Multiplexed Cytokine Assessment of T- and NK-cell Responses to Tumor Cells: Parallel Assessment of Biomarkers Associated with Efficacy, Safety, and Persistence

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1 Abstract

Engineered immune effector cell (IEC) therapies such as chimeric antigen receptor T cells (CAR T) have revolutionized the management of hematological malignancies and solid tumors. Those treated with CAR T often experience durable relapse-free survival. These IEC therapies demonstrate persistence, enhanced effector function, limited T cell exhaustion, and favorable safety profiles. Rational design of cellular immunotherapies must optimize for persistence and efficacy of the treatment while minimizing cellular exhaustion and adverse events that can result from excessive immune stimulation. Presently, limited human-relevant assays and tools exist to evaluate a cellular immunotherapy's mode of action in parallel with any potential safety liabilities. We thus created protein-based, translationally relevant, multiplexed immunoassays to rapidly assess the potential efficacy, persistence and safety of CAR T or CAR natural killer (NK) cellular immunotherapies using *in vitro* co-culture assays. To evaluate the translationally-relevant biomarkers of cellular immunotherapy associated with efficacy, persistence and safety, we first established a co-culture model using TALL-104 cells, NK-92 cells, or Jurkat cells (effector cells) with various target cell lines associated with solid tumors (breast: MCF-7; prostate: PC3; glioma: U87MG), hematological malignancies (CML: K562), and normal healthy cells (HEK293). TALL-104 cells were used as an allogeneic T cell that has previously demonstrated lytic capabilities to numerous cell types, including healthy cells due to non-restricted human leukocyte antigen (HLA) interactions, and NK-92 cells were used as an allogeneic NK cell line that spares select cell types due to selectivity for regulated killer-cell immunoglobulin-like receptor (KIR) ligands and receptors. We co-cultured the effector cells with the target cells at various effector:target (E:T) ratios for 24 hours.

Following co-incubation, we collected cell culture supernatants and cell lysates. Translationally-relevant biomarkers of efficacy, persistence, and safety were evaluated in multiplex using the MESO® SECTOR S 600MM instrument. The presence and concentration of multiple candidate biomarker proteins were assessed in multiplex using MESO SCALE DISCOVERY® U-PLEX CAR-T Combos related to (1) immune activation and cytotoxicity ("CAR-T Cell Efficacy Combo 1"; IFNγ, Granzyme B, IL-2, IL-12p70, Perforin), (2) persistence of cellular immunotherapy ("CAR-T Cell Persistence Combo 1"; IL-7, IL-15, IL-18, CD40L), and (3) cytokine release syndrome or immune effector cell-associated neurotoxicity syndrome ("CAR-T Cell Safety Combo 1"; IL-1β, IL-10, IL-6, MCP-1). Target cell death (via cleaved caspase 3) and total cell viability were also assessed. We found differing magnitudes of safety, persistence, and efficacy biomarkers that were effector cell-specific. Biomarkers from the CAR-T Cell Safety Combo such as MCP-1, IL-10 and IL-6 were significantly elevated in co-cultures of HEK293 with NK-92 cells compared to TALL-104. This may be due to the low density of KIR ligands and receptors on HEK293 versus MHC Class I. Moreover, we observed a significant increase in the cytotoxicity of HEK293 when co-cultured with NK-92 from increased levels of Cleaved Caspase 3 and percent cell lysis compared to TALL-104. Secreted biomarkers associated with efficacy and persistence (e.g. IFNγ, Granzyme B, IL-7, IL-15) were also detected in co-cultures of HEK293 with NK-92, further indicating that the NK-92 cells facilitate a cytolytic mechanism and persist due to innate cell engagement with target HEK293 cells. In the solid and blood tumor types, efficacy and persistence biomarkers were dependent on the E:T ratio of the co-culture. Namely, levels of Granzyme B, IFNγ, IL-2, IL-7, IL-15, and IL-18 were elevated based on the number of effector cells present with tumor cell lines. This indicates that HLA or KIR ligand presence and avidity facilitate efficacy and persistence programs. Safety biomarkers were also elevated in co-culture conditions, but this is expected as death of tumor cells via cytolysis increases pro-inflammatory markers like IL-10, IL-1β, MCP-1, and IL-6. Thus, the translationally-relevant protein biomarker assays were able to help inform the functional activity of our co-culture models.

