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Long-term stability of clinical-grade lentiviral vectors for cell therapy

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The use of lentiviral vectors in cell and gene therapy is steadily increasing, both in commercial and investigational therapies. Although existing data increasingly support the usefulness and safety of clinical-grade lentiviral vectors used in cell manufacturing, comprehensive studies specifically addressing their long-term stability are currently lacking. This is significant considering the high cost of producing and testing GMP-grade vectors, the limited number of production facilities, and lengthy queue for production slots. Therefore, an extended shelf life is a critical attribute to justify the investment in large vector lots for investigational cell therapies. This study offers a thorough examination of essential stability attributes, including vector titer, transduction efficiency, and potency for a series of clinical-grade vector lots, each assessed at a minimum of 36 months following their date of manufacture. The 13 vector lots included in this study were used for cell product manufacturing in 16 different clinical trials, and at the time of the analysis had a maximum storage time at −80°C of up to 8 years. The results emphasize the long-term durability and efficacy of GMP-grade lentiviral vectors for use in *ex vivo* cell therapy manufacturing.

INTRODUCTION

Lentiviral vectors (LVs) are widely used in cell therapies for treating various diseases due to their effectiveness in transferring genetic material. Compared to other vectors, LVs are particularly advantageous for clinical applications because of their ability to efficiently transduce nonproliferating or slowly proliferating cells. The use of LVs was initially confined to academic investigations, where they were first used in a clinical trial to transduce mature peripheral blood T cells for the treatment of HIV infection.[1] Since then, LVs have been used to effectively transduce multiple cell types for the treatment of myriad diseases. The number of gene therapy clinical trials worldwide using LVs for gene transfer has increased from 89 in 2014 to 364 as of May 2023.[2,3] Notably, LVs have been used for gene transfer into mature T cells for cancer immunotherapy. With this and the US Food and Drug Administration's approval of 6 chimeric antigen receptor (CAR) T products, the use of LVs has expanded significantly into the industrial sector.

Over the past 2 decades, the safety, targeting, and transduction efficiency of these LVs have improved significantly. However, one theoretical safety risk associated with LVs is the generation of novel replication-competent retrovirus or lentivirus (RCR/L) during cell product manufacturing or within a patient. To date, there are no confirmed reports of positive RCR/Ls in clinical vector lots, cell products, or patients infused with those cell products.[4–6] Another potential safety risk is insertional oncogenesis. In contrast to gammaretroviral vector systems used early on in gene therapy, which have a propensity to integrate near transcriptional start sites, the shift toward LV use is in part due to increased safety.[7] In addition, there is some clinical evidence indicating that gammaretroviral vectors may be subject to more silencing compared to LVs.[8] Overall, evidence supports LVs as a safe and effective method for generating therapies to treat various diseases.

Since treating our first patient with *ex vivo* LV-transduced T cells in 2003[1] at the University of Pennsylvania (UPenn), this approach to modifying cells has become a standard method in our Clinical Cell and Vaccine Production Facility (CVPF). We routinely use transgenes cloned into a modified derivative of the third generation, self-inactivating LV expression vector pRRL-SIN-CMV-EGFP-WPRE.[9] Along with the transgene plasmid, 3 packaging plasmids expressing gag/pol, vesicular stomatitis virus G (VSV-G), and Rev are used for LV production. This maximizes biosafety, because multiple recombination events are required to generate RCLs. In addition to a consistent vector production system, additional constants include using a

