

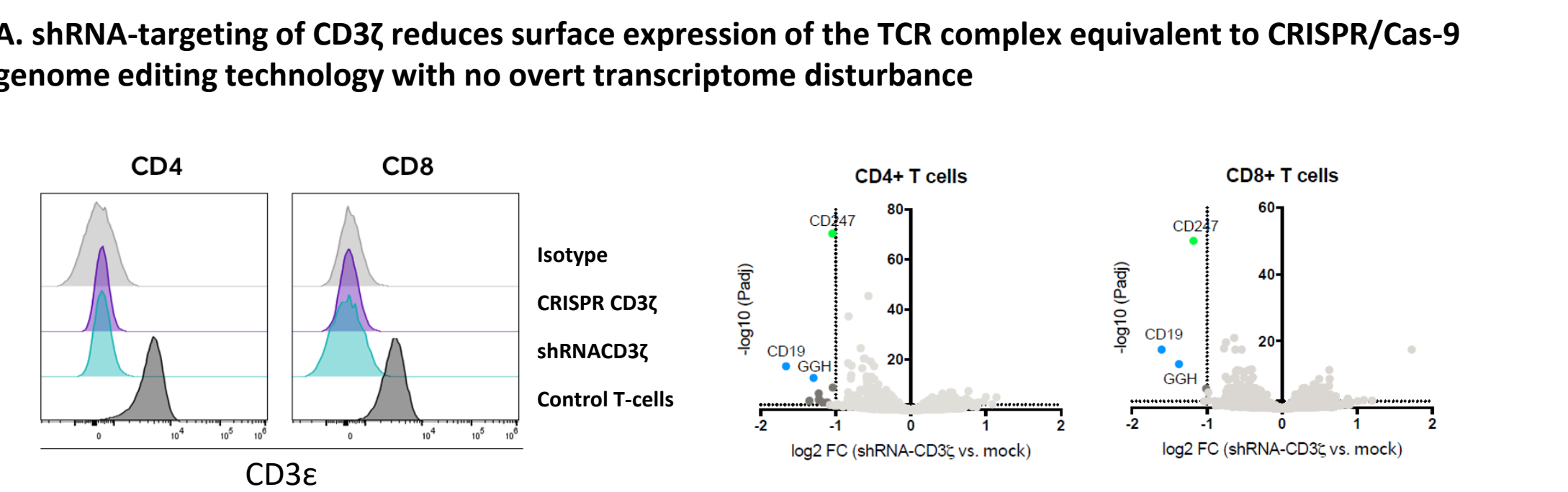
Abstract 3103: Single vector multiplexed shRNA provides a non-gene edited strategy to concurrently knockdown the expression of multiple genes in CAR T-cells

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Background:
Engineered T-cells expressing chimeric antigen receptors (CAR) are now delivering clinically relevant results in patients with advanced hematological malignancies. One critical area for future development is to additionally modulate gene expression using the CAR vector construct thereby endowing the engineered T-cell with specific desired features, including inhibiting alloreactivity, increased persistence, enhanced antitumor activity or improved safety. New data presented here show for the first-time that a multiplexed shRNA strategy can effectively decrease expression of multiple genes in a single CAR vector construct.

Methods
Short-hairpin RNA (shRNA) were cloned individually or multiplexed within micro-RNA scaffolds that enabled the co-expression of the individual shRNA with a CAR and a selectable marker all driven by the same PolIII promoter within a single vector. Human T-cells transduced with the CAR-shRNA vectors were selected, expanded *in vitro*, and examined for target gene expression and functional activity.

Figure 1. Expression of a single shRNA targeting CD3ζ confers prolonged TCR knockdown in primary T-cells



