Insights into chimeric antigen receptor (CAR) spatial organization and CAR T cell function revealed by imaging flow cytometry and transcriptome profiles

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Abstract

Background: CAR T cell therapies have revolutionized treatment for hematologic cancers, yet precise CAR detection and characterization by flow cytometry remain challenging. Using high-sensitivity 1-step CAR detection reagent, we depicted CAR expression and detailed spatial distribution in primary T cells.

Methods: Human T cells were isolated from peripheral blood mononuclear cells, stimulated with anti-CD3/CD28 Dynabeads and transduced with a lentiviral vector encoding a B-cell maturation antigen (BCMA)-specific CAR. The transduced cells were further expanded in culture for 2 weeks prior to cryopreservation. Upon thawing, cells were rested overnight in IL-2-supplemented media before being re-stimulated in co-cultures assays with the U266 target cell line and/or subjected to spectral and imaging flow cytometry.

Results: Spectral flow cytometry enabled clear discrimination of CAR T cells spiked into donor PBMCs from endogenous T cells, allowing detailed profiling of activation, exhaustion, and memory phenotypes. Imaging flow cytometry with BD FACSDiscover™ S8 and BD CellView™ Technology revealed two distinct CAR spatial patterns: circular and polarized. Functional assays with BCMA-CAR T cells showed antigen-specific degranulation, with CD107a externalization co-localizing with CAR in cells exhibiting the circular pattern. These cells also displayed G1-quiescent transcriptional signatures, indicating readiness for activation and enrichment of immune response pathways. In contrast, polarized CAR⁺ subsets expressed higher levels of *MKI67, HMGB1,* and cell cycle genes, consistent with proliferative activity. These findings suggest CAR spatial organization correlates with functional states and demonstrate the power of integrating advanced flow and imaging cytometry to resolve CAR T cell phenotypes and spatial dynamics with great precision.

Methods

Identification of functionally distinct CAR spatial patterns with BD CellView™ Image Technology and single-cell whole transcriptome analysis

Peripheral blood T cells from a healthy individual were activated with anti-CD3/CD28 Dynabeads in the presence of human recombinant IL-2. Following stimulation, the cells were transduced with a CAR construct targeting human B-cell maturation antigen (BCMA). After further cell expansion in IL-2, for 10 days the cells were cryopreserved for subsequent analysis. CAR spatial organization was examined by imaging flow cytometry using the BD FACDiscover™ S8 Cell Sorter with BD CellView™ Image Technology, followed by sorting of cells with distinct CAR spatial patterns for single-cell whole transcriptome analysis [Figure1].

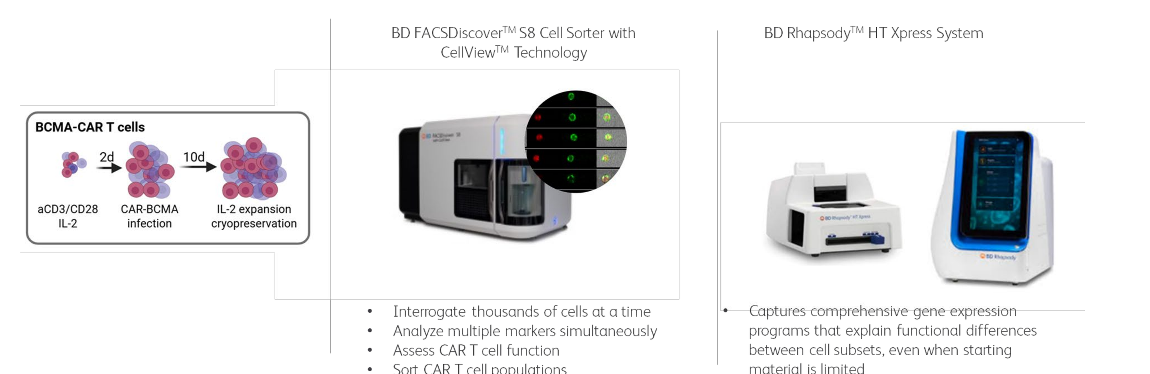


Figure 1: Whole transcriptome analysis of BCMA-CAR T cells with distinct CAR morphologies. BCMA-CAR T cells were analyzed and sorted in a FACSDiscover S8 Cell Sorter. The sorted populations expressing distinct CAR spatial distribution patterns were captured in the BD Rhapsody HT Xpress System prior to library preparation and sequencing.