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Table 1. Vector lot summary

Lot no.	Manufacturer	DOM	Initial SupT1 titer, TU/mL
LFP-14001N	CAROT	11/24/2014	2.09 × 10e9
LFP-1501G	CAROT	07/22/2015	1.33 × 10e9
LFP-1503C	CAROT	12/15/2015	7.29 × 10e7
LFP-1601C	CAROT	06/15/2016	3.11 × 10e7
LFP-1602C	CAROT	08/31/2016	3.07 × 10e8
LFP-1702C	CAROT	11/01/2017	1.90 × 10e8
LXFP1-C1801C	CHOP CVC	05/17/2018	4.86 × 10e8
LFP-1803C	CAROT	06/13/2018	2.22 × 10e8
LFP-1901C	CAROT	02/27/2019	9.07 × 10e8
LFP-1903C	CAROT	04/04/2019	4.69 × 10e8
LFP-1908C	CAROT	11/27/2019	9.02 × 10e8
LFP-2001C	CAROT	02/12/2020	1.15 × 10e9
LFP-2002C	CAROT	03/18/2020	1.61 × 10e8

limited number of LV suppliers, a standard cell manufacturing process, and dedicated, monitored storage conditions. These commonalities across clinical programs within the UPenn Center for Cellular Immunotherapies (CCI) uniquely position us to investigate the long-term stability of LVs for clinical cell manufacturing.

Currently, minimal data are available for the stability of clinical-grade or large-scale retroviral/LVs in long-term storage.[10,11] To evaluate the long-term stability of LVs for clinical cell therapy manufacturing, we conducted a comprehensive programmatic analysis of clinical-grade LV lots produced at either Children's Hospital of Pennsylvania Clinical Vector Core (CVC) or UPenn Center for Advanced Retinal & Ocular Therapy (CAROT) Good Manufacturing Practice (GMP) Core using similar production methods. Each lot included in this analysis was a minimum of 36 months past the date of manufacture (DOM) (Table 1). When feasible, these lots were assessed for titer stability, transduction efficiency in clinical T cell products at a given MOI over time, and indirect evaluation of potency using a coculture assay and interferon gamma (IFN-γ) ELISA.

RESULTS

Titer stability

Our analysis of titer stability incorporated 13 distinct clinical lentiviral vector lots, each encoding different CARs or T cell receptors (TCRs). These vectors were maintained at −80°C in the UPenn CVPF. Freezer temperature and function were continuously monitored by a qualified Rees system. Upon production, each vector lot underwent an initial titer determination using SupT1 cells as part of the vector release testing process. Titer determination for all vector lots was performed in the UPenn CCI according to established protocols, and each included the use of a single designated control vector with known titer for assay performance and normalization purposes. Figure 1 provides a visual representation of the additional titer determinations carried out at subsequent time points, aimed at assessing

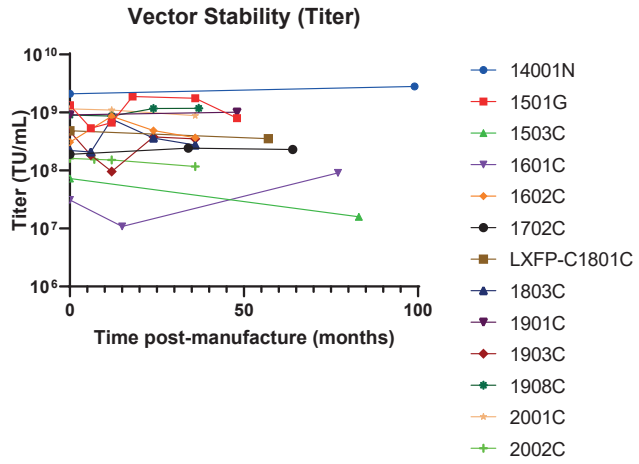

Figure 1. Clinical LV titers remain stable over time

Thirteen clinical LV lots were titered in SupT1 cells as part of vector release testing and at subsequent time points following storage at −80°C to assess long-term titer stability. SupT1 titers are expressed as transduction units per milliliter (TU/mL).

titer stability over time. The range for the final time points tested spanned from 36 to 99 months post-DOM. Using a linear mixed-effects model with random intercept that allows different vectors with their own starting titer values, there is insufficient evidence to support a statistically significant change over time (slope estimate: 0.5×10^{-3} log10 transduction units (TU)/mL per month, 95% confidence interval: -0.2×10^{-3} to 3.8×10^{-3} under robust SEs, p = 0.78). This suggests that the titer of clinical-grade LVs remains stable throughout long-term storage.

