Leveraging CRISPR/Cas9 and HDR to create an engineered CD33 CAR-T to target AML

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INTRODUCTION

► CD33-directed therapies for Acute Myeloid Leukemia (AML) are hampered by severe myelotoxicity due to on-target, off-tumor activity.

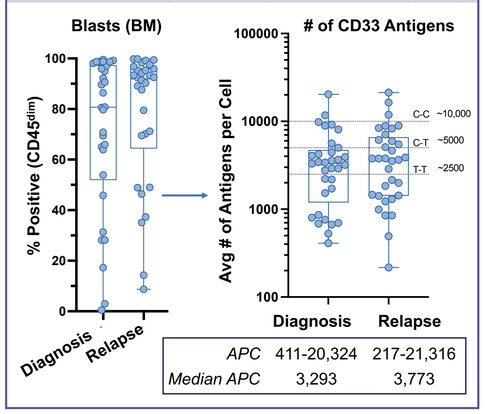
► Trem-cel is a HSPC transplant product designed to provide a reconstituted hematopoietic compartment that is resistant to anti-CD33 drug cytotoxicity[1].

► Patient AML blasts are heterogeneous and display a range of target antigen expression correlated with genotype (Fig 1).

AIM

► **To develop a more efficacious CD33-targeting therapy using CRISPR/Cas9 and Homology-Directed Repair (HDR).**

Fig 1. CD33 is expressed on patient AML blasts at a range of antigens per cell (APC)



	Diagnosis	Relapse
APC	411-20,324	217-21,316
Median APC	3,293	3,773

METHODS

Fig 2. CD33-directed Therapies

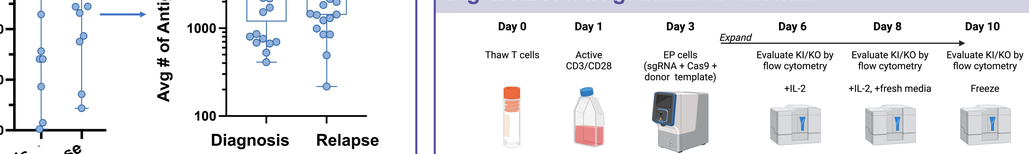
A ADC (Antibody Drug Conjugate)

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

B CAR-T (Chimeric Antigen Receptor)

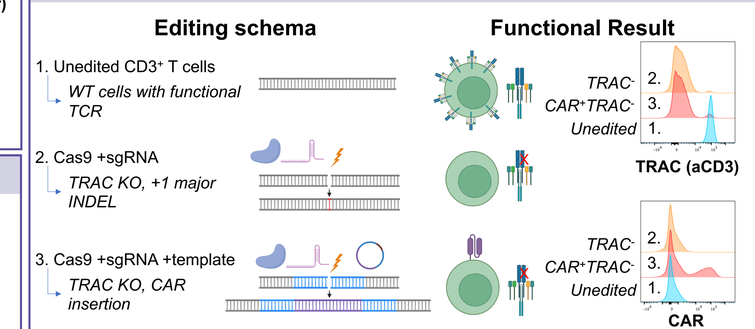
HDRCAR CD33bbz
- Lintuzumab CD33 binder
- CD28 transmembrane domain and hinge
- 4-1BB costimulatory domain
- High antigen sensitivity

Fig 4. HDRCAR generation schema



► Pan T (CD3⁺) cells were thawed at day 0 in media with IL-2 and activated on Day 1 for 48h. Cells were electroporated and expanded for 7 days[4,5].

Fig 3. CRISPR/Cas9 + HDR donor template yields CAR driven by endogenous TRAC promoter[2,3]



► The T Cell Receptor (TCR) is expressed on all T cells. TRAC KO disrupts TCR assembly and export to the cell surface, resulting in loss of CD3 expression. Donor template construct contains homology arms flanking the target locus; CAR knock in results in a full TRAC KO (>95%).

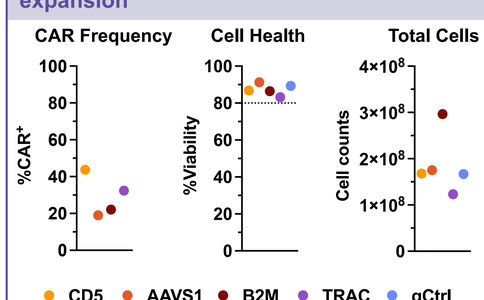
RESULTS

Fig 5. Non-viral templates evaluated show varying levels of viability and CAR expression

Donor Template Format	%CAR⁺	%Viability	Expansion
Nanoplasmid	35%	90%	⇑⇑
Minicircles	5%	68%	⇓
dsDNA - capped	42%	88%	⇓⇓
dsDNA – uncapped	12%	84%	⇓
Long ssDNA	1.5%	90%	⇓

