In vitro and *in vivo* cytolytic capacity of engineered CAR-T cells targeting CD4

372

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BACKGROUND

Chimeric Antigen Receptor (CAR) technology has shown significant potential in the pursuit of an HIV-1 cure. However, eliminating all latently infected cells by targeting gp120 on the cell surface depends on effective latency reversal, which remains a challenge. In a new approach to bypass this limitation, we have engineered CAR-T cells targeting CD4, the primary HIV receptor, with the aim of eliminating any cell harbouring HIV viral reservoir.

METHODS

- We generated two 2nd-generation, CD4-targeting CAR constructs—CAR-4 and Hu5a8-CAR—each with a distinct single-chain variable fragment (scFv) and a GFP reporter.
- A non-specific mock construct was also generated.
- CAR expression and functionality were validated by flow cytometry.

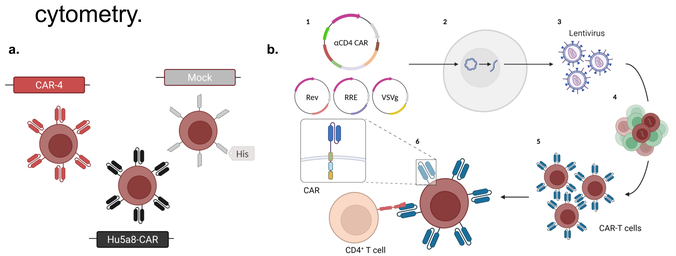


Figure 1. a. Schematic representation of the three different CAR. **b.** Schematic representation of CAR-T cells production through lentiviral transduction of CD8⁺ T cells. **1.** Reimann et al. 1997.

RESULTS:

1. Cytotoxicity in Cell Lines

➢ CAR-4 T cells efficiently eliminated 95–98% of CD4⁺ cell lines after 48h at a 1:1 effector to target (E:T) ratio.

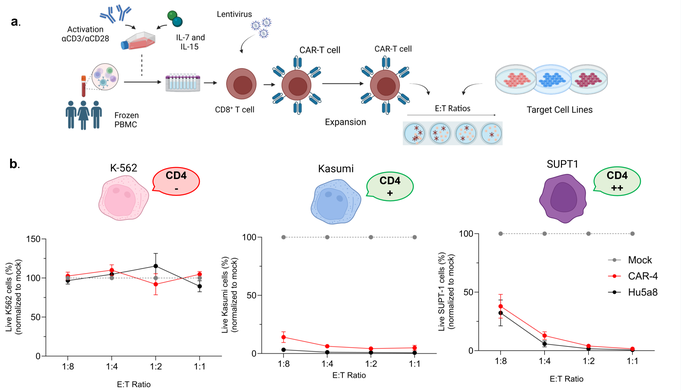


Figure 2. a. CAR-T cells production workflow. CAR-T cells were produced and co-cultured with different target cell lines. **b.** % of viable target cell lines normalized to mock in co-cultures with CAR-T cells at different E:T ratios at 48h. Mean (n=3) ± SEM are plotted.

Anti-CD4 CAR-T cells efficiently eliminated autologous CD4⁺ T cells from healthy donors both *in vitro* and in murine models.

However, reduced efficacy was observed *ex vivo* in cells from PWH warranting further investigation for clinical translation.

2. *In vitro* cytotoxicity in cells from healthy donors (HD)

➢ CAR-4 T cells consistently killed autologous CD4⁺ T cells (99.2±0.4%) at 48h of co-culture and 1:1 E:T ratio.

