# Design of biomarker multiplex assays to support research on CAR-T cell efficacy, safety, persistence and exhaustion

White Paper

## Background

Protein biomarker assays are well-established tools for interrogating biological responses to treatment. Modulation of context-relevant biomarkers allows researchers to gain insights on treatment-relevant dynamics. Herein, we discuss the development of four multiplex biomarker assays designed to monitor critical attributes associated with chimeric antigen receptor T cells (CAR-T). These U-PLEX® Combos include markers useful in research regarding efficacy, safety, persistence and exhaustion in cultured cell models and biofluids.

Engineered cellular therapies such as CAR-T have revolutionized the management of hematological malignancies and solid tumors. Multiple CAR-T therapies (including Kymriah, Yescarta, Breyanzi, Carteyva, and Tecartus) were approved by the FDA between 2017 and 2023. Patients treated with CAR-T often experience remarkable, relapse-free survival. These immune cell therapies demonstrate prolonged CAR-T cell persistence, enhanced CAR-T effector function, limited T cell exhaustion, and good safety profiles in many responding patients. The most common adverse reactions in patients receiving CAR-T therapy are cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS).[1-5] These adverse events may lead to patients being treated with highly immunosuppressive corticosteroids to mitigate effects, hospitalization, treatment discontinuation, and in rare instances, death.

**Table 1.** Definitions of CAR-T attributes.

| Term | Definition |
|---|---|
| **Efficacy** | CAR-T cell engagement, activation, and subsequent lysis of tumor cells |
| **Safety** | Avoidance of immune-related adverse events such as cytokine release syndrome and immune effector cell-associated neurotoxicity syndrome |
| **Persistence** | Proliferation, expansion, or presence of CAR-T cells |
| **Exhaustion** | Chronic antigen stimulation of T cells resulting in upregulation of co-inhibitory molecules |

CAR-T therapies must be optimized for persistence and efficacy while minimizing T cell exhaustion and adverse events (AE) that can result from excessive immune system stimulation (Figure 1). Recent studies have identified biomarkers associated with all of these key attributes. For example, the persistence of CAR-T cells is required to promote effective anti-tumor responses and lasting remission, and can be monitored by measuring cytokines such as IL-7, IL-15, IL-18, and IFN-γ. These markers correlate well with the number of CAR-T cells present in circulation.[4,6-8] CAR-T persistence is associated with a durable increase in T cell activity and secretion of proteins including IFN-γ, Perforin, and Granzyme B.[6-8]

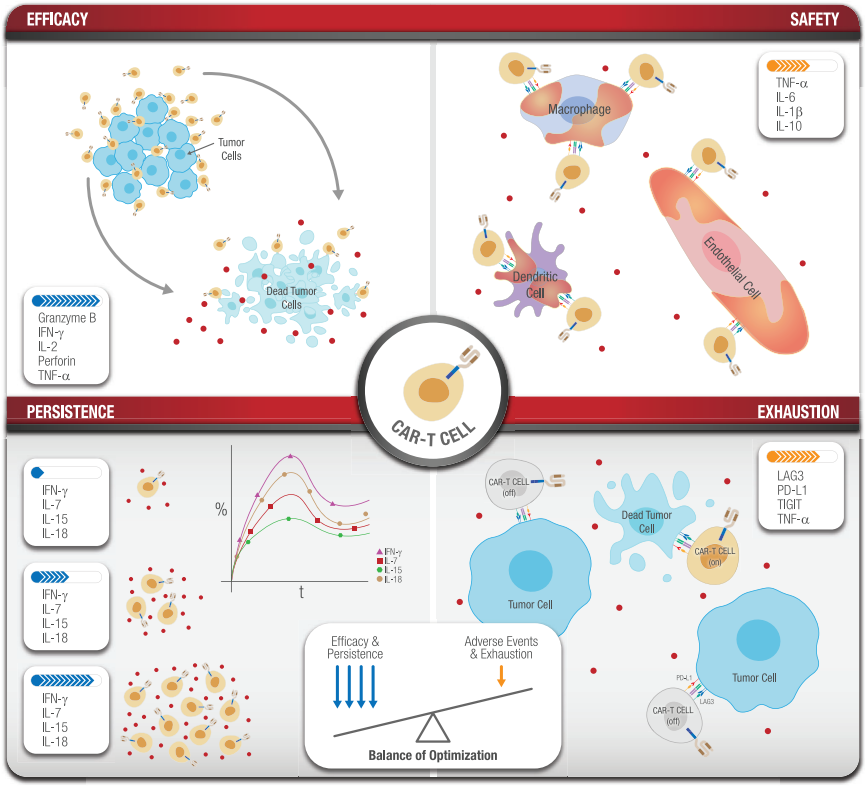


**Figure 1.** Efficacy biomarkers are surrogate indicators of tumor lysis and T cell function. Safety biomarkers are surrogates of AEs of CAR-T, such as CRS, ICANS, or on-target/off-tumor effects. Persistence and exhaustion biomarkers provide surrogate measures of T cell activity.

