Lentivector Producer Cell Lines with Stably Expressed Vesiculovirus Envelopes

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Retroviral and lentiviral vectors often use the envelope G protein from the vesicular stomatitis virus Indiana strain (VSVin.d.G). However, lentivector producer cell lines that stably express VSVind.G have not been reported, presumably because of its cytotoxicity, preventing simple scale-up of vector production. Interestingly, we showed that VSVind.G and other vesiculovirus G from the VSV New Jersey strain (VSVnj), Cocal virus (COCV), and Piry virus (PIRYV) could be constitutively expressed and supported lentivector production for up to 10 weeks. All G-enveloped particles were robust, allowing concentration and freeze-thawing. COCV.G and PIRYV.G were resistant to complement inactivation, and, using chimeras between VSVind.G and COCV.G, the determinant for complement inactivation of VSVind.G was mapped to amino acid residues 136–370. Clonal packaging cell lines using COCV.G could be generated; however, during attempts to establish LV producer cells, vector superinfection was observed following the introduction of a lentivector genome. This could be prevented by culturing the cells with the antiviral drug nevirapine. As an alternative countermeasure, we demonstrated that functional lentivectors could be reconstituted by admixing supernatant from stable cells producing unenveloped virus with supernatant containing envelopes harvested from cells stably expressing VSVind.G, COCV.G, or PIRYV.G.

INTRODUCTION

Progress in both gene therapy for inherited hematopoietic disorders and also T cell therapy for cancer relies on the use of lentiviral vectors (LVs). The LVs used so far in the clinic have been made via transient transfection (usually of 293 or 293T cells) with plasmids encoding HIV gag-pol, HIV rev, the viral vector, and the vesicular stomatitis virus Indiana strain (VSVind) envelope, a process similar to the laboratory method described 20 years ago. A similar transfection process using the VSVind envelope has also been described for the production of gammaretroviral vectors, where the robust nature of this G protein confers vectors the ability to be concentrated to a high titer by ultracentrifugation and purified using chromatography processes.

A robust and readily scalable process for LV production is becoming ever more necessary as clinical gene therapy becomes more successful. Because of reported problems with constitutive expression of the envelope G protein from VSVind (VSVind.G) on producer cells, LV producer cell lines with inducible VSVind.G expression have been constructed. However, these have not yet been employed for clinical LV production. Another approach has used a non-toxic gammaretroviral envelope from the feline endogenous virus RD114 with a modified cytoplasmic tail to allow LV incorporation. Although a producer cell clone for clinical use has been developed, this has not progressed to a clinical trial.

The RD114 envelope is suited to the transduction of lymphocytes and bone marrow stem cells. Also, when cells are transduced in vitro, the RD114 LV can be captured by retronectin to purify and concentrate the vectors. However, LV particles with RD114-derived envelopes are less infectious than those with VSVind.G in most cell lines tested. Also, RD114-enveloped LVs tend to shed the envelope during purification, resulting in loss of vector titer. Therefore, it is of interest to develop constitutive producer cell lines for LVs with the VSVind.G envelope as an alternative to inducible systems. Constitutive compared with inducible vector production would allow simpler manufacturing process; e.g., no requirement for induction or suppression chemicals and potential continuous culture and harvest. We tried to solve this problem by examining whether related vesiculovirus envelopes from the VSV New Jersey strain (VSVnj), Cocal virus (COCV), and Piry virus (PIRYV) could be stably expressed. There is limited information on receptor usage or the cellular infection mechanism of vesiculovirus envelope G proteins other than VSVind. It has been reported that, like VSVind.G, COCV.G has broad cell tropism, whereas Chandipura virus G (CHAV.G) and PIRYV.G
Investigation of the Fusogenic Cytotoxicity of VesGs

