Armoring ROR1 CAR T cells with IL18 improves CAR T cell function and cytotoxicity against pancreatic cancer cell-lines *in vitro* and *in vivo*

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Introduction

Background: ROR1 (Receptor tyrosine kinase-like Orphan Receptor 1) is a tumor-associated antigen widely expressed in solid tumors and is one of promising target for CAR T cell therapy in pancreatic cancer. A human second-generation ROR1-targeted CAR T-cell product was developed which demonstrated anti-tumor activity *in vitro* and *in vivo*[1]. This CAR T was further armored with IL18 to enhance expansion, persistence, and antitumor efficacy against pancreatic cell lines both *in vitro* and *in vivo*. To minimize the potential toxicity associated with constitutive IL18, an inducible NFAT promoter-based construct was developed.

CAR Design

Schematic representation of IL18-armored ROR1 CAR T-cell constructs

A second-generation CAR featuring an scFv targeting the extracellular domain of human ROR1 was designed with or without IL18.

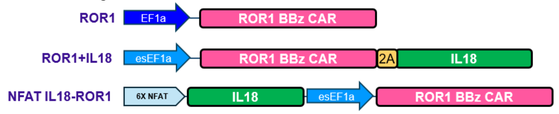


Figure 1. Structure of ROR1 and IL18-armored ROR1 CAR constructs. CAR constructs were generated by linking a single chain fragment variable (scFv) sequences targeting ROR1 in frame with a CD8 hinge (H) and transmembrane domain (TM), 4-1BB co-stimulatory domain and CD3 zeta activation domain. ROR1+IL18 (constitutively expressing IL18) was generated by incorporating IL18 sequence at the 3′ end of ROR1 CAR separated by P2A. NFAT IL18-ROR1 (inducible IL18 expressing cassette) was placed upstream of the internal CAR-promoter sequence. Lentiviral vectors (LV) encoding each construct, utilizing a EF1α promoter, were generated and used to transduce primary human T cells.

Results: CAR Ts Characterization

Manufacturing characteristics of ROR1 and ROR1+IL18 CAR T-cells

Human CD4+/CD8+ T cells were purified from leukopaks and transduced with ROR1-CAR lentiviral vector +/- IL18. Armored IL18 CAR T-cells showed increased expansion and percentage of CD8+ CAR at harvest, compared to unarmored CARs.

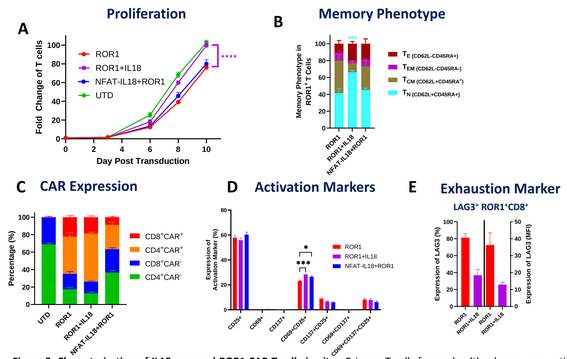


Figure 2. Characterization of IL18-armored ROR1 CAR T-cells *in vitro*. Primary T cells from a healthy donor were activated with TransAct™ in the presence of IL7/15 and transduced with LV encoding either ROR1 CAR alone (ROR1) or armored with IL18 (constitutive and NFAT-driven inducible). CAR T-cell expansion was analyzed by Vicell **(A)**. Flow cytometric analysis of memory phenotype **(B),** CAR expression **(C),** activation markers **(D),** and exhaustion markers **(E)** were evaluated. Bars signify the mean of three technical replicates (3 donors)± SEM. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001

Results: *In vitro* Cytotoxicity

IL18-armored ROR1 CAR T-cells showed comparable cytotoxic activity against AsPC-1 cell line

Cytotoxicity of CAR T cells against pancreatic tumor cell line AsPC-1 was accessed by xCELLigence serial killing assay. IL18-armored ROR1 CAR T-cells (constitutive or inducible) demonstrated comparable anti-tumor activity.