In parallel with efficacy, safety must also be tuned. Several markers associated with pro-inflammatory responses, including IL-6, IL-1β, and MCP-1 are commonly secreted following CAR-T treatment in subjects with Grade 2 or Grade 3 AEs or greater tumor burden.[8-10] A balance between the safety and efficacy of CAR-T therapy is desirable and specific for each patient. In a retrospective phase 2 study by Neelapu and colleagues[9], a biomarker signature associated with CRS and ICANS in patients dosed with axi-cel (Yescarta) was found. Specifically, peak levels of cytokines such as IL-6, GM-CSF, IFN-γ, and others were detected in the serum of patients within 8 days of dosing. The presence and magnitude were at least two times greater in patients who experienced AEs ≥ Grade 3 than those who did not. Interestingly, this study demonstrated an overall complete response rate of 78%, indicating that CAR-T therapy removed the tumor burden despite most patients experiencing AEs such as CRS. These findings indicate that efficacy and safety may be counterparts in determining response rates in current CAR-T clinical trials.

Lastly, T cell exhaustion limits the expansion, persistence, and efficacy of CAR-T products. Surface and shed biomarkers such as CTLA-4, PD-L1, and TIGIT are common inhibitory signals linked to the exhaustion of T cells following chronic antigen stimulation.[11] Patients in clinical trials with upregulation of these biomarkers typically relapse.[12] Deletion of the extracellular domain of surface markers such as PD-L1 or CTLA-4 on CAR-T cells has limited the exhaustion profile in preclinical models with increased persistence and expansion of CAR-T and improved efficacy,[13] indicating that monitoring and appropriately modifying the markers associated with exhaustion may improve clinical outcomes.

## Product Design and Characterization

Four U-PLEX Combos (multiplex assays) were designed to support research regarding the functions and mechanisms associated with CAR-T cell therapies. The sensitivity and specificity of individual U-PLEX assays are characterized using assay-specific reagents as well as serum, plasma, and cell culture supernatant samples. The four Combos noted in Table 2 combine multiple assays for translationally relevant biomarkers. Like all Combos, these assays were characterized for non-specific interactions between each assay at the component level. Non-specific binding (NSB) for each assay was calculated as a percentage of non-specific signal to specific signal. The observed NSB was less than 0.2% for all assays across all Combos, with the exception of CD28 and LAG3 in the CAR-T Exhaustion Combo. That pair-wise %NSB was 2.27%.

For further characterization, peripheral blood mononuclear cells (PBMC) from a healthy donor were cultured and stimulated using various conditions for 24-72 hours. Cell culture supernatants were collected and the Efficacy (Fig. 2A), Persistence (Fig. 2B), and Exhaustion (Fig. 2C) Combos were evaluated for their functional readouts. Stimulation with various Toll-like receptor (TLR) and T cell agonists resulted in the upregulation of interferon and checkpoint molecule secretion from immune cells over multiple logs of concentration range. These data emphasize the sensitivity and dynamic range integral to MSD's U-PLEX platform.

Potential research applications for these Combos include:

- Examination of CAR-T cell target-specific engagement and tumor cell killing in relevant human cell culture models.
- Elucidation of persistence and exhaustion biomarker profiles of CAR-T cell products in development.
- Linking exploratory biomarker profiles to adverse event findings.
- Rapid measurement of biomarkers commonly associated with efficacy in CAR-T therapy in relevant humanized animal models and translational studies.
- Justification of lymphodepletion regimens in pre-clinical applications.

