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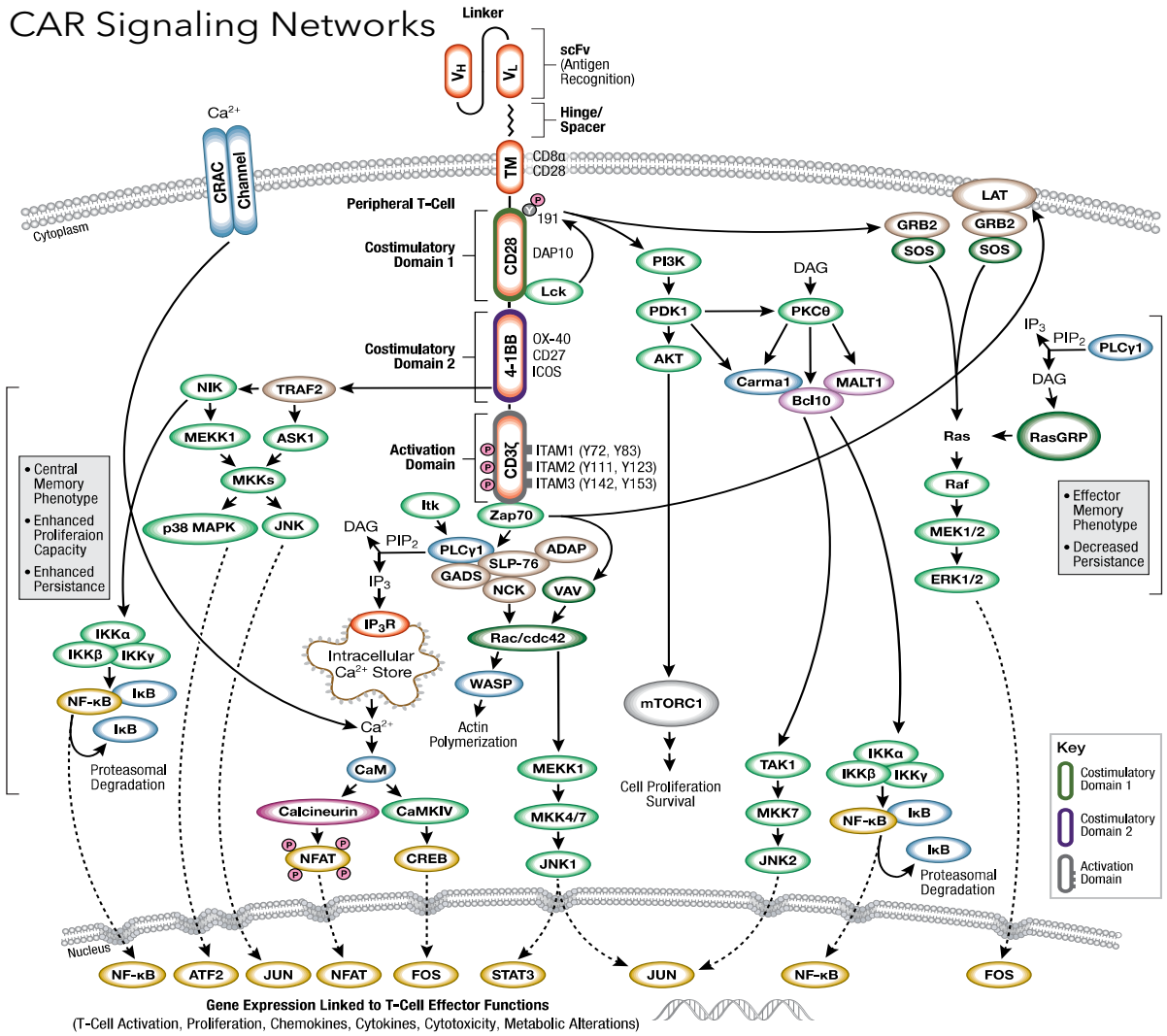
Application Note

