Developing CD33 CAR-T Cells Using CRISPR/Cas9-Mediated Genome Editing for Improved AML Therapy

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INTRODUCTION

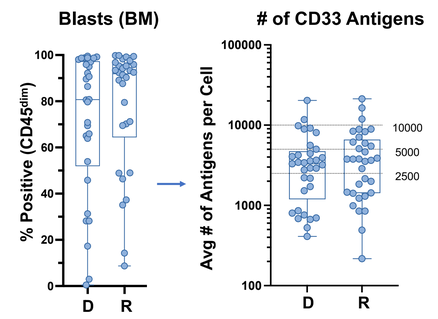
- CD33-directed therapies for Acute Myeloid Leukemia (AML) are hampered by on-target, off-tumor activity, resulting in severe myelotoxicity.
- Trem-cel (NCT04849910) is a HSPC transplant product designed to provide a reconstituted hematopoietic compartment that is resistant to anti-CD33 drug cytotoxicity[1].
- Empowered by the ability to create and target "cancer-specific antigens", we leveraged our genome engineering approaches to develop a more sensitive CD33-targeting therapy to address the range of target antigen expression found on patient cells.
- Recent advances in genome engineering demonstrated the feasibility of directed chimeric antigen receptor (CAR) insertion into T cells to enable next generation CAR-T therapies[2,3].
- Site-specific insertion of CAR into the *TRAC* locus has the potential to simultaneously yield a more uniform CAR-T product, minimize graft-versus-host disease, reduce potential risk of insertional oncogenesis, and enhance CAR potency[4].

OBJECTIVE

- Establish a genome engineered CAR-T platform that enables the development of immunotherapies with potent cytolytic activity, either as a stand-alone treatment, or in combination with Vor's platform of engineered transplant, to eliminate on-target, off-tumor toxicity of CAR-T cells to fully benefit high-risk AML patients.

METHODS

Fig 1. CD33 expression in primary AML bone marrow



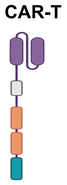
- D: Diagnosis, R: Relapse. CD33 antigen expression evaluated on patient sample blasts at diagnosis and relapse (QuantiBRITE) shows variable average CD33 antigens per cell (APC), potentially limiting patient cell targetability by current standard of care therapeutics in a traditional treatment setting.

Fig 2. CD33-directed Therapies

ADC (Antibody Drug Conjugate)

Gemtuzumab Ozogamicin (GO)
- FDA approved therapy for AML
- CD33 monoclonal antibody
- Linked to cytotoxic agent
- Moderate antigen sensitivity in a traditional treatment setting

CAR-T (Chimeric Antigen Receptor)

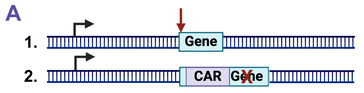


CD33-CAR
- Lintuzumab CD33 binder
- CD8 transmembrane domain and hinge
- 4-1BB (bbz) or CD28 (28z) co-stimulatory domain (co-stim)
- High antigen sensitivity

- CAR-T generated with CRISPR/Cas9 and homology dependent repair (HDR):
  - Expression driven by endogenous promoter
  - Functional impact of integration on T cell biology

Fig 3. CRISPR/Cas9 and HDR yields CAR driven by endogenous promoter of choice[2,3]

A



B

Platform-enabling loci of interest:

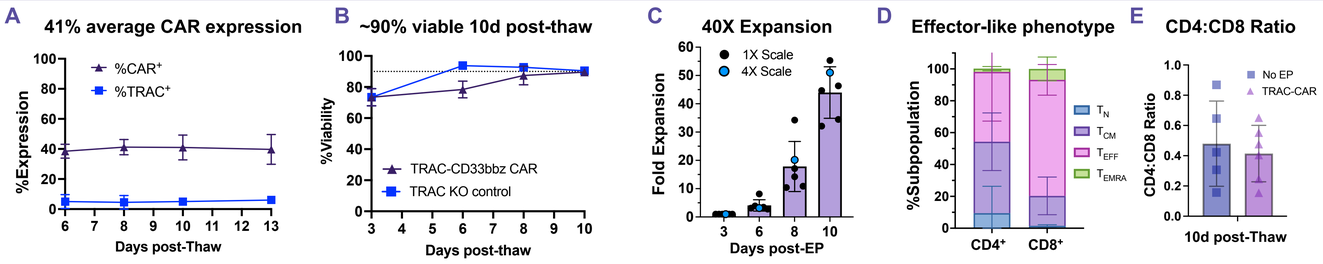
TRAC	Improved CAR expression and potency[2]
CD5	Functional enhancement[5]
B2M	Allo-enabling

