# Advancing CAR T Clinical Development with High-Parameter CyTOF Technology

Ling Wang, Shakir Hasan, Stephen Li, Michael Cohen, Deeqa Mahamed, Christina Loh

Standard BioTools Canada Inc., Markham, ON, Canada

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## Introduction

Adoptive immunotherapy using chimeric antigen receptor (CAR) T cells is considered a recent revolutionary treatment in cancer therapy. CAR T therapy has achieved great success in hematological B cell malignancies. However, it has faced significant challenges in solid tumors due to various factors, such as complex tumor microenvironments, restricted trafficking, impersistent antitumor activity and toxicities. True comprehensive profiling of CAR T cells requires not only phenotypic composition, but also functional capacity to elucidate the underlying mechanisms of antitumor activity. Cytokines and transcription factors (TF) play key roles in CAR T cell function, acting as indicators of T cell efficacy against tumor and master regulators of T cell differentiation and fitness. On the other hand, cytokine production by CAR T cells triggers systemic inflammatory responses, which mainly manifest as adverse events such as cytokine-release syndrome (CRS) and neurotoxicity. A better understanding of CAR T biology, including cytokine and TF profiling, will accelerate development of CAR T therapies with improved antitumor efficacy and durability and decreased systemic toxicities.

High-parameter flow cytometry has been a powerful tool to functionally characterize CAR T cells at multiple stages of clinical development, from product characterization during manufacturing to longitudinal evaluation of the infused product in patients. However, fluorescence-based cytometry faces significant challenges with signal overlap and autofluorescence, limiting sensitivity and the number of targets that can be detected in CAR T cells. Consequently, identification of rare cell population has always been challenging, and subsequent functional readouts of CAR T cells are questionable. CyTOF™ technology overcomes these limitations with low signal overlap and no autofluorescence. Further, CyTOF technology enables a streamlined and flexible workflow in clinical research using frozen antibody cocktails and stained cell samples. Here, we present a 43-marker CyTOF panel to simultaneously analyze phenotypic and functional protein expression in CAR T cells from *in vitro* co-culture with tumor cells.

## Materials and Methods

- **Co-culture:** CD19 CAR T cells (BPS Bioscience) were expanded (with 100 IU/mL IL-2) *in vitro* for three days followed by co-culturing with Nalm6 cells (CD19-expressing lymphoblastic leukemia cell line) for 2–4 days (Figure 1)
- **Staining:** A high-parameter CyTOF panel including 43 surface, cytoplasmic and nuclear markers was used to stain CAR T cells (Table 1). Samples co-cultured at different time points were stained using a pre-aliquoted frozen antibody cocktail. Surface staining was followed by intracellular staining, a modified nuclear protocol from the Maxpar™ Cell Staining with Fresh Fix User Guide (FLDM-01319), allowing for simultaneous staining of cytoplasmic and nuclear targets.
- **Acquisition:** Samples stained at different time points were frozen and simultaneously acquired on a CyTOF XT system at a later date