2 Methods

Co-culture mode set-up, optimization, and contrived sample testing

For co-culture model set-up, effector cells (TALL-104, NK-92, Jurkat) and target cells (MCF-7, PC3, U87MG, K562, HEK293) were cultured at various densities and effector-to-target (E:T) ratios in serum-depleted basal media conditions for 24 hours. The cell culture supernatant was collected to assess cellular viability using an ATP-based assay with glow-type luminescent signal (CellTiter-Glo, Promega Corp). The assay was optimized from conditions established using the CellTiter-Glo assay for density and E:T ratio.

Three E:T ratios were selected for testing and functional assay characterization using harvested cell culture lysates and supernatants using CellTiter-Glo as well as Meso Scale Discovery's assays for the following targets:

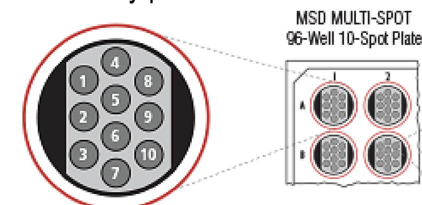
Matrix	Cell Lysate		Cell Supernatant		
Biology	Apoptosis	Proliferation	CAR-T Efficacy	CAR-T Safety	CAR-T Persistence
Biomarkers	Total Caspase 3 Cleaved Caspase 3	KI-67	IFNγ Granzyme B IL-2 IL-12p70 TNFα Perforin IL-6 IP-10	GM-CSF IFNγ IL-1β IL-6 IL-10 MCP-1 TNFα	CD40L Granzyme B IFNγ IL-7 IL-15 IL-18 LAG3 PD-L1 TNFα

Electrochemiluminescence (ECL) Technology

MSD's ECL detection technology uses SULFO-TAG™ labels that emit light upon electrochemical stimulation initiated at the electrode surfaces of MULTI-ARRAY® microplates.

- **High sensitivity:** Multiple rounds of label excitation and emission enhance light levels and improve sensitivity.
- **Broad dynamic range:** High and low abundance analytes can be measured without multiple sample dilutions.
- **Low background:** Electronic stimulation of detection label is decoupled from the output signal (light) resulting in extremely low background

Samples were assayed in biological triplicate. For cell lysate samples, the triplicates were pooled and assayed at 2.5 µg protein/well and assayed in technical duplicate. For cell culture supernatants, collected supernatant was diluted 10 to 40-fold in assay diluent and 25 µL of sample was added to the assay plate.