- **A. 1.** Cas9 + gRNA (red arrow) targets the integration site of interest creating a double stranded break that is repaired via HDR using a provided homology dependent repair template (HDRt). **2.** The resulting integrated CAR sequence is driven by the endogenous promoter (black arrow) of the targeted gene, and the gene itself is knocked out (red x).

RESULTS

Fig 4. CRISPR/Cas9 combined with HDR generates highly viable CAR-T cells with an effector-like phenotype at scale

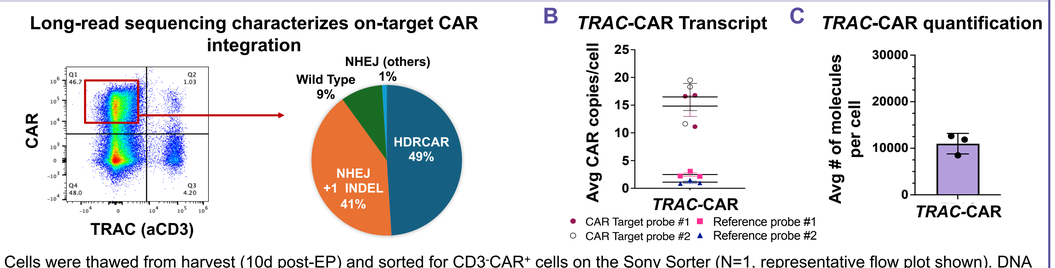
CAR-T Characterization



- Representative data of *TRAC*-CD33bbz-CAR generated at research-scale. **A.** Stable CAR expression and TRAC KO observed over 10d post-electroporation (EP, 13d post-thaw). **B.** Cells recover quickly post-EP to ~90% viability at day of harvest. **C.** Culturing process results in highly consistent and scalable cell expansion, with similar fold expansion observed at 1X and 4X cellular input to EP. **D.** *TRAC*-CAR cells show the expected effector-like phenotype, with CD8+ cells more differentiated and effector-like than CD4+ cells. **E.** The total and CAR+ cell populations show more CD8+ cells at the end of the process than CD4+ (representative *TRAC*-CD3328z-CAR). (N=4-6 donors).

Fig 5. *TRAC*-CD33-CAR molecular analysis yields on-target characterization and surface quantification

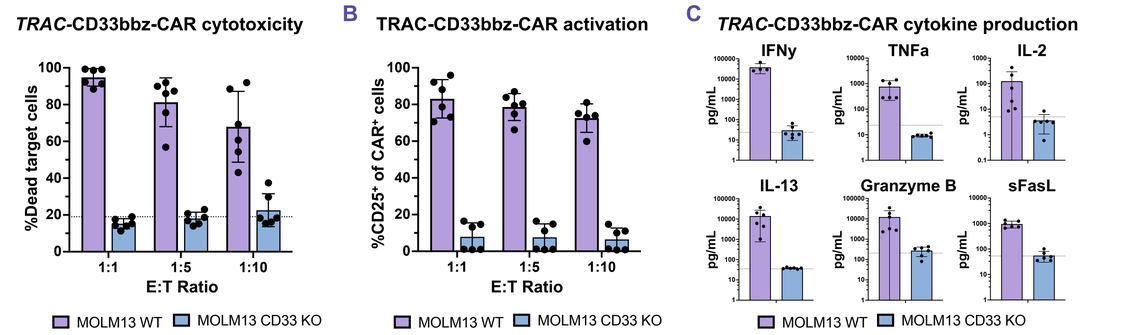
CAR-T Molecular Analysis



- **A.** Cells were thawed from harvest (10d post-EP) and sorted for CD3-CAR+ cells on the Sony Sorter (N=1, representative flow plot shown). DNA was isolated from collected cells and the region of interest was PCR amplified from primers that bind outside of the homology arms before being input to Nanopore library preparation protocol. Post-sequencing, the data was quality controlled and aligned to CAR and human genome reference sequences to generate editing frequencies. **B.** Transcript was evaluated using ddPCR with primers that bind to the scFv of the CAR construct (N=3). **C.** *TRAC*-CAR surface molecules were evaluated using a PE-tagged rCD33 antibody and quantified using QuantiBRITE (N=3).