A 24-color spectral flow cytometry panel was used for broad phenotypic characterization of BCMA-CAR T cells **[Table 1]**, while a reduced panel compatible with imaging fluorescence detection on the FACSDiscover platform was applied to examine CAR spatial distribution and for sorting of spatially distinct subsets **[Table 2 and Figure 2]**. A reduced panel containing BCMA-CAR AF488, CD107a-RB705, CD3-BUV395 and CD138-BV421 was also used to examine BCMA-CAR T cells stimulated with the multiple myeloma cell line U266 **[Figure 3]**.

Table 1: Immunophenotyping panel

Fluor	Marker
BUV395	CD27
BUV496	CD4
BUV615	CD197 (CCR7)
BUV737	CD56
BUV805	CD8
BV421	CX3CR1
V450	CD19
BV480	HLA-DR
FVS575v	Viability
BV650	CD185 (CXCR5)
BV786	CD3
BB515	CD45RA
RB545	CD57
RB613	CD95
RB670	CD183 (CXCR3)
RB705	CD196 (CCR6)
RB744	CD28
RB780	CD39
RY586	CD279 (PD-1)
RY610	TCRgd
RY703	CD25
RY775	CD127
AF647	BCMA-CAR
R718	CD194 (CCR4)

Table 2: Imaging compatible panel

Fluorochrome	Target	Purpose	Imaging
AF488	BCMA-CAR	CAR detection	Img1
RB705	LAG-3	T cell inhibitory receptor	Img 3
BUV395	CD3	T cell lineage	Minimal spills into imaging channels
BV421	Tigit	T cell inhibitory receptor	
BV605	PD-1	T cell inhibitory receptor	
BV786	CD8	Cytotoxic CD8 T cell	
APC	CD4	T helper CD4 T cell	
R718	TIM-3	T cell inhibitory receptor	
FVS780	NA	Live/dead discrimination	

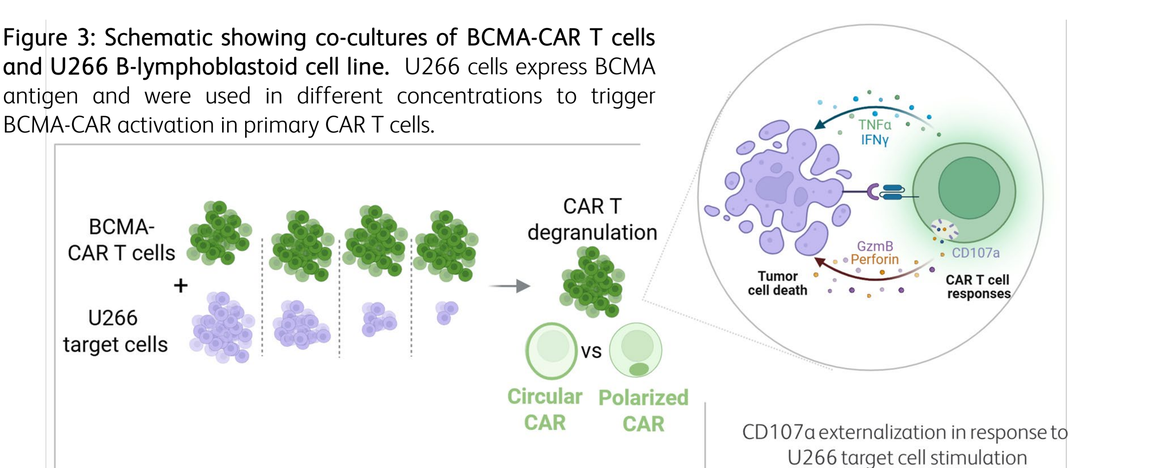
Sorting strategy defined with imaging parameters

High diffusivity BCMA-CAR	Low diffusivity BCMA-CAR
Uniform/Partially clustered	Clustered/polarized