To examine the cytotoxicity of vesiculovirus envelope glycoproteins (G proteins), we constructed vectors with the Streptolabiofacitis hinthustanus phleomycin inhibitor expressed as a fusion protein with the G proteins, separated by a foot-and-mouth disease virus (FMDV) 2A self-cleaving peptide\textsuperscript{14,23} (Figure 1). As a control envelope, we used that from the feline endogenous gammaretrovirus RD114 with a modified cytoplasmic tail (RDpro),\textsuperscript{8,10} which can be readily expressed on stable packaging cells.\textsuperscript{19,20} However, the use of this receptor cannot be generalized to the genus. In support of this, CHAV.G does not interact with LDLR.\textsuperscript{19} Furthermore, it has been postulated that all VesG go through similar structural and conformational changes during pH-dependent fusion.\textsuperscript{21} However, studies of wild-type virus infectivity and pre and post-fusion structures highlighted several differences regarding fusogenicity at acidic pH levels\textsuperscript{22} and the nature of pH-sensitive switches.\textsuperscript{23}

Here we looked at the cytotoxicity of the vesiculovirus envelopes, including VSVind.G and whether they could be stably expressed and sustain the construction of stable constitutive LV producer cell lines.

RESULTS

Establishment of Stable Envelope-Expressing Cells and Long-Term Vector Production

To confirm that the VesGs were indeed expressed on the transfected cells, we cultured bulk-selected populations for up to 10 weeks and detected envelope expression using the antiserum VSV-Poly, which has been reported to recognize VSVind.G.\textsuperscript{26} This antibody detected all VesGs on transiently transfected cells (Figure 3A, left); COCV.G was detected with intermediate efficiency, and VSVnj.G and PIRYV.G were weakly detected. After 10 weeks in culture, each bulk-selected population still expressed the respective G proteins, although, in each case, a proportion of the bulk-transfected and -selected, cells showed a lower level of expression than the transiently transfected population (Figure 3A, right). These bulk-selected populations expressing G proteins could produce LVs after transient supply of gag-pol, rev, and vector expression plasmids with titers of over $10^8$ transduction units (TU)/mL obtained with VSVind.G and COCV.G, with approximately $5 \times 10^5$ TU/mL for VSVnj.G and PIRYV.G 4 weeks after selection, the earliest time when sufficient cells could be obtained (Figure 3B). Over the course of 10 weeks, a steady drop in VesG expression and functional LV titers was observed. COCV.G and VSVind.G were able to retain relatively high titers compared with VSVnj.G and PIRYV.G, making them promising candidates as packaging cell line (PCL) envelopes. The titers in each case tended to decrease after longer selection, perhaps because of the enrichment of low G protein-expressing cells in the bulk populations noted in Figure 3A.

To establish which VesGs are most promising to generate a stable packaging cell line, their physical stability was tested using transiently produced LV. Figure 4A shows that LVs with all vesiculovirus envelopes were readily concentrated by centrifugation, as has been reported for VSVind.G.\textsuperscript{16} In addition, they were all stable during freeze-thawing and, in general, stable during incubation at 4°C or 37°C. VSVind.G, COCV.G, VSVnj.G, and PIRYV.G were stable after three cycles of freeze-thawing and incubation at 37°C, with a minimum recovery percentage of 80%. PIRYV.G was less stable during

pseudotyped LVs are suboptimal in transducing non-adherent cells, including lymphoid and hematopoietic stem cells.\textsuperscript{18} Recently, the 3D structure of the interaction between VSVind.G and its major receptor, low-density lipoprotein receptor (LDLR), has been elucidated.\textsuperscript{19,20} However, the isolated LDLR binding epitope is not conserved among vesiculovirus G protein (VesG), and, therefore, the use of this receptor cannot be generalized to the genus. In support of this, CHAV.G does not interact with LDLR.\textsuperscript{19} Furthermore, it has been postulated that all VesG go through similar structural and conformational changes during pH-dependent fusion.\textsuperscript{21} However, studies of wild-type virus infectivity and pre and post-fusion structures highlighted several differences regarding fusogenicity at acidic pH levels\textsuperscript{22} and the nature of pH-sensitive switches.\textsuperscript{23}