Transduction efficiency

As an additional function of vector stability analysis, transduction efficiencies were examined in clinical T cell products made with 1 of 11 different lentiviral vector lots. Of note, no products are included from lot LFP-1602C (used to generate a product with multiple modifications, and thus excluded due to unique manufacturing process) or lot LFP-1901C (no clinical products made). The objective was to assess whether transduction at a given MOI decreased as the vector lots were used further out from their DOM (Figure 2). These data were amassed from manufactured clinical T cell products across 16 different studies for a total of 258 products. For each vector lot, a minimum of 2 products (lots LFP-2001C and LFP-2002C) were summarized with a maximum of 54 products for the lentiviral lot LFP-1501G (MOI 0.76). Transduction efficiencies were determined by flow cytometry as part of their release testing, measuring CAR's single-chain fragment variable (scFv) surface expression in the final formulated clinical cell product. It should be noted that lot LFP-1501G was used to manufacture clinical products at 2 different MOIs. Of the 10 lots/MOIs evaluable by simple linear regression (≥3 clinical products manufactured), 60% (6/10) showed no significant change in transduction efficiency. The remaining 4 lots demonstrated small but significant changes in transduction efficiency over time: 30% (3/10) increased (LFP-1702C, MOI 0.33, slope = 0.02269,

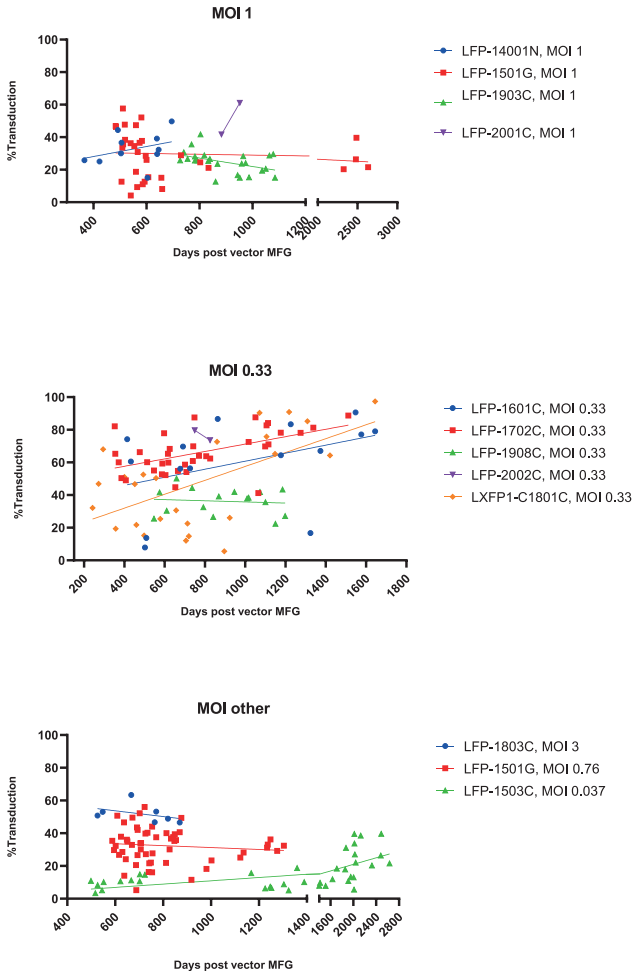

Figure 2. Transduction efficiency in clinical cell products does not decrease over time

The surface expression of each transgene expressed by the vector lot used for production of clinical cell therapy products was determined by flow cytometry. The transduction efficiency of each formulated product manufactured with a specific vector lot at a given MOI (MOI 1 [top], MOI 0.33 [center], or other MOI [bottom]) was plotted in relation to the time (in days) from the date of vector manufacture. Simple linear regression was used to generate a trend for each cell product produced. Two lots were not included in the simple linear regression analysis due to limited manufacture of clinical products (minimum required is 3).

