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THERAPY

Screening Clinical Cell Products for Replication Competent Retrovirus: The National Gene Vector Biorepository Experience

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Replication-competent retrovirus (RCR) is a safety concern for individuals treated with retroviral gene therapy. RCR detection assays are used to detect RCR in manufactured vector, transduced cell products infused into research subjects, and in the research subjects after treatment. In this study, we reviewed 286 control (n = 4) and transduced cell products (n = 282) screened for RCR in the National Gene Vector Biorepository. The transduced cell samples were submitted from 14 clinical trials. All vector products were previously shown to be negative for RCR prior to use in cell transduction. After transduction, all 282 transduced cell products were negative for RCR. In addition, 241 of the clinical trial participants were also screened for RCR by analyzing peripheral blood at least 1 month after infusion, all of which were also negative for evidence of RCR infection. The majority of vector products used in the clinical trials were generated in the PG13 packaging cell line. The findings suggest that screening of the retroviral vector product generated in PG13 cell line may be sufficient and that further screening of transduced cells does not provide added value.

INTRODUCTION

The use of genetically modified cells containing retroviral vectors is in advanced stages of clinical development. Retroviral-based gene therapy for adenosine deaminase deficiency was approved in Europe in 2016.[1] The US Food and Drug Administration approved the first retroviral *ex vivo* transduced T cell therapy in 2017 for the treatment of non-Hodgkin's lymphoma.[2]

Many retroviral vectors used in clinical applications are based on murine gammaretroviruses and are designed to be replication defective (subsequent use of the term retrovirus in this paper refers to gammaretroviruses). A potential risk of retroviral vectors is the development of replication-competent retroviruses (RCRs), which can arise by recombination of viral and cellular components during vector manufacture.[3] Unlike lentiviral vectors, which have not been shown to generate replication-competent viruses, retroviral vectors have been associated with RCR development. Most commonly, RCRs arose through recombination of vector and packaging sequences, and decreasing homology between these sequences has been shown to decrease virus formation.[4–10] Some recombinant retroviruses have also been shown to contain vector-packaging sequences and cellular-derived genetic sequecces.[11,12]

A major concern with RCR infection is treatment-related malignancy. Like the parent murine viruses, RCR generated during vector production has been shown to cause malignancies in mice and non-human primates.[13,14] Furthermore, retroviral gene therapy has been associated with leukemia development in a limited
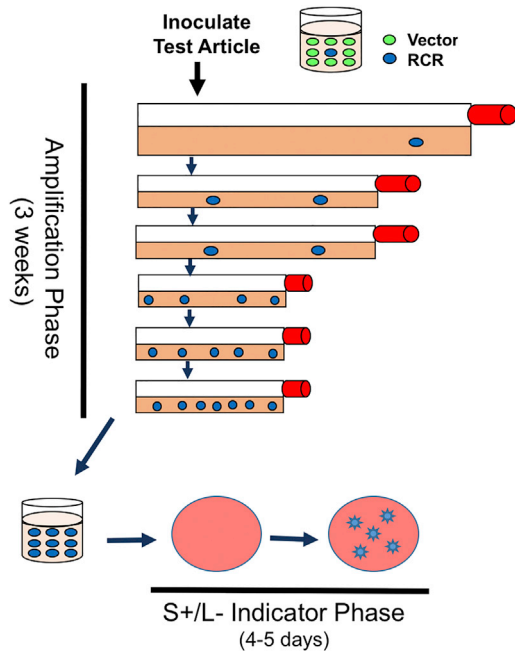
Figure 1. Schematic Representation of the Extended RCR Assay

The amplification phase allows any existing virus to increase. The presence of RCR in the culture media is confirmed by introducing cell-free media into the S⁺/L⁻ assay and analyzing for foci of transformed cells 4–5 days after inoculation.

number of clinical trials.[15–20] While research subjects who developed vector-associated malignancies did not have evidence of RCR, the mechanisms of insertional mutagenesis suggest that exposure to RCR would significantly increase the risk of treatment-related malignancy. This has influenced recommendations from the US FDA regarding RCR testing, which has mandated testing at three points.[21] First, RCR testing is a required release criterion for retroviral vector lots used in clinical applications.[22] Second, research subjects are to be monitored at various time points after treatment for the presence of RCR. The FDA has also imposed a third assessment: RCR testing must be performed on any cell product cultured *ex vivo* for more than 4 days. The number of cells to be tested is 1% of the cell product or 10^8 cells, whichever is less.[22,23] The rationale for testing an *ex vivo* cell product is to detect a low-level RCR that evaded detection in the vector product.

