CBLB, CISH and CD70 multiplexed gene knockout with CRISPR/Cas9 enhances cytotoxicity of CD70-CAR NK cells and provides greater resistance to TGF-β for cancer immunotherapy

Chao Guo, Ph.D.[1], Yanying Fan, Ph.D.[1], Alexander Aronov[1], Qi Zhang, M.S.[1] , Sombeet Sahu, M.S.[1], Mary-Lee Dequéant, Ph.D.[2], Changan Guo , Ph.D.[2], Sushant Karnik, M.S.[2], Glenn D. Leary[2], Chandirasegaran Massilamany, MVSc; Ph.D.[2], Parin Sripakdeevong, Ph.D.[2], Shashwat Deepali Nagar, Ph.D.[2], Nicole Flanagan[2], Ivan H. Chan, Ph.D.[1], James B. Trager, Ph.D. [1]*, [1]Nkarta Therapeutics, South San Francisco, CA, USA [2]CRISPR Therapeutics, Cambridge, MA *corresponding author

Introduction

Natural killer (NK) cells provide an attractive platform for development of effective cancer immunotherapies. NK cells are known for their ability to kill tumor cells and do not elicit graft-versus-host disease, making them a potential source of 'off-the-shelf' allogeneic cell therapy. NK cells are also amenable to CRISPR genomic engineering to enhance the antitumor activity of NK cells by increasing their cytotoxicity, overcoming suppression within the tumor microenvironment, or promoting their persistence and homing to tumor sites. CD70 is aberrantly expressed in a variety of malignant settings, including renal cell carcinoma (RCC) and adenocarcinoma, while its expression in normal tissues is restricted to a subset of lymphoid cell types. Cytokine inducible SH2-containing protein (CISH) is a NK cell checkpoint for IL-15 mediated NK survival, proliferation, cytotoxicity, and anti-tumor immunity. The E3 ubiquitin ligase CBLB is another negative regulator of NK cell function and has been shown to mediate TGF-β sensitivity by downregulating inhibitory SMAD7 in primary T cells. We hypothesized that knockout of both CISH and CBLB would not only improve NK cell effector function and but also render NK cells resistant to TGF-β mediated suppression.

Methods

In this study, we utilized CRISPR-Cas9 ribonucleoproteins (RNPs) to disrupt CISH and CBLB genes in isolated peripheral blood NK cells from healthy donors. CD70 was also knocked out on CAR NK cells to avoid fratricide due to CD70 expression on activated NK cells and specifically target CD70 overexpression on renal cell carcinoma (RCC) tumor cells. Western blotting, flow cytometry, and TIDE/Amplicon NGS Sequencing data confirmed all three genes were successfully disrupted. Then we expanded these edited NK cells by using IL-2 and stimulation using NKSTIM, a modified K562 stimulatory cell line expressing membrane-bound form of IL-15 (mbIL-15) and 4-1BBL. IL-12 and IL-18 were added during expansion to drive memory-like NK cell differentiation. Furthermore, we were able to transduce CRISPR/Cas9 edited NK cells to express a CD70-CAR construct and membrane bound IL-15. CAR expression was assessed by flow cytometry. In vitro cytotoxicity was measured using the IncuCyte S3 live cell analysis system.

Results

CD70/CISH/CBLB triple knockout CD70-CAR NK cells could be produced efficiently and exhibited similar persistence as CD70/CISH or CD70/CBLB double knockout CD70-CAR NK cells in culture. Cytotoxicity assays demonstrated that CD70/CISH/CBLB triple knockout CD70-CAR NK cells had greater tumor growth control after multiple rechallenges. In the presence of exogenous TGF-β, CD70/CISH/CBLB triple knockout CD70-CAR NK cells showed greater resistance to TGF-β mediated inhibition of cytotoxicity.