Analytical Tools to Evaluate CAR-T Cell Signaling & Activation

Tools to Characterize CAR-T Cells, Both Inside & Out

Chimeric antigen receptor (CAR)-T cell therapy is a revolutionary new pillar in cancer treatment with success in treating specific subsets of B cell leukemia, lymphoma, and multiple myeloma.[1] Much attention is focused on expanding CAR-T cell therapy to other hematologic malignancies and solid tumors. Central to engineering effective CAR-T cell therapies is a detailed understanding of the signaling cascades that regulate CAR-T cells, including trafficking and tumor infiltration, preventing antigen escape, resisting immunosuppressive responses, and ameliorating potentially fatal toxicity.[1] Detailed characterization of extracellular, membrane-bound, and intracellular signaling molecules is required, often with limited and complex sample types.



Protein Analytics Uniquely Suited for Cell Therapy Manufacturing

There is high demand across the cell therapy industry for automated methods capable of generating analytical-grade results that meet regulatory standards, are easy to transfer, and are amenable to scale with manufacturing. In FDA's 2024 Guidance for Industry on the Development of CAR-T Cell Products, the FDA recommends that the functionality of the signaling domain of CAR constructs be demonstrated and, if the CAR-T cell includes a cytokine transgene to enhance the CAR activity, sponsors should assess the production of the transgenic cytokine in a potency assay. Further, identity testing is recommended to include expression of the CAR construct.[2]

Flow cytometry is a workhorse tool for CAR-T cell analysis, but phospho-flow cytometry—used to detect phospho-proteins involved in intracellular signaling cascades—can be challenging to perform and requires a large sample volume, typically 500 µL to 1 mL of sample per run. This problem is compounded by the lack of quality validated antibodies, particularly for CARs and CAR signaling molecules like phospho-protein isoforms. While traditional Western blots provide size-based specificity, Western blots are poorly reproducible and require at least 50 µg of protein for detection.

As a result, cell therapy manufacturers are turning to orthogonal protein analysis solutions for their workflows, like the automated Simple Plex™ platform for microfluidic ELISAs and the Simple Western™ platform for capillary electrophoresis immunoassays. Supported by the growing number of patent citations and research articles by leading cell therapy R&D groups,[3-49] Simple Plex™ and Simple Western™ are tailored to meet industry needs for robust protein characterization assays with several benefits critical to cell therapy manufacturing, including speed, scalable automation, reproducible quantification, sensitivity, and small sample requirements.

Flow cytometry assays centered on CAR detection and identity have traditionally relied on detecting specific sequences within the variable heavy and variable light domains or the hinge region of CARs. However, available reagents can lack specificity, be difficult to combine into multi-parameter flow panels, or only be able to detect a single CAR. Cell Signaling Technology (CST) has developed first-to-market reagents called Anti-CAR linker antibodies, which can identify and confirm CAR expression, regardless of its antigen specificity.

Here, we demonstrate a streamlined workflow to engineer, identify, and characterize CAR-T cell signaling and activation status, revealing the kinetics of CAR-T cell activation and signal transduction with reproducible quantification. Specifically, we leveraged Bio-Techne's non-viral gene editing TcBuster™ platform to edit human primary T cells with a CD19 CAR. Following activation of the CAR using CD19 protein, we implemented automated protein analytical solutions to characterize cytokine secretion using the microfluidic ELISA Simple Plex™ platform and intracellular signaling events using the capillary immunoassay Simple Western™ platform with high-quality antibodies from CST.

The results presented here include granular time course information on CAR activation and stimulation of downstream signaling events using only 1-2 µg of lysate per capillary with hands-free automation, which would be difficult, if not impossible, to achieve with traditional immunoassays. Combining flow cytometry and Simple Western analyses, we observed CAR-T cells downregulate CAR expression in less than 10 minutes following antigen engagement. However, CAR presence at the cell surface may linger for 15 minutes or longer. Furthermore, the Simple Western results indicate CAR expression increases again at 4 hours, suggesting a 'recycling' of the CAR in T cells.

