Functional Characterization of CAR T Cell Activation, Exhaustion and Cytokine Production

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Introduction

CAR T cell therapy has emerged as a groundbreaking advancement in cancer treatment, offering new hope for patients with previously intractable forms of the disease. CAR T cell therapy involves the engineering of patients' T cells to target and eliminate cancer cells, a concept that has transformed the landscape of oncological therapeutics. Despite its promise, the effectiveness of CAR T cell therapy is intricately linked to the complex interplay between engineered T cells and the tumor microenvironment. In particular, CAR T therapy targeting solid tumors has shown limited efficacy due to various factors such as reduced trafficking, an immunosuppressive tumor milieu, reduced persistence and antigen escape. Thus, a better understanding of CAR T biology to maximize therapeutic outcomes while minimizing off-target effects – such as cytokine release syndrome and neurotoxicity – is necessary to overcome these limitations.

Objective

To develop an immunoprofiling solution that addresses the need for comprehensive characterization of CAR T cells, including surface marker expression, activation state, cytokine production and differentiation status

Conclusions

- Optimal CAR T detection and phenotyping was achieved with this comprehensive mass cytometry panel of 43 markers, ideal for immune profiling CAR T cells at all stages of CAR T therapy
- CAR T cells spiked into healthy donor peripheral blood mononuclear cells – to simulate immune profiling after infusion into patients– could be clearly resolved and immunoprofiled
- CAR T detection can be tailored to each CAR T product by combining a CAR T receptor detection reagent with a secondary antibody or probe conjugated to a metal tag
- The pre-optimized and validated Maxpar™ Direct Immune Profiling Assay has been used extensively for multi-site and longitudinal immune profiling studies due to its ease of use and reproducibility
- The lyophilized single-tube Maxpar Direct Immune Profiling Assay and Expansion Panels are optimized to work together while still allowing for expansion of the panel (for example, to incorporate sample barcoding for batched acquisitions)

Materials and Methods

Cell culture. Anti-CD19 CAR T cells (BPS Bioscience) were activated with ImmunoCult Human CD3/CD28/CD2 T Cell Activator (STEMCELL Technologies) and IL-2 (10 ng/mL) in ImmunoCult-XF T Cell Expansion Medium for two days, then passaged for an additional five days with IL-2. Cryopreserved PBMC (STEMCELL) and NALM6 cells (ATCC) were thawed and maintained in RPMI 1640 media supplemented with 10% fetal bovine serum, L-glutamine, penicillin and streptomycin.

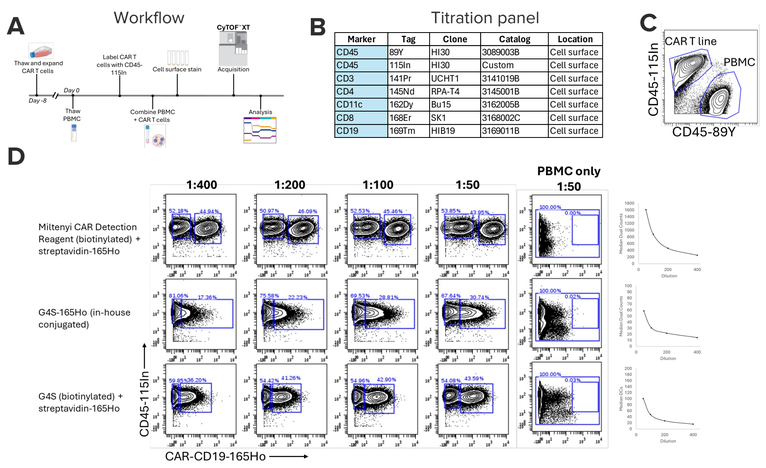
Panel design. The Maxpar Direct Immune Profiling Assay was supplemented with antibodies from the Maxpar Direct Basic Activation Expansion Panel (IL-2, IFNγ, TNFα, granzyme B, perforin and CD107a), five antibodies from the T Cell Expansion Panel 3 (OX40, TIGIT, CD69, TIM-3 and 4-1BB) and PD-1. Channel 165Ho was used for CAR CD19 detection, using either a directly conjugated or biotinylated G4S Linker antibody (Cell Signaling Technology) or biotinylated CD19 CAR Detection Reagent (Miltenyi) and streptavidin-165Ho as a secondary reagent. Samples were acquired on a CyTOF™ XT instrument in batch acquisition mode.

