A High-Throughput Screening Strategy for the Identification of Novel Lymphoproliferative Elements

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Key messages

- The current work describes a high-throughput method for screening lymphoproliferative elements *in vivo* for next generation CAR-T therapies
- By quantification of selectively enriched human PBMCs we can identify combinatorial elements that promote CAR-T cell expansion and survival in an *in vivo* mouse model
- Our platform is able to identify previously published lymphoproliferative elements as well as new, potent combinations of elements that are both dependent or independent of concomitant CAR signaling
- This semi-rational screening strategy allows us to continue to identify novel and potentially stronger synthetic lymphoproliferative peptides in combinations that can be used in future CAR-T strategies

Introduction

Chimeric Antigen Receptor (CAR) T cell therapy is effective against certain leukemias and lymphomas and shows promise for other incurable malignancies. Considerable challenges remain however to expand this platform technology beyond transplant-oriented hospital care. Centralized manufacturing of genetically modified T cells, lymphodepleting chemotherapy and patient management of current CAR-T therapies are associated with significant costs and treatment complexity. As a first step to reduce this treatment complexity, the present study describes a high throughput combinatorial domain library screening method to identify synthetic lymphoproliferative elements (SLE) capable of driving *in vivo* expansion and survival of CAR-T cells in a lymphoreplete host without the homeostatic proliferation signals generated by lymphodepleting chemotherapy.

Methods and experimental design

1. Library design

DNA barcoded high-diversity semi-rationally designed libraries of putative lymphoproliferative protein subdomains were assembled into a lentiviral vector co-expressing one of two different CARs. Each complete construct contained a dimerizing extracellular domain, a transmembrane domain, and an intracellular portion comprised of two independent signaling domains, that were assembled into a library with a theoretical diversity of almost 700,000 unique combinations.

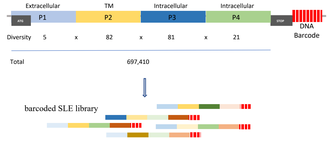


Fig 1. Construction of the combinatorial synthetic lymphoproliferative element (SLE) library with self-correcting DNA barcode identifiers.

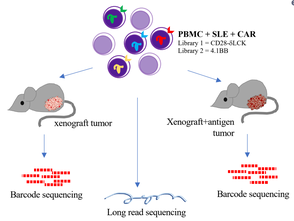


Fig 2. Experimental design and Next-Generation Sequencing data generation strategy. A combination of HiSeq and PacBio sequencing were used to allow high coverage barcode sequencing and identification of barcode-associated full length constructs of interest.

2. Experimental design

Human PBMCs were transduced with the library and cultured *in vitro* for several days. Expanded cells were injected into mice bearing xenograft tumors modified to express target antigens and compared to unmodified xenograft controls. The expansion rate of integrated cells was monitored weekly by quantitative PCR and after 21 days of exposure, genomic DNA was isolated from blood, spleen and xenograft tumor tissues.

3. Data Processing

Enriched barcodes were amplified using PCR and amplicons were subjected to HiSeq Next-Generation Sequencing. Barcode decoding was achieved using PacBio long read sequencing analysis to align full-length construct sequences with barcode quantitation.

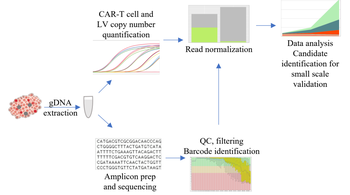


Fig 3. Next-generation sequencing data was processed using custom scripts and several R/Bioconductor packages [1,2,3,4] to identify individual barcodes in reads and map them to their associated full length SLE construct sequence. qPCR was used to quantify overall expansion in tissues and normalize counts appropriately. Data analysis was performed using R [1].

Results

4. Library QC and characterization

PBMCs libraries were characterized for species richness as well as size and GC content bias after *in vitro* expansion. Individual barcodes were identified by Next-Generation Sequencing and counted. Following artifact removal, observed and estimated species richness after *in vitro* expansion were determined based on the frequency and distribution of observed barcode species. Although not all possible combinations were obtained, the observed diversity in both libraries prior to injection was still above 100,000 individual species. Quantile plots of pre- and post-expansion distributions were used to evaluate any potential selection bias during *in vitro* expansion in terms of construct size or GC content. Little bias was observed with the exception of a slight under-representation of very large constructs after expansion.

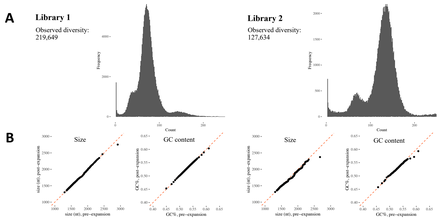
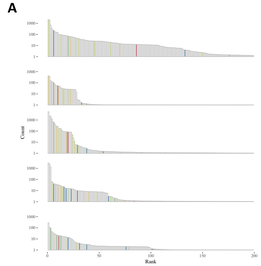


Fig 4. (A) Histogram of the distribution of unique barcodes in the post-*in vitro* expansion, pre-injection samples of libraries 1 (left) and 2. (right) Species richness was above 100,000 for both libraries. (B) Size and GC content selection biases after *in vitro* initial expansion in libraries 1 (left) and 2 (right), showing minimal bias as compared with pre-injection samples.

