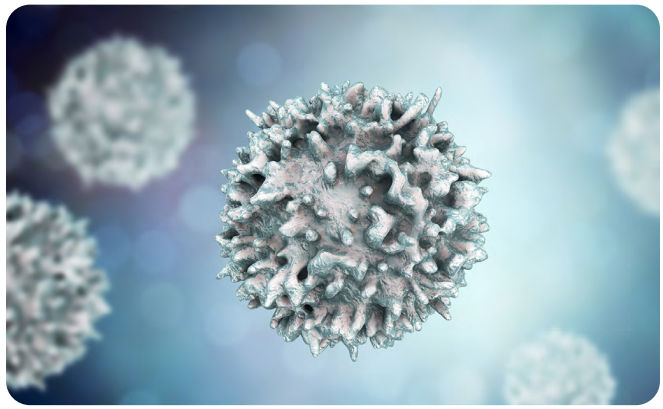
Review of cell-based methods to monitor CAR T activation and cell killing effectiveness

Introduction

Chimeric antigen receptor (CAR) T-cell therapy is at the forefront of adaptive immunotherapy efforts. CAR T-cells are engineered from T cells to specifically target tumor cells by promoting interactions on the cancer cell surface. Activation of CAR T-cells results in an increase in cell signaling from cytokines and chemokines produced by the CAR T-cells to aid in destruction of the targeted tumor cell. This review is aimed at demonstrating the use of different types of assays to gauge CAR T or T-cell activation and subsequent target tumor cell death.

The recent approval of CD19-specific CAR T therapies, Kymriah® (tisagenlecleucel) and Yescarta® (axicabtagene ciloleucel) for acute lymphoblastic leukemia (ALL) and non-Hodgkin's Lymphoma respectively, has opened the door for additional CAR T therapies with more diverse CAR T targets in the clinic. So far, the main successful targets have been blood malignancies which are easier to target than solid tumors which present a higher hurdle due to a more immunosuppressive microenvironment (Wang et al.). CAR T design includes an antigen binding site, hinge and spacer, and intracellular activation domains. First generation constructs expressed one intracellular signal domain for T-cell activation (CD3ζ) while third generation now commonly express additional co-stimulatory domains (CD28, or 4-1BB) (Androulla and Lefkothea).



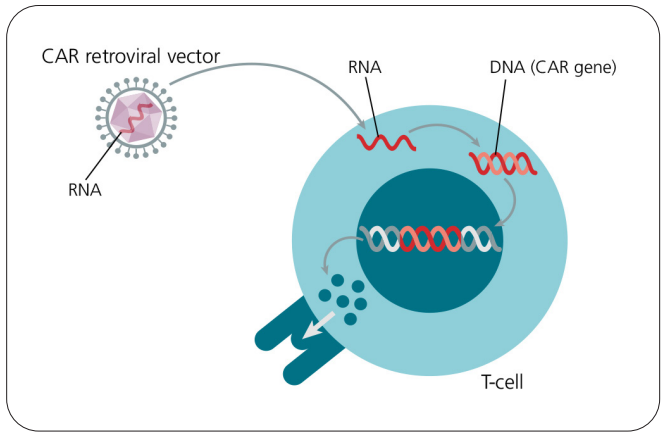


Figure 1. Retroviral transduction of the CAR gene in T cells to express a specific chimeric protein on their surface. This allows them to recognize cancer cells and activate to destroy those same cancer cells.

revvity

CAR-T cell activation

When CAR T-cells recognize the tumor antigen and bind to the tumor cell, a series of cell signaling events, including the release of cytokines, perforin and granzymes, leads to cell death in the tumor cells and surrounding stroma (Benmebarek et al.). The cytokines can include IFNγ, TNFα, IL-2 and IL-8. In co-culture *in vitro* model systems that mimic CAR T therapy these signaling molecules can easily be measured in the supernatant by methods such as AlphaLISA. Amplified luminescent proximity homogeneous assay (Alpha), is a no wash, bead-based assay format used to measure an analyte in cell supernatant or lysates as well as other complex matrices (serum, plasma, urine etc.). Figure 2 depicts the general AlphaLISA principle.

