Gamma-delta (γδ) CAR-T Cells Lacking the CD3ζ Signaling Domain Enhance Targeted Killing of AML Cells and Preserve Healthy Tissues

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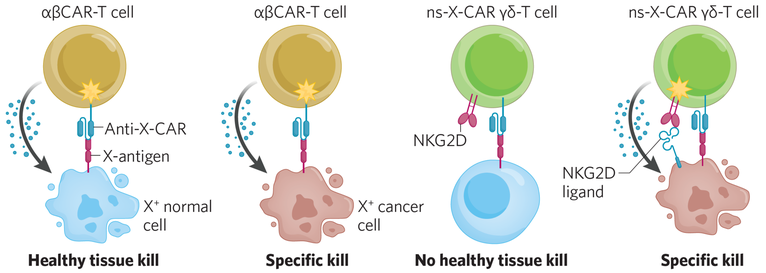
Introduction

Chimeric antigen receptor T cell (CAR-T) therapy has shown remarkable efficacy against B cell malignancies, offering hope to patients with limited treatment options. Extending this success to myeloid malignancies and solid tumors poses significant challenges due to the co-expression of targetable antigens on healthy tissues and hematopoietic progenitors (HSPCs). In this context, gamma-delta (γδ) T cells emerge as a promising alternative, equipped with the ability to recognize and eliminate malignant cells through the identification of multiple tumor-associated stress antigens, sparing normal tissues. We leveraged the tumor-sensing capabilities of γδ T cells with enhanced tumor localization by employing a non-signaling CAR (nsCAR) that excludes the CD3ζ domain, facilitating targeted tumor cell killing while preserving healthy tissues (Fig. 1). nsCAR constructs targeting CD33 were created to modify ex-vivo expanded and activated γδ T cells (nsCD33CAR) and evaluated against acute myeloid leukemia (AML) lines HL-60, KG-1a, and MOLM-13, as well as healthy donor CD34+ HPSCs, which also express CD33. Additionally, we tested three constructs including a CD33 targeting nsCAR (ns33-mCherry), a ns33CAR with co-expression of a membrane bound IL-15/IL15Ra fusion protein (ns33-mb15) to potentially augment cytotoxicity, and a CD33/CD123 dual-targeting nsCAR construct that also expresses IL-15 (ns-IL3-33-mb15).

Fig 1. Differentiating between healthy and cancer cells. Activation of nsCAR is mediated through the endogenous γδ T cell receptors and other surface molecules such as NKG2D and DNAM-1 and not through CAR-inducing signaling allowing this platform to distinguish healthy and cancer cells.



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Methods

Validation of CAR constructs by Jurkat T activation co-culture assay

- Jurkat T cells were transduced with sCAR or nsCAR lentivirus and subsequently co-cultured with CD33+ KG-1a cells at 1:1 ratio for 24 hours. Following the co-culture, the activation of CD69 was assessed using flow cytometry analysis.

γδ T mediated cytotoxicity assay

- Activated and expanded Vδ2+ γδ T cells from healthy donors were transduced with ns33CAR lentivirus and the transduction efficiency measured by flow cytometry two days post-transduction. For cytotoxicity assay, both untransduced (UTD) and nsCD33-CAR transduced γδ T cells were co-cultured with AML lines HL-60, KG-1a, MOLM13 and BM-CD34+ HSPCs obtained from healthy donor for 24h. γδ T cell mediated cytotoxicity was assessed via flow cytometry (CSFE+7AAD+/total CSFE+ cells).

Validation of ns33CARs

Signaling and non-signaling CD33CAR constructs

Fig 2. Singling and non-signaling CAR constructs

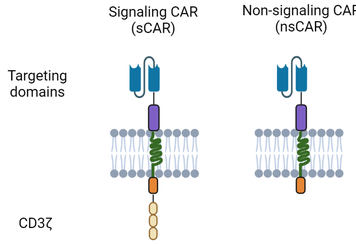
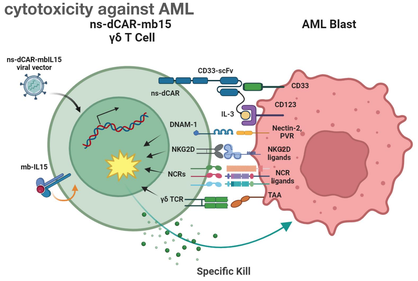
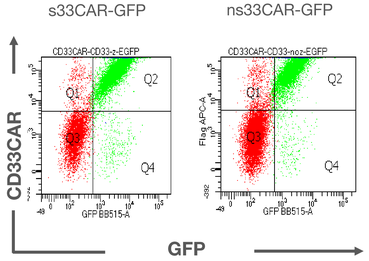


Fig 3. Proposed model for ns-dCAR-mb15 enhanced cytotoxicity against AML



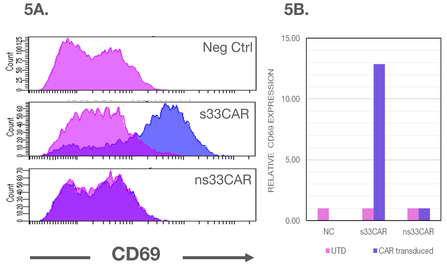
Validation of the s33CAR/ns33CAR expression

Fig 4. Flow analysis of s33CAR/ns33CAR transduced Jurkats



Activation of Jurkat-s33CAR/ns33CAR

Fig 5. Activation of CD69 after 24h-coculture with AML line KG-1a



ns33CAR-Jurkat Mitigate AICD with Extended AML Coculture

s33CAR and ns33CAR Jurkat cells cocultured with KG-1a AML cells for 7 days.