We anticipate these solutions will become standard methods in CAR-T cell therapy development, with applications including, but not limited to, CAR activation, such mechanism of action studies, and monitoring tonic signaling, a pivotal event controlling CAR-T cell efficacy.

Materials and Methods

Creation of CD19 CAR-T Cells

We engineered primary human T cells using a non-viral gene engineering TcBuster™ system from Bio-Techne. We designed an anti-CD19 CAR using the FMC63-scFv with the Whitlow linker, a CD8α hinge and transmembrane domain, 41BB intracellular domain, and CD3 signaling domain. We transposed 10 x 10^6 cells per sample with a tricistronic construct containing the CAR, green fluorescent protein (GFP), and dihydrofolate reductase (DHFR) to provide methotrexate resistance for selection. Cells were selected with methotrexate treatment and expanded for a total of 11 days to ensure a complete resting state. Cells were cultured in the presence of animal-free recombinant human IL-7 (Bio-Techne, PN BT-007-AFL) and IL-15 proteins (Bio-Techne, PN BT-015-AFL) using a G-Rex 6 well plate (ScaleReady, PN 80240M).

CAR-T cells were stimulated by treatment with biotinylated recombinant human CD19-antigen (Bio-Techne, PN AVI9269) conjugated to Dynabeads™ M-280 Streptavidin. As a negative control, we performed an identical treatment by replacing the antigen with recombinant human HER2 (Bio-Techne, PN AVI1129), which is not expected to stimulate signaling pathways downstream of the CD19 CAR. Following treatment, we analyzed cells by flow cytometry at time points of 5, 10, 15, 30, 60, and 240 minutes to examine CAR expression using the Whitlow/218 Linker (E3U7Q) Rabbit mAb (Alexa Fluor® 647 Conjugate) (CST, PN 69310). At each time point, cell pellets were frozen (3-5 million cells per pellet) to examine intracellular signaling molecules by Simple Western™ analysis (described below).

We also collected the culture supernatant at 240 minutes post-activation to confirm induction by secreted cytokines via Simple Plex™ analysis (described below).

Simple Western Analysis

We used Simple Western™ capillary immunoassays with the Jess™ instrument (ProteinSimple, a Bio-Techne brand, PN 004-650) for intracellular signaling analysis. We used the 12-230 kDa Separation Module (Bio-Techne, PN SM-W004) and the RePlex™ Module (Bio-Techne, PN RP-001) for sequential immunoprobing of phospho-protein and total-protein isoforms in the 1st and 2nd probing cycle, respectively. Because all primary antibodies were of rabbit origin for intracellular protein targets, we used the Anti-Rabbit Detection Module (Bio-Techne, PN DM-001), producing a sensitive chemiluminescence readout. Compass for Simple Western software (6.3.0) automatically calculated chemiluminescent peak areas to quantify intracellular expression.

We used primary monoclonal antibodies from CST (TABLE 1) for specific detection of intracellular signaling targets.

Target	Specificity	PN	SW kDa	Dil.
Whitlow/218 Linker	Total	69310	56	1:10
ERK1/2	Phospho-T202/Y204	4370	45	1:50
	Total	4695	43, 47	1:50
GADS	Phospho-T262	78972	47	1:25
	Total	95848	47	1:10
SLP-76	Phospho-S376	76384	88	1:100
	Total	70896	88	1:50

TABLE 1. Antibodies used for Simple Western analysis. CST provided all antibodies. PN: Part Number. SW: Simple Western. Dil: Dilution.

Simple Plex Analysis

To measure CAR-T cell activation by cytokine secretion, we used Simple Plex™ microfluidic immunoassays with the Ella™ instrument (ProteinSimple, a Bio-Techne brand, PN 600-100). We assessed activation by measuring the secretion of cytokines in the supernatants of cell cultures collected at 4 hours post-activation using the multi-analyte Simple Plex Cell Activation Panel 1 (Bio-Techne, PN ST01C-CS-003222), a 32-sample cartridge with 4 separate channels containing assays for Granzyme B, IL-2, IFN-γ, and TNF-α.