Here we looked at the cytotoxicity of the vesiculovirus envelopes, including VSVind.G and whether they could be stably expressed and sustain the construction of stable constitutive LV producer cell lines.
COCV.G and PIRYV.G Are More Resistant to Complement-Mediated Inactivation by Mammalian Sera

VSVind.G is known to be inactivated by complement, making it less suitable for in vivo gene therapy application, where exposure to the bloodstream will occur. When we tested LVs with other vesiculo-virus envelopes, we found that VSVPNj.G was also inactivated by human, mouse, guinea pig, and rabbit complement, whereas COCV.G and PIRYV.G were resistant, as was the control RDpro, as reported previously (Figure 5A). This differential sensitivity allowed us to map the region of the envelope responsible by constructing chimeras between VSVPNj.G and COCV.G; these were designed so that the protein junctions were made in regions of homology between the two G proteins (Figure 5B). These chimeras were expressed as detected by flow cytometric analysis of 8G5F11 (Kerafast, Boston, MA) immunostaining, an extracellular anti-VSVind.G mAb, and could produce infectious LVs at levels comparable with VSVPNj.G following transient transfection. LVs expressing the chimeric G proteins 4A, 1B, and 2B were resistant to human serum inactivation, suggesting that the region of VSVPNj.G between amino acids 136 and 370 confers complement sensitivity (Figure 5B). On the crystal structure of VSVPNj.G, this region corresponds to most of the pleckstrin homology domain and parts of the fusion, trimerization, and lateral domains (Figure S1).  

WinPac-COCV.G Packaging Cell Line: Establishment and Identification of Superinfection

These experiments indicated that COCV.G was a suitable envelope for lentivirus manufacture and in vivo use because it was thermostable and complement-resistant. We therefore transfected WinPac cells, which stably express HIV gag-pol and rev, with a COCV.G expression vector (Figure S2A) and isolated single-cell clones by selection in phleomycin. Functional clones were identified by transient transfection of all components (Figure 4). COCV.G expression on clone H4 was maintained after long-term culture for 40 passages (~3 months) (Figure S2C), albeit at lower levels compared with transient expression.

The final step in the construction of a producer cell line was to introduce a stable vector construct into the WinPac-COCV.G H4 clone. To do this, we employed a construct with a promoterless blasticidin resistance gene (BSR), pSIN-gfp-BSR, which confers BSR expression following integration next to a cellular promoter (Figure 6A). Transfection of WinPac-COCV.G cells with this construct yielded a polyclonal population that could be expanded to harvest LVs. LV yields from the WinPac-COCV.G bulk population and WinPac-RDpro stable producer cell clones were compared by seeding cells in Corning HYPERFlask and harvesting LVs each day for 5 days (Figure 6B). The stable WinPac-RDpro producer cell clone generated more LVs under these conditions than the WinPac-COCV.G H4 bulk population, in contrast to the relative titers following transient transfection, where COCV.G was higher (Figure 4).

When we examined cells after long-term culture for 10 weeks, the cell pellet of WinPac-COCV.G (H4) became visibly greener, whereas no changes were observed for WinPac-RDpro cells (Figure S3). Previous reports have described that cells expressing VSVPNj.G were not blocked for superinfection by VSVind. In contrast, expression of gammaretrovirus (GRV) envelopes blocks superinfection by viruses that use the same receptor. An infection assay demonstrated that WinPac-RDpro cells blocked RDpro-pseudotyped LV infection, whereas WinPac-COCV.G cells were permissive to infection by COCV.G-LV (Figure 7A). Furthermore, cells stably expressing each of the VesGs could be readily infected with LVs pseudotyped with Ves.G envelopes, whereas a superinfection block of RDpro-enveloped LVs was observed in RDpro envelope-expressing cells (Figure 7B). We therefore hypothesized that stable WinPac-COCV.G producer cells become superinfected with LV genomes when cultured for...
some time. To confirm this, early-passage stable WinPac-COCV.G producer cells were cultured in the presence of nevirapine, a non-nucleoside inhibitor of HIV reverse transcriptase. These cells had a lower LV genome number per cell than WinPac-COCV.G. Cells cultured in the absence of nevirapine accumulated high copies of the LV genome (Figure 7C).