The similarity between vector and viral particles complicates RCR detection. As both contain viral proteins such as capsid, integrase, and reverse transcriptase, protein-detection methods are generally not helpful. Similarly, assays for reverse transcriptase activity cannot distinguish RCR from vector particles. While replication-defective vectors lack the genetic material used in viral replication (i.e., gag, pol, and env gene regions), the sequences are present in the vector-producing cells. Carryover of cellular or plasmid DNA into the vector product will lead to false-positive molecular assays. To date, culture-based assays provide the highest level of sensitivity for RCR detection.

As a recombinant RCR may have a variety of viral and cellular components, the growth rate of a given RCR will be unknown at the time of analysis. Therefore, regulators have required biologic assays to have a minimum of five passages (approximately 3 weeks) of culture in order to amplify any slow-growing RCR.[22] A number of RCR culture assays have been described.[14,24–28] Most combine an amplification phase allowing a virus to grow to a high titer, followed by a detection method for viral particles.

While current RCR assays are sensitive, the large number of cells that must be tested along with the extended period of culture adds significant cost. Furthermore, it is unknown if screening of *ex vivo* transduced cells adds additional value when the vector product has been shown to be RCR negative. Revision in existing RCR testing requirements have been called for.[29] In this paper, we review the experience of the National Gene Vector Biorepository (NGVB), an NHLBI-funded resource (https://www.NGVBCC.org/), which assists investigators in RCR testing of clinical samples. As none of the products tested to date were found to contain RCR, the experience suggests re-evaluation of current requirements for testing *ex vivo* transduced products.

RESULTS

The RCR assay utilized in this study involves incubating test article (cells or culture media) with HEK293 cells (Figure 1). This cell line is highly infectible by retroviral vectors pseudotyped with gibbon ape leukemia virus (GALV) and RD114 envelopes and amplifies RCR to high titer.[27,28] After the amplification phase, media is then inoculated into cultures of PG4 cells. The PG4 line is an S⁺/L⁻ line (sarcoma positive/leukemia negative) cell line, which assumes a transformed phenotype in the presence of murine leukemia viruses (including RCR).[30] If an RCR is present in the test material, foci of transformed cells are evident in the culture. Any test article with foci detected in the indicator phase is considered positive for RCR.

Sample Analysis

This analysis reviewed RCR testing of cellular products from 14 clinical trials (Table 1). While the NGVB offers testing for any vector pseudotype, all the investigators for this study had independently selected the GALV envelope for their vector. All vectors were generated in the PG13 cell line,[10] except for one study that utilized the Phoenix-GALV cell line.[31] The majority of samples tested were T cells utilized in cancer immunotherapy trials. Autologous CD34⁺ hematopoietic progenitor cells and autologous keratinocytes were also evaluated. All vector products utilized in the clinical trials reported here tested negative for RCR prior to *ex vivo* transduction of target cells.

As shown in Table 2, there were 286 samples analyzed in this study. Four were control samples submitted by investigators (one cell sample and three supernatants). Of the remaining 282 samples, 266 were transduced cell samples and a total of 4.53×10^9 cells were analyzed. The median number of transduced cells per sample was 1.35×10^7. In addition, 16 supernatant test articles were submitted from transduced

Table 1. Clinical Trial Samples Evaluated for RCR

IU VPF Study Number	ClinicalTrial.Gov Study Number	Principle Investigator	Initial Assay Initiated	Final Assay Completed	Vector Generation Cell Line	Indication	Transduced Cell Type
10-8	NCT01723306, NCT00664196	Junghans	8/11/10	9/14/10	PG13	CAR-T	T cell
11-13	NCT01593696	Mackall	8/13/12	7/30/16	PG13	CAR-T	T cell
12-8	NCT00924326	Rosenberg	8/27/12	1/17/17	PG13	CAR-T	T cell
13-4	NCT01860937	Curran	10/23/13	9/19/19	PG13	CAR-T	T cell
13-7	NCT01044069	Davila	8/22/13	5/10/17	PG13	CAR-T	T cell
13-8	NCT01723306	Junghans	5/26/16	7/18/06	PG13	CAR-T	T cell
13-32	NCT01263379	Tang	11/4/13	5/10/17	PG13	epidermolysis bullosa	keratinocytes
14-4	NCT01593696	Mackall	4/30/14	11/6/16	PG13	CAR-T	T cell
14-5	NCT00669669	Kiem	1/14/16	9/16/16	Phoenix-GALV	glioblastoma multiforma	CD34+
15-9	NCT01723306	Junghans	5/26/16	7/18/16	PG13	CAR-T	T cell
15-10	NCT02215967	Kochenderfer	10/26/15	1/21/16	PG13	CAR-T	T cell
15-14	NCT02664363	Archer	1/7/16	2/12/16	PG13	CAR-T	T cell
15-15	NCT02498912	O'Cearbhaill	12/16/15	11/30/17	PG13	CAR-T	T cell

