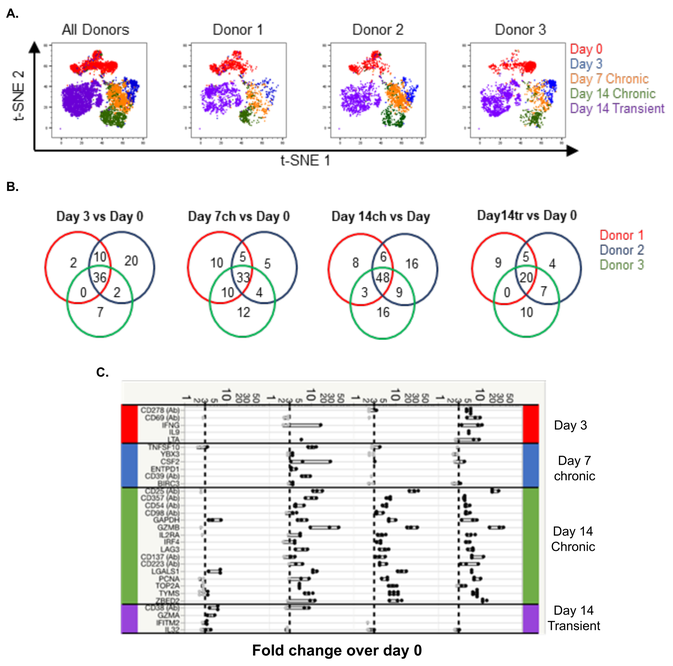


Figure 3. A) t-SNE visualization of CD8+ cells clusters at different time point and across 3 donors. (B) Venn diagrams indicate the number of shared or uniquely upregulated genes and proteins across the three donors at day 3, day 7, day 14 chronic, and day 14 transient stimulation, as compared to cells at day 0. (C) Identification of clusters of proteins and genes with unique expression patterns.

4. Relationship between inhibitory and proliferation markers

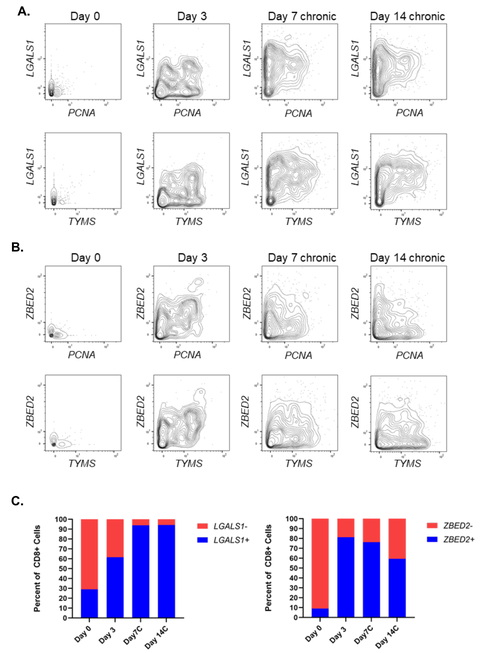


Figure 4. Bi-variate plots showing the relationship between the inhibitory markers *LGALS1* **(A)** or *ZBED2* **(B)** and the proliferation markers *TYMS* or *PCNA* throughout chronic stimulation. **(C)** Quantification of the frequency of cells expressing *LGALS1* and *ZBED2* throughout chronic stimulation. cells. Data were generated from concatenated CD8⁺ cells from 3 donors.

5. Correlation between gene and protein expression

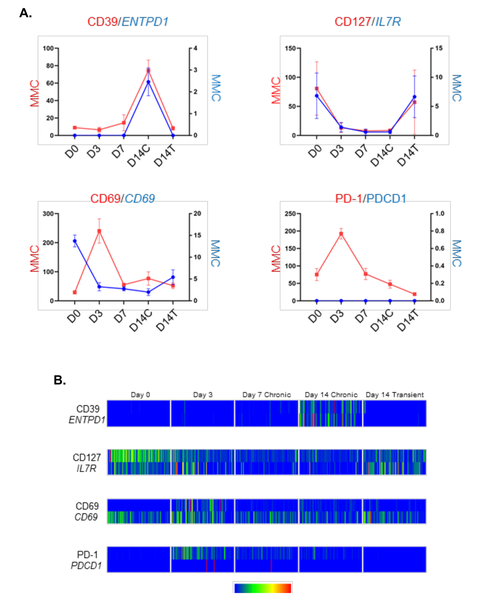


Figure 5. A) Kinetic analysis of mRNA and protein expression. mRNA and protein levels were measured as mean molecular count (MMC) and depicted by the red trace (mRNA) and blue trace (protein). The analysis was performed on three individual donors. Data are represented as mean ± standard deviation. **(B)** Single-cell heatmap enables visualization of the expression of genes and corresponding proteins in individual cells. A single cell is represented in each column.

Conclusions

- AbSeq enables protein analysis with sensitivity and specificity similar to flow cytometry, as demonstrated by overall equivalent quantitation of CD8⁺ T-cell subsets and measurement of expression levels for the majority of markers tested.
- A comprehensive and accurate characterization and classification of resting CD8+ T-cell subsets was achieved by virtue of simultaneously analyzing the expression of 38 surface markers and 399 targeted genes.
- In the context of *in vitro* chronic activation and expansion of T cells, this single-cell multiomic approach revealed distinct signatures shared by three different donors and characterized by unique expression patterns linked to the mode and duration of activation.
- Markers associated with T-cell inhibition/exhaustion of activation/proliferation were upregulated throughout the course of chronic stimulation, which prompted us to investigate the relationship between these markers at the single-cell level.
- Our study, for example, reports reciprocal expression of inhibitory *LGALS1* and *ZBED2*, in relationship to the expression of proliferation markers *PCNA* and *TYMS*.
- Analysis of kinetic expression of genes and their corresponding proteins was performed for 23 markers and showed an overall random distribution of marker for which correlation between the two modalities was observed.
- In conclusion, single-cell multiomic is a very powerful tool to identify immunophenotypic and molecular changes that occur during *in vitro* activation and expansion of T cells.
- In the context of CAR T cell generation, this approach may provide useful insight to monitor the exhaustion state of expanded cells in response to different manufacturing protocols and possibly linked to cell product efficiency.