A Mitigation to Superinfection: Cell-free in trans Pseudotyping
Genetic instability of vector producer cells caused by vector superinfection will be problematic in terms of product characterization. This problem would apply to the construction of LV producer cell lines with any of the VesGs we tested. However, stable expression of each VesG in the absence of the LV genome was viable. We therefore explored the possibility of admixing unenveloped LV particles with VesG preparations because this has previously been reported to yield infectious GRVs using VSVind.G. Figure 8A shows that this was possible using transiently produced LV particles without an envelope glycoprotein with transiently produced G protein preparations. Also, infectious particles could be generated with stably produced unenveloped LV particles and stably produced G protein preparations, with titers up to $4.0 \times 10^5$ TUs/mL (Figure 8B), highlighting a production process for LVs with VesGs that avoids transient plasmid transfection. By further optimization (e.g., use of clonal cells expressing a high level of vector genome), vector titer over $10^6$ TUs/mL should be achievable. This system would increase titers and reproducibility and remove the risk of plasmid DNA contamination of the LV preparation.

DISCUSSION
Perhaps one of the most striking findings of this study is that the VSVind.G protein is not demonstrably cytotoxic when stably expressed in cells. VSV is a lytic virus, but the viral M protein is the major cause of cell death. However, an M mutant virus retains some cytotoxicity because of the G protein. The assumption that VSVind.G alone cannot be stably expressed seems to come from brief observations reported by Burns et al. in 1993. Interestingly, Humbert et al. also demonstrated recently that stable expression of the VSVind.G and COCV.G proteins is possible. However, they found lower LV titers when VSVind.G-expressing cells were transfected with other components, which we did not observe (Figure 3). Similarly to Humbert et al., we proceeded to develop packaging cells based on COCV.G because it is resistant to complement inactivation (Figure 5A) and thermostable (Figure 4B). We also broadly mapped a determinant on VSVind.G that was responsible for complement inactivation (Figure 5), so chimeric envelopes or further defined mutants of VSVind.G, or indeed PIRYV.G, could also be used.

The construction of two COCV.G LV producer cell clones, for gfp and a TCR-encoding LV, has been published previously. Here we report the construction of a COCV.G gfp LV producer cell clone that could generate $1.3 \times 10^5$ TUs/mL LV particles every harvest after scaling up to a 560-mL Corning HYPERFlask (Figure 7). However, we believe that vector superinfection, which is not addressed by Humbert et al., may constitute a problem for clinical LV production from COCV.G producer cells. First, the extent of cell expansion we or Humbert et al. report is insufficient for clinical LV vector characterization and final production. It is possible that vector superinfection will prevent sufficient cell expansion. Second, the COCV.G producer cells will genetically change over time, making quality testing difficult. Finally, when using non-self-inactivating transfer vectors, the infidelity of HIV reverse transcriptase (approximately 1 substitution per 10,000 bases) will lead to accumulation of defective vector genomes within the producer cells. These will reduce the titer of the clinical LV and could generate mutant transgenes with potentially deleterious clinical effects.

Thus, the challenge in the generation of stable vesiculovirus envelope LV producer cell clones suitable for clinical use becomes that of inhibiting LV superinfection rather than that of envelope toxicity. One
receptors or the additional receptor Lgr4. Finally, it could also be possible to block LV/receptor interactions in the producer cell to be efficiently removed from the clinical LV. Second, it could be efficiently removed from the clinical LV. Consequently, and also remove the risk of plasmid DNA contamination in proliferation of two separate components may add cost and variability. Superinfection in stable vesiculovirus envelope LV producer cell clones could also be blocked in a number of ways. First, as we have shown, this can be prevented by expanding cells in an HIV inhibitor (Figure 7), which would have to be efficiently removed from the clinical LV. Second, it could be possible to block LV/receptor interactions in the producer cell cultures with the soluble receptor, receptor-associated protein (RAP), or antibodies, as described for the VSVind.G LDL family receptors or the additional receptor Lgr4. Finally, it could also be possible to generate vesiculovirus envelope LV producer cell clones in cell lines resistant to either vesiculovirus entry or LV replication at post-entry steps.