cell cultures. The total volume of media tested was 266 mL. All control and transduced test articles were negative for RCR.

Subject Follow-Up

Of the 282 transduced products analyzed, 271 were infused into 270 subjects (Table 3). The difference between analyzed products and infused subjects varied. One product was not infused due to a change in clinical status, and four subjects are awaiting infusion. Seven subjects had both cell and supernatant samples submitted and analyzed for the same product. One subject had two different products infused.

Of the 270 subjects treated, 241 (89%) had blood tested at least 30 days after infusion for evidence of RCR. The majority of subjects were evaluated by qPCR analysis using primers and probe for the GALV envelope (160 of 241, 66%). The remaining subjects were evaluated by the S^+/L^- assay (without amplification) representing 81 of 241subjects tested (34%). Regardless of testing method, all subjects tested were negative for RCR.

Assay Performance

The performance of the RCR assay was evaluated. As the assay is performed under Good Manufacturing Practice, any variation from the defined procedure is documented. We found out-of-specification or deviations in 17 of the 286 samples tested (5.9%). There were five contaminated samples in the amplification phase that required the investigator to send additional samples for repeated testing (1.7%). The 12 remaining tests were completed by repeating the S^+/L^- portion of the assay using reserve samples from the amplification phase. The need for repeating the S^+/L^- assays were as follows: six samples on the same assay required retesting due to a defective control flask. In five samples, the S^+/L^- assay control did not reach confluence within the time period required by the standard operating procedure. There was contamination of one sample in the S^+/L^- assay.

The assay sensitivity is 1 focus forming unit of RCR per 1 mL test article media incubated with $\geq 2 \times 10^5$ HEK293 cells or one test article cell co-cultured with ≥ 5 HEK293 cells. The ratio of test article to HEK293 cells was established to minimize receptor interference between vector and RCR; all assays reported were reviewed and met the appropriate ratio.

DISCUSSION

An important safety concern for patients seeking retroviral gene therapy is exposure to RCR. In this manuscript, we report a multi-institutional study including samples from 10 research groups involved with 14 clinical trials. No evidence of RCR was found in 282 transduced cell products tested in a rigorous RCR assay. Also, there was no evidence of RCR in the 241 research subjects screened for RCR post-treatment.

The NGVB recently reported on the lack of replication-competent lentivirus (RCL) in *ex vivo* transduced T cells used in cancer immunotherapy trials.[32] In that study, 460 cell products were tested with no evidence of RCL. Although the potential risk of RCR is likely higher than that of current third-generation lentiviral vectors, our current study provides additional support for the safety of retroviral vectors. RCL has not been reported with current HIV-1-based systems, and this finding likely relates to deletion of HIV-1 accessory genes, use of self-inactivating long terminal repeats (LTRs), the separation of packaging sequences on to different plasmids, and the use of transient transfection production methods that limit the time for vector generation to less than 1 week.[33] While methods for transient transduction of retroviral vectors are well described,[34] many retroviral vectors are generated in packaging cell lines, which require clone selection, expansion for master cell bank generation, and expansion for vector production, a process that typically takes weeks to months during which time recombinations could occur. Furthermore,