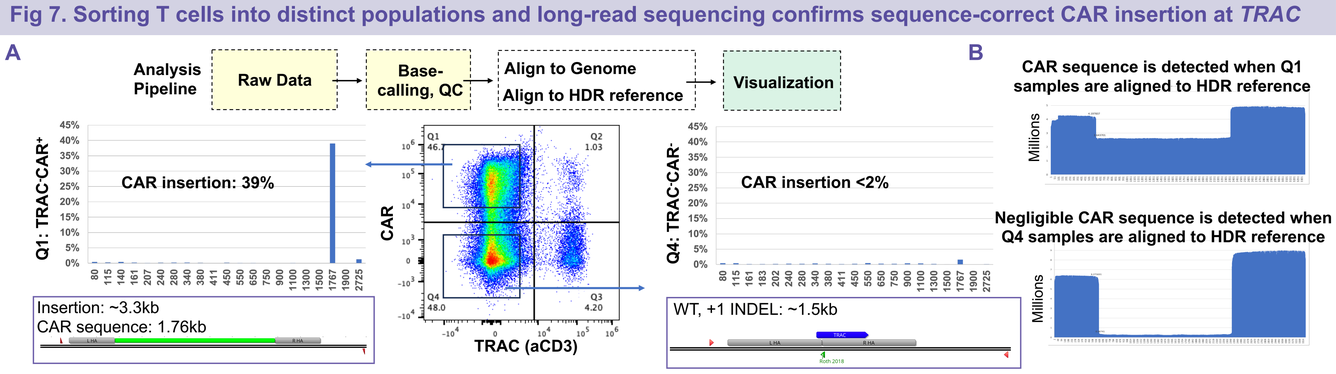
► Nanoplasmid donor template format yielded both the highest CAR knock-in frequency as well as cell viability and expansion of all the donor template formats tested (comparison data 5 and 7d post-EP; protocol optimized for Nanoplasmid).

Fig 6. Efficient CD33bbz CAR knock-in to 4 genomic loci with high viability and cell expansion



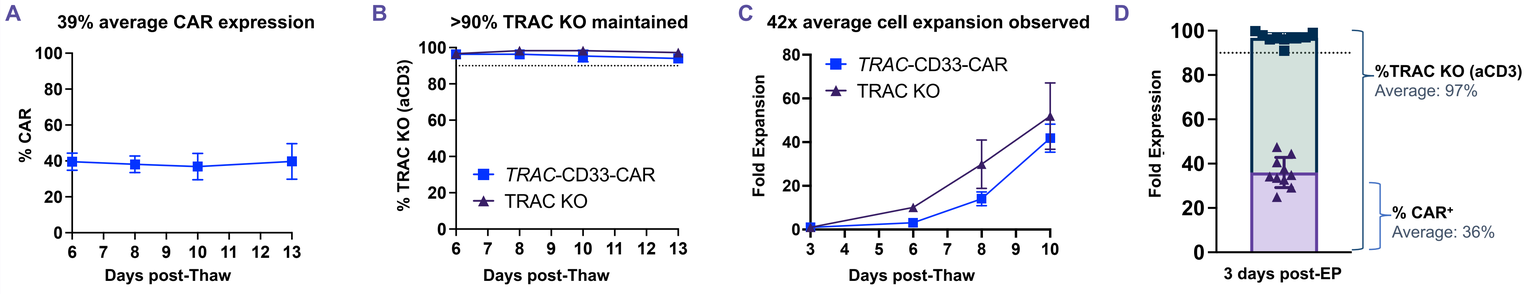
► 11 days post-thaw. Cells were healthy and expanding with similar levels of CAR expressed across different loci.

Fig 7. Sorting T cells into distinct populations and long-read sequencing confirms sequence-correct CAR insertion at *TRAC*



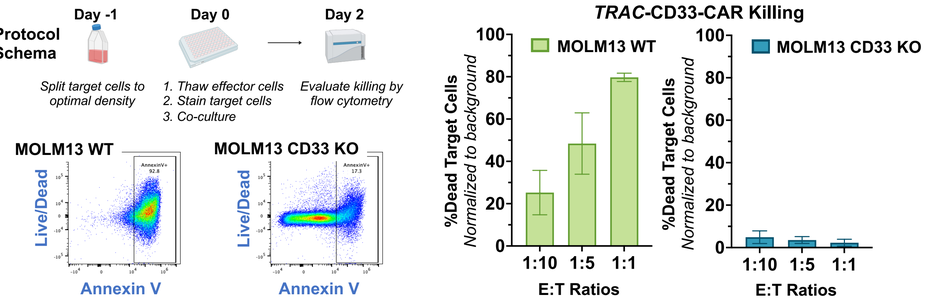
► A. Representative flow plot. Q1 (CAR⁺CD3⁻) and Q4 (CAR⁻CD3⁻) cells were collected via FACS. Target area of interest was amplified using primers outside the homology arms (1.5kb without insert; 3.3kb with insert). Amplified product then underwent library prep and long read sequencing via Nanopore.
► B. CAR sequence is only detected in Q1 sample

Fig 8. Research-optimized protocol yields highly viable CAR-T cells that expand and maintain TRAC KO and CAR expression



