Comprehensive Genomic Profiling of Primary Human Cells Modified by an Engineered Mammalian Transposon

#1212

Alisa O. Vershinina[1], Geneva Young[1], Francisco Navarro[1], Joshua LaMora[1], Rebecca Panitch[2], Hugues Bernard[1], Argus Athanas[2], Shanfu Xie[1], Joseph J. Higgins[1], Oleg Iartchouk[1]
[1]SalioGen Therapeutics, Lexington, MA USA; [2]Watershed Informatics, Cambridge, MA USA.

INTRODUCTION

Existing gene therapy methods have the potential to treat a wide range of diseases. However, gene editing imposes a risk of unintended off-target effects that promote genomic instability and oncogenesis through adverse events such as chromothripsis, random insertions, deletions, and chromosomal rearrangements (1, 2). To mitigate these safety concerns, we engineered a mammalian transposable element derived from *Myotis lucifugus* (MLT) to target specific sites in the human genome by a mechanism referred to as Gene Coding™ **(Fig. 1)**. Here, we evaluate the risks and implications of Gene Coding™ technology in primary human T-cells engineered to produce CD19 chimeric antigen receptor (CAR).

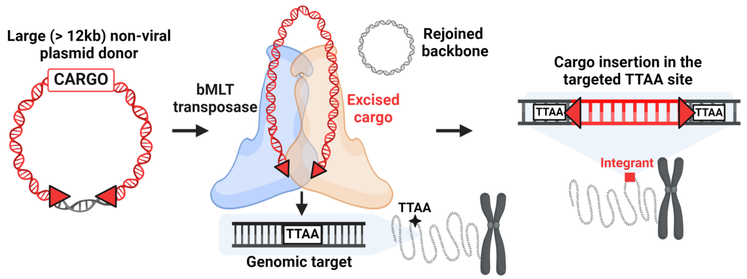


Fig. 1 Gene Coding™ technology. The programmable bioengineered mammalian transposase, bMLT, directs targeted insertion cargo from a non-viral plasmid donor into TTAAs in the human genome. A large cargo gene (CD19.CAR in this study) is integrated via a copy-paste mechanism that does not involve double stranded breaks in the targeted genome. The bMLT is well-adapted to the mammalian cell environment, thus it is able to function at high mammalian cell growth temperatures.

METHODS

Primary T-cells from three human donors were electroporated with both bMLT RNA and a CD19.CAR plasmid DNA (modified group), the CD19.CAR plasmid alone (control group), without any nucleic acid (control group), and left untreated (unmodified group) **(Fig. 2)**. CD19.CAR expression and copy number were measured by fluorescence-activated cell sorting (FACS) and ddPCR **(Fig. 3)**. Efficacy studies were conducted in Nalm6 xenografted NSG™ immunodeficient mice (presented at 2023 ASGCT by Navarro et al.). We characterized insertions of the CD19.CAR plasmid using a proprietary PCR-based assay targeting bMLT-specific terminal inverted repeats (TIRs) **(Fig. 4)**. To assess the genomic safety profile, we applied multiple levels of characterization including karyotyping, Illumina and PacBio sequencing, and hybridization capture enrichment of 127 oncogenes **(Fig. 5-6)**. We profiled lymphoma-derived Raji feeder cells along with T-cells as a positive control for cancer mutations **(Fig. 7)**.

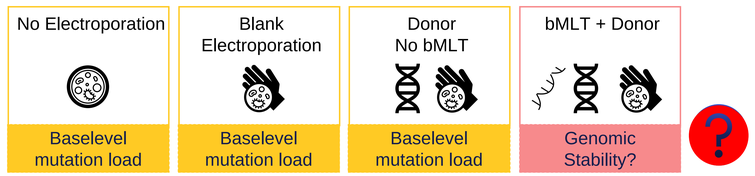


Fig. 2 Experimental conditions and expectations. We tested whether transposition by bMLT increased mutational burden leading to genome instability and raising potential safety concerns in the transfected T-cells.