B. Anti-BCMA CAR-T-cells with CD3ζ-targeting shRNA exhibit high efficacy and no signs of alloreactivity

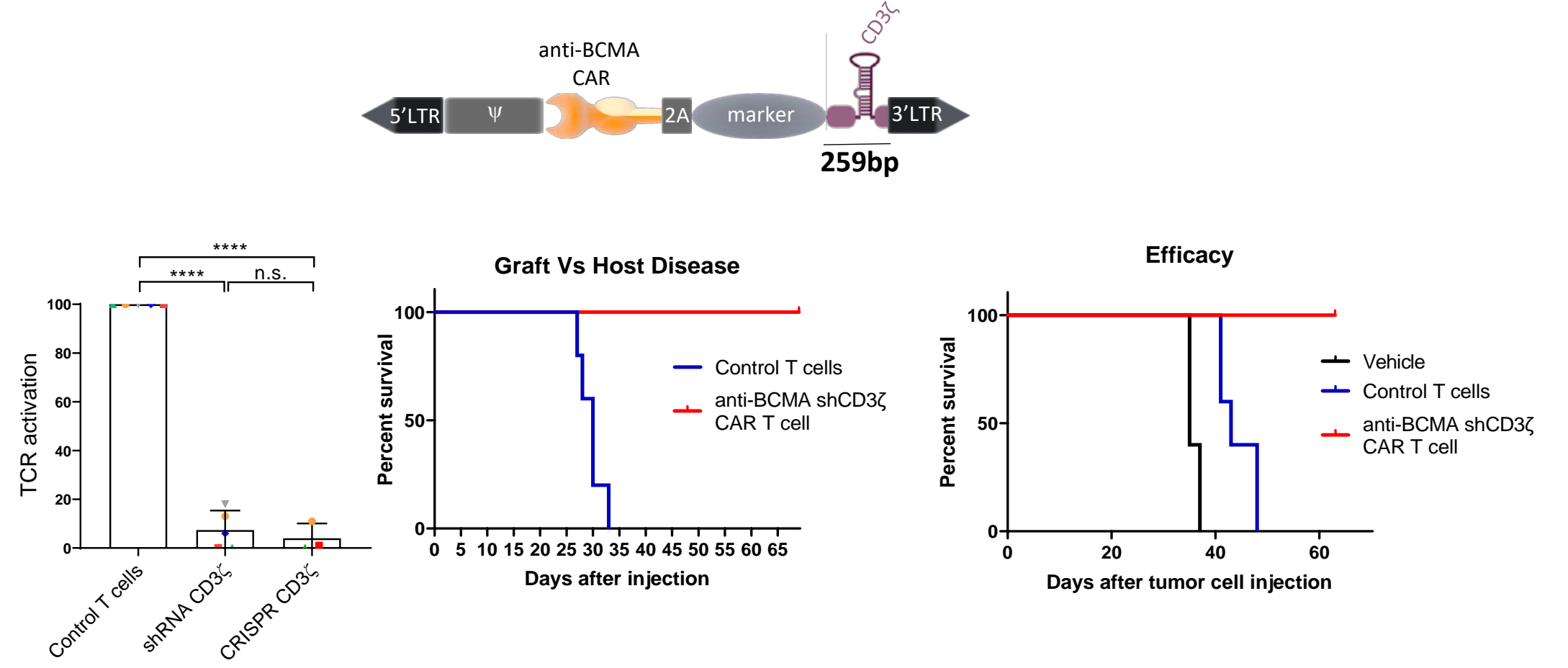
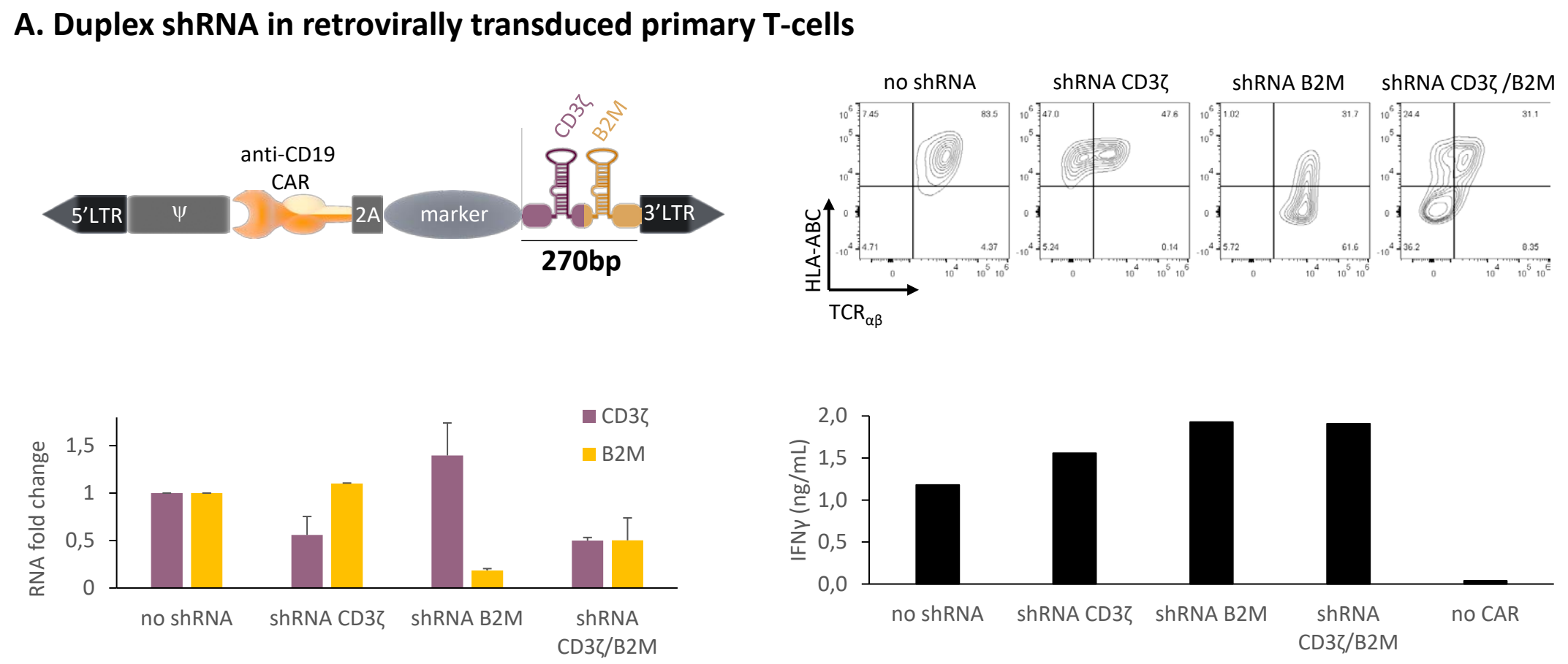


