Flow Cytometry-Based Monitoring of CAR-T cell Persistence and Immune Phenotypes Pre & Post-Infusion: Gating Challenges in Phase 1/2 Hematologic Malignancy Trials

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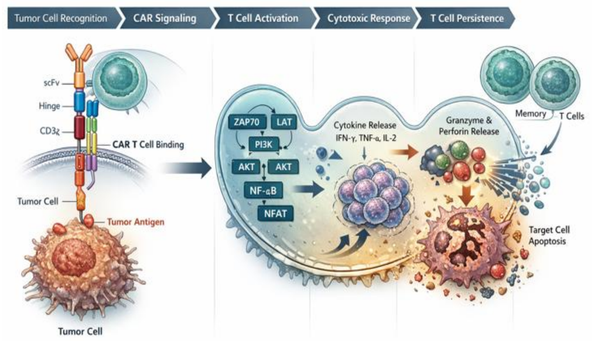
Introduction

Chimeric antigen receptor T-cell (CAR-T) therapies for hematological malignancies increasingly relied on both clinical outcomes and flow cytometry assessments of the CAR-T cells themselves to evaluate treatment efficacy. Flow assay development typically uses control CAR-T cells spiked into healthy blood or PBMCs; however, these controls do not fully replicate the phenotypic or scatter characteristics of patient-derived CAR-T cells following infusion. This discrepancy introduces challenges in gating accuracy and data interpretation.

Precision for Medicine (PfM) has developed cell therapy flow cytometry assays to quantify CAR-positive cell persistence and characterize immune phenotypes, including CD4, CD8 subsets, memory, effector differentiation, and activation/exhaustion. During assay development control CAR-positive cells were used to establish baseline gating strategy. However, post-infusion CAR-positive cells exhibited altered scatter profiles and marker expression compared to assay controls. While inter-assay controls ensure baseline reproducibility; however, adaptive gating strategies were required to accurately identify of CAR-positive populations in patient samples.

An adaptive gating strategy enables robust tracking of CAR-positive cell persistence and immune profiling post-infusion across both pre- and post-infusion clinical samples. Observed differences between inter-assay CAR controls and post-infusion CAR positive cells underscore the need for adaptive and dynamic gating strategies. Addressing these challenges is critical for reliable enumeration and immune monitoring of CAR cell persistence and for meaningful correlating with clinical outcomes.

CAR T cell Activation and Function



CAR-T Flow Method Summary

Whole blood was collected at clinical sites, shipped, and centralized for flow cytometry analysis.

The CAR-T flow assay profiled major immune subsets in lymphocytes including:

- T cells (CD4 & CD8 T cells)
- Memory T cell subsets
- B cells
- NK cells
- Monocytes
- Activation, Proliferation & Checkpoint/Exhaustion markers

CAR-T Flow Markers Included: Viability, CD45, CD3, CD4, CD8, CD14, CD19, CD56, CD45RA, CCR7, Ki67, CD28, CD25, LAG-3, TIM-3, and CAR Antigen

Antibodies were purchased from BioLegend, BD Biosciences or eBioscience. Assay controls included single color compensation controls and a healthy donor PBMC inter-assay control with full panel stains and fluorescence minus one or more markers (FMO/FMX) for objective setting of analysis gates.

Stained cells acquired with a BD LSRFortessa X-20™ cytometer using FACSDiva software. Data was analyzed with BD FlowJo software.