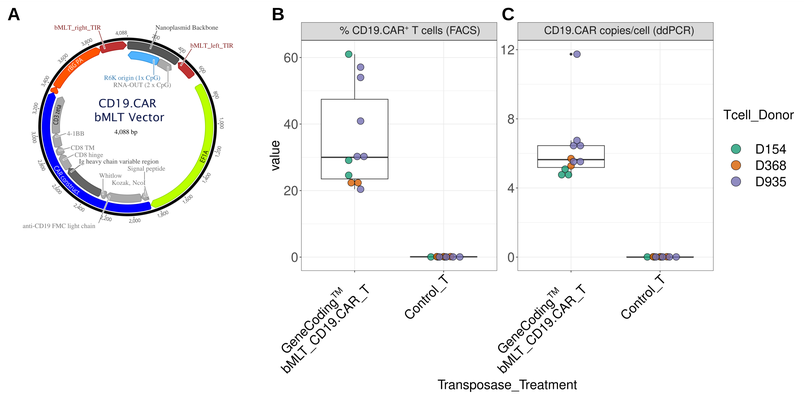
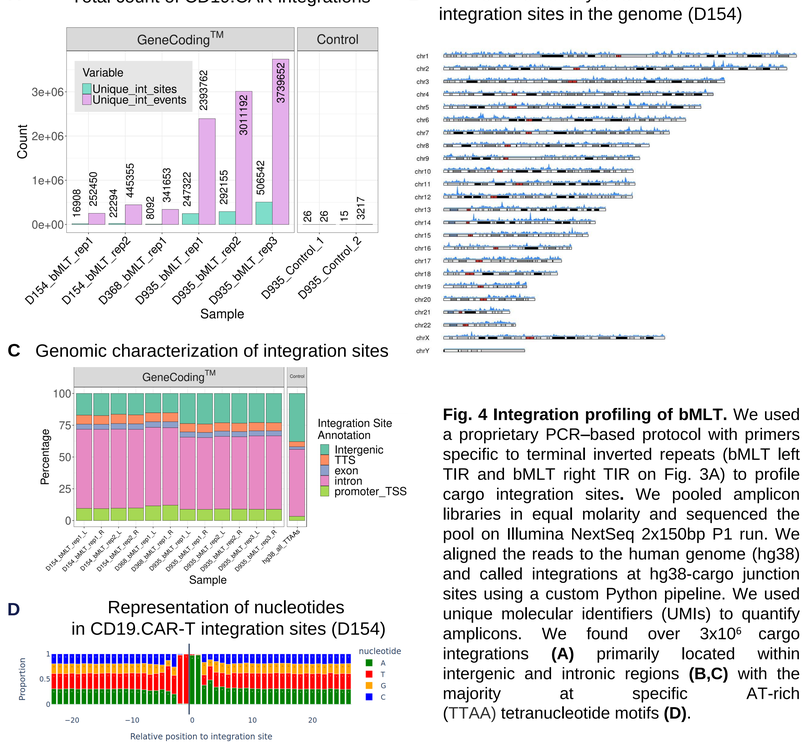


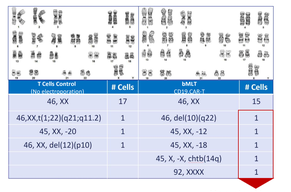
Fig. 3 Gene Coding™ technology results in expression of CD19.CAR cargo in bMLT-engineered T-cells. (A) Vector DNA plasmid used for primary T-cell transfection. **(B)** Modified T-cells showed up to 61% of CD19.CAR expression. **(C)** ddPCR detected an average of 6.19 cargo copies per cell. We detected persistent tumor suppression in xenograft mice treated for up to 70 days (data not shown, presented at 2023 ASGCT by Navarro et al.).

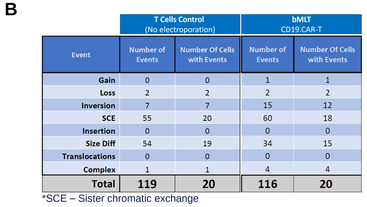
RESULTS



*No or low PCR amplification led to sample dropout. Samples amplified by primers to the left and right end of the transposase are combined.

