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Functional and single-cell assessment of CRISPR-modified CAR-T cells from NSCLC patients and healthy donors

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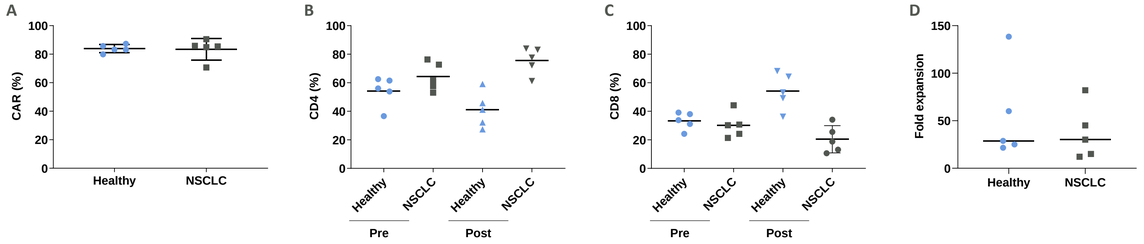
Abstract

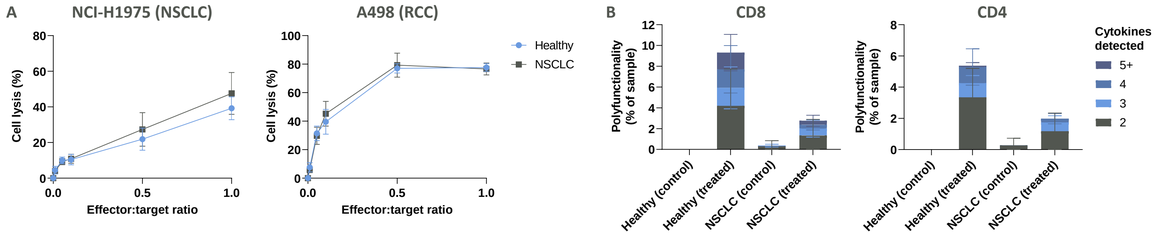
Cancer patients that have undergone multiple lines of treatment can have dysfunctional immune systems. In both solid tumors and hematological malignancies, T cells have been reported to be in various states of dysfunction including senescence or exhaustion. In order to assess the potency of CAR-T cells derived from cancer patients, we created CAR-T cells targeting CD70 from non-small cell lung cancer (NSCLC) patients as well as healthy donors. Anti-CD70 CAR-T cells were created by using CRISPR/Cas9 to insert the CAR cassette into a knocked-out *TRAC* locus. The *TRAC* knock-out eliminates any TCR-mediated effector functions. CAR-T preparations from either NSCLC patients or healthy donors were assessed *in vitro* for T cell effector functions including cell killing, cytokine secretion and expansion in the presence of lung tumor cells expressing antigen. CAR-T cells from NSCLC patients and healthy donors were also assessed for their ability to control human lung cancer-derived tumors in immunocompromised mice. Following these functional assessments, the heterogeneity of CAR-T preparations derived from different sources was characterized further by performing single-cell RNA sequencing analysis and single-cell protein analysis (CITE-Seq and single cell cytokine secretion). A superior understanding of CAR-T cell heterogeneity and donor variability could aid in producing more efficacious drug products for cancer.

Figure 1: CAR-T cells manufactured from T cell derived from healthy donors or NSCLC patients show comparable manufacturing attributes



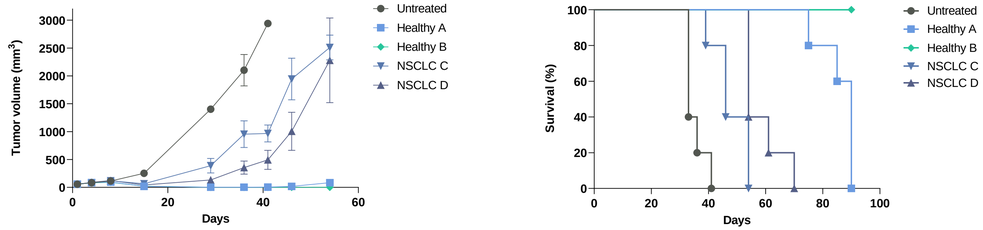
Production of CD70-targeted CAR-T cells from healthy and non-chemotherapy naïve lung cancer donor T cells. CAR-T cells were generated by knock in of a CAR construct into the *TRAC* locus in T cells from five healthy donors and five donors with NSCLC. **(A)** CAR expression and **(B)** percentage of CD4 cells and **(C)** CD8 cells pre- and post-manufacturing were assessed. **(D)** Fold expansion post manufacturing was also measured. Similar editing rates, T cell frequencies by flow cytometry, and *in vitro* expansion were observed across groups.

Figure 2: Healthy donor and NSCLC CAR-T cells show similar *in vitro* cytotoxicity, but healthy donor CAR-T cells have a much higher frequency of polyfunctional T cells



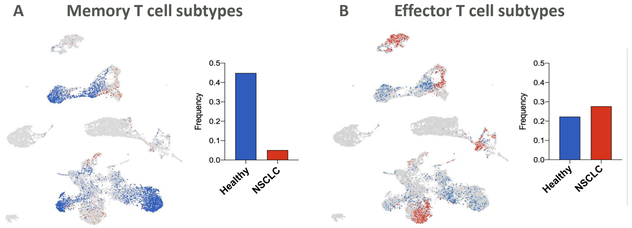
(A) Both healthy and NSCLC donor CAR-T cells efficiently lyse CD70-expressing cancer cell lines H1975 (NSCLC) or A498 (RCC) in cytotoxicity assays *in vitro*. **(B)** However, healthy donor CAR-T cells had a higher frequency of polyfunctional cytokine secreting T cells in both the CD4 and CD8 fractions following exposure to target cells expressing CD70 as compared to NSCLC donor CAR-T cells.