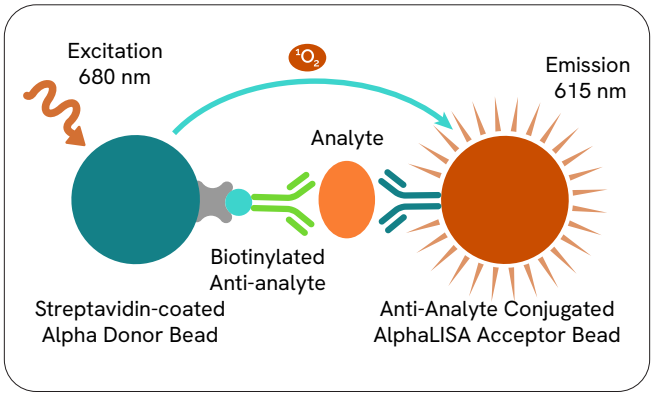


Figure 2. AlphaLISA Assay Principle. A biotinylated anti-analyte antibody is bound by the Streptavidin-coated AlphaLISA Donor beads, while another anti-analyte antibody is conjugated to AlphaLISA Acceptor beads. In the presence of analyte, the donor and acceptor beads come into proximity. Excitation of the donor beads at 680 nm provokes the release of singlet oxygen molecules that trigger a cascade of energy transfer in the nearby acceptor beads, resulting in a sharp peak of light emission at 615 nm. AlphaLISA signal is proportional to the amount of analyte present in the sample.

Von Scheidt et al. used AlphaLISA to measure IFNγ production from CAR T-cells in a co-culture setting in the presence of a bispecific cMyc/CD40 antibody. The cMyc tag is present only in CAR-T cells and CD40 is expressed on number of antigen presenting cells (APCs) including subsets of dendritic cells, monocytes, macrophages and B cells. In the presence of CD40+ APCs *in vitro* they saw an increase in CAR T-cell proliferation as measured by flow cytometry and IFNγ release that was not present in the mock transduced control cells or triggered by the addition of a mixture of cMyc and CD40 antibodies alone (Figure 3).

