Generation and validation of anti-linker monoclonal antibodies for the surface detection of scFv-based CARs

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INTRODUCTION

Chimeric Antigen Receptor (CAR)-T cell therapy is a highly innovative form of immunotherapy that has proven to be successful in the treatment of B cell malignancies and multiple myeloma. As this treatment modality continues to evolve toward the targeting of novel tumor antigens and engineering of cells with greater persistence, there is a need in multiple phases of the CAR-T development pipeline for highly specific detection reagents that can be leveraged to monitor the expression of CARs on the cell surface. Many commercially available CAR detection reagents, however, either lack specificity or are not versatile in their ability to detect CARs of differing antigen specificity. Here, we report on the generation and validation of rabbit monoclonal antibodies raised against two linker sequences that are commonly integrated into single-chain variable fragment (scFv)-based CARs. These antibodies serve as versatile detection reagents that can be used to interrogate the surface expression of CARs.

METHODS

The recombinant monoclonal antibodies, E7O2V and E3U7Q, were generated by rabbits immunized with peptide sequences most used for the linker region of scFv based CARs, Gly_4Ser and Whitlow/218, respectively (1,2). E7O2V and E3U7Q were directly conjugated to a panel of fluorophores and validated for specificity and versatility using live cell flow cytometric analysis of non-transduced versus CAR-transduced cells.

CONCLUSIONS

In a live cell flow cytometry assay, fluorophore conjugates of E7O2V and E3U7Q specifically detect surface expressed scFv-based CARs containing either a Gly_4Ser linker or a Whitlow linker/218, respectively. Furthermore, these recombinant monoclonal antibodies are versatile in that they can also detect their respective linker sequence independently of scFv specificity. The potential exists to leverage these antibodies for CAR-T cell enrichment and for incorporation into multiparametric flow cytometry panels used to phenotype CAR-T cells during the discovery, manufacturing, and subsequent phases of the development pipeline.

REFERENCES

1. Huston, J.S. et al. (1988) *Proc Natl Acad Sci U S A* 85, 5879-83.
2. Whitlow, M. et al. (1993) Pr*otein Eng* 6, 989-95.

Table 1: Commonly used reagents for flow cytometric detection of CAR surface expression

Staining Reagent	Limitations
Anti-Idiotype	Limited to detection of CARs with a scFv of given specificity
Protein L	1. Differential binding to κ light chain variable regions 2. Not amenable to incorporation into flow panels
Anti-Fab	1. Non-specificity 2. Not amenable to incorporation into flow panels
Anti-Fc	1. Non-specificity 2. Limited to detection of CARs with an Ig-derived hinge
CAR target antigen	Limited to detection of a CAR directed against a specific target antigen

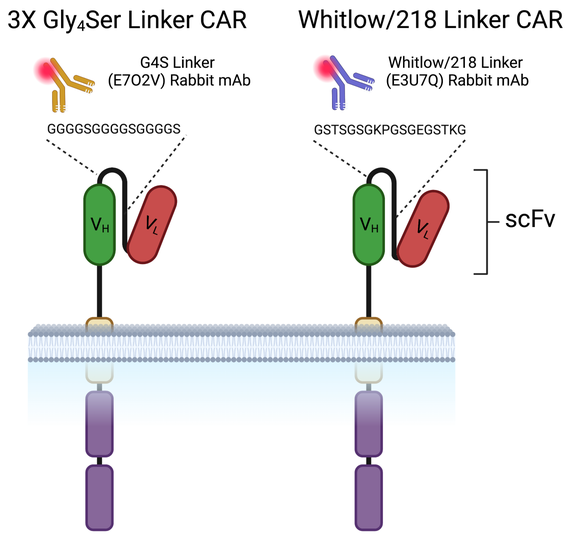
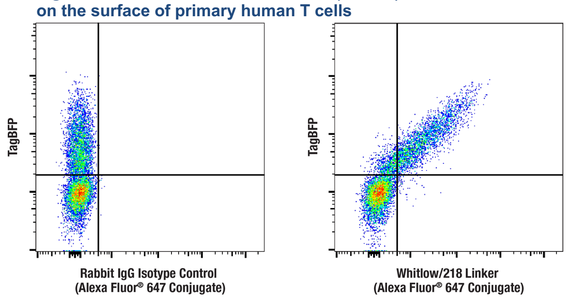


Diagram 1
Illustrated diagram showing the amino acid sequences of Gly_4Ser and Whitlow/218 linkers within the scFV of CARs that were targeted for discovery of recombinant rabbit monoclonal antibodies E7O2V and E3U7Q, respectively (created with BioRender.com).