Table 2. Number and Size of RCR Samples Analyzed by Study

NGVB Study Number	Principle Investigator	All Cell Samples	Negative Control Samples	Transduced Samples	Total Number of Control Cells Tested	Total Number of Transduced Cells Tested	Mean Number of Transduced Cells per Assay	Ratio of Test Cell to HEK293 Cell	Supernatant Samples Assayed	Negative Control Samples	Transduced Supernatant Samples	Total Volume of Negative Control Samples (mL)	Total Volume of Transduced Test Article (mL)	Mean Volume of Transduced Test Article (mL)	Ratio of Test Volume (mL) to 10^5 HEK293 Cell
10-8	Junghans	12	0	12	0	1.28E+07	1.07E+06	6.2							
11-13	Mackall	53	0	53	0	4.86E+08	9.16E+06	6.0							
12-8	Rosenberg	91	0	91	0	1.12E+09	1.23E+07	5.6							
13-4	Curran	24	0	24	0	9.20E+08	3.80E+07	5.3	2	0	2	0	173	86.5	2.3
13-7	Davila	39	0	39	0	1.51E+09	3.88E+07	5.3							
13-8	Junghans	1	0	1	0	1.00E+06	1.00E+06	5.0							
13-32	Tang	7	0	7	0	1.40E+07	2.00E+06	8.6	7	0	7	0	34	4.9	8.2
14-4	Mackall	13	0	13	0	1.22E+08	9.38E+06	6.1							
14-5	Kiem								4	0	4	0	45	11.5	4.4
15-9	Junghans	7	0	7	0	7.00E+06	1.00E+06	5.0							
15-10	Kochenderfer	10	0	10	0	1.40E+08	1.40E+07	5.4							
15-14	Archer								6	3	3	13	14	4.7	8.5
15-15	O'Cearbhaill	10	1	9	1.05E+07	2.01E+08	2.23E+07	5.4							
Total		267	1	266	1.05E+07	4.53E+09			19	3	16	13	266		
Mean							1.35E+07	5.8						26.9	5.9

Table 3. Follow-Up Testing for RCR by Study Infused with Transduced Cell Products

IU VPF Study Number	Principle Investigator	Number of Products Infused	Number of Subjects Infused	Subjects with RCR Follow-up	Method of RCR Detection	Level of Sensitivity per DNA
10-8	Junghans	12	12	5	qPCR	<10 copies/0.2 mcg DNA
11-13	Mackall	53	53	53	qPCR	<10 copies/0.2 mcg DNA
12-8	Rosenberg	91	90	80	S$^+$/L$^-$	1 FFU/mL
13-4	Curran	26	26	26	qPCR	<10 copies/0.2 mcg DNA
13-7	Davila	39	39	39	qPCR	<10 copies/0.2 mcg DNA
13-8	Junghans	1	1	1	qPCR	<10 copies/0.2 mcg DNA
13-32	Tang	7	7	7	qPCR	<10 copies/0.2 mcg DNA
14-4	Mackall	12	12	10	qPCR	<10 copies/0.2 mcg DNA
14-5	Kiem	4	4	4	qPCR	<10 copies/0.2 mcg DNA
15-9	Junghans	7	7	2	qPCR and S$^+$/L$^-$	<10 copies/0.2 mcg DNA, 1 FFU/mL
15-10	Kochenderfer	10	10	9	qPCR	<10 copies/0.2 mcg DNA
15-14	Archer	0	0	0	N/A	N/A
15-15	O'Cearbhaill	9	9	5	qPCR	<10 copies/0.2 mcg DNA
Total		271	270	241		

FFU, focus forming unit; mcg, microgram.

retroviral vector systems generally retain most of the components needed to generate a gammaretrovirus including the wild-type LTR.

The selection of the retroviral packaging cell line is an important factor in the risk of RCR development. A variety of packaging cell lines were developed in the 1980s and 1990s, and RCR was reported.[4–8,11,12] Subsequent modification in packaging cell lines appear to decrease the chance of recombination and PG13 cells appears to be the preferred cell line for many investigators.[10] One advantage of the PG13 cell line is that it was generated in NIH 3T3 cells, a murine-derived cell line. The GALV envelope used in PG13 cell lines is a feline-derived virus, and the receptor for the envelope is not present on murine cells. If an RCR did develop in a PG13 cell, the virus would not be expected to propagate within the culture.

Whether the finding of RCR free vector with the PG13 cell line applies to other methods and cell lines will depend on characteristics of the vector and cell line. Additional testing will require study before the safety data noted here can be extrapolated to other systems. One of the products included in this study utilized the Phoenix-GALV packaging cell line, which is based on the human HEK293 cell line. While the experience here is limited, it generated vector that was RCR free in the vector product, in transduced CD34$^+$ cells, and in research subjects.

The US FDA requires subjects treated with retroviral products to be monitored for RCR post-infusion. Currently, there is no guidance requiring a specific assay, and the majority of investigators chose a qPCR-based assay for the viral envelope, predicting that any RCR would contain the GALV envelope. One investigator chose the S$^+$/L$^-$ assay (without HEK293 amplification). All samples tested were negative providing further support for the safety of current retroviral packaging systems.