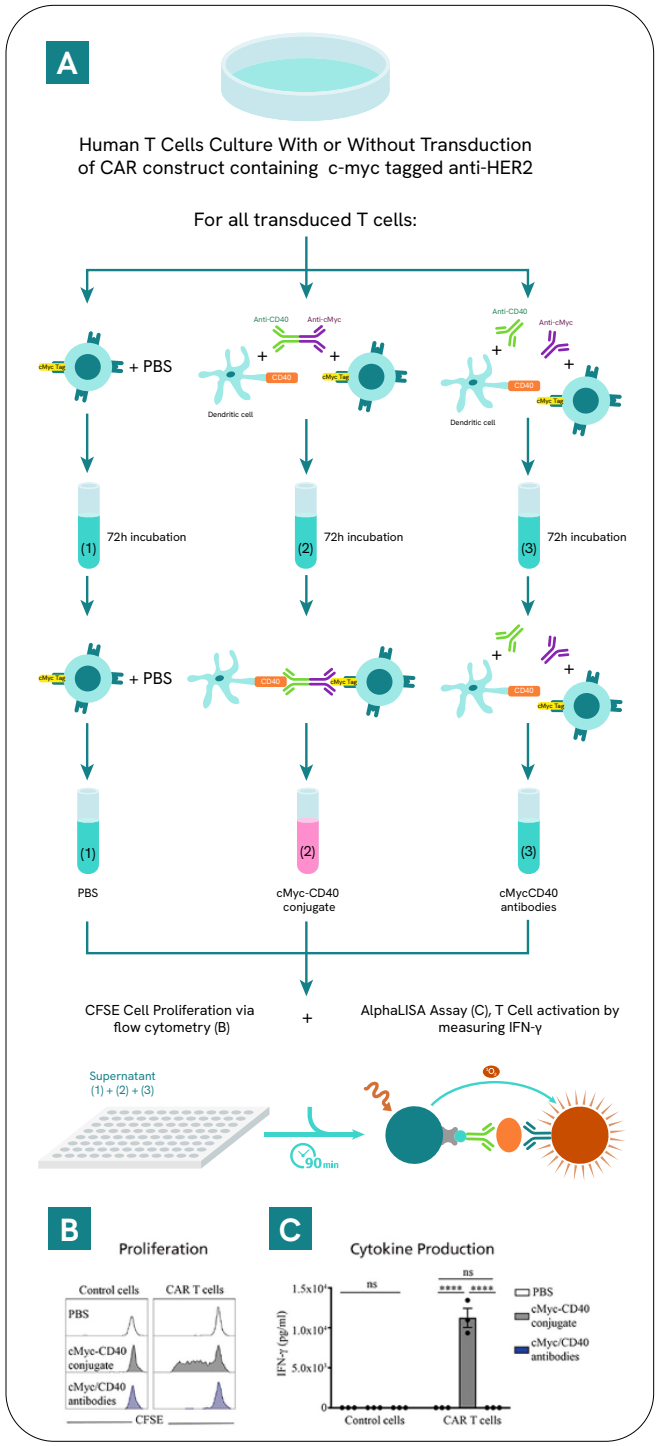


Figure 3. (A) The experimental method (B) Cell Proliferation via flow cytometry ; Carboxyfluorescein succinimidyl ester (CFSE) labelled CAR T-cell or empty vector transduced T-cells (control) were incubated with the anti-Human CD40/anti-cMyc tag-bispecific conjugate or unconjugated antibodies for 72 hours in the presence of monocyte-derived dendritic cells from the same donor. (C) Supernatant from the co-culture was analyzed for IFNγ using an AlphaLISA assay.

Copyright: von Scheidt, B. et al. Enterotoxins can support CAR T cells against solid tumors. PNAS 116:50 (2019).

Figure reproduced with permissions granted by PNAS.

CAR T phenotypic readout

In addition to using cytokine release to gauge CAR T-cell activity, measurements of specific cell lysis in the targeted tumor cell population demonstrate the cell killing activity of CAR T-cells. One method to measure target cell killing is by using a Europium labeling system. DELFIA EuTDA Cytotoxicity Reagents provides a safe and rapid way to label the target cell population with a BATDA reagent. Once inside the cell, intracellular esterases cleave the BATDA substrate to form TDA which can no longer cross the cell membrane barrier. Lysis of the labeled target cells release TDA in the supernatant which is then transferred to an assay plate and combined with a Europium solution to generate the fluorescent molecule EuTDA. Cell lysis leads to an increase of EuTDA and hence the increased signal correlates to the cell death of the labeled target cell population. Figure 4 shows the assay in the context of a CAR T co-culture experiment.

Du S-H et al. used the DELFIA EuTDA Cytotoxicity Reagents to compare multiple anti-CD19 CAR-T constructs by measuring specific lysis and cell death of the CD19 expressing target cell lines Raji and Daudi. Their work aimed to see the benefit of adding a CAR to a functional subset of T-cells called CIKZ by improving specificity and increasing cell killing. As seen in Figure 5, significant increased killing relative to the control Mock and GFP CAR-Ts is seen with almost all active CAR T constructs across both target cell types. This demonstrates the effectiveness of adding CAR T to T-cell subset of cells and improving cell killing capabilities.