Results

The Kinetics of CAR Trafficking Following Antigen Engagement

We engineered human primary T cells for CD19 CAR expression and activated CAR-T cells with CD19-conjugated beads, as described in the Materials and Methods. Our CAR design included the Whitlow/218 peptide linker, which affords the scFv with enhanced resistance to proteolysis and aggregation.[50] Our design also included constitutive GFP expression independent of CAR expression. Therefore, we monitored GFP and Whitlow/218 linker expression by flow cytometry in a time course following activation with CD19-conjugated beads (FIGURE 1). As negative controls, we included untreated (no beads) CAR(+) cells and CAR(+) cells treated with HER2-conjugated beads, which were not expected to activate our CAR-T cells. We also treated unmodified CAR(-) T cells with CD19 beads as a negative control.

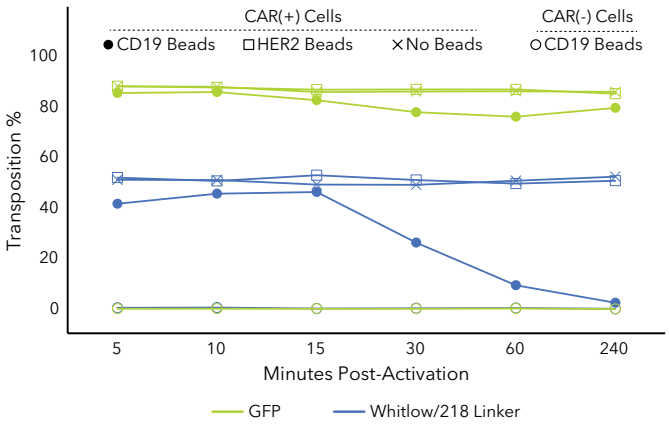


FIGURE 1. Flow cytometry analysis of GFP (green lines) and Whitlow Linker (blue lines) expression from 5 to 240 minutes post-activation/treatment with conjugated beads. CAR(+) cells activated with CD19-conjugated beads (filled circle markers); CAR(+) cells treated with HER2-conjugated beads (square markers); CAR(+) cells treated with no beads (x markers); CAR(-) cells treated with CD19-conjugated beads (empty circle markers).

The flow cytometry results showed a 50% decrease in expression of the Whitlow/218 linker at 30 minutes post-activation, which was only observed in CAR(+) cells in the presence of CD19 beads. At 60 minutes post-activation, the Whitlow/218 linker was barely detectable above background levels and was undetectable after 4 hours. Conversely, the expression of the Whitlow/218 linker remained stable in CAR(+) cells in the presence of HER2 beads and without beads. We also observed stable expression of GFP throughout the time course experiment for all CAR(+) cells and no expression for CAR(-) cells, as expected (**FIGURE 1**). These results indicate that CAR expression at the cell surface begins to disappear approximately 30 minutes after engagement with the target antigen, likely initiating an intracellular signaling cascade for T cell activation.[51]

Because flow cytometry staining is limited to cell surface proteins under the conditions tested here, we next asked whether this pattern of Whitlow/218 linker expression observed at the cell surface occurs in whole-cell lysate. To test this, we used Simple Western to measure Whitlow/218 linker expression in whole-cell lysates from cells harvested at the same time points as the flow cytometry analysis using the same anti-Whitlow linker antibody.

Like the flow cytometry results, the Simple Western results show Whitlow/218 linker expression diminished after activation (**FIGURE 2**). However, Simple Western analysis showed a faster Whitlow/218 linker expression reduction at 10 minutes post-activation, with maximum reduction at 60 minutes. Interestingly, Whitlow linker expression increased again at 4 hours post-activation, suggesting a cellular 'recycling' of the CAR. Simple Western provides a more sensitive readout and, thus, more detailed kinetics of CAR regulation in T cells.