**Phylogenetic Analysis of Vesiculovirus and Rabies Virus G Proteins Based on Amino Acid Sequences**

G proteins of the major vesiculoviruses (VSVind, UniProt: P03522; COCV, UniProt: O56677; VSVnj, UniProt: P04882; PIRYV, UniProt: Q85213; Maraba virus, UniProt: F8SFP4; VSV Alagoas strain [VSVala], UniProt: B3FRL4; Chandipura virus, UniProt: P13180; Carajas virus, UniProt: A0A0D3R1Y6; and Isfahan virus, UniProt: Q5K2K4) as well as the G protein of the rabies virus (UniProt: P8JXF6) were included in the analysis. The amino acid sequences were aligned using the ClustalOmega online multiple sequence alignment tool (EMBL-EBI). The evolutionary analyses were conducted in MEGA7. The evolutionary history was inferred by using the maximum likelihood method based on the Jones-Taylor-Thornton matrix-based model. The tree with the highest likelihood is shown, with the bootstrap confidence values (out of 100) indicated at the nodes. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site, depicted in the linear scale.

**Materials and Methods**

**Cell Culture**

HEK293T and WinPac cells were cultured in DMEM (Sigma-Aldrich, St. Louis, MO) containing 2 mM L-glutamine (Gibco, Carlsbad, CA) supplemented with 50 U/mL penicillin, 50 mg/mL streptomycin (Gibco), and 10% fetal calf serum (FCS) (Sigma-Aldrich or Gibco) at 37°C and 5% CO2. When indicated, antibiotics were added to the culture medium (antibiotics and their working concentration are listed in Table S1). The stable cell lines derived from WinPac cells as well as HEK293Ts are listed in Table S2.

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removed, and cells were washed with PBS and fixed with absolute methanol for 5 min. Wells were air-dried, and the cells were stained with Giemsa (Sigma-Aldrich) for 30 min, washed with water, and imaged using bright-field microscopy (Hitachi, Tokyo, Japan).

**Extracellular Antibody Binding Assay**

G protein-expressing HEK293T and WinPac-COCV.G cells were harvested, washed twice with PBS, and plated in U-bottom 96-well plates at identical densities. Cells were then incubated with 1:500 or 1:200 dilutions of 8G5F11 (Kerafast, Boston, MA) or VSV-Poly,

respectively, in 1% BSA (Sigma-Aldrich) in PBS in a total reaction volume of 200 μL for 30 min at 4°C. After washing twice with PBS (Sigma-Aldrich) to remove any unbound antibodies, the cells were incubated for another 30 min at 4°C with a 1:500 dilution of their respective fluorophore-conjugated secondary antibodies (the list of antibodies utilized is shown in Table S4) in 1% BSA in PBS in a total reaction volume of 200 μL. Cells were then washed twice, fixed in 2% paraformaldehyde (PFA), and analyzed by flow cytometry.

**Transient LV Production**

Three-plasmid co-transfection into HEK293T cells was used to make pseudotyped LVs as described previously. Briefly, 4 x 10^6 293FT cells were seeded in 10-cm plates. 24 hr later, they were transfected using FuGene6 (Promega, Madison, WI) with the following plasmids: self-inactivating (SIN) pHV (a gfp expressing vector plasmid), p8.91 (a Gag-Pol-Rev expression plasmid), p8.91 (a Gag-Pol-Rev expression plasmid), and envelope expression plasmids (the details of these plasmids are listed in the Table S3). The medium was changed after 24 hr, and then vector-containing medium (VCM) was collected over 24-hr periods for 2 days. Following collection, VCM was passed through a 0.45-μm cellulose acetate filter and either concentrated or directly stored at −80°C.