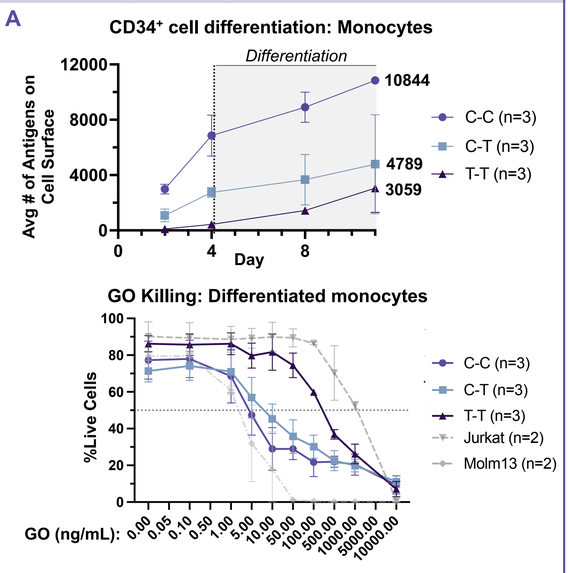
► A. Stable CAR expression observed post-EP (N=3).
► B. High (>90%) and stable TRAC KO observed with and without HDR template (N=3).
► C. Robust cell expansion seen (200E6 CD3⁻; 75E6 CAR⁺CD3⁻ at 10d post-thaw; N=3).
► D. Reproducible TRAC KO and CAR expression across donors (N=10 healthy donors).

Fig 9. *TRAC*-CD33-CAR is functional and specifically kills CD33⁺ MOLM13 WT target cells



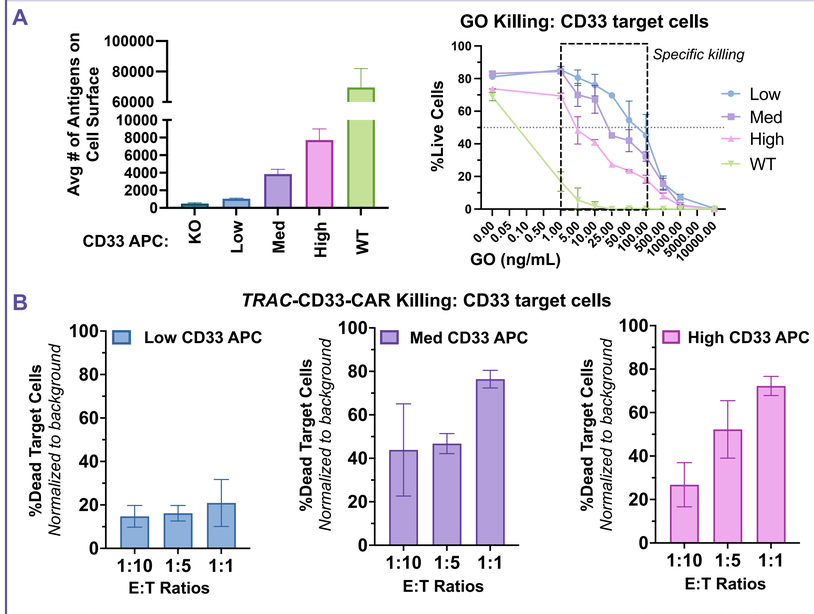
► *TRAC*-CD33-CAR demonstrates highly specific killing in a 48h cytotoxicity assay at all Effector : Target (E:T) ratios tested (N=3 healthy donors).

Fig 10. CD33 cell surface expression impacts GO-mediated cytotoxicity



► A. *In* vitro differentiated CD34⁺-derived monocytes (healthy donors, N=3 per genotype) show a distribution of average CD33 APC.
► B. GO does not target and kill differentiated CD34⁺ cells from T-T donors.

Fig 11. *TRAC*-CD33-CAR effectively kills tumor cells of varying CD33 surface expression



► A. *TRAC*-CD33-CAR demonstrates specific killing of target cells with as few as 1000 average CD33 APC.
► B. Significant IC50 right shift is seen with fewer than WT CD33 antigen expression
► C. CD33 Antigen Density Platform enables modeling for therapeutic targetability

CONCLUSIONS

► Robust knock-in of CD33bbz CAR to the *TRAC* locus (*TRAC*-CD33-CAR) was demonstrated using CRISPR/Cas9 and HDR (>95% TRAC KO with 36% average %CAR⁺ cells; N=10).

► Research-scale protocol optimization generated highly viable (>85%) CAR-T cells that expand similarly to non-HDR manipulated cells yielding 42x expansion over 7 days.

► Full length CAR sequence insertion to *TRAC* locus was validated by long read sequencing.

► *In vitro* antigen density platforms were established to model the range of genotype-dependent CD33 antigen expression found on patient AML blasts.

► *TRAC*-CD33-CAR cells specifically kill CD33⁺ target cells at therapeutic ranges of CD33 antigen expression (range of average CD33 APC evaluated: 1k-70k).

► **This work enables pre-clinical development of CAR-T cell therapies with selective tumor targeting and the potential 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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