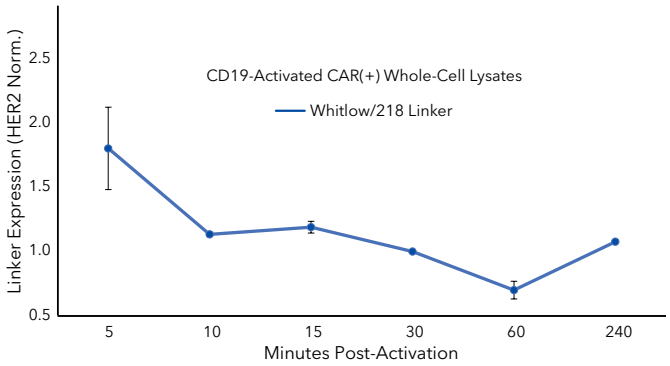


FIGURE 2. Simple Western analysis of Whitlow/218 linker expression in CD19-activated CAR(+) whole-cell lysates from 5 to 240 minutes post-activation. Error bars represent the standard errors from the means (n=2 except for the 240-minute timepoint of n=1).

The flow cytometry and Simple Western results demonstrate that CAR-engineered T cells downregulate CAR expression immediately following antigen engagement. However, CAR presence at the cell surface may linger for 15 minutes or longer. Also, CAR expression increases again at 4 hours, suggesting a 'recycling' of the CAR in T cells.

The Kinetics of Downstream Intracellular Signaling Events

Next, we investigated the intracellular signaling events following the activation of CAR-T cells. To do so, we analyzed whole-cell lysates on the Simple Western platform using specific antibodies targeting phospho- and total-protein isoforms of central T cell signaling molecules, including GADS, pGADS, SLP-76, pSLP-76, ERK1/2, and pERK1/2 (**FIGURE 3**). Electropherograms resulting from Simple Western analysis of GADS/pGADS show a 2.5X increase in pGADS from 5 to 240 minutes post-activation with CD19, which was not observed with HER2 treatment (**FIGURE 3**).

We quantified the phospho/total protein ratios normalized to HER2 for GADS, SLP-76, and ERK1/2 (**FIGURE 4**). 15 minutes passed before activation of GADS and SLP-76, as represented by phospho/total protein ratios, was observed and continually increased up to 4 hours. Meanwhile, maximal ERK1/2 activation (2-fold increase) was seen at 15 minutes, then decreased steadily. As with the analysis of the Whitlow/218 linker (**FIGURE 2**), here Simple Western results revealed detailed kinetics of intracellular CAR-T cell signaling events with reproducible quantitation.

Simultaneous Quantification of Four Secreted Cytokines

We assessed functional activation of CAR-T cells by measuring the secretion of cytokines using Simple Plex (**FIGURE 5**). All cytokines tested here, including Granzyme B, IL-2, IFN-γ, and TNF-α, were detectable at ng/mL levels 4 hours post-CD19-activation in CAR-T cells. IL-2, IFN-γ, and TNF-α were not detected in our negative control samples, though a low level of Granzyme B remained in CAR(-) cells and CAR(+) cells without CD19 activation, but not in the media-only control.

