Efficient Generation of Anti-HIV *TRAC*-CAR T cells to enable High-Throughput Binder Screening

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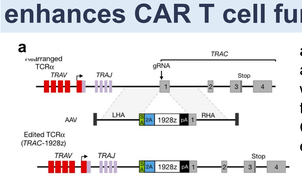
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Background

- First generation anti-HIV CAR T cells were insufficiently potent to mediate a therapeutic effect in early clinical trials, but have demonstrated safety in participants for decades.[1]
- Recent advances in the cancer CAR T cell field have brought renewed interest in the development of CAR T cels for HIV, including enhanced persistence and proliferation[2], increased antigen sensitivity[3], and multiplexed cellular edits including logic gates and precise genomic edits to knockdown or knock-in key regulators of T cell exhaustion.[4]
- Manufacturing CAR T cells using lentiviral vectors results in heterogeneity within and across different donor products, making it difficult to compare efficacy between recipients, and safety concerns have been raised over insertional mutagenesis.[5] Additionally, screening new CAR binders or architectures typically requires individual arrayed functional testing of each receptor.
- We have previously demonstrated that the precise genomic integration of a CAR at the T-cell receptor alpha constant locus (*TRAC*) using CRISPR/Cas9 results in a uniform and more potent CAR product, and enables high throughput screening of hundred**s** of receptors at once.[6]

Methods

Precise genomic knock-in of a CAR at the *TRAC* locus enhances CAR T cell function

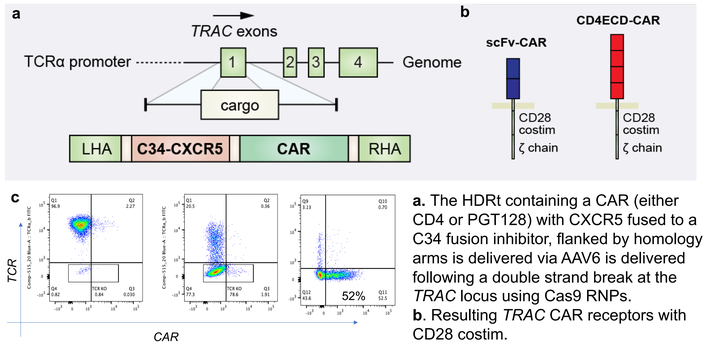


From Eyquem et al. Nature, 2017

a. Ribonuculeoproteins (RNPs) of Cas9 protein + sgRNA are electroporated into T cells, followed by transduction with AAV delivering the homology directed repair template that contains the CAR transgene, shown here as a 1928z CAR which recognizes CD19⁺ tumors. Successfully engineered CARs will be TCR negative and CAR positive.

b, c. *TRAC*-1928z CARs outperform those with knock-in at other loci *in vivo*, and those generated by retroviral (non-targeted) CAR delivery.

Generation of anti-HIV *TRAC* CAR T cells



a. The HDRt containing a CAR (either CD4 or PGT128) with CXCR5 fused to a C34 fusion inhibitor, flanked by homology arms is delivered via AAV6 is delivered following a double strand break at the *TRAC* locus using Cas9 RNPs.
b. Resulting *TRAC* CAR receptors with CD28 costim.

c. Example CAR knock-in shown by flow cytometry, from left to right: unedited cells, *TRAC* ko cells, and the CD4 *TRAC* CAR, achieving above 50% receptor knock-in.

We successfully generated functional, *TRAC*-targeted, anti-HIV CAR T cells using bnAb- and CD4-derived binders, which will enable large pooled screens of hundreds of binders to identify those optimal for use in anti-HIV CAR T cells.

Incucyte live-cell imaging to measure *in vitro* CAR killing capacity

Assay: Target cells containing a red or green fluorescent reporter are co-cultured with effectors (*TRAC* CAR T cells) in triplicate and imaged by the Incucyte every 2-4 hours. After imaging, 'masks' are created using a fluorescent threshold per cell to obtain the target cell counts per image. A reduction in target cell proliferation is used as a proxy for cell killing, compared to 'mock' effectors that contain no CAR. For the cell line assays below, K562 Env⁺ cells[7] express an mKate red reporter. For the live virus assays, we used primary human CD4⁺ T cells infected with an R5-tropic GFP reporter strain, NL-GI JRFL[8], and resulting infected cell counts (10-20% of CD4⁺s) are used to determine the effector : target ratio.

Results

TRAC CARs can kill Env⁺ cells, and exert some control on infected CD4⁺ T cells

Figure 1: PGT128 *TRAC* CARs maintain some control of Env+ cell line at lower effector:target ratios than CD4 *TRAC* CARs.