6. Top candidate composition analysis

Further analysis was performed to identify common key protein subdomains and pathways that support selective *in vivo* expansion and survival of human lymphocytes in a tumor-bearing mouse model, dependently or independently of a CAR-mediated signaling.

Based on their overall performance in both libraries, all parts were attributed a score that was used to rank and select SLE candidates for further testing.



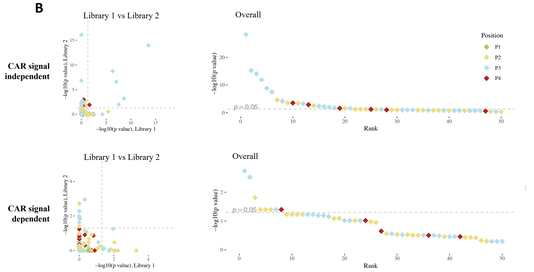


Fig 6. Analysis of the subcomponents of the SLE library (A) Example count data where constructs containing different P3 parts were highlighted (colors). This data, collected in multiple tissues and across multiple replicates, was used to evaluate individual part performance. (B) FDR-adjusted p values for all parts in the presence of absence of antigen in library 1 vs library 2 (left) and for overall top ranking parts combining both libraries (right). (C) Pathway analysis and evaluation of the implication of specific signaling pathways in the performance of intracellular domains. ag, antigen.

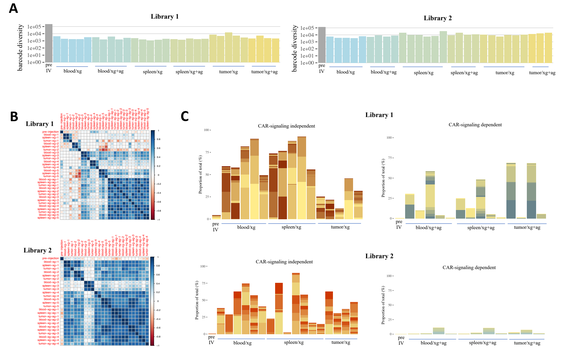


Fig 5. (A) Species richness in multiple samples as compared with pre-injection sample. (B) Correlation plots for all tested samples. (C) Contribution of top CAR-signaling independent constructs to the overall population in samples obtained from xenografts with no antigen (left panel) and of the top CAR-signaling dependent constructs to the overall population in samples obtained from xenograft overexpressing the antigen of interest (right panel) in libraries 1 and 2. xg, xenograft; ag, antigen.

5. Assessment of *in vivo* expansion and identification of top candidates

Barcodes were quantified in blood, spleen and tumor using NGS. In order to take the variability of overall expansion in each animal into account, counts were scaled according to qPCR-measured lentivirus copy numbers, so that each sample was representative of one µg of original tissue. Associated constructs were identified by long read sequencing and top candidates with consistent prevalence among replicates were selected for further characterization in a small scale library. Comparison of samples obtained from animals whose xenograft tumor expressed the target antigen or not allowed us to evaluate CAR-mediated signaling dependency.

7. *In vivo* and *in vitro characterization of selected lymphoproliferative elements*

A small scale library was built from top ranking candidates from both libraries 1 and 2 and tested for its ability to induce CAR-T cell expansion in a xenograft mouse model in the presence or absence of antigen, dependently or independently of CD28-dLCK CAR signaling. Significant expansion was obtained in both cases, and a few candidates with consistent expansion across replicates were identified for further characterization. Select SLEs were also tested for their ability to induce T cell expansion *in vitro* in the absence of cytokines and antigen. Two constructs showed consistent expansion in three different donors.

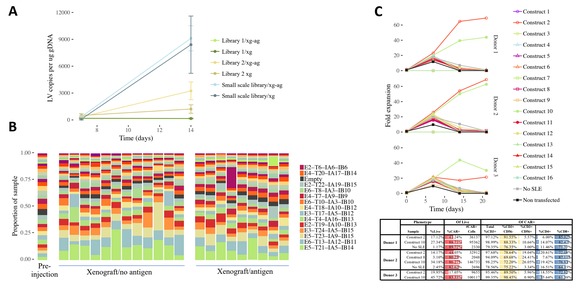


Fig 7. (A) A small scale library using 30 selected candidates induced enhanced *in vitro* expansion at 14 days post-injection as compared with the original libraries. (B) Identification of several SLE that persisted and/or expanded in a CAR/antigen signaling independent in multiple replicates (B) for further evaluation and characterization. (C) Antigen-independent cytokine-free *in vitro* expansion of PBMC transduced with a few selected constructs in three different healthy blood donors. Two constructs induced expansion in all tested donors.

Conclusions

Taken together, these results demonstrate that a high throughput combinatorial screening strategy with quantitative bioinformatics is a viable method for identifying protein domain combinations capable of selectively driving human CAR-T cells *in vivo*. These small synthetic combinatorial protein domains may facilitate lymphodepleting chemotherapy-free regimens and lower CAR-T cell doses in the future.

References

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