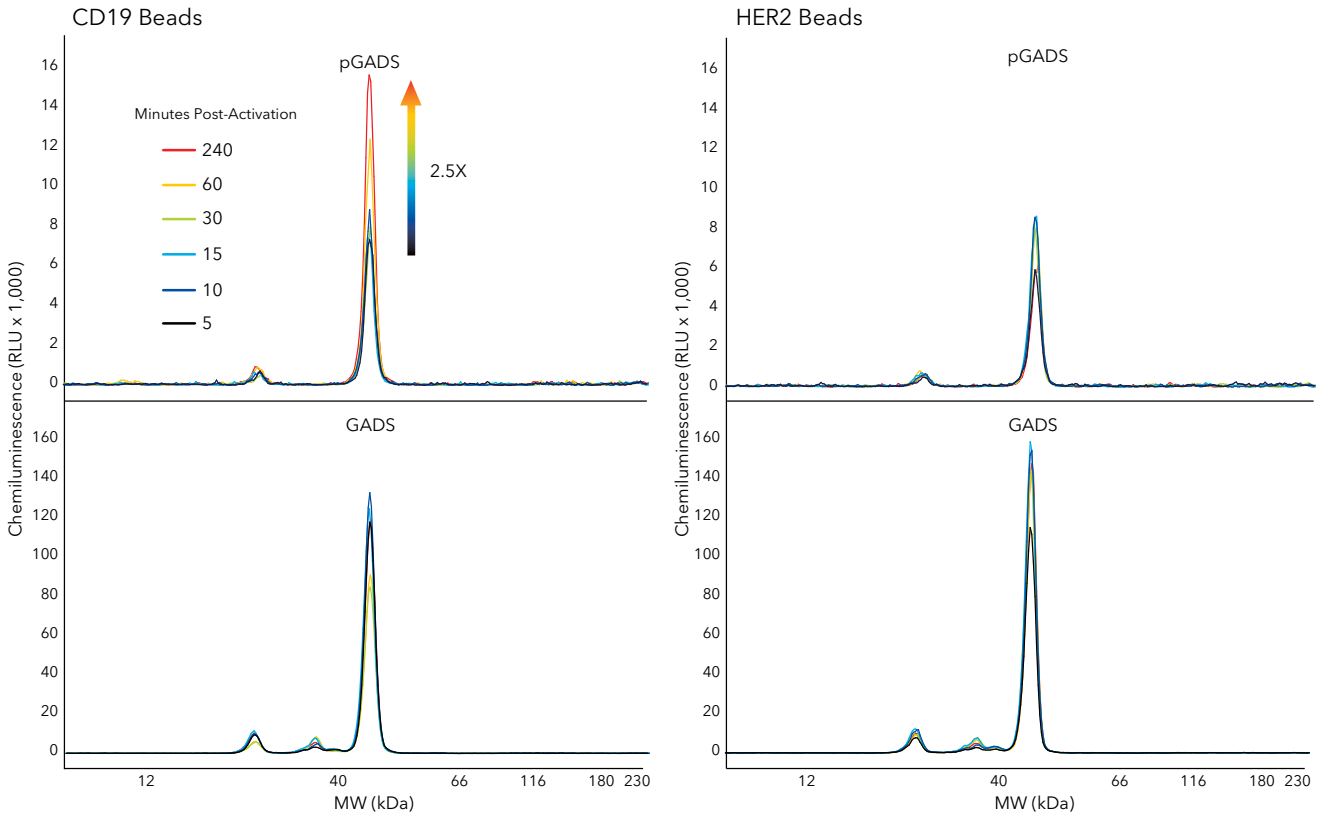


FIGURE 3. Simple Western analysis of CAR(+) cells activated with CD19 beads (left panels) or treated with HER2 beads (right panels) from 5 to 240 minutes post-activation/treatment.