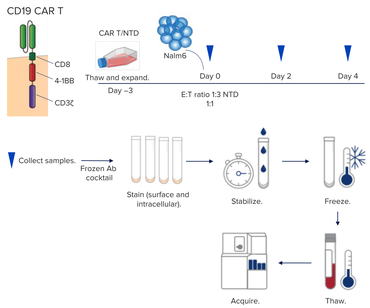


**Figure 1. Experimental workflow.** E:T ratio – effector:target ratio; NTD – non-transduced T cells

**Table 1.** CAR T CyTOF panel

| Category | Marker | Function | Inventory |
|---|---|---|---|
| Phenotypic marker (16) | CD45 | Phenotyping | Catalog |
| | CD14 | | Catalog |
| | CD16 | | Catalog |
| | CD19 | | Catalog |
| | CD56 | | Catalog |
| | CD3 | | Catalog |
| | CD4 | | Catalog |
| | CD8 | | Catalog |
| | CD27 | | Catalog |
| | CD28 | | Catalog |
| | CD161 | | Catalog |
| | CD45RA | | Catalog |
| | CD45RO | | Catalog |
| | TCRαβ | | Catalog |
| | CD25 | | Catalog |
| | CD127 | | Catalog |
| Immune checkpoint marker (10) | 4-1BB | Activation | Catalog |
| | CD154 | | Catalog |
| | CD95 | Exhaustion | Catalog |
| | CTLA-4 | | Catalog |
| | PD-1 | | Catalog |
| | PD-L1 | | Catalog |
| | TIM-3 | | Catalog |
| | TIGIT | | Catalog |
| | LAG-3 | | Catalog |
| | BTLA | | Catalog |
| Core cytokine (5) | TNFα | Effector | Catalog |
| | IFNγ | | Catalog |
| | IL-2 | Regulatory | Catalog |
| | IL-4 | | Catalog |
| | IL-17 | Inflammatory | Catalog |
| | IL-6 | | Catalog |
| Cytotoxic mediator (3) | Granzyme B | Effector | Catalog |
| | Perforin | | Catalog |
| Treg cytokine (2) | IL-10 | Treg | Catalog |
| | TGF-β | | Catalog |
| Transcription factor | FoxP3 | | Catalog |
| Drop-in | CD69 | Activation | Catalog |
| Transcription factor | T-bet | | Catalog |
| | TOX | Exhaustion | Customized |
| | CD39 | | Customized |
| Drop-in | Ki-67 | Proliferation | Catalog |
| | Biotinylated CD19 + Cytavidin | CAR T detection | Customized |

## Conclusions

- **Comprehensive CAR T profiling** by this 43-marker **CyTOF panel** revealed that CAR T cells became activated, proliferated and produced cytokines in *in vitro* co-culture with tumor cells and exhibited an exhaustive-like Treg phenotype at the end of a four-day co-culture with target cells
- **Exceptional signal resolution of cytokines and TFs** revealed a diverse polyfunctional antitumor signature of CAR T cells in the CD8 TEMRA cell subset
- The CAR molecule was conveniently detected by biotinylated CD19-CAR reagent and **Cytavidin** (metal-conjugated streptavidin)
- Effortless panel design, the **frozen antibody cocktail** and **simultaneous staining of cytokines and TFs** warranted accelerating time to insights with minimized experimental variability

## Results

### CAR T displayed effector phenotype following *in vitro* tumor cell co-culture

#### Specific and sensitive detection of CAR T cells enabled thorough phenotypic analysis of differentiation

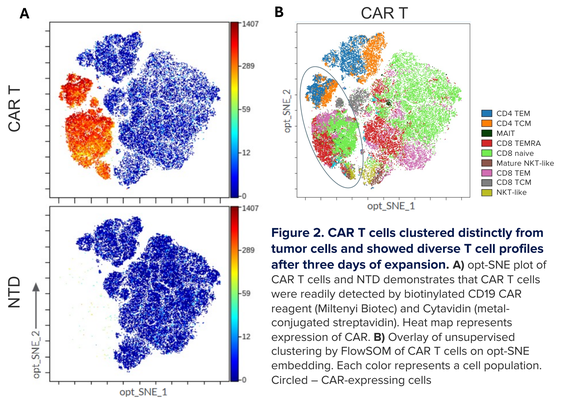


**Figure 2. CAR T cells clustered distinctly from tumor cells and showed diverse T cell profiles after three days of expansion. A)** opt-SNE plot of CAR T cells and NTD demonstrates that CAR T cells were readily detected by biotinylated CD19 CAR reagent (Miltenyi Biotec) and Cytavidin (metal-conjugated streptavidin). Heat map represents expression of CAR. **B)** Overlay of unsupervised clustering by FlowSOM of CAR T cells on opt-SNE embedding. Each color represents a cell population. Circled – CAR-expressing cells

#### CAR T showed cytotoxicity against tumor cells

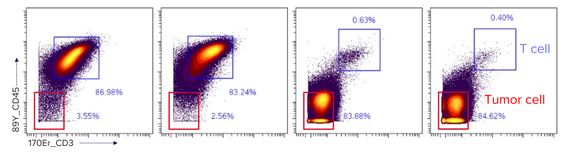


**Figure 3. CAR T cells showed cytotoxicity at two days and four days after co-culture with tumor cells.** CAR T or NTD were co-cultured with Nalm6 for 2–4 days. Diminished tumor cell population was observed after two days and four days co-culture with CAR T, while tumor cells dominated the co-culture with NTD. The biaxial plots present singlets of co-cultured T cell-tumor cell mix. T cell – CD45+CD3+; tumor cell – CD45–CD3–