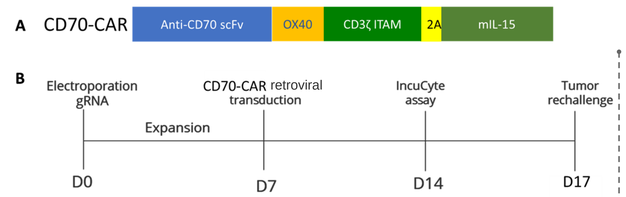
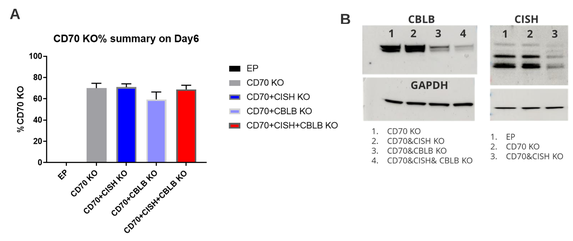


Figure 1. (A) Schematic map of retroviral vector encoding CD70-CAR and membrane bound IL-15. (B) Schematic timeline for producing gene-knockout CD70-CAR NK cells



C

TIDE or NGS on target INDEL frequency			
Group	CD70	CISH	CBLB
EP only	0	0	0
CD70 KO	74.2%	N/A	N/A
CD70+CISH KO	76.5%	68.9%	N/A
CD70+CBLB KO	72.8%	N/A	87.8%
CD70+CISH+ CBLBKO	75.7%	68.7%	81.0%

Figure 2. Cas9-RNP results in efficient gene deletion in primary human NK cells. (A) CD70 knockout efficiency is between 60-70% before CD70-CAR transduction. (B) Western blot for CISH and CBLB proteins from control or gene KO NK cells. (C) Summary table for indel frequency of CD70, CISH and CBLB based on TIDE analysis or targeted NGS of primary human NK cells.

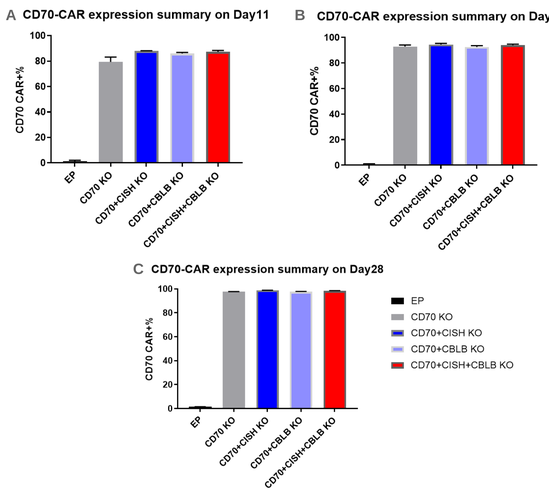


Figure 3. CD70-CAR+ NK cells are enriched during in vitro culture.
CD70-CAR expression on transduced NK cells at (A) day 11, 4 day post transduction, (B) day 21 and (C) day 28.

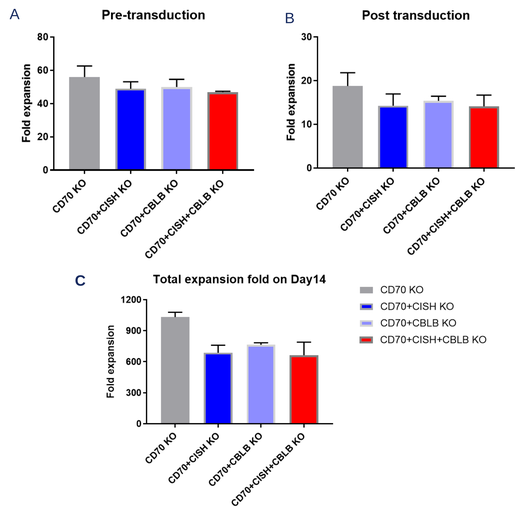


Figure 4. CD70/CISH/CBLB triple knockout CD70-CAR NK cells could be produced efficiently and exhibited similar expansion as CD70/CISH or CD70/CBLB double knockout CD70-CAR NK cells in culture.
(A) Fold expansion of NK cells on day 7 (pre-transduction) (B) Fold expansion of NK cells on day 14 (7 days post transduction) (C) Total fold expansion of NK cells (day 0-14).