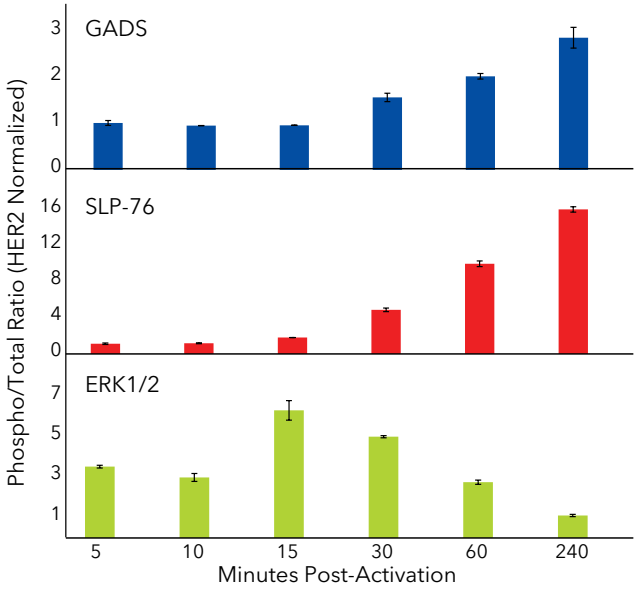


FIGURE 4. Quantification of phospho/total protein isoform ratios of intracellular signaling molecules, GADS, SLP-76, and ERK1/2, detected by Simple Western in CAR(+) cells activated with CD19-conjugated beads. Data are normalized to CAR(+) cells treated with HER2-conjugated beads. Error bars represent the standard errors from the means (*n*=2).

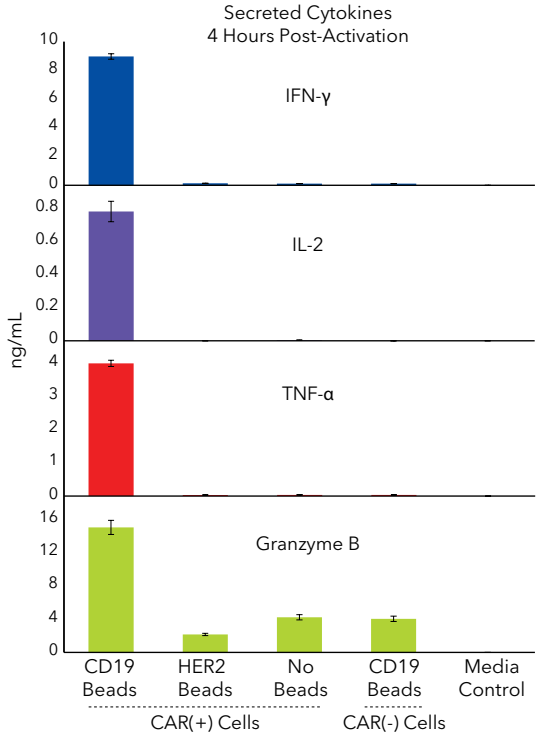


FIGURE 5. Simple Plex quantification of secreted cytokines in the supernatant of CAR(+) and CAR(-) cell cultures harvested at 4 hours post-treatment with beads. Error bars represent the standard deviations from the means (*n*=3).

Navigate Your CAR Challenges with Fit-For-Purpose Analytical Tools

Using living cells as therapeutics brings unique challenges compared to non-living drugs. Without the right analytical tools, understanding the complex protein network that creates safe and effective CAR-T cells can jam the development pipeline and prevent therapies from reaching their destination, a patient needing treatment. Here, we leveraged non-viral genome editing and advanced protein analytical solutions to shine new light on CAR-T cell activation, CAR trafficking, and signal transduction.

We used an antibody targeting the Whitlow/218 linker (CST, PN 69310), a common component in CAR design. While the antibody is widely used for analysis by flow cytometry, we also validated the antibody for analysis using the Simple Western platform, enabling the measurement of CAR expression in whole-CAR-T cell lysates. Taking both flow cytometry and Simple Western results together, we observed that CAR-T cells downregulate CAR expression in less than 10 minutes following antigen engagement. However, CAR presence at the cell surface may linger for 15 minutes or longer. Furthermore, the Simple Western results indicate CAR expression increases again at 4 hours, suggesting a 'recycling' of the CAR in T cells.

Intracellular signaling molecules downstream of CAR activation, including GADS and SLP-76, required at least 15 minutes to demonstrate a stimulatory modification as represented by phospho/total protein ratios, which continually increased up to 4 hours. Meanwhile, the ERK1/2 activation spiked by 2-fold at 15 minutes, then decreased steadily. Simple Western analysis revealed detailed kinetics of intracellular CAR-T cell signaling events with reproducible quantitation for both CAR expression and intracellular signaling molecules. Finally, in a single hands-free run in less than 90 minutes, the Simple Plex assay provided quantification results of 4 secreted cytokine concentrations ranging in ng/mL levels at 4 hours post-activation.

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