LL BCMA-CAR Merge — High CAR Diffusivity, Low DCoM SSC_CAR | LL BCMA-CAR Merge — Low CAR Diffusivity, High DCoM SSC_CAR

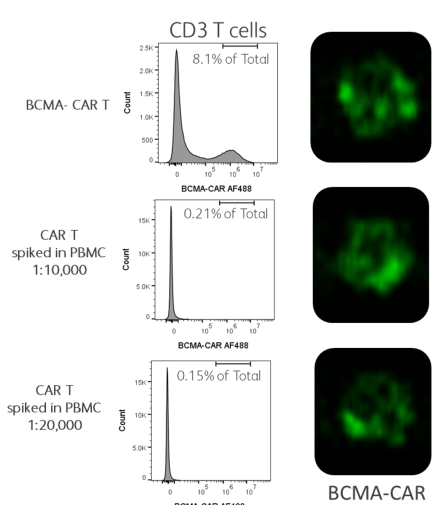
Figure 2: Image-derived features support the sorting of BCMA-CAR T cells populations with distinct CAR spatial distribution patterns.

Figure 3: Schematic showing co-cultures of BCMA-CAR T cells and U266 B-lymphoblastoid cell line. U266 cells express BCMA antigen and were used in different concentrations to trigger BCMA-CAR activation in primary CAR T cells.

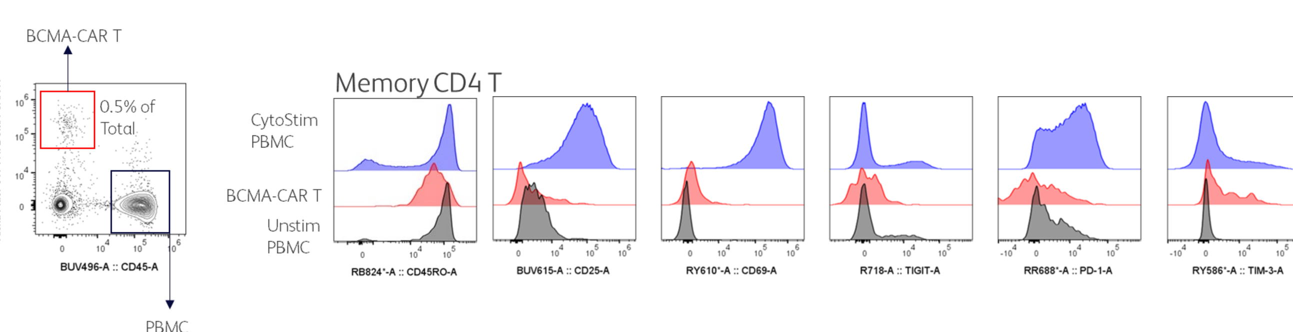


Results – BCMA-CAR detection in heterogenous samples and visualization of CAR spatial organization