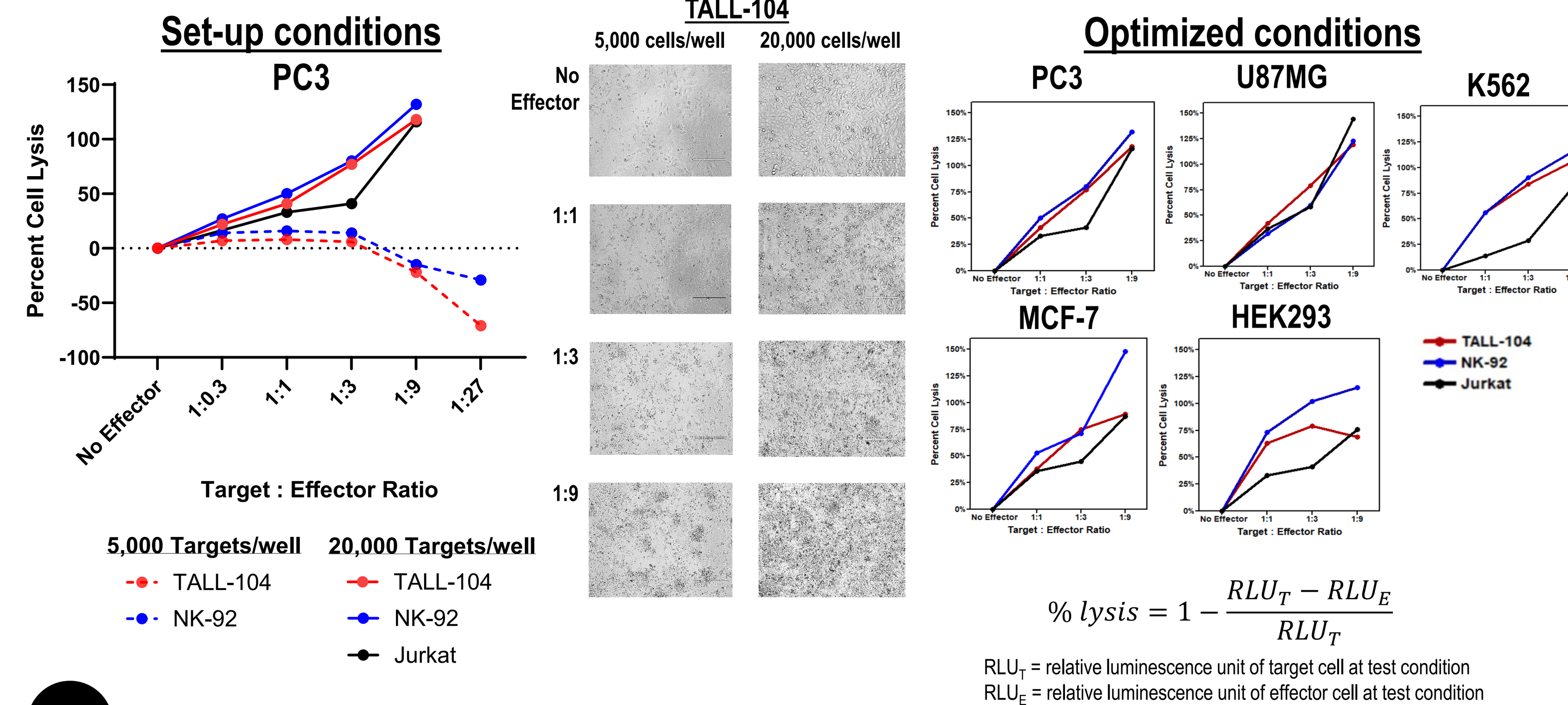


Multiplex U-PLEX® CAR-T Combos (Multiplex Assays)

Biotinylated capture antibodies are each coupled to U-PLEX Linkers, which self-assemble onto unique array elements (or "spots") on the U-PLEX plate. Leveraging U-PLEX's multiplexing platform conserves valuable samples by allowing up to ten determinations per well.

3 Co-culture set-up and optimization

The co-culture assays were optimized at multiple cell densities and E:T ratios using the CellTiter-Glo assay and visual inspection. We found the operating range of the co-culture assay to be optimal when seeding 20,000 target cells per well and between a 0.3-9:1 E:T ratio for the assay. All effector cell types tested impacted viability of the target cell in the co-culture model in a dose-dependent manner at the highest E:T ratio ranging between 69%-148% percent cell lysis after 24 hours co-culture.