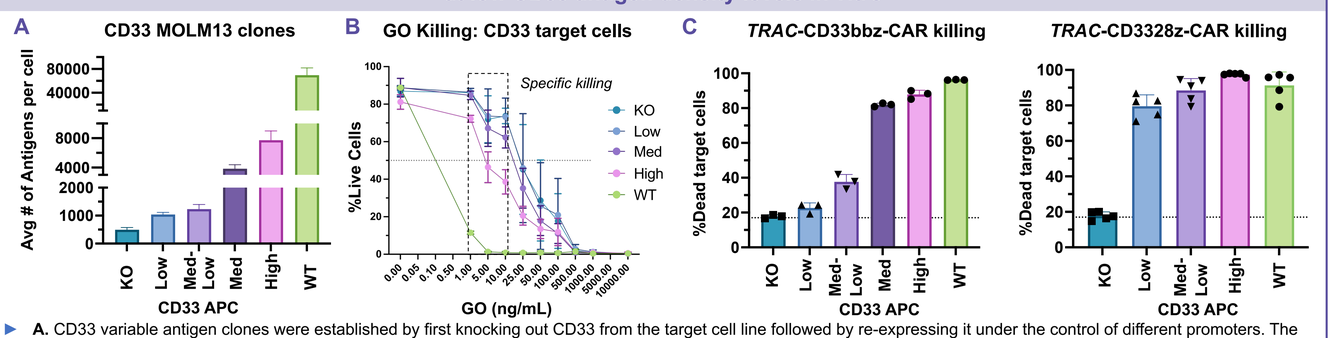
Fig 6. *TRAC*-CD33bbz-CAR exhibits antigen-dependent cytolytic activity and cytokine secretion *in vitro*

CAR-T Function



- **A.** *TRAC*-CD33bbz-CAR cells were co-cultured in a 48h cytotoxicity assay with WT (CD33+) and CD33 KO MOLM13 target cells; specificity of killing was evaluated at multiple Effector to Target (E:T) Ratios (N=6 donors, line denotes background target cell health). **B.** CAR+ cell activation status was evaluated in the same assay by CD25 status. **C.** *TRAC*-CAR cells produce relevant cytokine above background levels (line) only when cultured with WT cells (1:1 E:T).

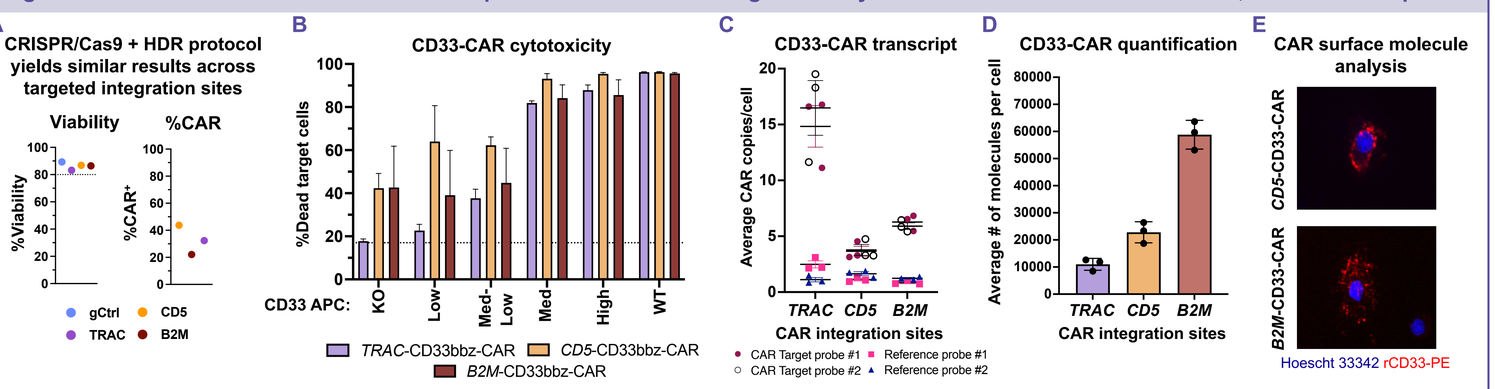
Fig 7. *TRAC*-CD33-CAR constructed with a 4-1BB or CD28 co-stim domain demonstrate potent cytolytic activity at low CD33 antigen density levels *in vitro*