Figure 2. Efficient expression of two shRNAs from a single vector confers multiple gene knockdown in primary human CAR T-cells



B. shRNA multiplexing from a single vector enables generation of a homogenous CAR T-cell population

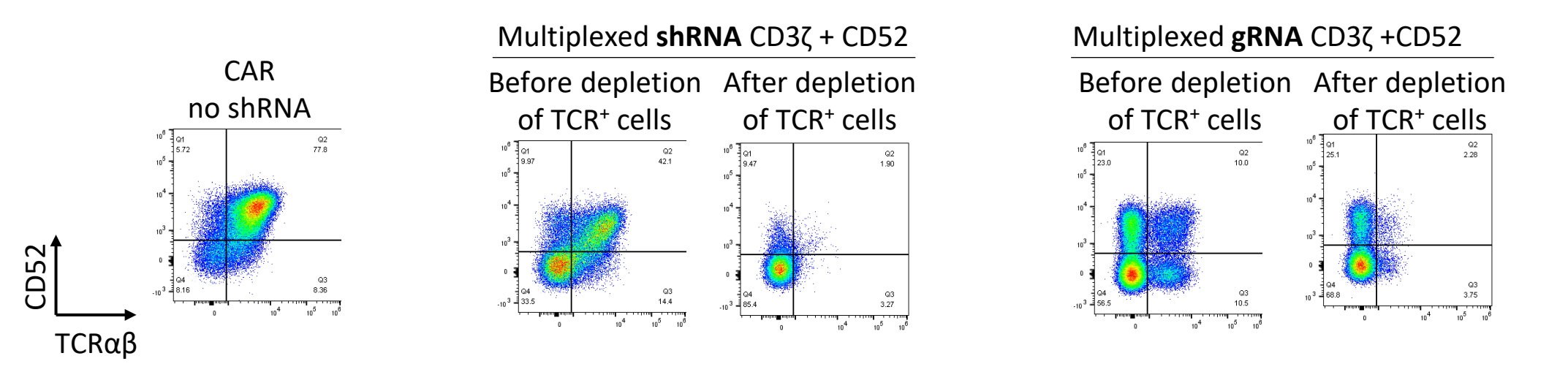
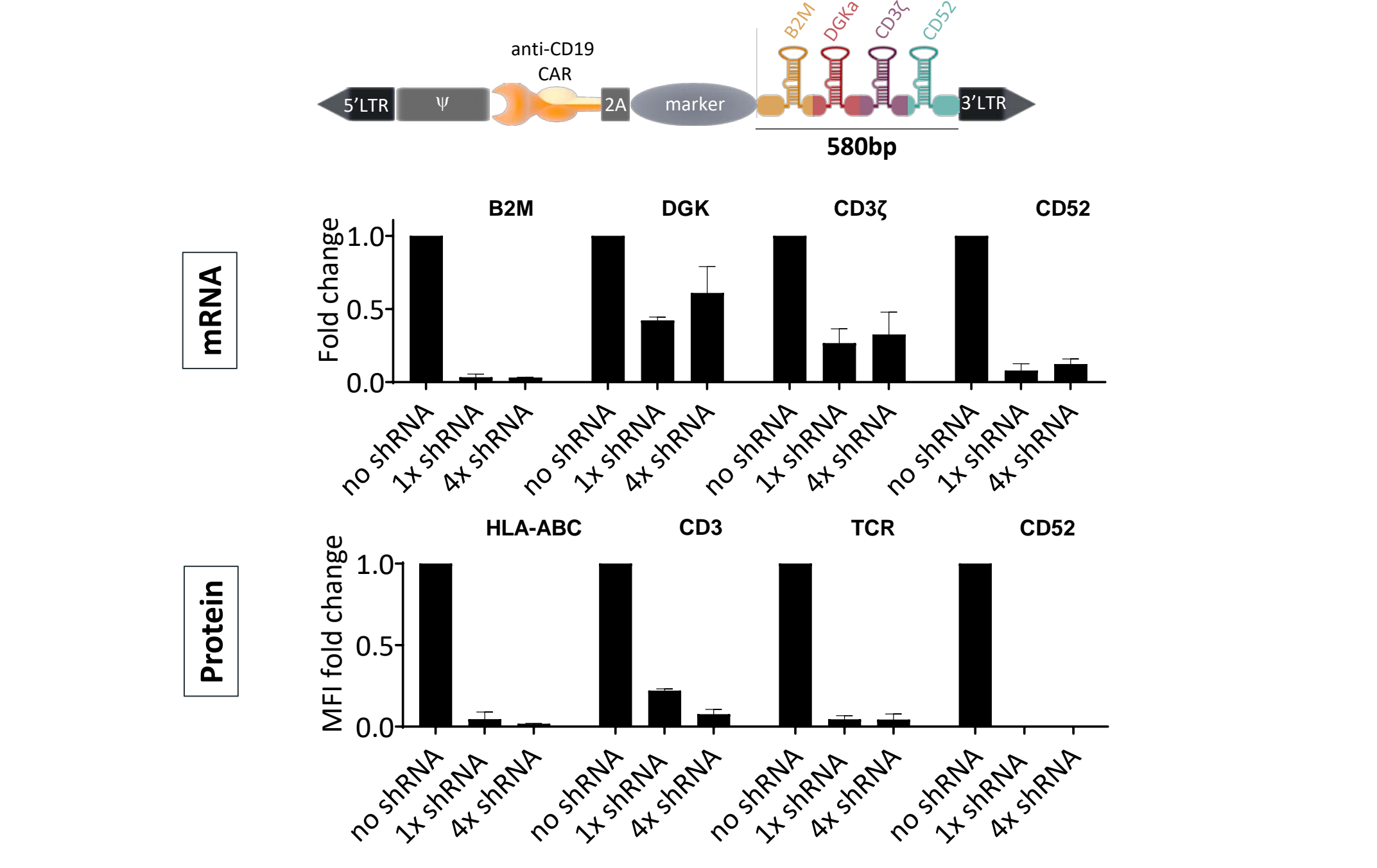


Figure 3. A single multiplexed vector enables knockdown of 4 genes simultaneously in Jurkat T-cells



Results and Figure Legends:

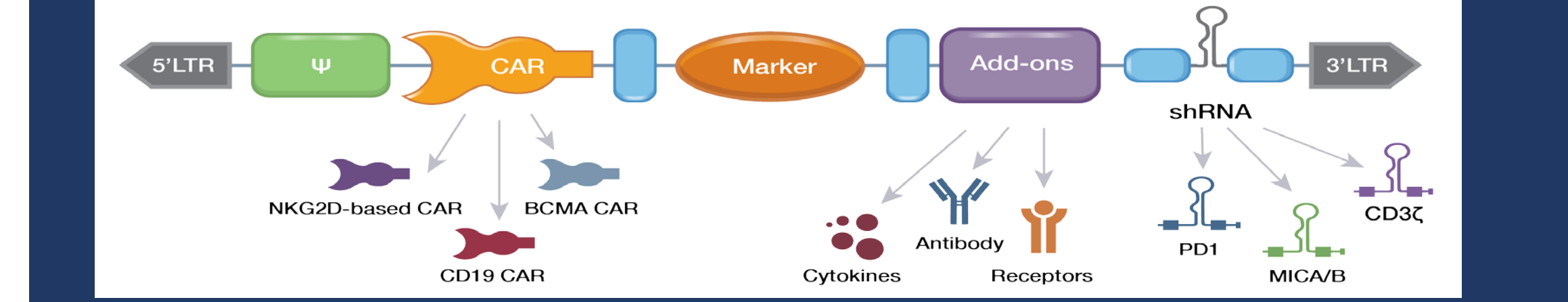
Figure 1: Expression of a single shRNA targeting CD3ζ confers prolonged TCR knockdown in primary T-cells. A 259bp DNA fragment incorporating a CD3ζ-specific shRNA was cloned into a retroviral vector, effectively knocked down expression of CD3 to levels observed with CRISPR/Cas-9 technology (Figure 1A left). Off-targets screening by transcriptional profiling of isolated T-cells revealed only one differentially regulated gene (gamma-glutamyl hydrolase, GGH) besides the expected CD3ζ (CD247) and reporter (truncated CD19) genes (Figure 1A right). The shRNA targeting CD3ζ was incorporated into a retroviral vector containing an anti-BCMA CAR. The reduction in TCR expression resulted in minimal cytokine production upon TCR stimulation *in vitro* (Figure 1B). When injected in NSG mice these CAR T-cells showed no evidence of graft-versus-host disease (GvHD) induction yet maintained BCMA-specific CAR activity in established myeloma xenograft models (Figure 1B, KMS model).

Figure 2: Efficient expression of two shRNAs from a single vector confers multiple gene knockdown in primary human CAR T-cells. Subsequent studies confirmed that two shRNAs could be expressed from a single retroviral vector to modulate the expression of multiple genes (Figure 2A). shRNAs specific for CD3ζ and β-microglobulin (B2M) were engineered downstream of an anti-CD19 CAR and efficiently knocked down mRNA and protein expression of CD3ζ and B2M, while maintaining CAR activity (Figure 2A). Head to head comparison highlights the advantage of shRNA multiplexing over CRISPR/Cas-9 genome editing technology by generating a more homogeneous population of cells, thereby facilitating simple manufacturing of complex CAR T-cell designs through single step enrichment (Figure 2B).

Figure 3: A single multiplexed vector enables knockdown of 4 genes simultaneously in Jurkat T-cells. Further engineering of the microRNA framework reduced the size of shRNA-incorporating DNA fragment to 580bp while enabling the incorporation of up to 4 shRNAs. shRNAs specific for CD3ζ, B2M, CD52 and diacylglycerol kinase alpha (DGK) were engineered into the framework downstream of an anti-CD19 CAR. Transduced Jurkat cells showed concurrent knockdown of the respective gene products at the mRNA and protein levels. There was near equivalent knockdown of each gene in the multiplexed format as that achieved with the single shRNA (Figure 3).

Conclusions:

- Celyad's multiplexed shRNA platform provides a next-generation, allogeneic approach to CAR T-cell development, enabling flexibility, versatility and single step engineering based on an all-in-one-vector approach
- CYAD-211, incorporating an anti-BCMA CAR and shRNA targeting CD3ζ, is expected to move into clinical testing by end-2020.
- Clinical candidates exploiting multiple shRNA knockdowns are in the next wave of clinical development.
- Continued preclinical development has now demonstrated proof of principle that the concurrent knockdown of up to four genes is feasible using an optimized shRNA that is only 580bp in size and knocked down a diverse array of targets, with distinct mRNA abundance and protein localization.
- Combining multiplexed shRNA knockdown with additional genes (add-ons) will provide potential for additional therapeutic functionality to our non-gene edited allogeneic CAR T approach being used in all 200-series products.



Any questions? Please contact us at contactus@celyad.com