Summary of Results

Fig. 1A Linearity Study: Adaptive Gating Strategy for CAR+ T cells

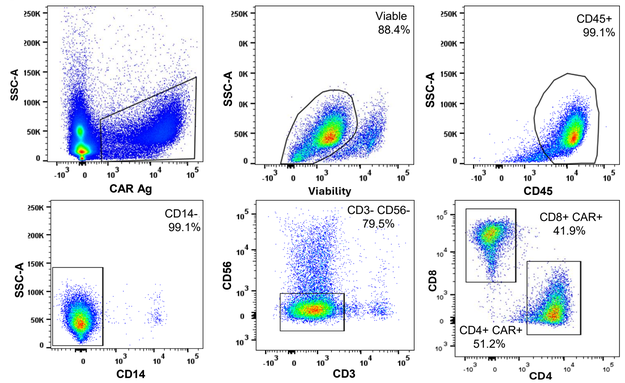


Fig. 1B Linearity Study: Original Gating Strategy for CAR+ T cells

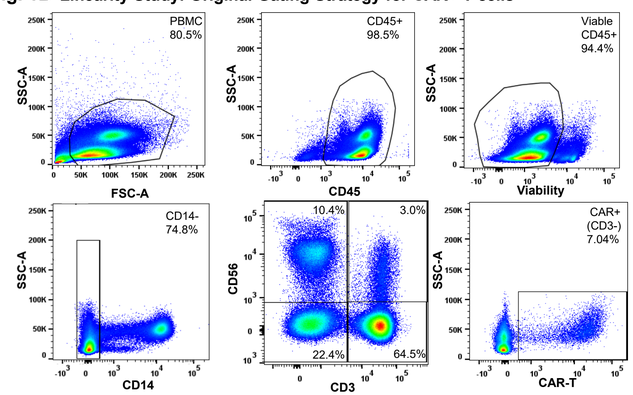
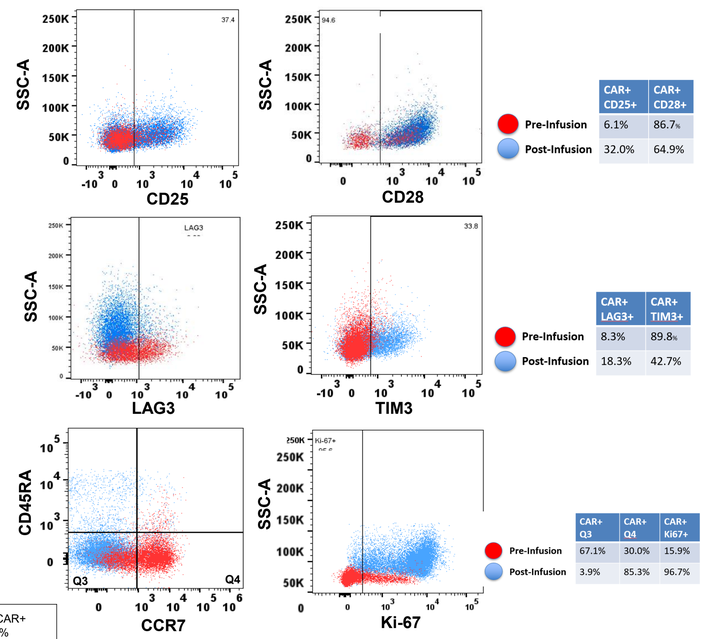


Fig. 1C CAR T-cell Pre & Post Infusion Activation & Exhaustion State



	CAR+ CD25+	CAR+ CD28+
Pre-Infusion	6.1%	86.7%
Post-Infusion	32.0%	64.9%

	CAR+ LAG3+	CAR+ TIM3+
Pre-Infusion	8.3%	89.8%
Post-Infusion	18.3%	42.7%

	CAR+ Q3	CAR+ Q4	CAR+ Ki67+
Pre-Infusion	67.1%	30.0%	15.9%
Post-Infusion	3.9%	85.3%	96.7%

Figure 1. Comparison of CAR-T Cell Gating Strategies, and Pre & Post Infusion Activation/ Exhaustion Status

(A.) Adaptive Gating Strategy for CAR+ T cells: In healthy donor linearity study, 9 dilutions of CAR+ T cells were spiked into one million healthy donor PBMCs and stained. This data is representative of 25K CAR+ T cell spike. The gating of the CAR+ T-cells is first in the gating strategy and subsequently gating of the CAR+ T cells within the immune cell subsets increased the efficiency & recovery of identifying CAR+ T cells.