p = 0.0007; LXFP1-C1801C, MOI 0.33, slope = 0.04257, p = 0.0020; LFP-1503C, MOI 0.037, slope = 0.01005, p < 0.0001) increased, whereas only 10% (1/10) decreased (LFP-1903C, MOI 1, slope = −0.02591, p = 0.0289) in transduction efficiency over time. It is important to note that the transduction efficiencies reported here are from clinical products manufactured using autologous cells from patients with multiple factors that may affect the quality of CAR T cells, including disease type, age, prior therapies, and peripheral blood cell phenotype and health[12]; the small changes in transduction efficiencies observed over time, in the absence of corresponding changes in titer and potency, may be attributed to donor-to-donor variability, rather than vector stability. Overall, the data demonstrate that 90% of evaluable vector lots used to manufacture clinical products demonstrated stable or increased transduction efficiencies up to >7 years post-DOM (latest time point tested was 2,633 days post-DOM).

Potency

To further assess long-term stability of LVs, 11 clinical viral vector lots were tested for potency at a time point ranging from 36 to 92 months post-DOM. Lot LFP-1602 was exhausted and thus not included. Lot 14001N was not tested due to unavailable detection reagent. The potency of these vectors was evaluated using an ELISA that measured the release of IFN-γ when T cells transduced at the same MOI used for clinical manufacturing were cocultured for 24 h (±1 h) with cell lines expressing the corresponding target antigen at a 1:1 viable cell ratio. This is a generally accepted assay that is appropriate for testing the activity of a variety of transgene constructs. Although elements such as variation in the CARs/TCRs expressed, differences in target cells, and variation in transduction efficiency result in a significant breadth of response, Table 2 demonstrates that all 11 vector lots tested were able to induce measurable IFN-γ release (i.e., 100% success probability). We have high confidence, with a probability of 0.9, that cell products generated from a vector lot at least 36 months from DOM can successfully produce measurable IFN-γ release in response to the recognition of target antigen at least 81% of the time (1-sided 90% exact confidence interval, 0.81–1.00).

DISCUSSION

LVs serve a fundamental role in myriad approved and experimental cell and gene therapies in current clinical use. Gaining a deep understanding of the long-term stability of these vectors is essential for several reasons. A considerable length of time may pass between the initial use of the vector in establishing the feasibility of manufacturing a cell product for an Investigational New Drug (IND) application, and the eventual completion of a clinical trial. It is ideal to minimize the number of vector lots used for a study due to the substantial cost associated with clinical-grade LVs and to minimize the potential for lot-to-lot variation. Furthermore, the high demand for vector production slots and limited capacity result in lengthy queues. Therefore, it is crucial to be able to procure high-quality vector on a large scale that can meet clinical needs for several years.

To assess the functional stability and potency of clinical-grade LVs, we leveraged the historical data generated in the UPenn CCI spanning nearly a decade. This dataset includes 13 different LV lots produced by 2 vector manufacturers under similar conditions, using a 4-plasmid transfection approach. It should be noted, however, that one of those plasmids is the VSV-G envelope, which has been shown to improve stability as compared to other envelope pseudotypes.[13] Each vector lot underwent titer determination in SupT1 cells, a prerequisite for release, with stability titers assessed at several subsequent time points. Every vector included in this analysis was subjected to stability testing conducted at least 36 months postmanufacture. The