Results

Optimal CAR T detection

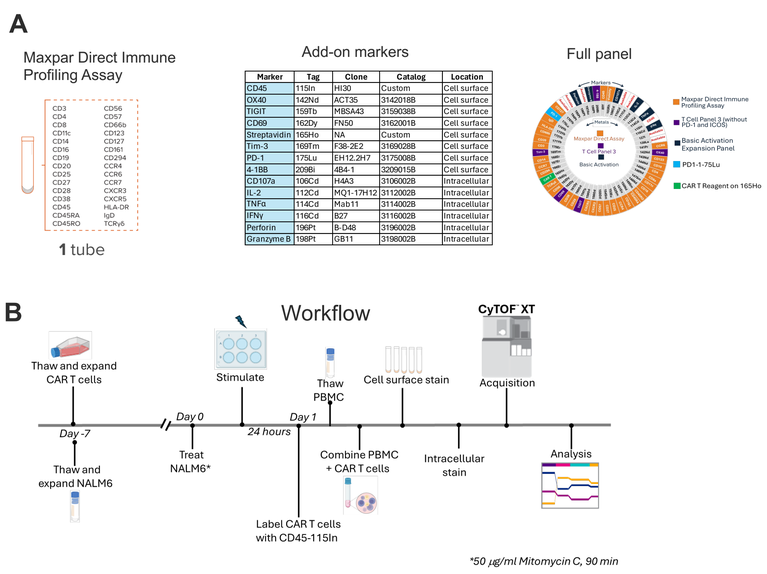
We first compared CAR T detection options for sensitivity and specificity using a CD19 CAR-transduced cell line spiked into healthy donor PBMC. Indirect staining with Miltenyi Biotec biotinylated CD19 CAR Detection Reagent followed by streptavidin-conjugated 165Ho was compared against a directly conjugated or biotinylated anti-G4S E702V Linker antibody from Cell Signaling Technology.

- Indirect staining with the Miltenyi CD19 CAR Detection Reagent yielded the best resolution and lowest background
- The anti-G4S Linker antibody demonstrated higher signal and reduced nonspecific staining when directly conjugated to 165Ho (using a Maxpar X8 Antibody Labeling Kit) compared with indirect staining
- About 50% of the CD19 CAR T cell line lacked CAR-CD19 receptor expression, necessitating pre-labeling of the cell line with CD45 conjugated to 115In
- However, CD45-115In was not required for dimensionality reduction and FlowSOM clustering as most (about 87%) of the CAR T cells clustered together based on their distinct expression profile of the full panel of markers (Figure 3)



Marker	Tag	Clone	Catalog	Location
CD45	89Y	HI30	3089003B	Cell surface
CD45	115In	HI30	Custom	Cell surface
CD3	141Pr	UCHT1	3141019B	Cell surface
CD4	145Nd	RPA-T4	3145001B	Cell surface
CD11c	162Dy	Bu15	3162005B	Cell surface
CD8	168Er	SK1	3168002C	Cell surface
CD19	169Tm	HIB19	3169011B	Cell surface

Figure 1. A) Workflow of activation and staining of CAR T cells. **B)** Panel for titration of CAR T detection reagents. **C)** Representative staining of a 1:1 spike-in sample of PBMC:CAR T cells showing pre-labeling of the CAR T cell line with CD45-115In allows for differentiation from the PBMC, which stain with CD45-89Y in the panel. **D)** Titration of CAR T detection reagents. CAR T cells and healthy donor PBMC were mixed 1:1 after labeling the CAR T cells with CD45-115In. The cell mixtures were then stained with different concentrations of probes against the CAR-CD19 receptor and a backbone panel of CD3, CD4, CD8, CD11c and CD19, followed by secondary staining with streptavidin-165Ho (sAv) for the biotinylated detection reagents.



Marker	Tag	Clone	Catalog	Location
CD45	115In	HI30	Custom	Cell surface
OX40	142Nd	ACT35	3142018B	Cell surface
TIGIT	159Tb	MBSA43	3159038B	Cell surface
CD69	162Dy	FN50	3162001B	Cell surface
Streptavidin	165Ho	NA	Custom	Cell surface
Tim-3	169Tm	F38-2E2	3169028B	Cell surface
PD-1	175Lu	EH12.2H7	3175008B	Cell surface
4-1BB	209Bi	4B4-1	3209015B	Cell surface
CD107a	106Cd	H4A3	3106002B	Intracellular
IL-2	112Cd	MQ1-17H12	3112002B	Intracellular
TNFα	114Cd	Mab11	3114002B	Intracellular
IFNγ	116Cd	B27	3116002B	Intracellular
Perforin	196Pt	B-D48	3196002B	Intracellular
Granzyme B	198Pt	GB11	3198002B	Intracellular

Figure 2. A) Panel design and **B)** workflow of activation and staining of CAR T cells. CAR T cells were activated with CD3/CD28/CD2 beads for two days, then expanded for five days with IL-2. NALM6 target cells were treated with 50 µg/mL mitomycin C for 90 minutes to inhibit cellular proliferation, then co-cultured with CAR T cells for 24 hours at a 1:1 ratio. CD107a and the protein trafficking inhibitors monensin and brefeldin A were added in the last six hours of co-culture.