**LV Titration, Envelope Stability, and Infection Assays**

The functional titer of each vector preparation was determined by flow cytometric analysis for GFP expression following transduction of HEK293T cells. Briefly, 2 x 10^4 293T cells were infected with LVs plus 8 μg/mL Polybrene (Merck Millipore, Billerica, MA) for 24 hr. Infected cells were detected by EGFP expression using

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**Figure 5. LVs Bearing COCV.G and PIRYV.G Envelope Are Resistant to Inactivation by Complement**

Vectors were diluted 1:20 (v/v) with serum-free medium or heat-inactivated (HI) or fresh human and mammalian serum, incubated at 37°C for 1 hr, and then plated on HEK293T cells. The percentage of GFP-positive cells was measured 2 days later via flow cytometry. Measured titters were normalized to those of serum-free samples. (A) The reduction of titters by sera after heat-inactivation at 56°C for 1 hr was less than 30% for all LVs. (B) Substantial proportions of LVs with VSVind.G, but not COCV.G, PIRYV.G, or RDpro env, were inactivated by complement. The data shown represent relative titters ± SD for four experiments performed in duplicate. (C) The design and the cross-over points of the chimeric G proteins are represented in linear diagrams, in which white bars stand for wild-type (WT) VSVind.G sequences, and WT COCV.G sequences are represented by black hatched bars. The cross-over point between the two WT sequences is indicated by the amino acid number above the bar. (D) Sensitivity of LVs pseudotyped with chimeric G proteins to inactivation by several mammalian sera. Reductions of titters by HI sera were less than 30% for all LVs (data not shown). The data shown represent relative titters ± SD for four experiments performed in duplicate.
titer of serum mixed sample

and storage at OptiMEM on ice and incubated on ice for 1 hr before aliquoting. The virus was resuspended in cold plain Optima LK-90 ultracentrifuge using the SW-28 swinging bucket rotor (radius, 16.1 cm). The virus was resuspended in cold plain OptiMEM on ice and incubated on ice for 1 hr before aliquoting and storage at −80°C.

Figure 6. Construction of the WinPac-COCV.G Producer Cell Line
(A) Schematic representation of pSIN-GFP-BSR (promoterless blasticidin vector genome). (B) Total transduction units of COCV.G- and RDpro-enveloped LVs harvested from stable producers in Corning HYPERFlask 2D culture. 560 mL of LV-containing medium was collected every 24 hr over 5 days. The data presented represent the mean yield from a single large-scale production. Average titers of COCV.G-LV and RDpro-LV harvested were 8.2 × 10^5 and 2.9 × 10^5 transduction units (TU)/mL, respectively. Titrations to determine the functional titers were performed in triplicate. Data were obtained from cells 25–30 passages following antibiotic selection.

FACSCanto II (BD Biosciences, San Jose, CA) and Flowjo software 48 hr following the start of transduction. Titer were calculated from virus dilutions, where 1%–20% of the cell population was EGFP-positive, using the following formula:

\[
\text{Titer} = \frac{\text{transduction units (TU)}}{\text{mL}} = \left( \frac{\text{no. of cells at transduction}}{\text{the volume of virus preparation added (mL)}} \right) \times \left( \frac{\% \text{ of GFP positive cells}}{\text{dilution factor}} \right)
\]

Infection assays for superinfection detection and envelope stability were carried out similarly. For envelope stability, vectors were titrated on HEK293T cells after either incubation at different temperatures or freeze-thawing cycles. The percentage of recovery was calculated according to the volume, and titers of the recovered LVs were compared with the volume and titers of the crude ones. On the other hand, to detect superinfection, stable envelope-expressing cells, WinPac-RDpro, or WinPac-COCV.G cells were challenged with VesG-pseudotyped LVs at MOI 0.5 or 3.0.

LV Concentration
Harvested VCM was concentrated ~100-fold by ultra-centrifugation at 22,000 rpm (87,119 × g) for 2 hr at 4°C in a Beckmann Optima LK-90 ultracentrifuge using the SW-28 swinging bucket rotor (radius, 16.1 cm). The virus was resuspended in cold plain OptiMEM on ice and incubated on ice for 1 hr before aliquoting and storage at −80°C.