Table 2. Potency assessment by IFN-γ ELISA

Vector lot	Transgene	Transduction efficiency, %	Net IFN-γ release (target -control), pg/mL	Fold increase over control (target)	∼Months between test and DOM
LFP-1501G	muCD19 CAR	38.5	256,603	31.36	92
LFP-1503C	Mesothelin CAR	19.5	161,993	791.21	75
LFP-1601C	PSMA CAR	64.4	245,063	1,461.44	81
LFP-1702C	CD22 CAR	57.2	239,141	1,020.05	64
LXFP1-C1801C	CD123 CAR	89.0	37,334	194.10	58
LFP-1803C	Muc1 CAR	56.6	677	19.86	57
LFP-1901C	NY-ESO-1 TCR	40.9	1,475	23.92	49
LFP-1903C	Muc1 CAR	31.2	416	8.59	48
LFP-1908C	CD19-IL18 CAR	40.9	123,539	200.26	38
LFP-2001C	GFRa4 CAR	63.4	43,101	35.84	37
LFP-2002C	PSMA CAR	40.4	237,070	193.12	36

compiled data suggest a noteworthy stability of these vectors when stored at −80°C.

In addition to titer, the clinical cellular products generated using these vectors were evaluated as a surrogate for stability. By assessing products made with a given vector lot at a specific MOI, we were able to conclude that there is no trend indicative of decreasing transduction efficiency with increasing time from the DOM for vector use in cell manufacturing in 90% of evaluable vector lots/MOIs. To evaluate potency, a coculture assay was performed in which vector-transduced T cells were combined with cell lines expressing their cognate antigen, and IFN-γ production was assessed by ELISA. Although no acceptance criteria were set for positivity given the uniqueness and limited number of each vector lot, potency was assessed for information only, and results illustrated that all 11 vector lots tested, ranging from 37 to 92 months post-DOM, were able to generate cells which released IFN-γ in response to antigen recognition.

The vectors analyzed in this work have various targets of interest, including CD19, CD22, CD123, prostate-specific membrane antigen (PSMA), and mesothelin, among others. The most efficacious CAR T cells to date are those that target the B cell antigens CD19 and CD22. Because these CARs have shown the ability to induce clinical remissions, clinical responses can indirectly support vector stability and potency. Across 3 studies (NCT02650414, NCT02906371, NCT04684563) in which CD19 or CD22 targeting vectors were used at least 36 months post-DOM to make clinical cell products, 12 of 16 such products resulted in complete remission, complete remission with incomplete blood counts, or partial remission at the time of disease response assessment.

In summary, our comprehensive analysis corroborates the long-term stability of clinical-grade LVs when stored under suitable conditions. The data gathered from evaluating titer stability, transduction efficiency, and potency demonstrate compelling evidence of these vectors' maintaining their functionality and potency for several years postmanufacture. The consistent titer, the lack of diminished transduction efficiency over time in the majority of the lots tested, and the successful clinical responses further endorse the reliability and efficacy of these vectors. We believe these findings would largely hold true and be applicable to other clinical-grade vector suppliers provided that vector lots were maintained under similar GMP-compliant storage conditions. These conclusions carry substantial significance for the cell and gene therapy field, offering reassurance to researchers, clinicians, and manufacturers about the dependability and durability of LVs.

MATERIALS AND METHODS

Vector manufacture and storage

Vectors were manufactured using a transient transduction system in which HEK293T cells are transfected with separate plasmids encoding VSV-G, gag/pol, RSV-REV and the transgene of interest. Vector supernatant was clarified and concentrated using tangential flow filtration, treated with benzonase, and further concentrated by centrifugation. Vector lots were formulated in buffered saline containing trehalose and aliquots were stored in the UPenn CVPF in −80°C freezers with continuous automated temperature monitoring (Rees Scientific, Trenton, NJ).