4A CAR T detection sensitivity



4B CAR T phenotypic profile



4C Image-derived CAR spatial profiles

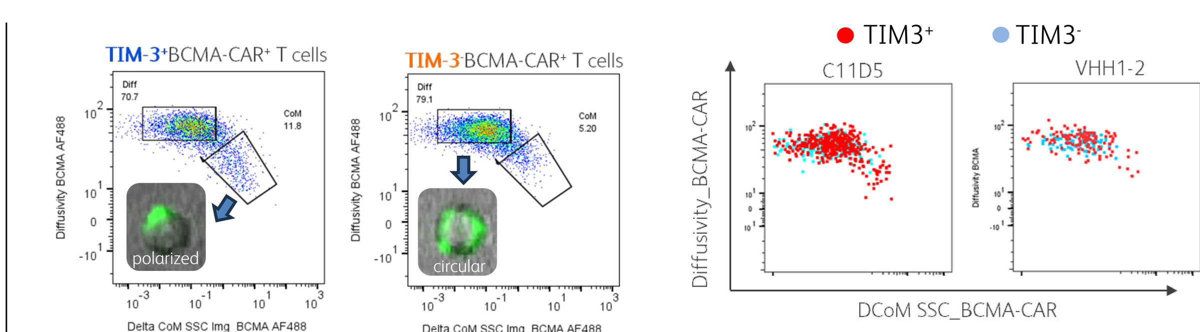
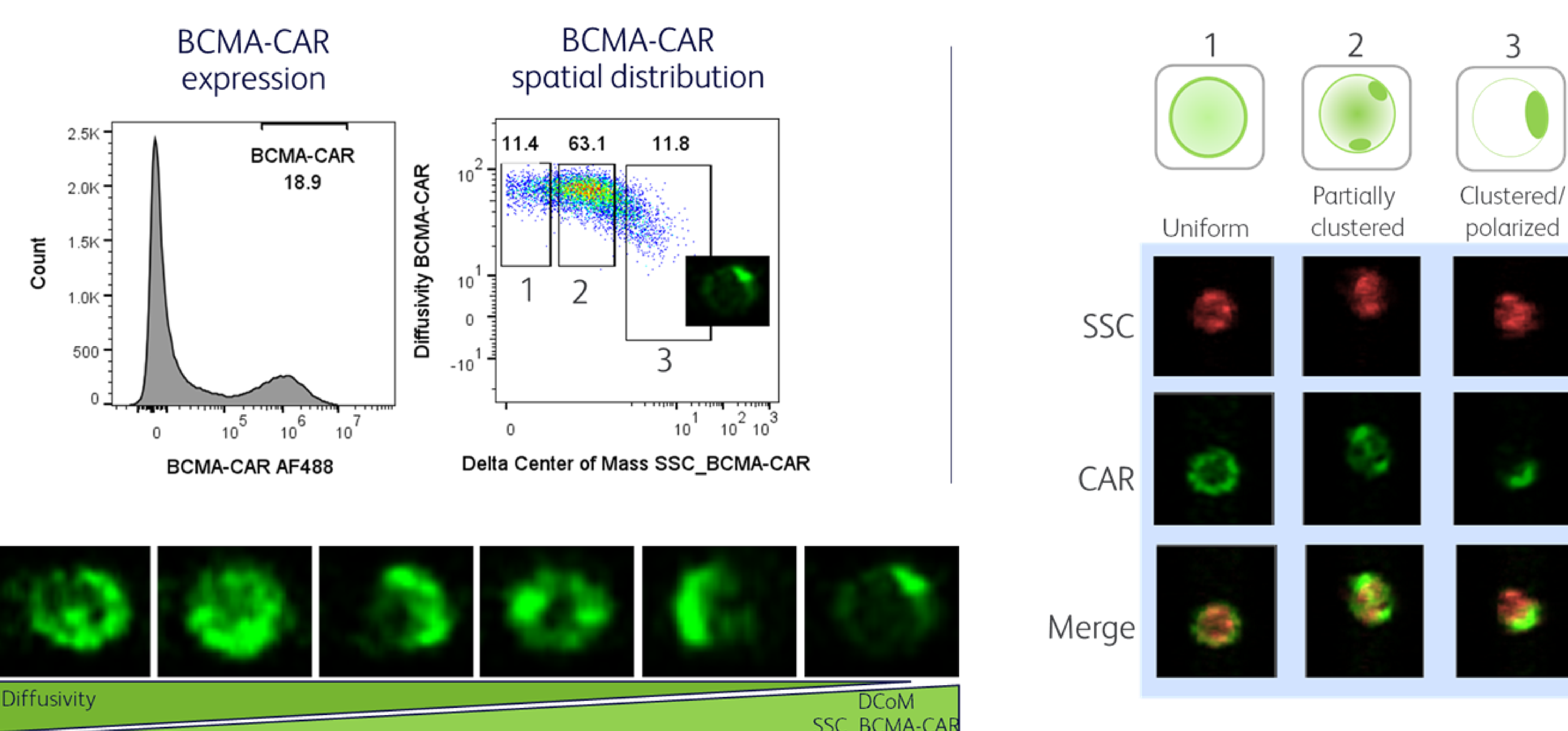