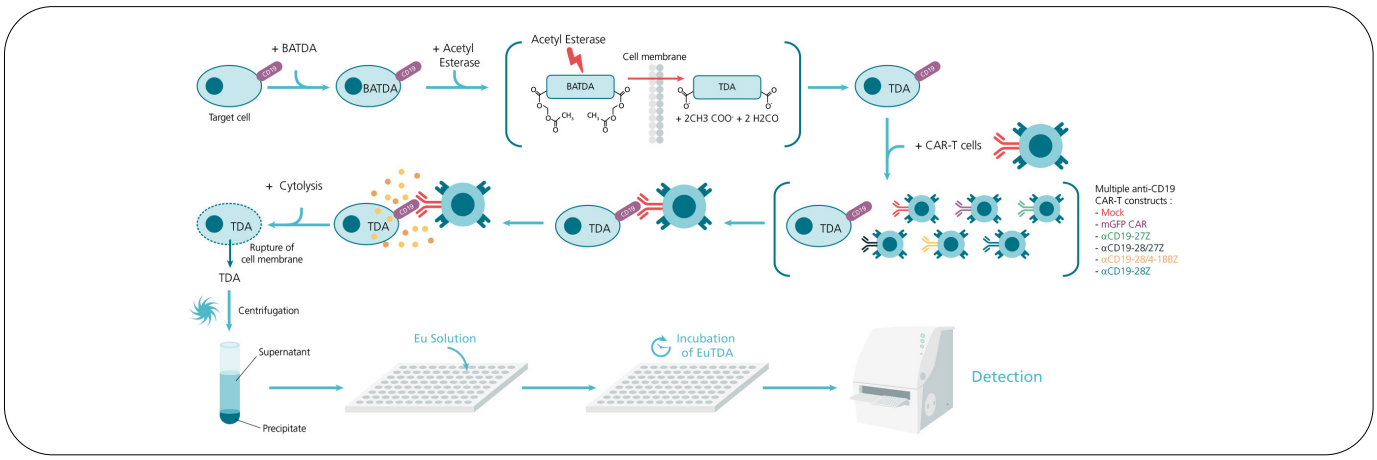


Figure 4. DELFIA EuTDA Cytotoxicity Assay in a CAR T Co-culture Format. After loading target cells with BATDA reagent the cells rapidly process the molecule into TDA and are then co-cultured with the selected CAR T-cell line. Activation of the CAR T-cells leads to cytokine release and cytolysis of the target cell line. TDA is then collected in the supernatant and mixed with a Europium solution to form EuTDA which is highly fluorescent. The final level of EuTDA is proportional to the amount of cell death from the target cell population. Maximum release control wells are used to calculate specific release and are established by using lysis buffer to fully lyse the target cells.

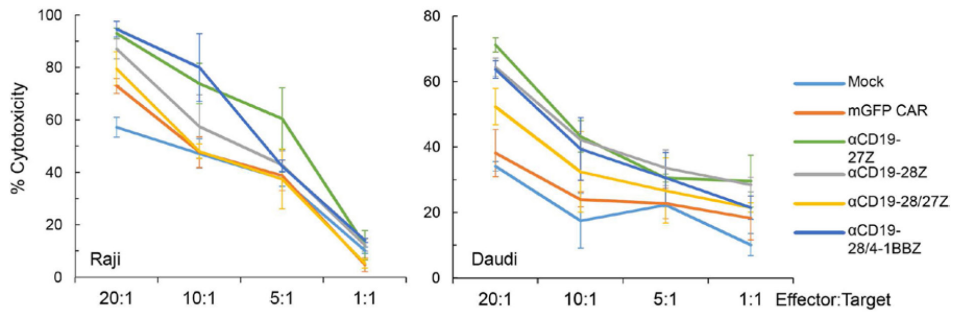


Figure 5. Tumor-targeting efficiencies and cytotoxicity of CIKZ cells modified with various anti-CD19 mRNA CARs. DELFIA EuTDA Cytotoxicity Reagents were used to measure cytolysis in target CD19 expressing cell lines Raji and Daudi. Percent cytotoxicity is shown as relative to the cell lysis buffer control (100% lysis).

Copyright: Du S-H, Li Z, Chen C, Tan W-K, Chi Z, Kwang TW, et al. (2016) Co-Expansion of Cytokine-Induced Killer Cells and Vγ9Vδ2 T Cells for CAR T Cell Therapy. PLoS ONE 11(9): e0161820.

Figure reproduced with permissions under a Creative Commons Attribution License.