While all NGVB investigators within the RCR study time frame were invited to participate in this study, four did not provide data on patient follow-up and the 93 RCR assays performed for their trials were not included in this analysis. While we excluded these samples due to lack of follow-up, the cell products tested in the NGVB were all negative for RCR.

In summary, RCR has not been detected in cell products manufactured for clinical use. Participants evaluated post-infusion were also without evidence of RCR exposure. It should be noted all but one study utilized the PG13 cell line. Given the known development of RCR in other cell lines and envelopes, additional studies would be required before extending these findings to other packaging cell lines. Given this caveat, screening cell products for RCR does not add additional assurance of safety and should no longer be required when a well-characterized cell line and the resulting retroviral vector product have been successfully screened for RCR.

MATERIALS AND METHODS
Collection of Study Data
The NGVB is an NIH-sponsored resource that assists gene-therapy investigators in meeting FDA-required testing (https://www.NGVBCC.org/). Only samples intended for *in vivo* administration were included in the analysis. Investigators with study agreements agreeing to participate were sent a list of their test articles with the dates of assay initiation and completion. Participants were asked to supply the following information: (1) production method (stable packaging cell line versus transient transduction); (2) envelope

Table 4. Passage Methodology and Ratio of Test Article to HEK293 Cells

Test Supernatant Volume	HEK293 Cells	Flask Size	Split 1	Split 2	Split $\geq$ 3
1–4 mL	2×10^6	$25\ cm^2$	1:5 $25\ cm^2$	1:5 $25\ cm^2$	1:5 $25\ cm^2$
10–15 mL	5×10^6	$75\ cm^2$	1:5 $75\ cm^2$	1:5 $75\ cm^2$	1:5 $75\ cm^2$
30–50 mL	1.5×10^7	$175\ cm^2$	1:10 $75\ cm^2$	1:5 $75\ cm^2$	1:5 $75\ cm^2$
60–90 mL	2×10^7	$300\ cm^2$	1:10 $175\ cm^2$	1:10 $175\ cm^2$	1:5 $75\ cm^2$
90–120 mL	3×10^7	$450\ cm^2$	1:10 $175\ cm^2$	1:10 $175\ cm^2$	1:5 $75\ cm^2$
Test Cell Number	HEK293 Cells	Flask Size	Split 1	Split 2	Split $\geq$ 3
2 to 4×10^5	2×10^6	$25\ cm^2$	1:5 $25\ cm^2$	1:5 $25\ cm^2$	1:5 $25\ cm^2$
5 to 10×10^5	5×10^6	$75\ cm^2$	1:5 $75\ cm^2$	1:5 $75\ cm^2$	1:5 $75\ cm^2$
1.5 to 3×10^6	1.5×10^7	$175\ cm^2$	1:10 $75\ cm^2$	1:5 $75\ cm^2$	1:5 $75\ cm^2$
2 to 4×10^6	2×10^7	$300\ cm^2$	1:10 $175\ cm^2$	1:10 $175\ cm^2$	1:5 $75\ cm^2$
3 to 6×10^6	3×10^7	$450\ cm^2$	1:10 $175\ cm^2$	1:10 $175\ cm^2$	1:5 $75\ cm^2$

pseudotype; (3) clinical indication; (4) clinicaltrial.gov identifier; (5) if the product was administered to the subject; (6) if the clinical vector product was shown to be RCR free prior to use in the clinical trial; (7) the results of post-infusion RCR screening (if performed $\geq$ 30 days infusion); and (8) the method of post-infusion testing and the level of sensitivity of the assay. For research subjects screened for RCR after product administration, the site and method of screening was at the investigators' discretion.

Cell Line and Positive Control Preparation

HEK293 (American Type Culture Collection [ATCC], Manassas, VA; catalog CRL-1573) and Mink GALV SEATO cells (a kind gift of M. Eiden and C. Wilson, NIH, Bethesda, MD) were maintained in D10 medium (DMEM; Invitrogen, Carlsbad, CA) with 10% fetal bovine serum (FBS; Hyclone, Logan, UT), 2 mM L-glutamine (Invitrogen), 1 mM sodium pyruvate, and 100 units/mL penicillin and 100 μg/mL streptomycin (Invitrogen). PG4 cells (ATCC, CRL-2032) were maintained in MyCoys 5A media with 10% FBS and 100 units/mL penicillin and 100 μg/mL streptomycin. All cell lines were maintained at 37°C and 5% CO_2.