Figure 4: BCMA-CAR detection and visualization with BD CellView™ imaging parameters. A) BCMA-CAR T cells were analyzed after being spiked into CD45-stained PBMC preparations. The BD 1-step BCMA-CAR Detection Reagent reliably labeled CAR T cells at frequencies as low as 0.15% of the total. Images from cell alone (top) or heterogenous samples (middle and bottom) showed comparable quality. **B)** Cell analysis with a 24-color panel revealed that this particular BCMA-CAR T cell culture was enriched with memory CD4 T cells, which exhibited elevated expression of CD336 (TIM-3) compared to resting T cells. **C)** Using a 9-color panel, BCMA-CAR T cells were further stratified based on BCMA-CAR spatial distribution. Within cells expressing similar BCMA-CAR total intensity, one subset displayed a strong, punctate BCMA-CAR AF488 signal, characterized by high delta center of mass between side-scatter (SSC) imaging and BCMA-CAR AF488 fluorescence. In contrast, a second subset exhibited a more diffuse and uniform CAR signal, indicative of distinct patterns of CAR spatial organization on the surface. Additionally, this panel revealed higher frequencies of cells displaying the polarized morphology among TIM-3 positive cells derived from either BCMA-CAR (clone C11D5) or BCMA-CAR (clone VHH-12) cultures.

Results – Relationship between CAR spatial organization and CAR T cell functional states

5A Using BD CellView™ imaging parameters to uncover CAR spatial distribution patterns on cell surface



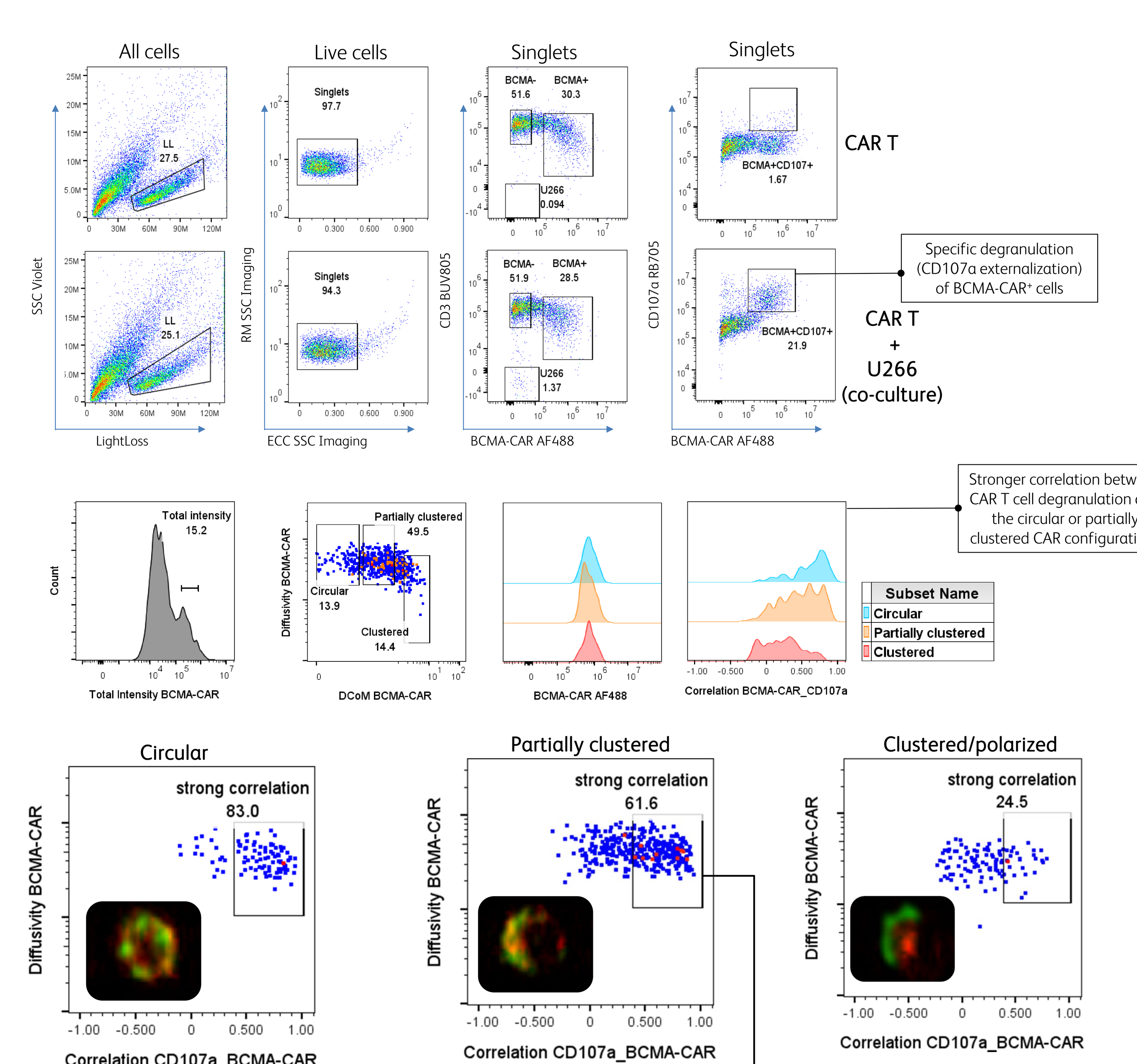
Imaging parameters:

- DCoM SSC_BCMA-CAR : Measure the separation between SSC and BCMA-CAR
- Diffusivity: Measure how spread out the BCMA-CAR AF488 signal is
- Max intensity : Display the intensity of the brightest BCMA-CAR pixels in the image

Subset Name: BCMA-; Clustered_polarized; Partially clustered; Circular

Total intensity BCMA-CAR | Max intensity BCMA-CAR AF488

5B CAR circular conformation correlates with T cell degranulation



Imaging parameters:

- Correlation: Show fluorescence signal overlaps between CD107a and BCMA-CAR

Stronger correlation between CD107a and BCMA-CAR signals suggests their co-localization during CAR T cell degranulation

Figure 5: Imaging parameters reveal relationships between CAR spatial patterns and CAR T cell functional states. A) The delta center of mass parameter which quantifies the separation of BCMA-CAR surface fluorescence and side scatter (SSC)-derived intracellular granularity, distinguished punctate (clustered/polarized) from uniform (circular) BCMA-CAR distributions. Cells with uniform CAR patterns exhibited higher BCMA-CAR diffusivity whereas punctate patterns were enriched for cells with the highest BCMA-CAR max intensity signals, as shown by histogram overlays. **B)** CAR T functional activation was assessed by re-stimulation with BCMA-positive U266 cells. BCMA-CAR T cells were cultured alone or co-cultured with U266 cells for 5h in the presence of brefeldin, monensin and anti-CD107a -RB705. Co-culture with U266 induced specific CD107a surface externalization in BCMA-CAR T cells, indicating antigen-dependent degranulation. Notably, uniform BCMA-CAR spatial patterns correlated strongly with CD107a signal intensity, suggesting co-localization of BCMA-CAR and CD107a at the cell surface during CAR T cell activation.