Combination therapies

Another strategy for immunotherapies is the search for small molecules or biologics that enhance T-cell activity. A good example is checkpoint inhibitor molecules that target cytotoxic T lymphocyte antigen 4 (CTLA4) or the programmed cell death 1 (PD1) pathway. Checkpoint inhibitors target inhibitory receptors on T-cells and reinvigorate anti-tumor immune responses (Havel et al.). Dextras et al. used this approach to screen for small molecules that could boost T-Cell activity directed against melanoma by synergistically enabling T-Cell recognition of melanoma cells. They used homogeneous time-resolved fluorescence (HTRF) technology to screen for an increase in IFNγ as a marker for T-Cell activation. IFNγ was detected in a sandwich assay format using 2 different specific antibodies, one labeled with Europium Cryptate (donor) and the second with d2 (acceptor). When the labeled antibodies bind to the same antigen, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength (665 nm). The two antibodies bind to the IFNγ present in the sample, thereby generating FRET. Signal intensity is proportional to the number of antigen-antibody complexes formed and therefore to the IFNγ concentration (Figure 6).

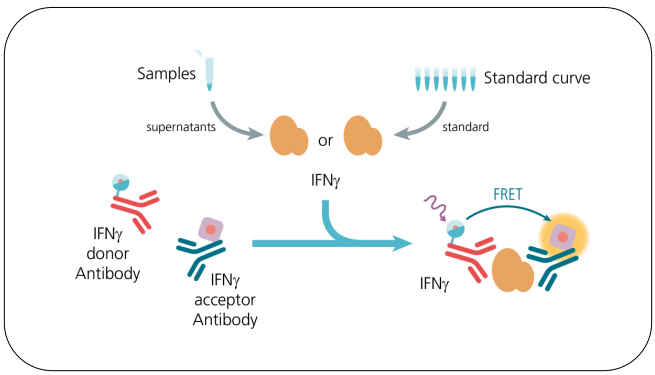


Figure 6. HTRF IFNγ Assay Principle. Samples of unknown concentration are compared to a standard curve for quantification of the level of IFNγ.

Dextras et al. used the IFNγ HTRF assay to screen a focused library of approximately 500 small molecules targeting a broad range of cellular mechanisms. Thirty six active molecules were found that increased IFNγ production. Screening workflow and hits are shown in Figure 7. These hits were tested in orthogonal assays such as cell proliferation and by qRT-PCR to reach a final set of 4 compounds that they felt warranted further investigation for patients with BRAF mutant melanoma resistant to immunotherapy.

