Evolving Multiplexed shRNA to generate tailored CAR T cell therapy

Mikhail Steklov[1], Eytan Breman[1], Benjamin Le Calve[1], Hannes Iserentant[1], Peggy Sotiropoulou[1], David E. Gilham [1]

BACKGROUND

Manipulating protein expression to generate cells with a specific desired phenotype is one of the central goals of engineered cell therapy. Short Hairpin RNA (shRNA) is a well-established approach to reduce protein expression through the targeted degradation of messenger RNA transcripts. Through the addition of an shRNA targeting CD3zeta we were able to knockdown the T cell receptor (TCR) from the cell surface and used this principle to create allogeneic Chimeric Antigen Receptor (CAR) T cells targeting B-cell maturation antigen (BCMA), which we are currently assessing in a phase I clinical trial (Clinical trials.gov: NCT0461355).

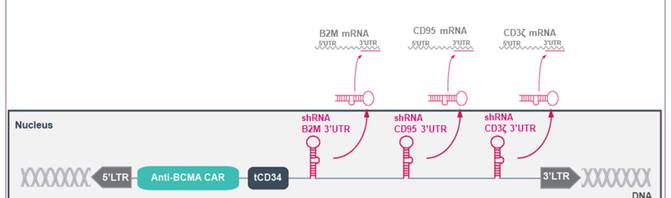
The attraction of the shRNA approach is to express multiple shRNA within the same vector that can regulate protein expression of multiple targets thereby optimizing CAR T cell phenotype, persistence and efficacy.

Transition from Single shRNA to Multiplexing

- Given the effectiveness of a single shRNA targeting approach, the obvious next step would be to investigate targeting of multiple genes with shRNA to optimize cell phenotype and function.
- We have previously demonstrated concurrent knockdown of up to four different gene products (multiplexing) in T lymphocyte cell line with our first-generation shRNA scaffold based on miR196a2 (Figure 1).
- Even though the multiplexing of shRNAs based on our first-generation shRNA scaffold allowed the knockdown of multiple targets (beta 2 microglobulin (B2M), DGK, CD3zeta and CD52) and was successful in terms of target knockdown in human primary T cells, an obvious reduction in retroviral titer was observed (Figure 2).
- These titer reductions were variable between the duplex and triplex shRNA constructs examined but were uniformly low when compared to the single shRNA construct.
- Therefore, **the goal of this study** was to engineer a second generation of shRNA scaffold that could enable consistent expression of duplexed and triplexed shRNAs while also elevating vector titer making it suitable for future clinical application.

Materials & Methods

Retroviral vectors encoding a CAR targeting BCMA, a well-studied antigen co expressing a tag (tCD34) for cell enrichment and identification along with shRNA multiplexed, were generated. The shRNA multiplexed were inserted within a microRNA (miR) framework to enable expression from a single Pol II promoter (the retroviral LTR promoter, see schematic diagram below). Assessment of protein target knockdowns in human primary T cells along with retroviral titers were determined.
For the creation of CAR T cells, PBMCs were isolated from healthy donors and T cells activated using CD3/CD28 beads. Following activation cells were transduced with the retroviral vectors and subsequently the tag positive cells purified and harvested after an expansion period.
CAR T cells shRNA targeted knockdown was assessed by flow cytometric analysis.
shRNA against b2M was assessed functionally, by incubating NK cells with allogeneic anti-BCMA (aBCMA) CAR T cells expressing the shRNA against B2M, B2M KO or no shRNA. NK cell degranulation was then assessed by CD107 expression. The functional assessment of CD95 (FAS) shRNA was conducted by titrating FAS activating antibody with CAR T cells containing the shRNA, or not. Cells transduced only with the truncated marker were used as an additional control. Cellular viability was assessed after 24h of incubation.



RESULTS

Figure 1. Knockdown of 4 separate genes in Jurkat cell line.

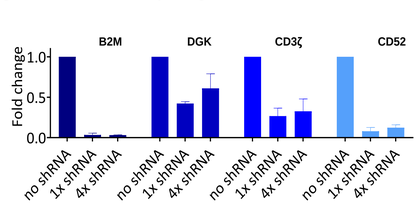
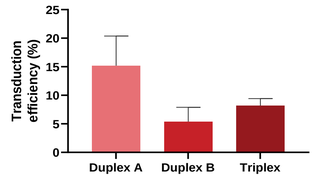


Figure 2. Transduction efficiency of first generation shRNA multiplexing



Ongoing shRNA Multiplex Research Focused on Key Targets

CD3ζ (CD247)

- Component of the TCR complex
- Knockdown of TCR designed to prevent Graft versus Host Disease (GvHD)
- shRNA targeting approach validated through CYAD-211 program

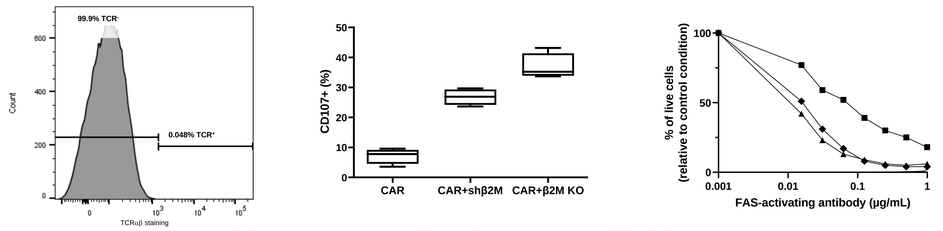
Beta-2-microglobulin (β2M)

- Component of the MHC Class I molecules
- Knockdown of β2M designed to prevent Host versus Graft disease (HvG) allowing for greater cell persistence

FAS (CD95)

- Cell surface death receptor whose activation leads to apoptosis
- Knockdown of FAS designed to prevent apoptosis and improve persistence

Figure 3. Assessment of shRNA KD and functionality. A) TCR expression of BCMA CAR T cell containing an shRNA against CD3zeta. **B)** Functional assessment of CAR T cells containing or not an shRNA against B2M, by incubating the cells with allogeneic NK cells and measuring NK-cell degranulation. **C)** Functional assessment of shRNA against CD95 by incubating CAR T cells with a FAS activating antibody. Cell viability was assessed after 24h of incubation. Squares are anti-BCMA CAR T cells containing shRNA against CD95, diamonds represent BCMA CAR without the shRNA and triangles depict a Mock transduced control.