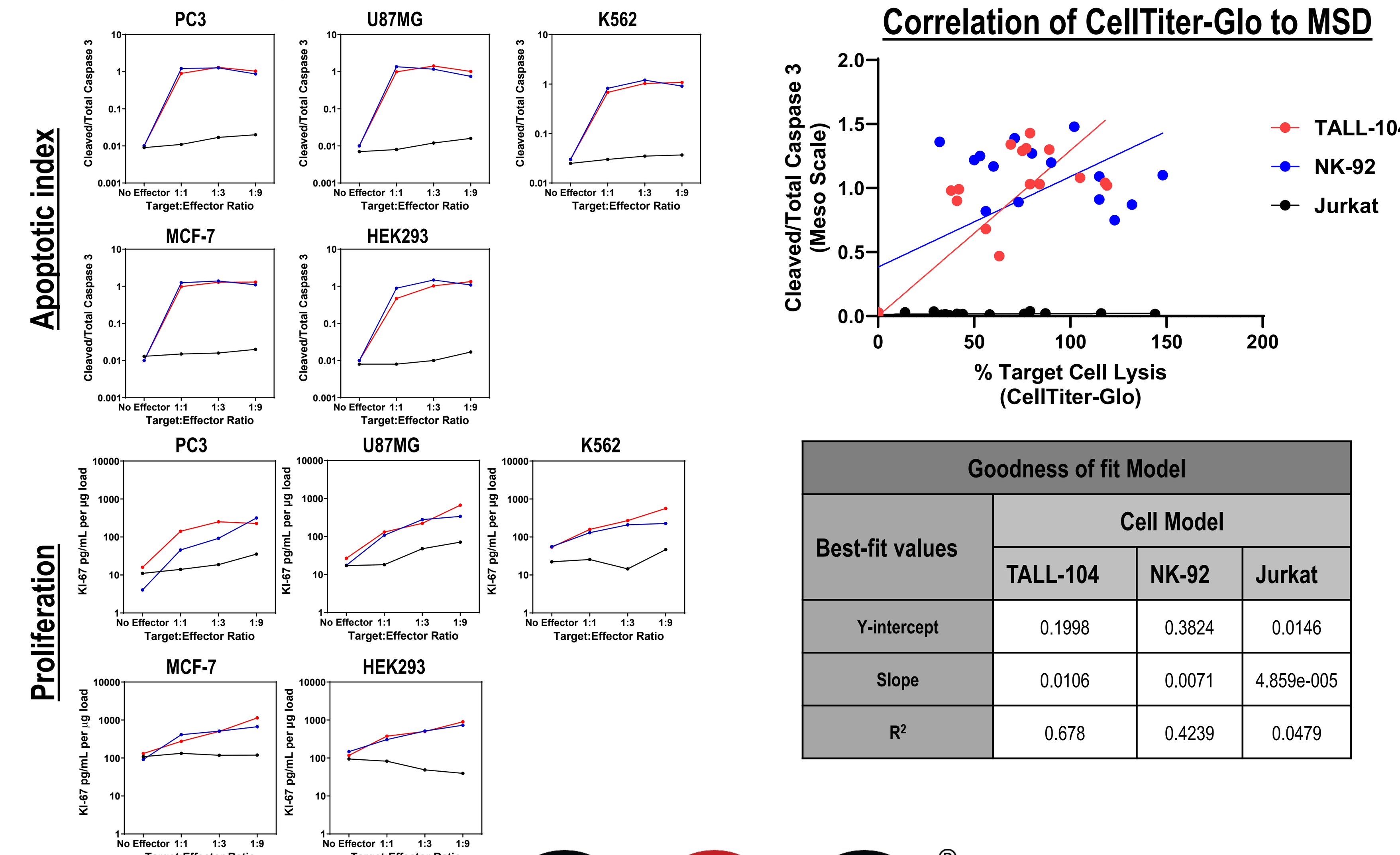


$$\% \; lysis = 1 - \frac{RLU_T - RLU_E}{RLU_T}$$

RLU_T = relative luminescence unit of target cell at test condition
RLU_E = relative luminescence unit of effector cell at test condition

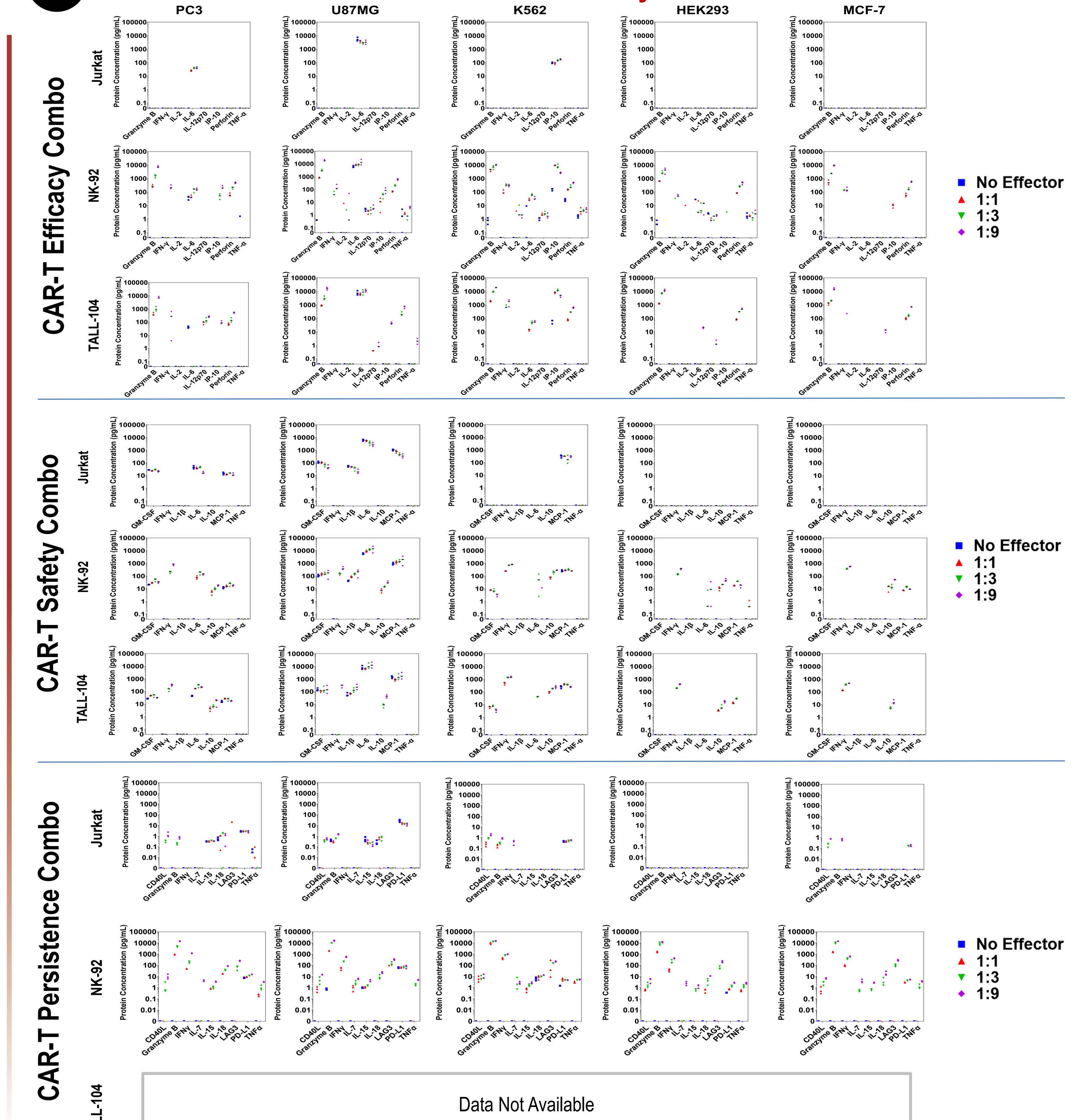
4 Apoptotic index, KI-67, and CellTiter-Glo correlation