#### CAR T shifted to mainly effector phenotype post-co-culture with tumor cells

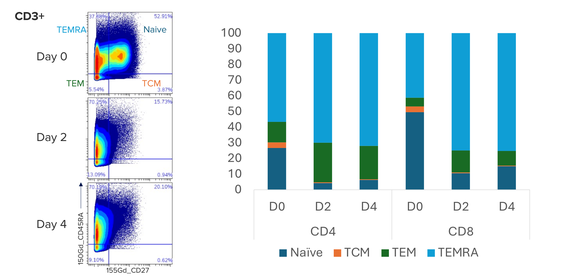


**Figure 4. CAR T differentiated toward effector phenotype after co-culture with tumor cells.** CAR T was co-cultured with Nalm6 for 2–4 days. The memory phenotypes of CD4+ and CD8+ CAR T were evaluated using surface marker CD45RA and CD27. Naive – CD45RA+CD27+; TCM (central memory) – CD45RA–CD27+; TEM (effector memory) – CD45RA–CD27–; TEMRA ( terminal effector) – CD45RA+CD27–

### Dynamic functional signatures of CAR T cells were revealed by simultaneous high-parameter detection of cytokines, transcription factors and surface markers

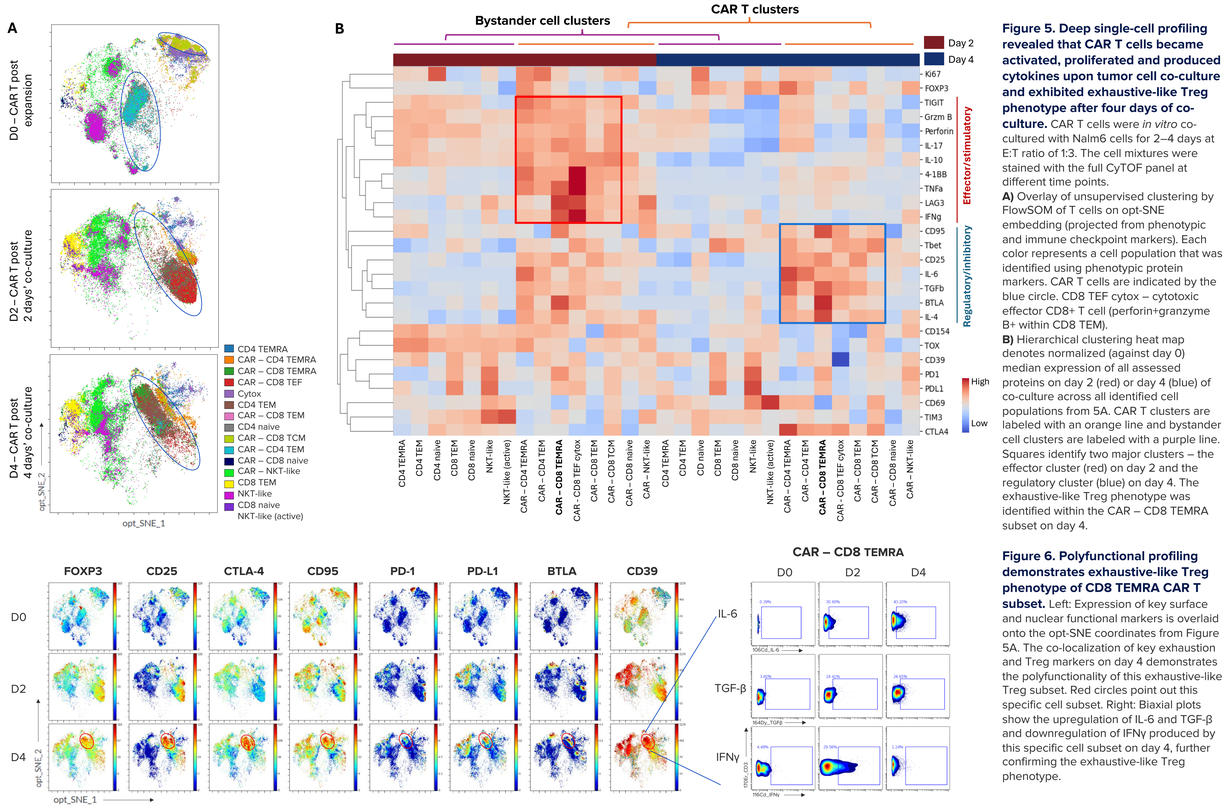


**Figure 5. Deep single-cell profiling revealed that CAR T cells became activated, proliferated and produced cytokines upon tumor cell co-culture and exhibited exhaustive-like Treg phenotype after four days of co-culture.** CAR T cells were *in vitro* co-cultured with Nalm6 cells for 2–4 days at E:T ratio of 1:3. The cell mixtures were stained with the full CyTOF panel at different time points.
**A)** Overlay of unsupervised clustering by FlowSOM of T cells on opt-SNE embedding (projected from phenotypic and immune checkpoint markers). Each color represents a cell population that was identified using phenotypic protein markers. CAR T cells are indicated by the blue circle. CD8 TEF cytox – cytotoxic effector CD8+ T cell (perforin+granzyme B+ within CD8 TEM).
**B)** Hierarchical clustering heat map denotes normalized (against day 0) median expression of all assessed proteins on day 2 (red) or day 4 (blue) of co-culture across all identified cell populations from 5A. CAR T clusters are labeled with an orange line and bystander cell clusters are labeled with a purple line. Squares identify two major clusters – the effector cluster (red) on day 2 and the regulatory cluster (blue) on day 4. The exhaustive-like Treg phenotype was identified within the CAR – CD8 TEMRA subset on day 4.

