Poster #21

MEIRAGTx

Riboswitch-regulated chimeric antigen receptor (RiboCAR) enhances CAR-T cell anti-cancer efficacy

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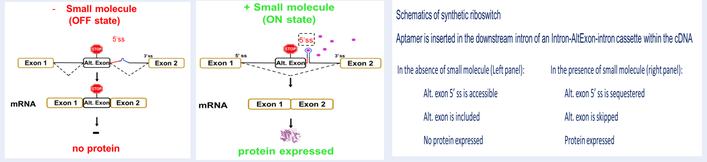
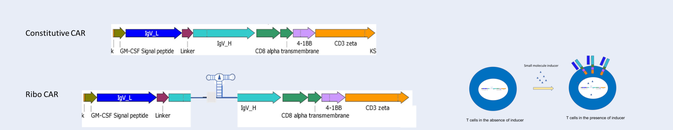
ABSTRACT

Chimeric antigen receptor (CAR)-T cell therapy is a promising treatment for certain cancers. However, it is increasingly evident that the level and timing of CAR molecule expression is important for CAR-T cell activation, durability and anti-cancer activities. Here, we present the development of RiboCAR, a system for precise control of CAR expression via orally available small molecule inducers. Unlike previously reported regulatable CAR platforms that utilize viral protease or chemical-induced protein dimerization, RiboCAR contains a synthetic mammalian ON riboswitch in the coding sequence of the CAR transgene, in which the aptamer functions as a sensor for a specific novel small molecule inducer. The expression level of the CAR gene is precisely dependent on the level of the riboswitch inducer. CAR is undetectable in the absence of the small molecule, and a precise dose response in CAR levels is achieved with increasing dose of small molecule, reaching levels higher than constitutively active CAR upon maximal small molecule dose. Induced CAR expression diminishes following withdrawal of the small molecule. Consistent with small molecule induced expression of the CAR molecule, we controlled CAR-T cell activity by the small molecule inducer. Additionally, T cells with low levels of CAR expression via RiboCAR show enhanced target cell-stimulated T cell activation, reduced markers of exhaustion and greater cytotoxicity when compared with T cells expressing CAR constitutively. CAR levels can be activated to the most effective levels and can be switched on and off according to the presence of the small molecules. With a bioavailable small molecule inducer, CAR-T activity can be precisely tuned and "remotely" controlled *in vivo*. This precise control of CAR levels with its impact on CAR-T activity and durability provides a system for significantly improving the efficacy of CAR-T therapy in comparison to current CAR-T with constitutively active CAR expression.

Aptamer-modulated alternative splicing riboswitch



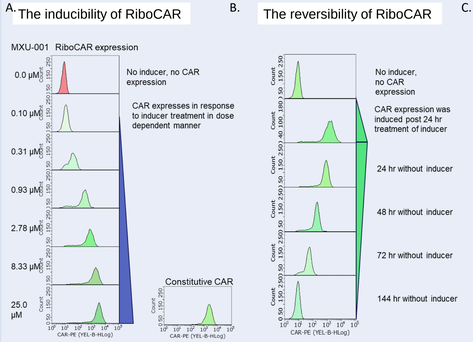


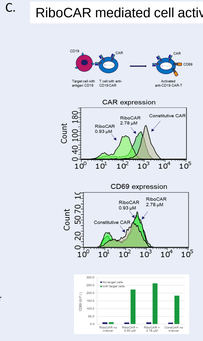
A. Schematics of synthetic aptamer riboswitch

B. Schematics of the RiboCAR gene: riboswitch cassette is inserted in the coding sequence of CAR gene.

- In the absence of the small molecule inducer, CAR is not expressed on cell surface.
- In the presence of the small molecule inducer, CAR is expressed on the cell surface.

Jurkat T cells with RiboCAR knocked into the TRAC locus express higher levels of CD69 in response to target antigen stimulation compared to CAR-T with constitutive CAR knocked into the TRAC locus



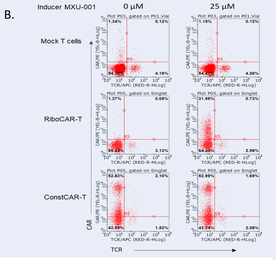


A. Jurkat T cells with RiboCAR knocked into the TRAC locus were treated with a novel small molecule inducer MXU-001 at various concentrations for 48 hrs.

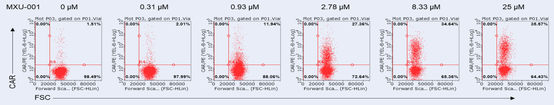
B. 24 hours post MXU-001 treatment, RiboCAR transfected Jurkat T cells were withdrawn from MXU-001 treatment and CAR expression was monitored every 24 hours.