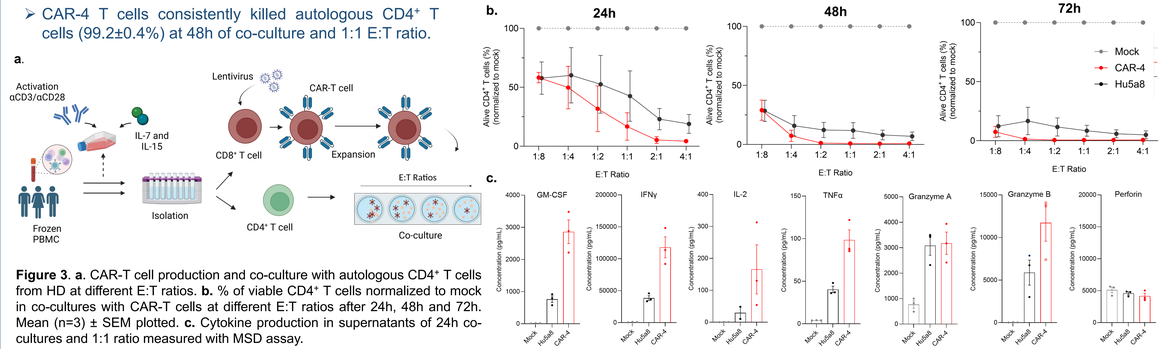


Figure 3. a. CAR-T cell production and co-culture with autologous CD4⁺ T cells from HD at different E:T ratios. **b.** % of viable CD4⁺ T cells normalized to mock in co-cultures with CAR-T cells at different E:T ratios after 24h, 48h and 72h. Mean (n=3) ± SEM plotted. **c.** Cytokine production in supernatants of 24h co-cultures and 1:1 ratio measured with MSD assay.

3. *In vitro* cytotoxicity in cells from HD infected with HIV-1

➢ CAR-4 T cells displayed comparable cytotoxic activity at 48h (97.3±0.5%), although reduced levels of cytokines in plasma were detected.

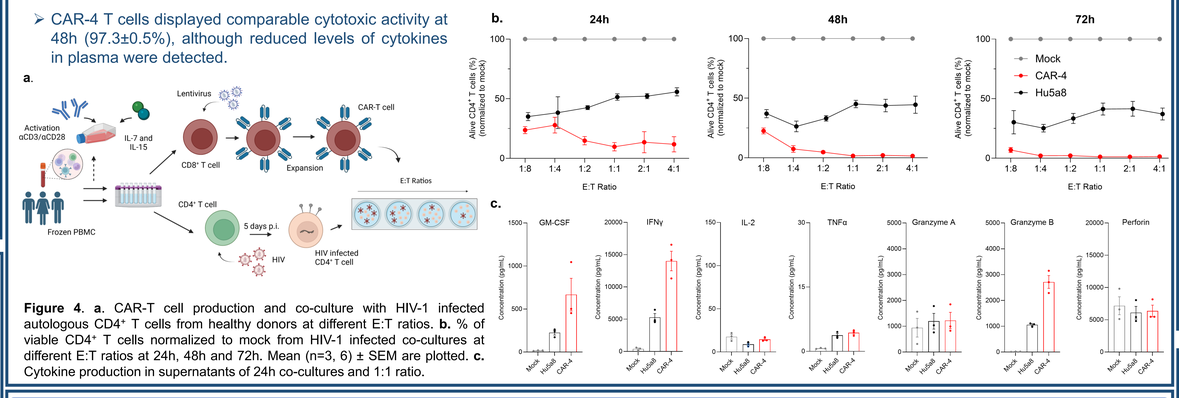


Figure 4. a. CAR-T cell production and co-culture with HIV-1 infected autologous CD4⁺ T cells from healthy donors at different E:T ratios. **b.** % of viable CD4⁺ T cells normalized to mock from HIV-1 infected co-cultures at different E:T ratios at 24h, 48h and 72h. Mean (n=3, 6) ± SEM are plotted. **c.** Cytokine production in supernatants of 24h co-cultures and 1:1 ratio.

4. *In vivo* cytotoxicity in a humanized murine model

➢ Infusion of CAR-4 T cells into uninfected mice resulted in an 89.9±7.4% reduction of hCD4⁺ T cells at week 3 post-infusion.