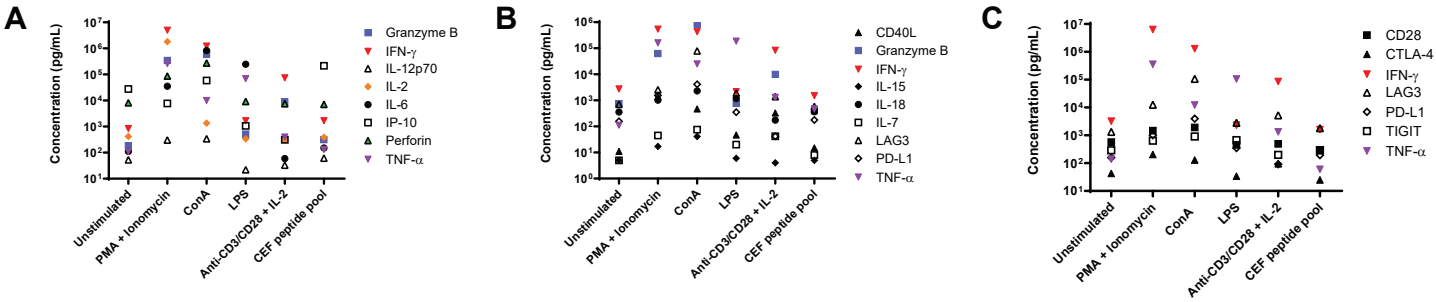


**Figure 2.** The concentration of analytes in the Efficacy (A), Persistence (B), and Exhaustion (C) U-PLEX Combos were tested from cell culture supernatants that were diluted 40-fold. PBMCs from an individual donor were cultured and stimulated under various conditions for 24-72 hours. Concentrations of each analyte are reported for each stimulation. (PMA = phorbol-12-myristate-13-acetate; LPS = lipopolysaccharide; IL = interleukin; ConA = Concanavalin A; CEF = cytomegalovirus (C), Epstein-Barr virus (E), and influenza virus (F)).

## Conclusion

These assays are designed to simultaneously measure multiple biomarkers relevant to CAR-T cell research and development. They perform robustly with minimal non-specific binding with broad dynamic range and sensitivity.

**Table 2.** List of biomarkers in U-PLEX CAR-T cell Combos and their relevance to CAR-T cell research.

| Panel | Assay | Description |
|---|---|---|
| **Persistence** | CD40L | Co-stimulatory molecule to prime and expand CAR-T cells |
| | Granzyme B | Effector molecules secreted by T cells upon target engagement, induces apoptosis in target cells |
| | IFN-γ | Secreted by proliferating CAR-T cells |
| | IL-7 | Promotes CAR-T cell survival through up-regulation of anti-apoptotic genes |
| | IL-15 | Regulates CAR-T cell effector function |
| | IL-18 | Enhances the proliferative capacity of CAR-T cells |
| | LAG3 | Inhibitory ligand resulting in reduced CAR-T cell activation and regulation of immune cell homeostasis |
| | PD-L1 | Promotes T cell exhaustion and self-tolerance |
| | TNF-α | Pro-inflammatory cytokine secreted by numerous cells upon inflammatory stimuli, promoting activation and proliferation of naïve and effector CAR-T cells |
| **Efficacy** | Granzyme B | Effector molecule secreted by T cells upon target engagement, induces apoptosis in target cells |
| | IFN-γ | Secreted by proliferating CAR-T cells |
| | IL-2 | Promotes CAR-T cell proliferation and effector function |
| | IL-6 | Pro-inflammatory cytokine secreted by macrophages in response to microbial molecules following engagement with T cells |
| | IL-12p70 | Stimulates CAR-T cells to improve cytotoxic capacity |
| | IP-10 | Stimulates chemotaxis of CAR-T cells to tumors |
| | Perforin | Pore-forming cytolytic protein expressed in granules of cytotoxic CAR-T cells |
| | TNF-α | Pro-inflammatory cytokine secreted by numerous cells upon inflammatory stimuli promoting activation and proliferation of naïve and effector CAR-T cells |
| **Exhaustion** | CD28 | Activates CAR-T cells by increasing their sensitivity to the antigen of interest |
| | CTLA-4 | Inhibits CAR-T activation and upregulates co-inhibitory molecules |
| | IFN-γ | Secreted by proliferating CAR-T cells |
| | LAG3 | Inhibitory ligand resulting in reduced CAR-T cell activation and regulation of immune cell homeostasis |
| | PD-L1 | Promotes CAR-T cell exhaustion and self-tolerance |
| | TIGIT | Co-inhibitory ligand for T cells impacting CAR-T function |
| | TNF-α | Pro-inflammatory cytokine sectored by numerous cells upon inflammatory stimuli promoting activation and proliferation of naïve and effector CAR-T cells |
| **Safety** | GM-CSF | Affects myeloid cell proliferation and activation, activates CD4+ T cells |
| | IFN-γ | Secreted by proliferating CAR-T cells |
| | IL-1β | Pro-inflammatory cytokine that is secreted following CAR-T engagement with endothelial cells |
| | IL-6 | Pro-inflammatory cytokine secreted by macrophages in response to stimulus following engagement with CAR-T cells |
| | IL-10 | Inhibits tumor-specific function while upregulating pro-inflammatory effects following on-target, off-tumor CAR-T cell engagement |
| | MCP-1 | Enhances macrophage responses to pro-inflammatory stimuli following CAR-T cell engagement |
| | TNF-α | Pro-inflammatory cytokine secreted by numerous cells upon inflammatory stimuli promoting activation and proliferation of naïve and effector CAR-T cells |

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