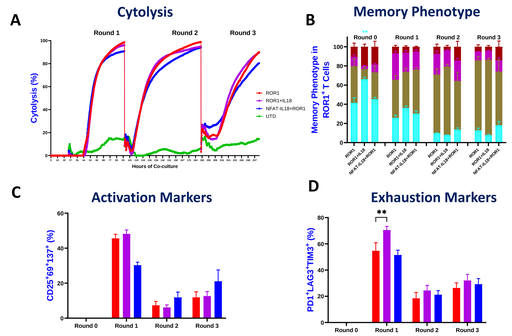


Figure 3. IL18-armored ROR1 CAR showed similar cytoxicity against AsPC-1 *in vitro*. Armored or non-armored CAR T-cells were co-cultured with AsPC-1 cell line; cytotoxicity was monitored by xCELLigence (**A**); T cell memory phenotype (**B**), activation (**C**) and exhaustion (**D**) markers were assessed by flow cytometry. Bars signify the mean of three technical replicates (3 donors)+ SEM. **p<0.01

IL18-armored ROR1 CAR T-cells demonstrated enhanced IFNγ and/or killing in Ovarian cancer (OVCAR3) and Glioblastoma (A-172) cell lines when compared to unarmored CARs

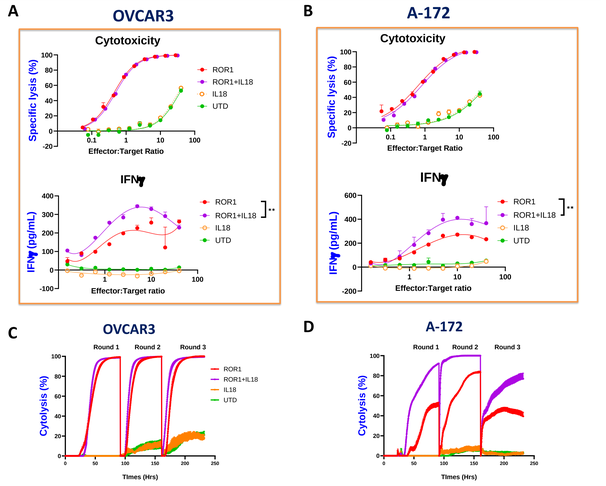


Figure 4. *In vitro* cytoxicity of IL18-armored ROR1 CAR T-cells against OVCAR3 ad A-172 cell lines. Armored or non-armored CAR T-cells were co-cultured with OVCAR-3 or A-172 cell line either overnight (**A**,**B**) or long-term and re-challenge (**C**,**D**). Cytotoxicity was assessed by Luciferase-based assay (**A**,**B**) or x-celligence (**C**,**D**). Secreted INFγ was detected by ELISA (**A**,**B**). Each symbol signifies the mean of three technical replicates (3 donor)+ SEM. **p<0.01

Results: *In vivo* Efficacy

IL18-armored ROR1 CAR T-cells reduced tumor burden in Pancreatic cancer xenograft model AsPC-1

IL18-armored ROR1-CAR efficacy was evaluated *in vivo* using NSG mice xenografted by subcutaneous implantation of AsPC-1 and then treated with ROR1 CAR T-cells, with or without armoring. CAR-T and cytokine level in peripheral blood were determined by flow cytometry and MSD U-plex assay, respectively. Caliper measurement of xenograft tumor size was performed to assess tumor burden.

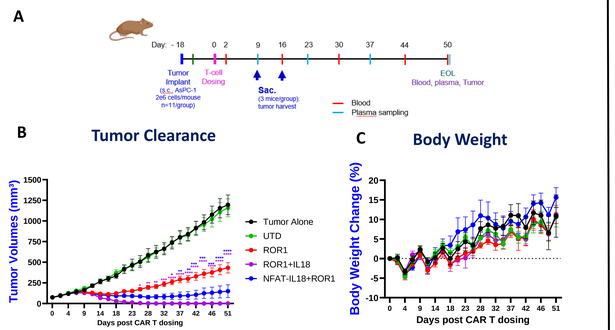
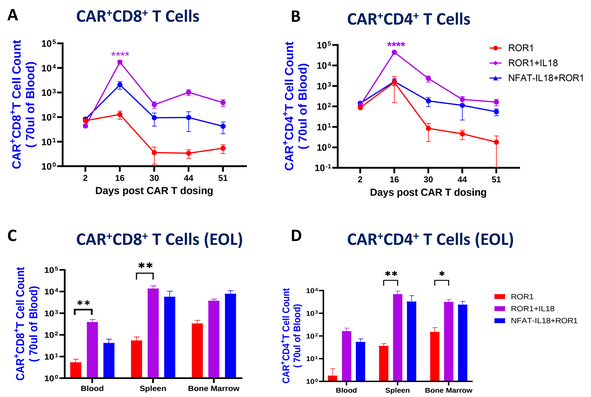