Phenotypic and functional characterization of CAR T cells

To simulate post-infusion immune profiling of CAR T treated patients, unstimulated or NALM6-activated CAR T cells were spiked into healthy donor PBMC at 1:1, 2:1, 5:1 and 10:1 (PBMC:CAR T) ratios. This admixture was then stained with the full 43-marker panel and evaluated for lineage marker expression. The CAR T cells were easily resolved from the PBMC background even at 10-fold lower frequency. UMAP projection using the full panel also easily grouped the majority of CAR T cells into a distinct region, while FlowSOM clustering led to 11 out of 30 metaclusters highly enriched for CAR T cells.

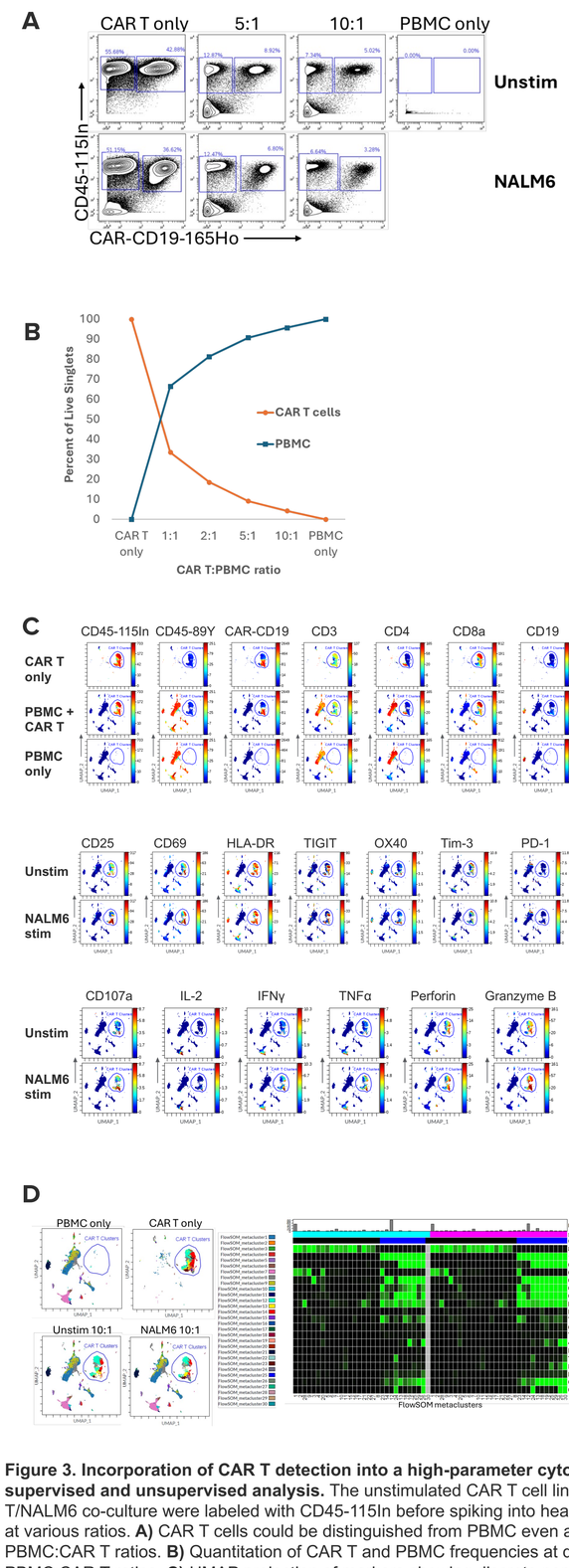


Figure 3. Incorporation of CAR T detection into a high-parameter cytometry panel for supervised and unsupervised analysis. The unstimulated CAR T cell line and the CAR T/NALM6 co-culture were labeled with CD45-115In before spiking into healthy donor PBMC at various ratios. **A)** CAR T cells could be distinguished from PBMC even at high PBMC:CAR T ratios. **B)** Quantitation of CAR T and PBMC frequencies at different PBMC:CAR T ratios. **C)** UMAP projection of markers showing discrete populations of CAR T cells in a 10:1 PBMC:CAR T mix after subsampling each file to 50,000 events. **D)** FlowSOM clustering overlaid on UMAP embeddings. **E)** Expression heat maps of key markers across FlowSOM clusters. All protein targets in the panel except CD45-89Y and CD45-115In were used for UMAP projection and FlowSOM clustering.

Maxpar Pathsetter for the automated quantitation and characterization of CAR T cells

Maxpar Pathsetter is an automated and complete analysis solution for samples stained with the Maxpar Direct Immune Profiling Assay and Expansion Panels.

