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Commentary

Chimeric miRNA cluster enables multiplex and titratable gene inhibition in CAR-T cells

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Chimeric antigen receptor (CAR) T cells have revolutionized the treatment of hematological malignancies. Yet, primary and secondary resistance,[1] severe toxicities,[2] and low activity in solid tumors[3] continue to hamper the advancement of CAR-T cell technologies. Therefore, modifications of molecular precision are increasingly used to enhance CAR-T cell potency and to overcome the above challenges.[4] The CAR construct is shuttled into T cells via lentivirus or a retrovirus in approved CAR-T cell products. Modifying these viral vectors offers a straightforward way to introduce additional potency enhancers and/or toxicity controllers into CAR-T cells. An attractive option is to use small RNA inhibitors to block CAR-T cell exhaustion and CAR-T cell toxicity and generally to modulate CAR-T cell phenotype.

The article in the recent of issue of *Molecular Therapy Nucleic Acids* by Rossi et al.[5] shows a promising approach to integrate multiplex short hairpin RNA (shRNA)-expressing loci into the viral vector used to manufacture CAR-T cells. In particular, the authors screened 48 human microRNA (miRNA) clusters and their miRNA expressing scaffolds and finally developed a chimeric expression cluster with four scaffolds originating from different natural miRNA clusters. The authors optimized the order of the scaffolds as well as the linkers and restriction sites between the scaffolds to enable the incorporation of various shRNA sequences into a fourplex expression system in a plug-and-play fashion. The authors went on to show how target mRNA levels can be titrated depending on the efficacy of the corresponding shRNA guide sequence. Biologically relevant examples are also presented in the article and include the functional knockout of CD3ζ or CD95 (to inhibit TCR activation or CD95L-mediated apoptosis, respectively) and a 70% knockdown of β2M in order to prevent natural killer (NK) cell activation but to enable the use of allogeneic CAR-T cells.

Although the utility of multiplexing two shRNAs in viral vectors carrying a CAR has been demonstrated before,[6,7] Rossi et al.[5] report the first fourplex shRNA expression system. Rossi et al.[5] are also the first to mechanistically analyze and use natural miRNA cluster scaffolds to power shRNA multiplexing. Furthermore, the possibility to fine-tune the silencing potency of each scaffold in the fourplex separately is a biologically meaningful and attractive way to modulate CAR-T cell phenotype and distinguishes the shRNA approach from a gene editing approach the most: target gene expression cannot be precisely titrated using current gene editing technologies. Successful and precise setting of the CAR-T cell phenotype pre-administration is likely to require modulating multiple targets at the same time.

One limitation of the study is the need to screen a large number of shRNA sequences in order to identify sequences active in a miRNA scaffold environment. Better understanding of miRNA scaffold biology would enable better sequence prediction algorithms.

The use of the CAR-encoding viral vector to shuttle CAR-T cell modifiers has general advantages and drawbacks. First, modifiers will only be expressed in CAR-T cells and not in untransduced T cells in the product. This may be advantageous when inhibiting CAR-T cell exhaustion or enhancing CAR-T cell penetration into a solid tumor but may be problematic when modifying allogeneic T cells as a CAR-T cell source, where alloreactivity must be inhibited in every single T cell transduced to a patient. CAR transduction efficiency in currently approved products is heterogeneous and not routinely monitored during CAR-T cell manufacturing.[8] Current CAR-T cell products also show a variability of copy number per cell and a heterogeneity of genomic integration sites[9] influencing expression levels of the CAR construct. These phenomena affect the expression of CAR-T cell modifiers encoded in the CAR-shuttling viral vector, and therefore, the precise setting of shRNA silencing levels in individual CAR-T cells would probably be challenging in the clinical setting. Importantly, the therapeutic window of CAR-T cells is not only defined by the initial CAR-T cell dose but also by CAR-T cell proliferation rates *in vivo*. Therefore, the timing of a CAR-T cell modulation strategy is important and ideally should take the proliferation rate into account.[10] Temporary RNA inhibitors, such as chemically synthesized siRNAs, may offer a CAR-T cell modifying strategy[11] sensitive to CAR-T cell proliferations rates. Whereas, RNA inhibitors expressed from a genomic locus such as the fourplex shRNA cluster reported by Rossi et al.[5] are expected to exert permanent effects within the CAR-T cell life cycle.

The article by Rossi et al.[5] represents an important addition to the tool library to be used when choosing a CAR-T cell modification strategy. Multiplexing four shRNAs enables the combination of several modification goals, such as exhaustion prevention, toxicity control, enrichment for memory phenotype, and potency enhancement. Especially, solid tumor indications would benefit from more potent and better penetrating CAR-T cells. The multiplex shRNA

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expression system may also facilitate the development of allogeneic, off-the-shelf CAR-T cell products and contribute to optimize other cell therapy modalities, such as TCR-engineered T cells, donor lymphocyte infusions, and NK cell therapies. It will be interesting to see how the technology reported by Rossi et al.[5] will perform in clinical settings. Another important question is whether a chimeric miRNA cluster can be further expanded to include even more shRNA sequences.

DECLARATION OF INTERESTS

I am co-inventor on a provisional patent application concerning nucleic-acid-modified cell therapies.

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