Whole cell lysate was collected from co-cultured conditions using Trypsin-EDTA for adherent cell lines and centrifugation. For suspension cells, co-cultures were centrifuged. Ice cold lysis buffer was added to cell lysates to extract intracellular protein. 2.5 µg protein per well from test conditions was assayed for cleaved and total caspase 3 and for KI-67. Dose-dependent responses in apoptotic cell death, expressed as the apoptotic index (ratio of cleaved to total caspase 3), were observed for TALL-104 for Jurkat cells. Proliferation of effector cells, measured by KI-67 levels, also increased in a dose-dependent fashion indicating that target engagement activated TALL-104 and NK-92 cell proliferation. Some correlation was observed between the CellTiter-Glo results and MSD's apoptosis assay suggesting that it is critical to monitor cell death in cellular immunotherapy assays. Surprisingly, CellTiter-Glo predicted cell death of numerous target cell types co-cultured with Jurkat cells whereas the Meso Scale assays did not, as demonstrated by an R^2 value < 0.05.



Goodness of fit Model			
Best-fit values	Cell Model		
	TALL-104	NK-92	Jurkat
Y-intercept	0.1998	0.3824	0.0146
Slope	0.0106	0.0071	4.859e-005
R^2	0.678	0.4239	0.0479

5 Functional assessment of translationally-relevant biomarkers: CAR-T Combos



Supernatant was collected from co-cultures and assayed using U-PLEX CAR T Efficacy, Safety, and Persistence Combos. Biomarkers elevated in co-cultures with Jurkat cells were target-cell line dependent with no dose-dependent changes observed. Co-cultures of Jurkat with HEK293 and MCF-7 had no impact on Efficacy, Safety, or Persistence cytokines or chemokines measured (below LLOD).

Target cell effects as well as dose-dependent effects were observed for TALL-104 and NK-92 cell types on biomarkers associated with Efficacy, Safety, and Persistence. With respect to efficacy, NK-92 cells induced secretion of at least 5 biomarkers (e.g., IFNγ, Granzyme B, Perforin, IL-12p70, and IP-10) in multiple co-culture models whereas TALL-104 commonly activated only 3 biomarkers (Granzyme B, IL-12p70 and Perforin) indicating different modes of action between NK-92 and TALL-104.

Similar observations are noted for CAR-T Safety where biomarkers associated with safety in co-cultures with Jurkat cells were target cell dependent. Interestingly, co-cultures of NK-92 and TALL-104 with HEK293 (assumed healthy) induced expression of IFNγ, IL-10, and MCP-1 indicating an inflammatory response which was unexpected for NK-92, but verifies the efficacy data. These results for TALL-104 and NK-92 were also dose-dependent.

Persistence of effector-cell function is demonstrated by significant increases in CD40L, IL-7, IL-15, and IL-18 when NK-92 are cultured with target cells. These data indicate that the cells are active and producing effector functions related to persistence and proliferation, confirming the KI-67 result.

7 Conclusions

- Cellular immunotherapy co-culture models are robust, reproducible and require multiple assays to down-select potential engineered candidates.
- The ratio of cleaved to total caspase 3 (apoptotic index) may be superior to other cell death assays in assessing a cellular immunotherapy's ability to kill target cells in co-culture settings.
- Dose-dependent effects of a cellular immunotherapy can be rapidly assessed using MSD's CAR-T Combos from *in vitro* screens related to efficacy, safety, and persistence.

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