C. Jurkat T cells with RiboCAR or constitutive CAR knocked into the TRAC locus were co-cultured with CD19+ Raji cells at 1:1 ratio in the presence of MXU-001 at the indicated concentrations for 24 hours.

Schematics of RiboCAR gene and its expression



C. Dose dependent expression of CAR in RiboCAR-T cells in response to riboswitch inducer



A. Schematics of RiboCAR gene targeting to TRAC locus in primary T cells using CRISPR/cas9 and AAV6 as HDR donor vector., and the timeline of RiboCAR targeting to primary T cells.

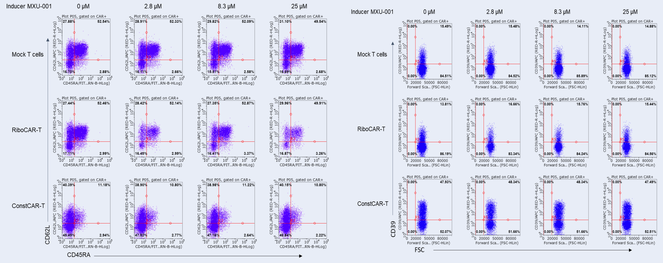
B. Engineered T cells were treated with riboswitch small molecule inducer 3 days post gene targeting to Induce the expression of CAR in RiboCAR-T cells.

C. RiboCAR-T cells were treated with MXU-001 at the indicated concentrations to induce CAR expression

RiboCAR-T cells are less differentiated and enriched in naïve/stem cell-like memory T cells

A. RiboCAR-T cells are less differentiated and exhibit indistinguishable phenotype from Mock T cells

B. RiboCAR does not increase the expression of inhibitory exhaustion marker

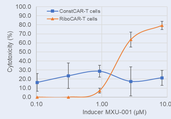


A. RiboCAR-T and ConstCAR-T cells were treated with or without MXU-001 at the indicated concentrations 6 days post CRISPR/cas9 transfection and AAV transduction and staining with anti-human CD62L and CD45RA antibodies.

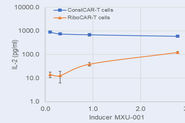
B. RiboCAR-T and ConstCAR-T cells were treated with or without MXU-001 at the indicated concentrations 6 days post CRISPR/cas9 transfection and AAV transduction and staining with anti-human CD39 antibody.

RiboCAR-T cells are more potent than ConstCAR-T cells in anti-cancer activity *in vitro*

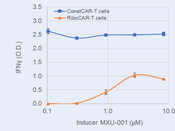
A. RiboCAR-T cells have small molecule dose dependent, superior cytotoxic activity



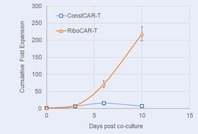
B. RiboCAR-T cells release dose dependent, lower level of IL-2 following tumor cell stimulation



C. RiboCAR-T cells release dose dependent, lower level of IFNγ following tumor cell stimulation



D. RiboCAR-T cells exhibit superior expansion capacity following repeated tumor cell stimulation



A. RiboCAR-T cells or ConstCAR-T cells were co-cultured with Raji-ffLuc cells at 2:1 E:T ratio in the presence of various concentration of MXU-001 for 48 hours. Luciferase activity was measured for cytotoxicity assessment.
B. Supernatants were collected from the cytotoxicity assay in A for IL-2 cytokine ELISA.
C. Supernatants were collected from the Cytotoxicity assay in A for IFNγ cytokine ELISA.
D. CAR-T cells were stimulated with MMC-treated Raji cells at 1:1 ratio with the presence of MXU-001. The stimulation was repeated every 3 days later at the same condition.

Summary

- CAR expression is tightly regulated by riboswitch via a small molecule inducer in dose dependent manner.
- Small molecule dose can fine-tune the level of RiboCAR expression. This precise control of RiboCAR via small molecule inducer improves the activity of CAR-T cells.
- RiboCAR-T cells appear to have more naïve/stem cell memory T cell phenotype in culture compared to ConstCAR-T cells.
- RiboCAR-T cells appear more potent in cancer cell killing activity in cell culture.
- RiboCAR-T cells release lower levels of cytokines following tumor cell stimulation *in vitro*.
- RiboCAR-T cells have higher tumor cell stimulated expansion capacity *in vitro*.
- Riboswitch regulated CAR provides tightly regulated CAR-Ts which have potential safety benefits in addition to the potential for increased potency.