- **A.** CD33 variable antigen clones were established by first knocking out CD33 from the target cell line followed by re-expressing it under the control of different promoters. The cells were then single cell sorted on CD33 expression by FACS and expanded. A range of CD33 expressing clones (low to high) were chosen for further evaluation in cytotoxicity assays. **B.** The ADC GO shows limited killing of cells that have antigens <4000 average CD33 molecules on the cell surface in this *in vitro* model. **C.** *TRAC*-CD33 CAR shows co-stim dependent sensitivity to CD33 antigen density, with *TRAC*-CD3328z-CAR showing the highest sensitivity to CD33 even at the lowest CD33 density levels.

Fig 8. *B2M*- and *CD5*- CD33bbz-CAR are more potent at lower CD33 antigen density levels *in vitro* than *TRAC*-CD33bbz, but also less specific

Genomic Loci Expansion



- **A.** CD33-CAR shows similar CAR frequency and overall cell viability when additional loci are targeted for integration. **B.** CD33-CAR integrated at *CD5* and *B2M* demonstrate increased CD33 sensitivity and killing of CD33 low and med-low antigen expressing cells over *TRAC* integrated CAR, though with a subsequent increase in non-specific activity (N=3, line denotes target cell background). **C.** CD33-CAR transcript is tightly expressed across N=3 donors and shows locus-dependent levels (N=3, ddPCR). **D.** CD33-CAR surface molecules were evaluated using PE-tagged rCD33 antibody and quantified by QuantiBRITE. **E.** Functional CAR surface molecules were stained with rCD33 antibody and imaged with a Nikon SoRa Spinning Disc Confocal AX series, 40X oil immersion.

Fig 9. CD33 Antigen Density Platform enables *in vitro* therapeutic modeling

Antigen Density Platform

A

Avg. CD33 antigen density:	Neg	Low	Med-low	High	WT
ADC	*Non-specific killing*	-	-	++	+++
TRAC-CAR (4-1BB)	-	-	+	+++	+++
TRAC-CAR (CD28)	-	+++	+++	+++	+++
B2M-, CD5- 4-1BB CAR	*Non-specific killing*	++	+++	+++	+++

Targetability

B

CAR-T Architecture

Insertion site

Therapeutic Modality

- **A.** ADC modality shows the least sensitivity and most non-specific killing in the CD33 antigen density platform. *TRAC*-CAR demonstrates co-stim dependent antigen sensitivity, with CAR containing a CD28 co-stim domain showing the highest sensitivity and specificity of modalities evaluated. CAR integrated at *B2M* or *CD5* show increased antigen sensitivity as well as increased non-specific killing. **B.** CD33 antigen density platforms enables evaluation of insertion site impact to T cell biology and CAR expression, CAR-T architecture and design, and therapeutic modality choice.

CONCLUSIONS

- Robust knock-in of CD33-CAR to the TRAC locus (*TRAC*-CD33-CAR) was achieved using CRISPR/Cas9 and HDR, resulting in consistent CAR expression, high viability, and scalable expansion.
- Molecular analysis of *TRAC*-CD33-CAR revealed high TRAC knock out with simultaneous on-target integration, and quantified CAR transcripts and surface molecules driven by the endogenous *TRAC* promoter.
- *TRAC*-CD33-CAR is specific and sensitive to low CD33 target antigen densities in a co-stim dependent manner, efficiently killing CD33+ low density target cells.
- Targeted insertion to loci with varied endogenous promoter activity can be used to tune CAR surface expression and sensitivity to low antigen density target cells.
- **This work enables pre-clinical development of next-generation CAR-T cell therapies with enhanced safety and potency to improve AML patient outcomes**

References

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Disclosures
All authors listed above are current or former employees of Vor Bio

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