**Figure 6. Polyfunctional profiling demonstrates exhaustive-like Treg phenotype of CD8 TEMRA CAR T subset.** Left: Expression of key surface and nuclear functional markers is overlaid onto the opt-SNE coordinates from Figure 5A. The co-localization of key exhaustion and Treg markers on day 4 demonstrates the polyfunctionality of this exhaustive-like Treg subset. Red circles point out this specific cell subset. Right: Biaxial plots show the upregulation of IL-6 and TGF-β and downregulation of IFNγ produced by this specific cell subset on day 4, further confirming the exhaustive-like Treg phenotype.

### CAR T cells exhibited diverse cytokine profiling during *in vitro* co-culture with tumor cells

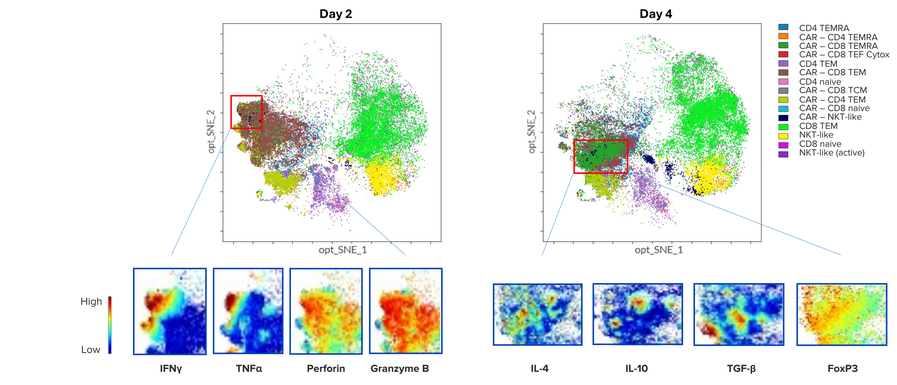


**Figure 7. Deep cytokine profiling of CAR T cells during tumor cell co-culture unveiled effector and regulatory cytokine signatures.** Co-culture cell mixtures at day 2 and 4 were stained and projected into opt-SNE space using phenotypic markers and cytokines. Each color represents a cell population. The plot gradient (cold to warm) represents expression levels of cytokines within each small cell cluster area (red squares) extracted from the whole opt-SNE map. The warmer color signifies higher expression. Diverse cytokine signatures are identified: Effector cytokines IFNγ, TNFα, perforin and granzyme B are co-expressed in the same cell cluster area on day 2 co-culture. Regulatory cytokines IL-4, IL-10 and TGF-β are co-expressed in the same cell cluster on day 4 co-culture. Note that FoxP3 is co-localized with regulatory cytokines, implicating Treg phenotype of this specific cluster (CAR – CD8 TEMRA).

Standard BioTools Inc.
2 Tower Place, Suite 2000, South San Francisco, CA 94080 USA
+1 650 266 6000 • Toll-free in the US and Canada: 866 359 4354
standardbio.com



CyTOF XT PRO

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