Next Generation Protein Characterization of CD19-CAR Signaling Cascades

Francisco Ramírez[1], G. Dalton Smedley[2], **Charles Haitjema[1]**, Amrik Singh[3], David Hermanson[2], Chris Heger[1]
[1]Bio-Techne, San Jose, CA
[2]Bio-Techne, Minneapolis, MN
[3]Cell Signaling Technology, Danvers, MA

Abstract

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 and intracellular signaling molecules is required, often with limited and complex sample types.

To study CAR-T signaling, we leveraged a non-viral gene editing platform (TcBuster) to introduce a CD19-CAR into T-cells. Chemically selected cells were isolated and CAR surface expression was verified by flow cytometry analysis using an antibody raised against the peptide linker within the CAR scFv. We then stimulated the CAR-T cells with immobilized CD19 and implemented automated solutions to characterize IFN-γ secretion using automated ELISA (Simple Plex) and intracellular signaling events using a capillary immunoassay platform (Simple Western). The capillary immunoassay provided multiplex protein expression measurements of multiple signaling molecules, including phosphorylated and total protein isoforms, with specific molecular weight characterization, and required only 3 µL of lysate for analysis. Together, these data show the importance of understanding the delicate interplay between signaling molecules that regulate CAR-T function.

Materials and Methods

TcBuster™
Non-viral gene delivery to engineer CAR-T cells

We engineered primary human T cells using a non-viral gene engineering TcBuster™ system (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.

ScaleReady™
G-Rex® Bioreactors for cell expansion

Cells were cultured in the presence of animal-free recombinant human IL-7 (Bio-Techne) and IL-15 proteins (Bio-Techne) using a G-Rex 6 well plate (ScaleReady). CAR-T cells were stimulated by treatment with biotinylated recombinant human CD19-antigen (Bio-Techne) conjugated to beads.

Jess™ instrument, powered by Simple Western™ technology
CAR trafficking and intracellular signaling measurements

We used Simple Western™ capillary immunoassays with the Jess™ instrument (ProteinSimple, a Bio-Techne brand) for intracellular signaling analysis. We used primary monoclonal antibodies from CST (**TABLE 1**) for specific detection of intracellular signaling targets.

Simple Plex™ assays on the Ella™ platform
Secreted cytokine detection

We assessed activation by measuring the secretion of cytokines in the supernatant of cell cultures collected at 4 hours post-activation using the multi-analyte Simple Plex Cell Activation Panel 1 (ProteinSimple, a Bio-Techne brand) with 4 separate channels containing assays for Granzyme B, IL-2, IFN-γ, and TNF-α.

Target	Specificity	PN	Observed kDa
Whitlow/218 Linker	Total	69310	56
ERK1/2	Phospho-T202/Y204	4370	45
	Total	4695	43, 47
GADS	Phospho-T262	78972	47
	Total	95848	47
SLP-76	Phospho-S376	76384	88
	Total	70896	88

TABLE 1. Antibodies used on the Jess instrument for Simple Western analysis. CST provided all antibodies.

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Results

The Kinetics of CAR Trafficking Following Antigen Engagement

Our CAR design included the Whitlow/218 peptide linker, which affords the scFv with enhanced resistance to proteolysis and aggregation.[2]

Flow cytometry analysis showed CAR expression at the cell surface began to disappear approximately 30 minutes after engagement with the target antigen, likely initiating an intracellular signaling cascade for T cell activation (**FIGURE 1**).[3]

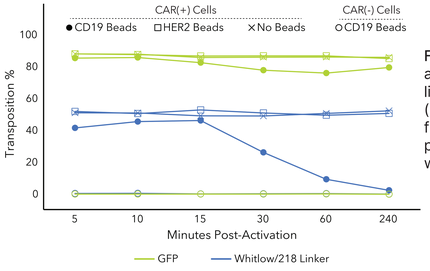

FIGURE 1. Flow cytometry analysis of GFP (green lines) and Whitlow Linker (blue lines) expression from 5 to 240 minutes post-activation/treatment with conjugated beads.

Simple Western analysis of whole-cell lysates showed a faster Whitlow/218 linker expression reduction at 10 minutes post-activation. Interestingly, Whitlow linker expression increased again at 4 hours post-activation, suggesting a cellular 'recycling' of the CAR (**FIGURE 2**).