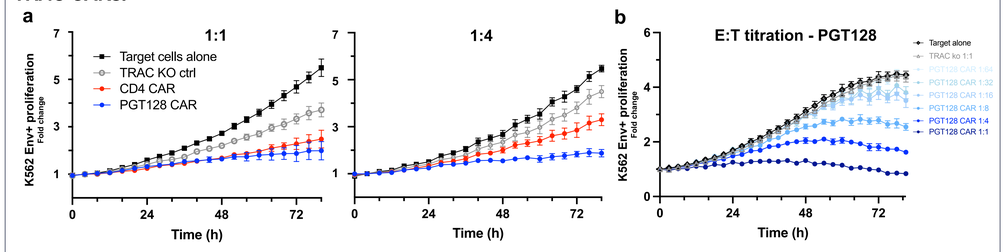


Figure 1: K562 Env growth fold change from 0h is displayed over 80h. **a.** CD4 CARs (red), PGT128 CARs (blue) and *TRAC* ko control T cells (grey) were cocultured with K562s expressing Env, at a 1:1 (left) and 1:4 (right) effector:target ratios. **b.** PGT128 CARs were co-cultured with the K562 Env+ mKate+ cell line at a range of E:T ratios from 1:1 to 1: 64. Values displayed represent the mean of 3 replicates, ±SD.

Figure 2: CD4 *TRAC* CAR killing of autologous HIV⁺ CD4 T cells appears donor dependent with killing observed in 1 of 2 donors, at a 1:1 E:T ratio, while PGT128 *TRAC* CARs exert some control in 2 of 2 donors.

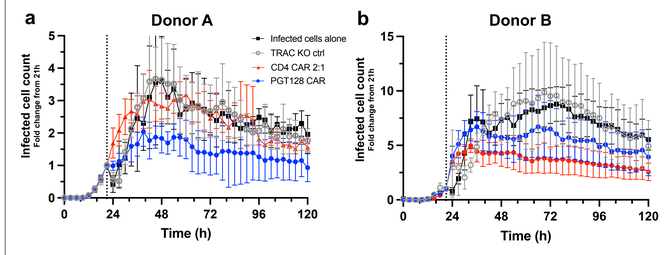


Figure 2: Effectors were added 21 hours post infection (dotted line) at a 1:1 E:T ratio. Infected cell counts are displayed as the fold change per well from 21h. CD4 CARs (red), PGT128 CARs (blue) and *TRAC* ko control T cells (grey) are shown vs infected cells alone (black). **a.** Values displayed represent the mean of 3 replicates, ±SEM. **b.** Values displayed for the CARs represent a mean of 3 replicates ±SEM, while the controls represent the mean of 2 replicates.

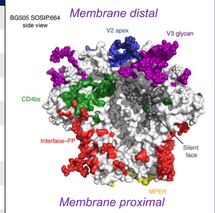
Future directions: pooled binder screens

Binders: bnAbs, controller-derived Abs, and CD4 variants

Source	Type	Epitope	#	Tested as CAR
CATNAP (LANL)	scFv	CD4bs	54	5
CATNAP (LANL)	scFv	V1/V2 APEX	98	3
CATNAP (LANL)	scFv	V3	56	4
CATNAP (LANL)	scFv	MPER	21	1
CATNAP (LANL)	scFv	interface	26	-
non-nAbs (CATNAP)	scFv	co-RBS/internal residues	41	3
controller-derived Abs[9]	scFv	unknown	185	-
CD4 WT	natural ligand	CD4bs	1	1
CD4 mutants[10]	natural ligand	CD4bs	4	1
		TOTAL	474	18

Table 1: Env-targeting binders that we plan to evaluate in *TRAC* CARs in a pooled screen, derived from a variety of sources. 'Tested as CAR' reflect those found in publications.

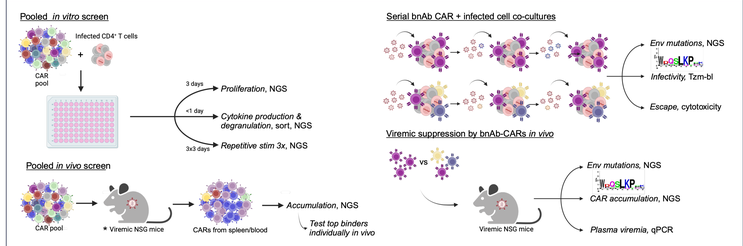
BnAb Epitopes



Env depiction from Burton & Sok *Nature Immunology,* 2018.

Hypotheses:
- ❖ Cell-distal epitopes on Env may be most accessible to target with a CAR.
- ❖ Binders must have broad activity, but do not have to be neutralizing to be effective CARs.

Pooled screening and CAR escape assays:



*PDX mouse model as described by McCan and Jones, *JEM* et al. 2021.

Conclusions

- Anti-HIV *TRAC* CARs can be generated using CD4-based and bnAb-derived binders.
- bNAb-derived PGT128 outperforms CD4-derived binder in high antigen contexts, but CD4 binder efficacy may be donor-dependent in lower antigen settings, as seen in the NL-GI JRFL killing assay.
- CAR architectures with more sensitivity to low antigen densities should be explored in the context of HIV, given that Env is present in very low levels at the surface of infected cells *in vivo*, even after ART is stopped.

Acknowledgements

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References:

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