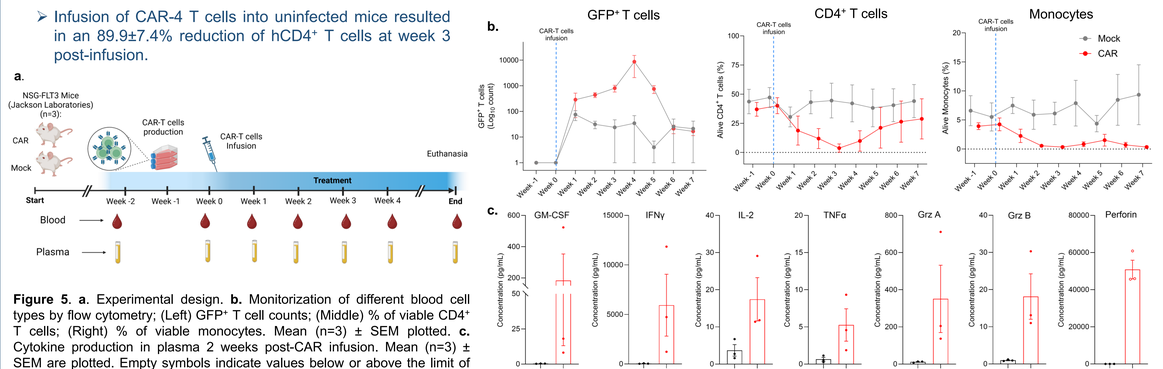


Figure 5. a. Experimental design. **b.** Monitorization of different blood cell types by flow cytometry; (Left) GFP⁺ T cell counts; (Middle) % of viable CD4⁺ T cells; (Right) % of viable monocytes. Mean (n=3) ± SEM plotted. **c.** Cytokine production in plasma 2 weeks post-CAR infusion. Mean (n=3) ± SEM are plotted. Empty symbols indicate values below or above the limit of quantification.

5. *In vitro* cytotoxicity in cells from people with HIV (PWH)

➢ CAR-4 T cells generated from PWH eliminated autologous CD4⁺ T cells after 48h (51±14%), showing reduced efficacy vs. CAR-4 T cells derived from healthy donors.

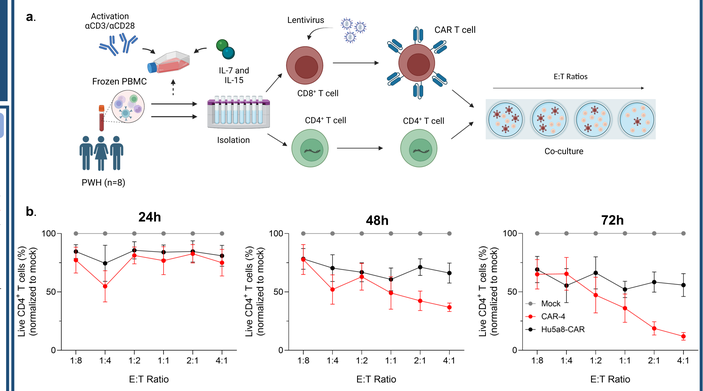


Figure 6. a. CAR-T cell generation with PBMC from PWH and co-culture with autologous CD4⁺ T cells from healthy donors at different E:T ratios. **b.** % of viable CD4⁺ T cells normalized to mock from co-cultures with autologous CAR-T cells at different E:T ratios at 24h, 48h and 72h. Mean (n=3) and SEM are plotted.

CONCLUSIONS

➢ We designed anti-CD4 CAR-T cells that efficiently and specifically eliminate both uninfected and HIV-1–infected CD4⁺ T cells *in vitro*, achieving complete cell depletion after 48h of co-culture at high E:T ratios.

➢ We further demonstrated that CAR-4 T cells exert potent *in vivo* activity, effectively depleting CD4⁺ cells in humanized mouse models.

➢ We observed that CAR-T cells generated from CD8⁺ T cells of PWH displayed reduced cytotoxic activity, warranting further investigation.

ACKNOWLEDGEMENTS

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PLAIN LANGUAGE SUMMARY

We engineered immune cells (CAR-T cells) designed to recognize and eliminate CD4 cells, which are the main hiding place of HIV in the body. In pre-clinical studies, these CAR-T cells were highly effective, although their activity was lower when using cells from people living with HIV, highlighting the need for further research before clinical use.