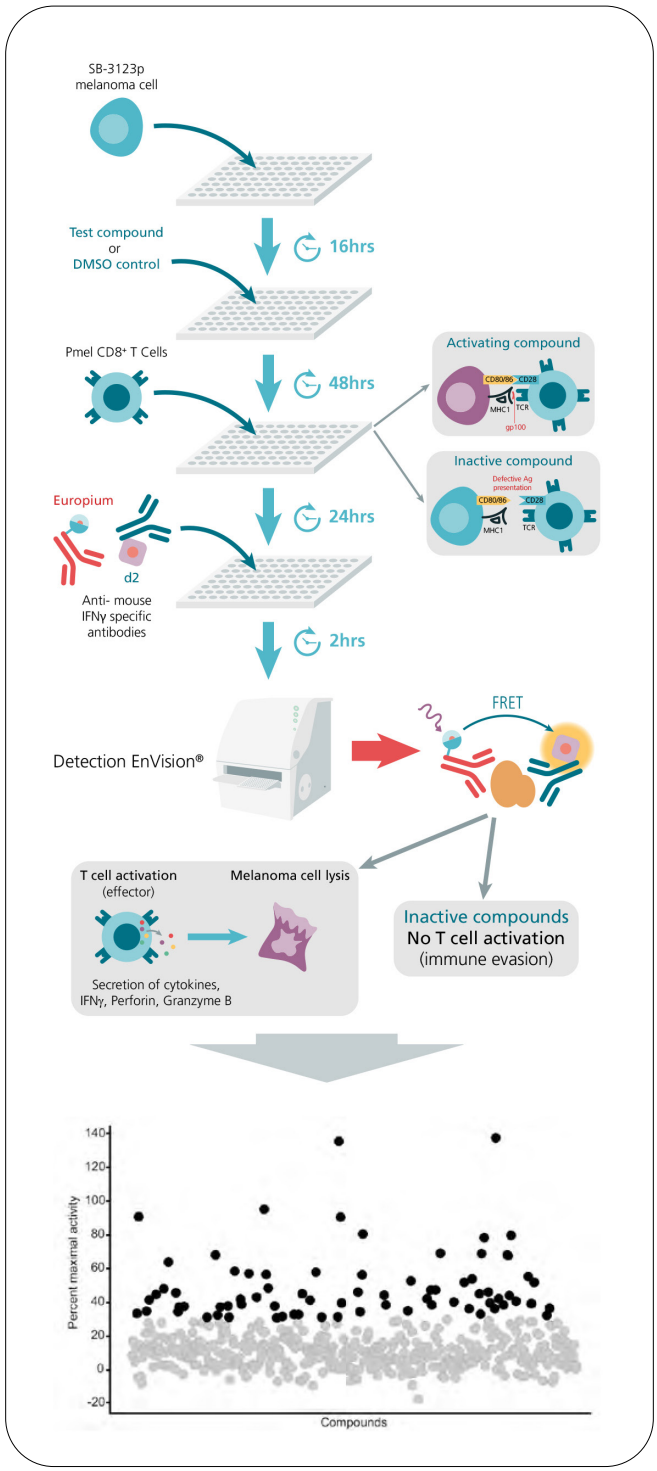


Figure 7. HTRF screen for small molecules increasing IFNγ production and release from Pmel CD8+ T-cells co-cultured with melanoma cells. A 1536-well HTS screen was performed with approximately 500 small molecules narrowed down to 36 hits from the primary HTRF screen.

Copyright: Dextras, C. et al. (2020). Identification of small molecule enhancers of immunotherapy for melanoma. Scientific Reports. 10:5688

Figure reproduced with permissions under a Creative Commons Attribution 4.0 International License.

Conclusion

Activation of CAR T or T-Cells in an *in vitro* setting can easily be monitored using homogeneous assay technologies such as AlphaLISA and HTRF. These methods are simple, sensitive, and robust for measuring cytokine molecules associated with T-cell activation while consuming minimal sample and providing faster time-to-results compared to ELISA assays. In addition, target cell death in a co-culture experiment can be easily measured using DELFIA EuTDA Cytotoxicity Reagents, by pre-labeling only the target cells and therefore excluding measurement of any cell death from the effector cells. DELFIA cytotoxicity reagents provide a bright and stable fluorescent alternative to more traditional chromium-51 release assays. The publications highlighted here demonstrate the capability of these assay technologies to aid in the discovery of cell-based immunotherapies.

References

Wang, Z. et al. (2017). Current status and perspectives of chimeric antigen receptor modified T cells for cancer treatment. Protein Cell, 8:896-925

Androulla and Lefkothea (2018). CAR T-cell Therapy: A New Era in Cancer Immunotherapy. Current Pharmaceutical Biotechnology, 19 p5-18

Benmebarek, M. et al. (2019). Killing Mechanisms of Chimeric Antigen Receptor (CAR) T Cells. International Journal of Molecular Sciences, 20:1283

Von Scheidt, B. et al. (2019) Enterotoxins can support CAR T cells against solid tumors. PNAS 116:50.

Du S-H, Li Z, Chen C, Tan W-K, Chi Z, Kwang TW, et al. (2016) Co-Expansion of Cytokine-Induced Killer Cells and Vγ9Vδ2 T Cells for CAR T Cell Therapy. PLoS ONE 11(9): e0161820.

Havel, J. et al. (2019) The evolving landscape of biomarkers for checkpoint inhibitor immunotherapy. Nat Rev Cancer 19(3): 133–150

Dextras, C. et al. (2020). Identification of small molecule enhancers of immunotherapy for melanoma. Scientific Reports. 10:5688

Copyright ©2023, Revvity, Inc. All rights reserved.