6A Association between CAR spatial organization and functional transcriptome

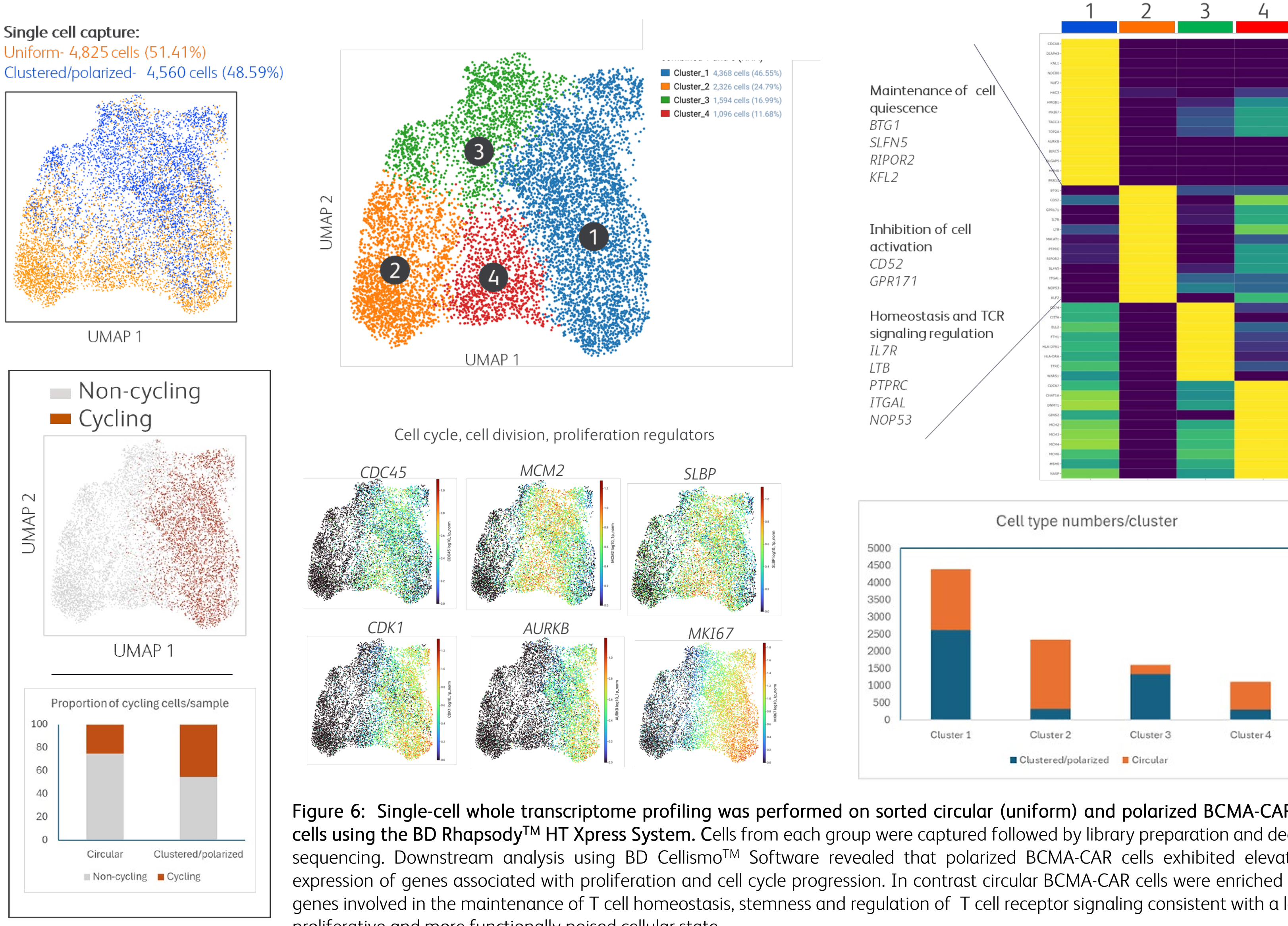


Figure 6: Single-cell whole transcriptome profiling was performed on sorted circular (uniform) and polarized BCMA-CAR T cells using the BD Rhapsody™ HT Xpress System. Cells from each group were captured followed by library preparation and deep-sequencing. Downstream analysis using BD Cellismo™ Software revealed that polarized BCMA-CAR cells exhibited elevated expression of genes associated with proliferation and cell cycle progression. In contrast circular BCMA-CAR cells were enriched for genes involved in the maintenance of T cell homeostasis, stemness and regulation of T cell receptor signaling consistent with a less proliferative and more functionally poised cellular state.

Conclusions

- BCMA-CAR T cells exhibited distinct spatial patterns, appearing either uniform (circular) and punctate (clustered/polarized), independent of overall CAR expression levels.
- Imaging parameters distinguished these spatial patterns, in which circular distributions show higher diffusivity, while punctate distributions exhibited higher max intensity signals.
- Functional assays revealed a link between CAR organization and activity, in which circular BCMA-CAR patterns correlated strongly with CD107a externalization upon target engagement, indicating active degranulation.
- Single-cell transcriptomics confirmed functional differences, with circular BCMA-CAR T cells enriched for immune response-activating signaling pathways compared to clustered/polarized cells.
- Together, these results suggest that CAR spatial organization, rather than expression level alone, emerges as a marker of CAR T cell functional states. Insights into how CAR distribution relates to function may inform engineering strategies to promote favorable spatial organization, enhancing efficacy as well as enable enrichment of CAR T cells with higher activity before infusion. It may also serve as an early biomarker of functional state, supporting patient stratification and real-time assessment of therapeutic potential.

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