Titer determination

SupT1 cells were transduced with control and experimental vectors in a series of dilutions in 96-well plates and incubated at 37°C. A total of 24–26 h later, cells were fed with R10 culture medium (RPMI 1640, 10% fetal bovine serum [FBS], 2% HEPES, 1% penicillin/streptomycin). After an additional 72–76 h, cells were collected by centrifugation at 2,000 rpm for 3 min and resuspended in fluorescence-activated cell sorting buffer (2.5% FBS in PBS) and repeated for a total of 3 washes. Cells were then stained for CAR or TCR expression using specific primary and secondary (as needed) antibodies. The surface

expression of CAR or TCR was analyzed by flow cytometry. The first dilution at which the percentage of CAR/TCR$^+$ SupT1 cells was less than 20% typically represents the most accurate limiting-dilution titer for the vector and was used for titer calculation according to the formula: titer (TU/mL) = (% positive cells/100) $\times$ 2E4 $\times$ 20 $\times$ dilution. A correction factor based on the established titer of the control vector was calculated and applied to determine the final titer of each vector lot.

Transduction efficiency determination

As part of product release testing, clinical cell products were stained by product-defined flow cytometry panels to determine scFv or TCR surface expression before the final formulation. In brief: fresh cells from harvest day -1 or harvest day of manufacturing culture were stained with specific reagent for detection of the transgene, as well as phenotypic monoclonal antibodies (i.e., CD2, CD3, and CD45) and viability marker. The stained cells were acquired on a qualified flow cytometer. Transduced cells were gated from the CD45$^+$CD3$^+$ (or CD45$^+$CD2$^+$) cell population following gating of intact singlets and live cells. The percentage of transduced cells was reported and used for dose formulation.

Potency assessment by IFN-γ ELISA

Potency of vector lots was tested using human elutriated lymphocytes from a single normal donor who had been previously qualified. Lymphocytes were thawed, washed, and resuspended in either (1) OpTmizer media supplemented with interleukin (IL)-7 and IL-15, (2) X-Vivo with IL-2, or (3) X-Vivo with IL-7 and IL-15, depending on product-defined clinical manufacturing conditions, and seeded in T25 flasks. Cells were activated using Dynabeads CD3/CD28 CTS (Thermo Fisher Scientific Baltics, Vilnius, Lithuania) at a cell:bead ratio of 1:3. The following day, the cells were transduced at the established clinical MOI. Cells were then maintained by feeding, counting, and density control up to 5×10^5 cells/mL every other day. On day 10, the cultures were harvested and transduced cells were co-incubated with the target tumor cells or control tumor cells for potency testing. Supernatant was collected approximately 24 h (±1 h) later and frozen. IFN-γ ELISA was performed on thawed supernatants using human IFN-γ DuoSet ELISA (DY285B) from R&D Systems (Minneapolis, MN) following the manufacturer's protocol. Plates were read on an FLUO STAR OMEGA instrument and data were analyzed using Omega Data Analysis software.

DATA AND CODE AVAILABILITY

All of the study data are included in the article.

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AUTHOR CONTRIBUTIONS

Conceptualization: J.K.J.; C.H.J., A.C., and J.K.J. supervised the project. Design, execution, and/or interpretation of the reported experiments or results: Y.M., G.P., J.A.F., V.E.G., A.D., F.C., K.A., J.P., and N.G. Statistical analysis: W.-T.H. Writing – review & editing: all authors.

DECLARATION OF INTERESTS

C.H.J is an inventor on patents and/or patent applications licensed to Novartis Institutes of Biomedical Research and receives license revenue from such licenses. C.H.J. is a scientific cofounder of Capstan Therapeutics, Bluewhale Bio, and Tceleron; is a consultant to Kite Pharma; and is a member of the Scientific Advisory Boards of AC Immune, Alaunos, BluesphereBio, Cabaletta, Carisma, Cartography Biosciences, Cellares, Celldex, Decheng, Poseida, Replay Bio, Verismo, and WIRB-Copernicus Group. A.C. is a scientific cofounder of Tceleron, and is a consultant to Kite Pharma and Bluewhale Bio. J.A.F. has received grants and personal fees from Cartography Biosciences, grants from Tmunity Therapeutics, and personal fees from Retro Bio and Shennon Bio outside the submitted work. In addition, J.A.F. holds patents related to CAR T cells for cancer that are licensed and associated with royalties.

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