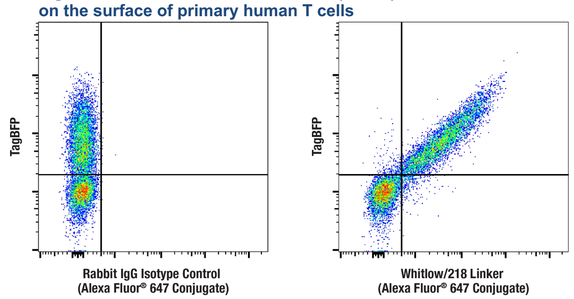
Validation of Whitlow Linker/218 (E3U7Q) Rabbit mAb

Figure 1A: Detection of an anti-CD19 (FMC63) CAR on the surface of primary human T cells



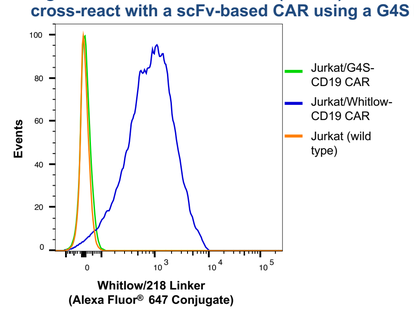
Flow cytometric analysis of live pan-CD3+ T cells engineered to express a scFv-based Anti-CD19 (FMC63) CAR containing a Whitlow/218 linker, using Whitlow/218 Linker (E3U7Q) Rabbit mAb (Alexa Fluor® 647 Conjugate) (right) or concentration-matched Rabbit (DA1E) mAb IgG XP® Isotype Control (Alexa Fluor® 647 Conjugate) #2985 (left). Tag Blue fluorescent protein (TagBFP) is a transduction marker co-expressed with the CAR.

Figure 1B: Detection of an anti-CD20 (Leu16) CAR on the surface of primary human T cells



Flow cytometric analysis of live pan-CD3+ T cells engineered to express a scFv-based Anti-CD20 (Leu16) CAR containing a Whitlow/218 linker, using Whitlow/218 Linker (E3U7Q) Rabbit mAb (Alexa Fluor® 647 Conjugate) (right) or concentration-matched Rabbit (DA1E) mAb IgG XP® Isotype Control (Alexa Fluor® 647 Conjugate) #2985 (left). Tag Blue fluorescent protein (TagBFP) is a transduction marker co-expressed with the CAR.

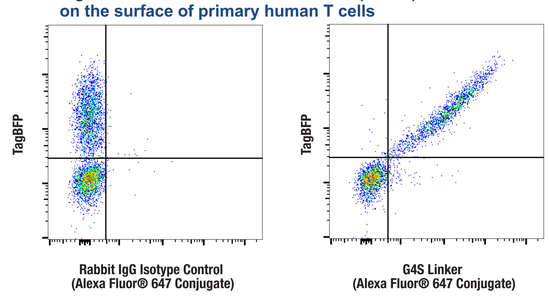
Figure 1C: Whitlow Linker/218 (E3U7Q) Rabbit mAb does not cross-react with a scFv-based CAR using a G4S linker



Live wild type Jurkat cells, and Jurkat cells stably expressing a scFv-based CD19 (FMC63) CAR using either a 3X G4S linker or a Whitlow/218 linker, were immunostained with Whitlow/218 Linker (E3U7Q) Rabbit mAb (Alexa Fluor® 647 Conjugate) and analyzed by flow cytometry.

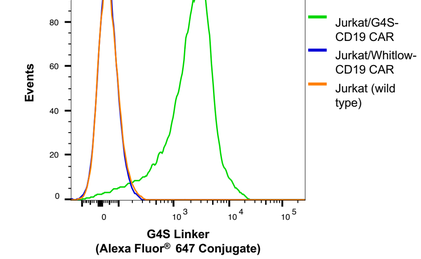
Validation of G4S Linker (E7O2V) Rabbit mAb

Figure 2A: Detection of an anti-CD20 (Leu16) CAR on the surface of primary human T cells



Flow cytometric analysis of live pan-CD3+ T cells engineered to express a scFv-based Anti-CD20 (Leu16) CAR containing a G4S linker, using G4S Linker (E7O2V) Rabbit mAb (Alexa Fluor® 647 Conjugate) (right) or concentration-matched Rabbit (DA1E) mAb IgG XP® Isotype Control (Alexa Fluor® 647 Conjugate) #2985 (left). Tag Blue fluorescent protein (TagBFP) is a transduction marker co-expressed with the CAR.

Figure 2B: G4S Linker/218 (E7O2V) Rabbit mAb does not cross-react with a scFv-based CAR using a Whitlow/218 linker



Live wild type Jurkat cells, and Jurkat cells stably expressing a scFv-based CD19 (FMC63) CAR using either a 3X G4S linker or a Whitlow/218 linker, were immunostained with G4S Linker (E7O2V) Rabbit mAb (Alexa Fluor® 647 Conjugate) and analyzed by flow cytometry.

Table 2: Selected anti-CAR linker rabbit mAb conjugates

Product Name	Catalog #
G4S Linker (E7O2V) Rabbit mAb (PE Conjugate)	#38907
G4S Linker (E7O2V) Rabbit mAb (Alexa Fluor® 647 Conjugate)	#69782
G4S Linker (E7O2V) Rabbit mAb (Alexa Fluor® 488 Conjugate)	#50515
G4S Linker (E7O2V) Rabbit mAb (Biotinylated)	#17621
Whitlow/218 Linker (E3U7Q) Rabbit mAb (PE Conjugate)	#62405
Whitlow/218 Linker (E3U7Q) Rabbit mAb (Alexa Fluor® 647 Conjugate)	#69310
Whitlow/218 Linker (E7O2V) Rabbit mAb (Alexa Fluor® 488 Conjugate)	#55809
Whitlow/218 Linker (E3U7Q) Rabbit mAb (Biotinylated)	#32523

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