The positive control for the assay utilizes the GALV SEATO virus. The NGVB maintains this cell bank, and a vial of cells are thawed, placed in a 75-cm^2 flask, and expanded over three passages into three 175-cm^2 flasks. After three passages, media is changed on confluent flasks and media is harvested after approximately 24 hr and filtered through a 0.45-μm filter. The media is aliquoted into cryovials, and the virus stock is stored at $\leq$ –70°C for no more than 5 years. The tissue culture infective dose 50 ($TCID_{50}$) of the virus stock is determined on PG4 cells.

RCR Assays

Amplification Phase

HEK293 cells are expanded to the number required based on the test articles to be assayed. On day −1, assay control flasks are prepared by plating eight 25-cm^2 flasks with 2×10^6 HEK293 cells per flask; three flasks will be used for negative control and five flasks will be used for the positive control. For liquid test articles, HEK293 cells are plated in flasks of different sizes to attain a test-volume-to-HEK293-cell ratio within a specified range (Table 4). Similarly, cell test articles are plated at specific test-cell-to-HEK293-cell ratio, also shown in Table 4.

On day 0, medium is removed from the supernatant test article and control flasks and control and test articles are added. For the negative control, 1 mL of D10 medium is added. For the positive control, GALV SEATO virus at the $TCID_{50}$ (in 1 mL of media) is added to five 25-cm^2 flasks. Test articles are added to the appropriate flasks at specified ratios according to Table 4. Polybrene at a final concentration of 8 μg/mL is added to all controls and supernatant test articles. Cultures are then incubated for 2–4 hr, after which time medium is removed from control and supernatant flask, fresh medium is added, and cultures are returned to the incubator. Cells are split at a 1:5 ratio, decreasing the size of flasks at split 1 as shown in Table 4.

Cultures are maintained in log-phase growth, passaging a minimum of five times over a 3-week period. At the end of this period, a media change is performed when cells are confluent and the media is collected 24 hr after the exchange. The media is filtered through a 0.45-μm filter and either used immediately or frozen at −70°C to 80°C for later testing. Reserve samples are also stored at −70°C to 80°C in case repeat testing is required.

Indicator Phase

On days 14–15 of the amplification phase, a vial of PG4 cells are thawed and expanded. On day −1 of the assay, cells are plated in 6-well dishes with 4 mL of media and 1×10^5 cells per well. On day 0, the media is removed from the wells. There are two sets of controls. The "amplified" negative and positive controls are set up using the respective media from the corresponding amplification-phase negative and positive controls. 1 mL from the respective negative control cultures are filtered (0.45 μm) and added to two wells of PG4 undiluted. These control for adequate amplification of virus during the 3 weeks of culture.

In addition, a new set of "direct" negative controls and "direct" positive controls are evaluated using diluted GALV SEATO virus. 1 mL of media is added to three "direct" negative control wells. The "direct" positive control consists of GALV SEATO virus that is of a known potency and is plated at five serial log dilutions, with at least two dilutions below the $TCID_{50}$.

For each test article culture, three wells are evaluated, two wells containing 1 mL of undiluted amplification phase media and one well containing 1 mL of amplification media diluted 1:100. Polybrene at a final concentration of 8 µg/mL is added to all control and test articles.

Cultures are incubated from 2 to 4 hr, and the media is removed and replaced with 4 mL of media. On day 2, the medium is removed from each well and replaced. On day 4, wells are inspected for foci using an inverted microscope (if wells are not confluent they are re-fed and read on days 5–6). The foci are enumerated independently by two technicians. The PG4 assay is acceptable if the following three criteria are met: (1) no foci are observed in the negative control wells; (2) foci are noted at a dilution which is 2-log more concentrated than the $TCID_{50}$ dilution in the direct positive control; and (3) one or more wells of the five amplification-positive controls originally inoculated at the $TCID_{50}$ contain foci. A test article is considered positive for RCR if one or more foci are detected.

AUTHOR CONTRIBUTIONS

CONFLICTS OF INTEREST

J.N.K. has research funding from Kite, a Gilead company, and Celgene and also holds patents on CAR T-cell technology. M.L.D. is a consultant for Celyad, Novartis, Precision Biosciences, and Servier and receives research funds from Celgene.

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