The anti-Whitlow/218 antibody (CST) used here is not validated for traditional Western blot, but is validated for Simple Western, with the benefits of 3 µL of sample required, automation, and reproducible quantification.

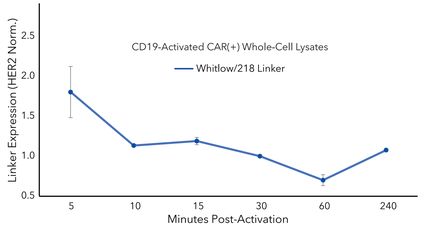

FIGURE 2. Simple Western analysis of Whitlow/218 linker expression in CD19-activated CAR(+) whole-cell lysates from 5 to 240 minutes post- activation.

The Kinetics of Downstream Intracellular Signaling Events

We investigated the intracellular signaling events using the Jess instrument, powered by Simple Western technology, using specific antibodies targeting phospho- and total-protein isoforms of central T cell signaling molecules.

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**).

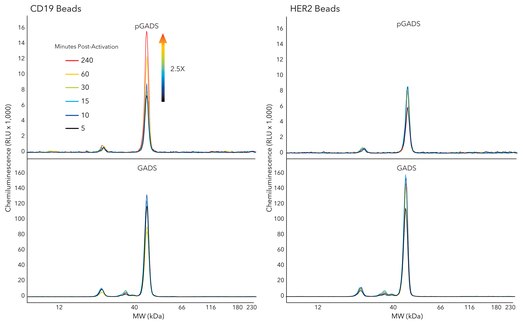

FIGURE 3. Simple Western analysis of CAR(+) cells activated with CD19 beads (left panels) or treated with HER2 beads (right panels) from 5 to 240 minutes post-activation/ treatment.

We quantified the phospho/total protein ratios normalized to HER2 for GADS, SLP-76, and ERK1/2 using the Simple Western Jess instrument (**FIGURE 4**).

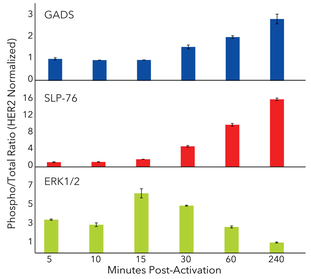

FIGURE 4. Simple Western assays on the Jess instrument quantified phospho/total protein isoform ratios of intracellular signaling molecules, GADS, SLP-76, and ERK1/2, in CAR(+) cells activated with CD19-conjugated beads.

Simultaneous Quantification of Four Secreted Cytokines

We assessed functional activation of CAR-T cells by measuring the secretion of cytokines using Simple Plex assays on the Ella platform.

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 (**FIGURE 5**).

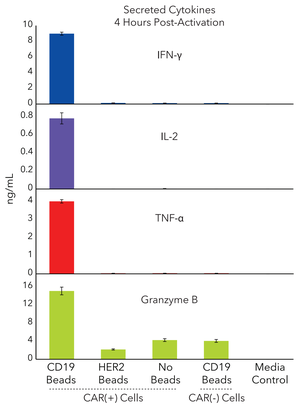

FIGURE 5. Simple Plex assays on the Ella platform simultaneously quantified 4 secreted cytokines in the supernatant of CAR(+) and CAR(-) cell cultures harvested at 4 hours post-treatment with beads.

Conclusion

Towards Fit-For-Purpose Analytical Tools

- 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 validated an antibody targeting the Whitlow/218 linker (CST) using Simple Western technology, enabling the measurement of CAR expression in whole-CAR-T cell lysates in addition to flow cytometry.
- The flow cytometry and Simple Western results showed 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.
- Simple Western analysis revealed detailed kinetics of intracellular CAR-T cell signaling events with reproducible quantification for both CAR expression and intracellular signaling molecules.
- Finally, the Simple Plex assay provided quantitative results of 4 secreted cytokine concentrations ranging in ng/mL levels at 4 hours post-activation in a single hands-free run in less than 90 minutes.

References

1. *Blood Cancer J.* 2021 Apr 6;11(4):69. 2. *Protein Eng.* 1993 Nov;6(8):989-95. 3. *Immunity.* 2020 Aug 18;53(2):456-470.e6.