Figure 5. IL18-armored ROR1 CAR T-cells enhanced anti-tumor activity in AsPC-1 xenograft model. Schematic diagram of *in vivo* study design **(A)**. Measurement of tumor volume **(B)**,and body weight change during the study **(C).** Five NSG mice per group were enrolled with 2e6 AsPC-1 dose were engrafted subcutaneously. 1e6 CAR⁺T cells/mouse were administered intravenously (IV). Data were analyzed statistically with mean ± SEM represented in graphs. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001

IL18-armored ROR1 CAR T-cells showed increased number and persistence against Pancreatic cancer xenograft model AsPC-1

T cell number (CAR⁺CD4/8⁺) were analyzed from peripheral blood of AsPC-1 xenografted NSG mice treated with ROR1 CAR T-cells with/out armor using flow cytometry. Blood, spleen and bone marrow were also processed and analyzed at end of life (EOL) study.



Results: *In vivo* Efficacy

Figure 6. IL18-armored ROR1 CAR T-cells showed higher proliferation and persistence in AsPC-1 *in vivo* study. Constitutive IL18 and NFAT-driven inducible IL18 armored CAR-T enhanced proliferation and persistence of CD8⁺/CD4⁺ ROR1 CAR⁺T-cells compared to non-armored ROR1 CAR Ts in peripheral blood samples **(A, B)**, as well as spleen and bone marrow samples at take down (EOL, end of life) (**C,D**). Mean counts ± SEM is displayed. *p<0.05, **p<0.01, ****p<0.0001

IL18-armored ROR1 CAR Ts showed increased effector cell phenotype compared to ROR1 CAR alone early (day 2 post-CAR T infusion) in AsPC-1 Pancreatic cancer xenograft model

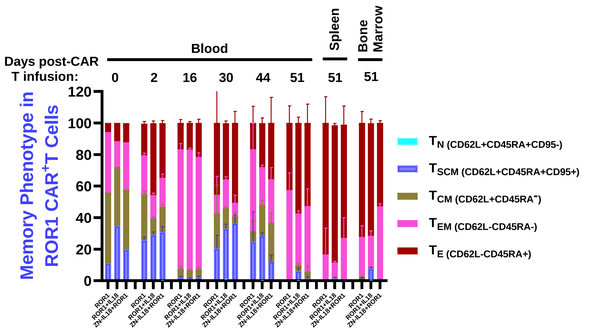


Figure 7. IL18-armored ROR1 CAR T-cells showed higher effector cell phenotype at early stage (i.e. day 2 post-CAR T infusion) in AsPC-1 xenograft *in vivo* study. Peripheral blood of AsPC-1 xenografted NSG mice treated with ROR1 CAR T-cells with/out armor were collected at the indicated time points; memory phenotypes of ROR1 CAR+T cells were analyzed by flow cytometry. At day 51 post-CAR T infusion (i.e. EOL), spleen and bone marrow were also processed and analyzed. Data were analyzed statistically with mean ± SEM represented in graphs

Conclusions

- IL18 armor augmented ROR1 CAR T-cell proliferation during manufacturing and demonstrated superior anti-tumor activity *in vitro* with regard to cytokine production and long-term re-challenge.
- In xenograft studies, IL18 CAR T-cells enhanced CAR-T cell number, persistence, and anti-tumor activity.
- Armoring of CAR T cells with NFAT-inducible IL18 performed nearly as well in *in vivo* studies, and provided the option for greater control of potential IL18-associated toxicity with constitutive expression-based LV constructs.

References

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3. Li, Y. R., Zhu, Y., Chen, Y., & Yang, L. (2025). The clinical landscape of CAR-engineered unconventional T cells. Trends in cancer, 11(6), 520-539.