Multiplexing with First Generation shRNA Scaffold

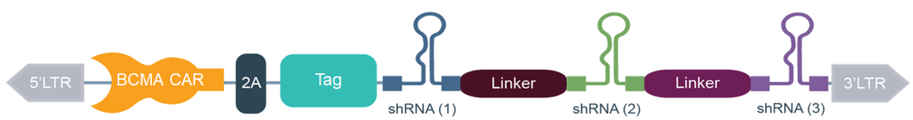
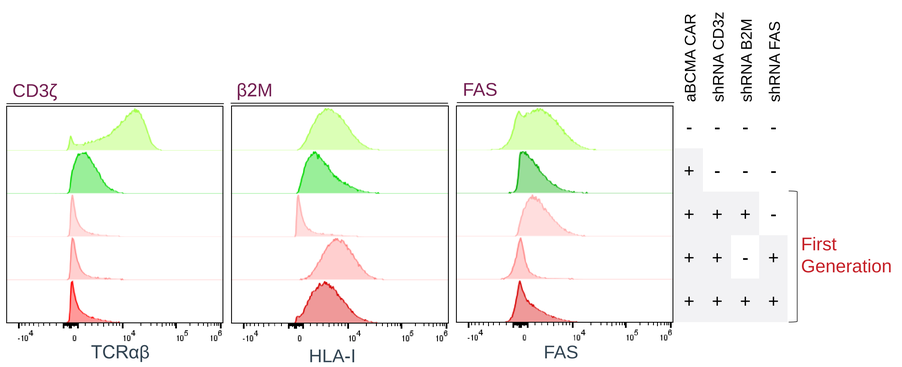


Figure 4. Assessment of shRNA KD in the first generation scaffold. Expression of either TCRab, HLA-I or FAS protein levels on the cell surface of anti-BCMA CAR T cells, containing shRNAs against CD3z, B2M and FAS/CD95 as depicted in the legend to the right.



Multiplexing with Novel Second Generation shRNA Scaffold

Figure 5. Assessment of shRNA KD in the first generation and second-generation scaffold. Expression of either TCRqb, HLA-I or FAS protein levels on the cell surface of anti-BCMA CAR T cells, containing shRNAs against CD3z, B2M and FAS/CD95 as depicted in the legend to the right.

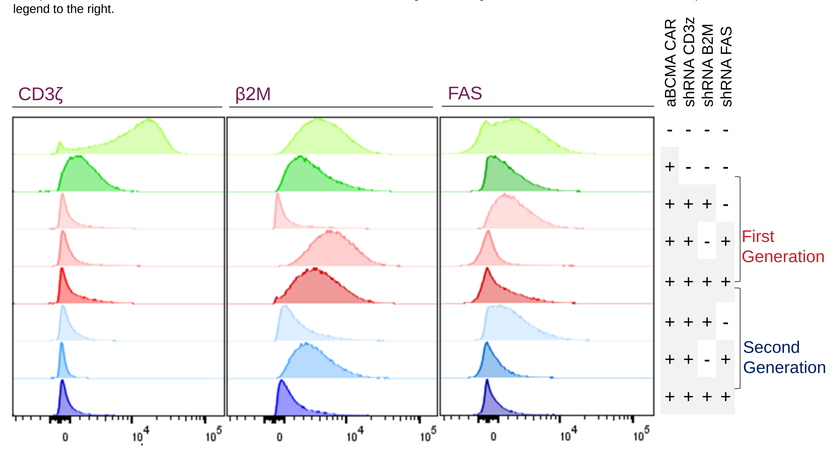
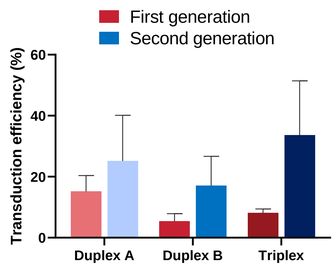


Figure 6. Comparison of transduction efficiency in the first generation and second generation scaffold. A side-by-side comparison of an anti-BCMA CAR T cell containing two different duplexes or a triplex shRNA from either the first- or second-generation scaffold. Transduction efficiency was assessed by percentage expression of the transgene.

- Increase in transduction efficiencies achieved with the second generation shRNA scaffold



CONCLUSIONS

- Multiplexing shRNA within a single vector format with scaffolds that ensure co-linked expression of the shRNA, with therapeutic transgenes, is a highly attractive approach to generate CAR T cells with bespoke, desired phenotypes. However, multiplexing the first-generation shRNA resulted in reductions in vector titer and therefore a significantly decreased transduction efficiency of the vectors obtained.
- A second generation scaffold was engineered that allows for at least a similar knockdown efficiency as previously observed coupled with maintenance of retroviral titer to a level which supported acceptable levels of primary T cell transduction efficiency.
- These developments now provide the opportunity to develop second generation clinical candidates using shRNA multiplexed technology.

AFFILIATIONS, DISCLOSURES & ACKNOWLEDGMENTS

1. Celyad Oncology, Mont-Saint-Guibert, Belgium
All authors are employees of Celyad Oncology.

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