Fig. 4 Integration profiling of bMLT. We used a proprietary PCR–based protocol with primers specific to terminal inverted repeats (bMLT left TIR and bMLT right TIR on Fig. 3A) to profile cargo integration sites**.** We pooled amplicon libraries in equal molarity and sequenced the pool on Illumina NextSeq 2x150bp P1 run. We aligned the reads to the human genome (hg38) and called integrations at hg38-cargo junction sites using a custom Python pipeline. We used unique molecular identifiers (UMIs) to quantify amplicons. We found over $3x10^6$ cargo integrations **(A)** primarily located within intergenic and intronic regions **(B,C)** with the majority at specific AT-rich (TTAA) tetranucleotide motifs **(D)**.





Event	T Cells Control (No electroporation) Number of Events	T Cells Control (No electroporation) Number Of Cells with Events	bMLT CD19.CAR-T Number of Events	bMLT CD19.CAR-T Number Of Cells with Events
Gain	0	0	1	1
Loss	2	2	2	2
Inversion	7	7	15	12
SCE	55	20	60	18
Insertion	0	0	0	0
Size Diff	54	19	34	15
Translocations	0	0	0	0
Complex	1	1	4	4
Total	119	20	116	20

*SCE – Sister chromatic exchange

Fig. 5 Metaphase karyotype analysis (KromaTiD). Normal 46, XX female karyotypes were shown in control and bMLT-treated T-cells without evidence of chromosome loss, chromothripsis or rearrangements. **(A)** Giemsa-banding control and CD19.CAR-T cells in D935. **(B)** Chromosomal events detected using directional genomic hybridization.

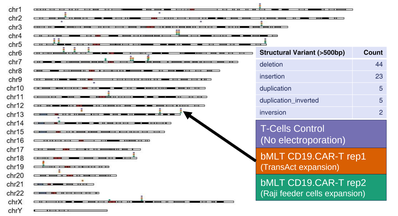


Fig. 6 Optical genome mapping (Bionano Genomics). Control and bMLT-engineered T-cells shared nearly all structural variants longer than 500bp. Karyotype plot with genomic coordinates of rearrangements detected in D935-derived CD19.CAR-T and control T-cells (total counts are shown in the table to the right).

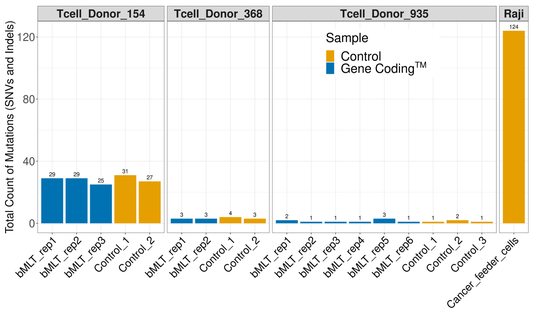


Fig. 7 Somatic variant calling (GATK) in 127 oncogenes (IDT xGen™ Pan-Cancer Hyb Panel). No differences in oncogenic potential between controls and bMLT-generated CD19.CAR-T cells were identified. Total counts of SNPs passing quality control filters are shown for T-cell donors. All oncogenes were enriched up to 2000-fold read coverage.

In addition to SNPs, we analyzed copy number variation in the oncogenes (CNVkit). We did not detect CNVs with log2 copy ratio above 4 and did not detect any copy number expansions regardless of experimental treatment (data not shown).

CONCLUSIONS

- We created a comprehensive genomic safety profile of CD19.CAR-T cells engineered using a novel transposon system called Gene Coding™ technology.
- Gene Coding™ technology does not exacerbate the risk of unexpected and unintended genomic effects in T-cells, thus does not lead to safety concerns.
- Future directions include RNA sequencing, profiling of other primary human cell types, and further evaluation of copy number variations.

REFERENCES

1. Leibowitz ML, Papathanasiou S, Doerfler PA, et al. Chromothripsis as an on-target consequence of CRISPR-Cas9 genome editing. Nat Genet. 2021;53(6):895-905. 2. Bailey SM, Cross EM, Kinner-Bibeau L, Sebesta HC, Bedford JS, Tompkins CJ. Monitoring Genomic Structural Rearrangements Resulting from Gene Editing. J Pers Med. 2024;14(1):110.

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- Conflicts: AOV, GY, FN, JLM, HB, SX, JJH, and OL are currently full-time employees and stock option holders of SalioGen Therapeutics, Inc., a private biopharmaceutical company. RP and AA , are contractors of SalioGen Therapeutics, Inc. and full-time employees and stock option holders of Watershed Informatics, Inc.