Each sample file is analyzed and summarized in five minutes using a probability state modeling approach to identify cell types and cell differentiation states. This approach enables unbiased and robust quantitation of cell types and functional phenotypes. CD4 and CD8 CAR T cells were added as cell types and their differentiation states and phenotypes defined.

- The CAR T cells were mapped to a distinct Cen-se′ region based on expression of markers defined in the expression profile
- In comparison to PBMC CD4 and CD8 T cells, CAR CD4 and CD8 T cells were predominantly effector T cells and responded to NALM6 activation
- The response to NALM6 activation was mainly observed in the CAR-CD19+ population, confirming CD19 expression on the NALM6 cells induced antigen-specific CAR+ T cell activation and cytokine production

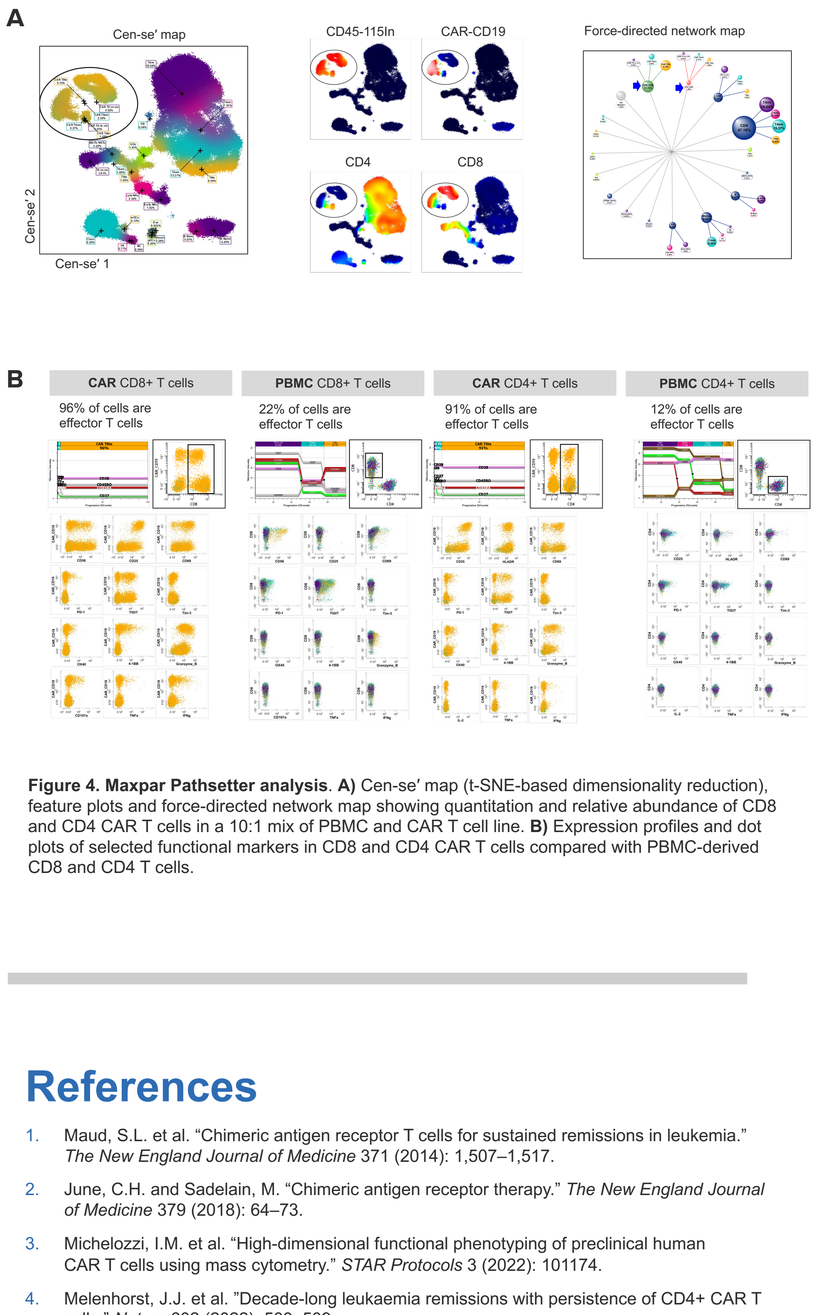


Figure 4. Maxpar Pathsetter analysis. **A)** Cen-se′ map (t-SNE-based dimensionality reduction), feature plots and force-directed network map showing quantitation and relative abundance of CD8 and CD4 CAR T cells in a 10:1 mix of PBMC and CAR T cell line. **B)** Expression profiles and dot plots of selected functional markers in CD8 and CD4 CAR T cells compared with PBMC-derived CD8 and CD4 T cells.

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