(B.) Original Gating Strategy for CAR+ T cells: In the same healthy donor sample, spiked 25K CAR+ T cells the viable immune cell subsets are gated first and then the CAR-T+ cells are gated after the monocyte and CD3 exclusion. This does yield CAR+ T cells however in Figure 2A the efficiency is lower compared to the gating strategy described above in Fig. 1A.

(C) CAR+ T cells Pre & Post Infusion: Data here is representative of one donor, the CAR-T cells are gated on their activation/exhaustion and T cell memory status by evaluating these markers (CD28, CD25, LAG3, TIM3, Ki67, and CD45RA & CCR7), pre-infusion and one month post infusion. The frequency data of each markers is provided in the table.

Conclusion

Static gating approaches fail to fully capture the biological variability of post-infusion CAR-positive cells. An adaptive gating strategy improves the accuracy and robustness of CAR cell enumeration and immune profiling, strengthening the assessment of CAR persistence and its relationship to clinical response.

Precision would like to extend our sincere appreciation to the entire Flow Cytometry team in our Frederick, MD laboratory and to Precision's Biomaterials team for their continued partnership and for providing high-quality healthy and disease-state PBMCs which makes these studies possible.

Fig. 2A Linearity Study: CAR+ T cell Recovery using Adaptive Gating

Spiked CAR+ T cells	Original Gating Recovered CAR	Adapted Gating Recovered CAR	(%) Efficiency Original Gating	(%) Efficiency Adapted Gating	(Δ %) Improvement	(Fold) Improvement
0	1	0	-	-	-	-
11	5	6	45.5	54.6	9.1	1.20
34	9	10	26.5	29.4	2.9	1.11
103	28	33	27.2	32.0	4.9	1.18
309	87	108	28.2	35.0	6.8	1.24
926	191	254	20.6	27.4	6.8	1.33
3,700	526	756	14.2	20.4	6.2	1.44
8,300	1,716	2,300	20.7	27.7	7.0	1.34
25,000	5,031	6,502	20.1	26.0	5.9	1.29
75,000	15,022	18,809	20.0	25.1	5.1	1.25

Fig. 2B Recovery Efficiency: Original vs. Adaptive Gating

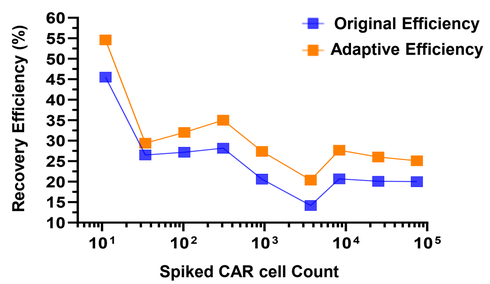


Figure 2. Analysis of Improved CAR cell Recovery

(A.) CAR+ T cell Recovery using Adaptive Gating: Recovery efficiency (percent of recovered relative to spiked CAR cells) is shown for original and adapted gating across a range of CAR cell inputs. Adapted gating consistently improves CAR cell recovery efficiency across the full dynamic range, increasing average efficiency from ~24.8% to ~30.9% (average Δ = +6.1 percentage points; average fold improvement = 1.26×). False-positive recovery at zero input is eliminated. Largest absolute improvement at low input (11 cells, Δ = +9.1%).

(B.) Recovery Efficiency Original vs. Adaptive Gating: The plot displays recovery efficiency (percentage recovered relative to spiked CAR count) for both original and adjusted gating methods across a range of spiked CAR cell counts. The data points show that adjusted gating consistently yields improved recovery efficiency compared to original gating. The highest improvement in difference occurs at a low input of 11 cells with 54.6% efficiency, a 20% increase. The graph uses a logarithmic scale on the x-axis for clarity across several orders of magnitude of spiked cell counts.

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