Figure 3: Differences in healthy donor and NSCLC CAR-T cell functionality become evident in *in vivo* models, where healthy donor CAR-T cells can show more robust efficacy



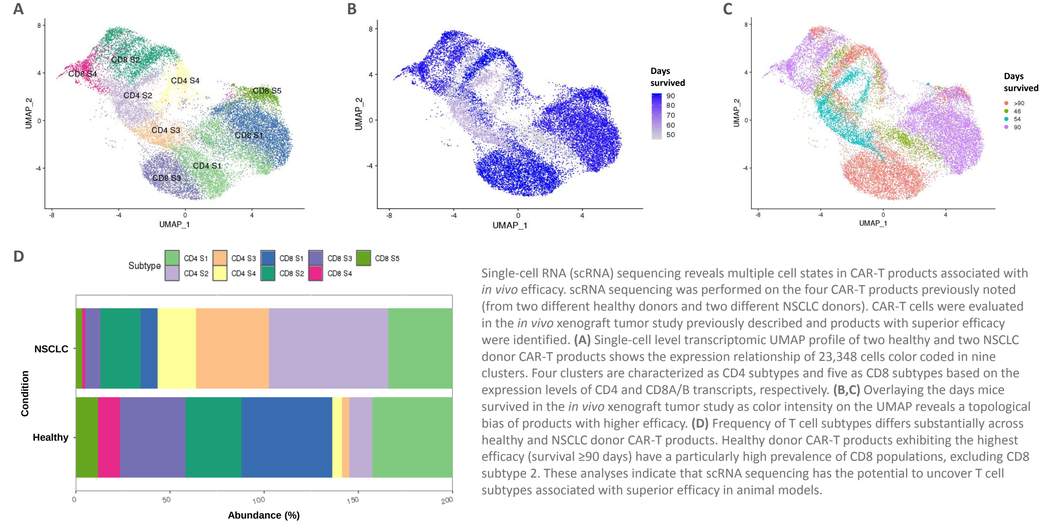
In order to identify donors that had superior anti-tumor activity in a mouse model of NSCLC. NCI-H1975 cells were injected subcutaneously into the flanks of NOG mice and once the tumors had grown to ~50 mm^3 in size CAR-T cells were dosed. Tumor size and the survival of mice are shown. CAR-T cells from two healthy donors ("A" and "B") show tumor control, while CAR-T cells from two NSCLC donors ("C" and "D") show poor function *in vivo*. These four donors were used in subsequent single-cell experiments to identify product attributes associated with superior *in vivo* function.

Figure 4: Healthy donor CAR-T cells tested had a higher proportion of memory phenotypes than NSCLC donor CAR-T cells



Single-cell surface epitope sequencing can reveal cellular phenotypes associated with *in vivo* activity. **(A)** CITE-Seq (Cellular Indexing of Transcriptomes and Epitopes by Sequencing) analysis was performed on CAR-T cells from two healthy donors, which had performed well in an *in vivo* xenograft tumor study, and two NSCLC donors, which had performed poorly in the same study. **(A)** Memory and **(B)** effector T cell subtypes were selected (colored vs. gray cells within each UMAP) using proprietary selection criteria and evaluated for the distribution between well performing (blue) and poor performing (red) CAR-T cell products (insets). Single cell protein epitope sequencing revealed that a greater proportion of healthy donor CAR-T cells, which performed well *in vivo*, had a memory phenotype as compared to NSCLC donor CAR-T cells. Effector phenotypes did not appear different.

Figure 5: Single-cell RNA sequencing can distinguish T cell subtypes associated with higher *in vivo* efficacy



Single-cell RNA (scRNA) sequencing reveals multiple cell states in CAR-T products associated with *in vivo* efficacy. scRNA sequencing was performed on the four CAR-T products previously noted (from two different healthy donors and two different NSCLC donors). CAR-T cells were evaluated in the *in vivo* xenograft tumor study previously described and products with superior efficacy were identified. **(A)** Single-cell level transcriptomic UMAP profile of two healthy and two NSCLC donor CAR-T products shows the expression relationship of 23,348 cells color coded in nine clusters. Four clusters are characterized as CD4 subtypes and five as CD8 subtypes based on the expression levels of CD4 and CD8A/B transcripts, respectively. **(B,C)** Overlaying the days mice survived in the *in vivo* xenograft tumor study as color intensity on the UMAP reveals a topological bias of products with higher efficacy. **(D)** Frequency of T cell subtypes differs substantially across healthy and NSCLC donor CAR-T products. Healthy donor CAR-T products exhibiting the highest efficacy (survival ≥90 days) have a particularly high prevalence of CD8 populations, excluding CD8 subtype 2. These analyses indicate that scRNA sequencing has the potential to uncover T cell subtypes associated with superior efficacy in animal models.

Conclusions from preclinical studies

- CD70-directed CAR-T cells can be produced from both healthy donors and lung cancer donors using CRISPR/Cas9
- Most *in vitro* assays do not distinguish between healthy and NSCLC donor CAR-T cells, but differences in CAR-T functionality become visible in more challenging *in vivo* xenograft tumor models
- Healthy donor CAR-T cells show a higher proportion of polyfunctional cytokine secreting CAR-T cells
- Single-cell protein sequencing can identify phenotypes associated with superior performance in lung cancer models
- Single-cell RNA sequencing can identify T cell subsets associated with superior *in vivo* performance in an unbiased manner