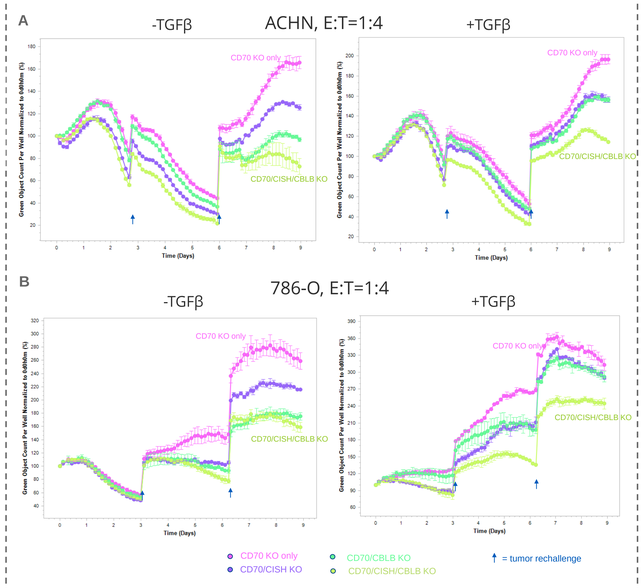


Figure 5. CISH/CBLB combination KO is highly active in controlling tumor growth after multiple tumor rechallenges. Cytotoxicity of gene-knockout CD70-CAR NK cells against renal cell carcinoma (RCC) cell line: (A) ACHN cells or (B) 786-O cells at a 1:4 E:T ratio in the absence or presence of TGFβ (20ng/ml) via Incucyte at day 14.

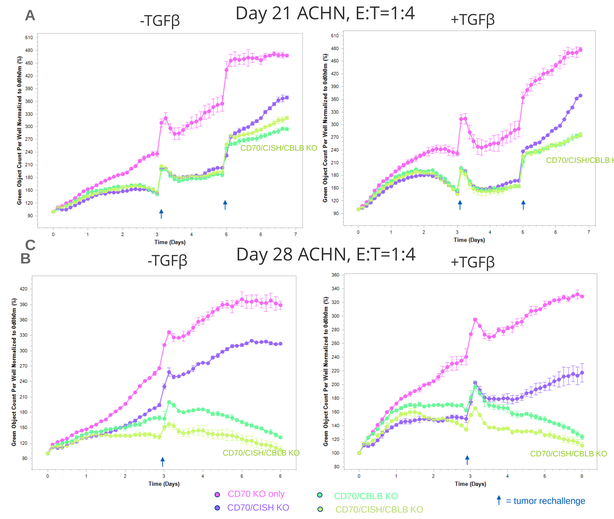


Figure 6. Even after extended culture, CISH/CBLB combination KO shows greater tumor growth control upon multiple tumor rechallenges.
Cytotoxicity of gene-knockout CD70-CAR NK cells against ACHN cells at a 1:4 E:T ratio in the absence or presence of TGFβ (20ng/ml) via Incucyte at (A) day 21 or (B) day 28.

Conclusion

In summary, we show CD70/CISH/CBLB triple knockout CD70-CAR NK cells demonstrate enhanced anti-tumor activity against renal cell carcinoma (RCC) solid tumor cell lines and provide greater resistance to TGF-β mediated inhibition. These data support the further exploration of CD70/CISH/CBLB triple gene knockout CD70 CAR NK cells for clinical application.

References

1. Rautela et al, *bioRxiv* 2018 Sep 6
2. Delconte et al, *Nature immunol* 2016 Jul;17(7):816-24.
3. Viel et al, *Sci Signal* 2016 Feb 16;9(415):ra19.
4. Gruber et al, *J Mol Cell Biol.* 2013 Dec;5(6):358-68.
5. Al Sayed et al, Blood 2017 Jul 20;130(3):297-309.
6. Ran et al, *Nat Protoc* 2013 8, 2281-2308.
7. Lu et al, *J Immunol.* 2021 Feb 15;206(4):677-685.

Contact

James Trager, PhD
jtrager@nkartatx.com
www.nkartatx.com