The CAR surface expression decreased with length of co-culture in s33CAR+ constructs compared with no change in ns33CAR+ population after extended co-culture with KG-1a.

Fig 6A.

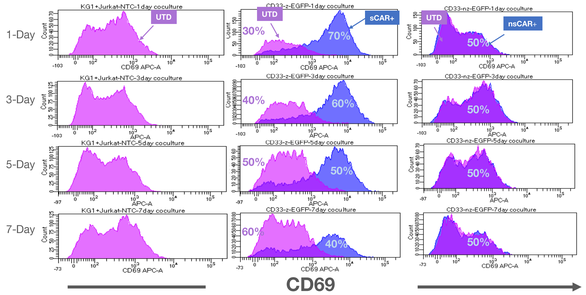
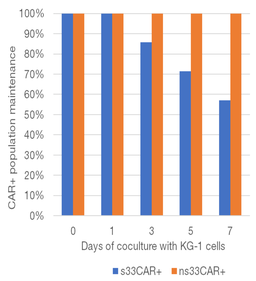


Fig 6B.



ns33CARs Enhance Cytotoxicity And Spare Healthy Tissues

Transduction of γδ T cells with 3 different nsCD33-CAR lentiviral vectors

Fig 7A.

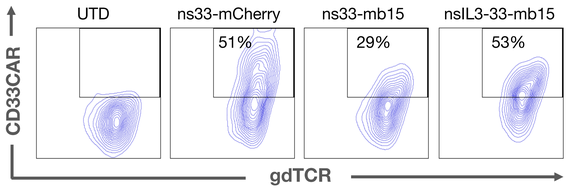
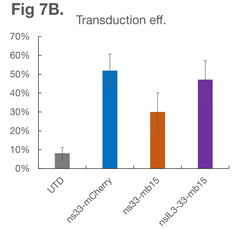
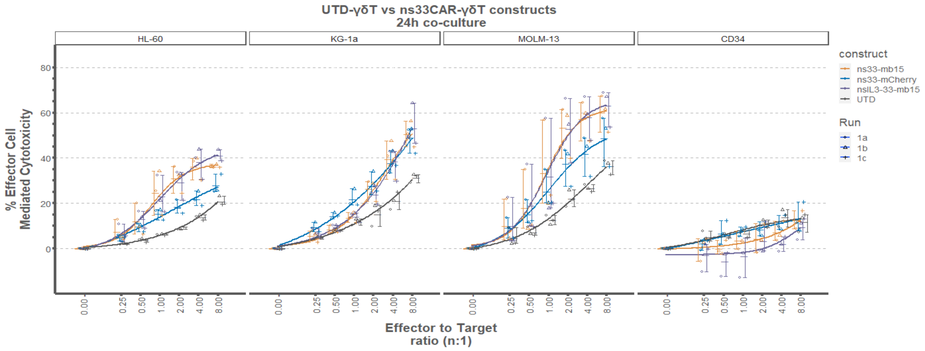


Fig 7B.



Cytotoxicity assay: nsCD33-CAR-γδ T vs AML and healthy donor CD34+ HSPCs

Fig 8. nsCD33-CAR-γδ T vs AML and CD34, 24h coculture, in triplicates



- ns33CAR-γδT cells exhibited enhanced cytotoxicity, with an increase of up to 3x
- Membrane-bound IL15/IL15Ra (mb15) showed greater cytotoxicity compared to without it
- Minimal killing of healthy donor CD34+ HSPCs that express CD33 with UTD and all ns33CAR-γδT cells

Conclusions

- The nsCAR platform demonstrates the ability to distinguish between healthy and leukemic cells even when the CAR antigen targets are expressed on the healthy tissues, widening the therapeutic index and reducing the risk of "on-target, off-tumor" toxicity.
- The nsCAR constructs likely increase the immune synapse resulting in cells that exhibit greater killing against various AML lines expressing CD33 and CD123. Importantly, these engineered cells exhibit minimal killing against healthy donor CD34+ HSPCs. Similar cells utilized in an on-going Phase 1 trial of INB-100 (NCT03533816) have not demonstrated significant hematopoietic toxicities to date.
- Co-expression of membrane-bound IL15-IL15Ra enhances the cytotoxicity of nsCD33CAR-γδ T cells against AML cells. Ongoing assessment aims to evaluate the impact of this modification on the persistence and fitness of nsCAR-γδ T cells.
- While the IL3/CD33 dual-nsCAR did not show additional enhanced cytotoxicity *in vitro* against AML lines compared to mono-nsCD33CAR, it holds potential for *in vivo* therapy targeting leukemic stem cells (LSCs) and primary AML cells with heterogeneous phenotypes.
- Overall, the nsCAR platform for γδ T cells emerges as a promising candidate for myeloid leukemias and solid tumor cancers. Current efforts focus on optimizing the constructs for maximum cytotoxicity against highly resistant AML and advance towards IND enabling studies.