Serum Sensitivity Assay
Approximately 3 hr prior to infection, HEK293T cells were seeded in 12-well plates at a density of 2 × 10^5 cells/well in 2 mL of complete medium containing 8 μg/mL Polybrene (Merck Millipore). Later, 2.5 μL of VesG-LV at a 1.6 × 10^7 TUs/mL titer were mixed with plain OptiMEM (Gibco) and heat-inactivated mammalian sera (human [catalog number S1764], guinea pig [catalog number S1639], and rabbit [catalog number S7764] from Sigma-Aldrich and mouse [catalog number IMS-C57BL6-COMPL] from Patricell, Nottingham, UK) by incubation at 56°C for 1 hr and fresh mammalian sera (1:20 v/v), incubated at 37°C for 1 hr, and plated on the cells. 48 hr later, cells were harvested and analyzed via flow cytometry for EGFP expression. Relative infection rates for all samples were normalized to that of OptiMEM samples within individual VesG-LV using the following equation:

\[
\text{Infection\%} = \frac{\text{titer of serum mixed sample}}{\text{titer of OptiMEM mixed sample}} \times 100
\]

LV Production from Producer Cells
Cells were seeded at a density of 2.1–2.3 × 10^5 cells/cm^2. After 72 hr, cells were washed with medium, and 0.08–0.1 mL/cm^2 of medium was replaced. 24 hr later, VCM was collected, passed through a 0.45-μm cellulose acetate filter (SL, Nottingham, UK), and stored at −80°C. Fresh medium was added to the cells for collection after 24 hr. This process was repeated up to three times. For HYPERFlask production, cells were expanded under antibiotic selection and 10 μM nevirapine in the case of WinPac-COCV.G and seeded at a density of 1.16 × 10^6 cells/cm^2. 24 hr later, VCM was collected, passed through a 0.45-μm cellulose acetate filter, and stored at −80°C.

qPCR for Time-Dependent Superinfection Study
To determine infection copies in WinPac-COCV.G cultured in the presence and absence of 10 μM nevirapine, SYBR Green-based qPCR was used. Initially, genomic DNA (gDNA) was extracted from 2 × 10^6 cells using the DNeasy Blood and Tissue Kit (QIAGEN, Crawley, UK) following the manufacturer’s instructions. gDNA concentration was determined by spectrophotometry and adjusted to 50 ng/mL. 100 ng of gDNA was used as a template for qPCR reactions using the QuantiTect SYBR Green PCR Kit (QIAGEN) and ABI 7500 real-time PCR system (Applied Biosystems, Warrington, UK). PCR reactions were performed at 95°C for 15 min, followed by 40 cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. A melting curve was run following each assay.
All qPCR reactions were performed in triplicate. Infection was quantified by amplification of the LV genome via qPCR and normalized to human beta (HB)-actin copy numbers per cell. The data presented represent mean ± SEM of the qPCR assay performed in triplicate. The standards and primers used, the map of the LV vector with the primer locations, and the expected fragments before and after integration are shown in Figure S4.

To calculate the DNA copy number per cell, β-actin was quantified in parallel to any gene of interest and divided by 6 to give the number of cells per reaction. This was done assuming HEK293T cells are triploid and that the primer pair used (HB-actin-F and HB-actin-RC) detect the β-actin gene (on chromosome 7) and β-actin pseudogene (on chromosome 11).

The standards used in all qPCRs were 10^5, 10^4, 10^3, 10^2, and 10^1 plasmids/reaction. For β-actin and superinfection copies, the standards were made by cloning the PCR product from HB-actin-F and RC or 3LTR_fw_INFCross_Cp and 5LTR_INFCross_Cp, respectively, into pJet (Thermo Fisher Scientific).

**Cell-free in trans Pseudotyping**

Bald, unenveloped LV and soluble G proteins were produced either transiently or stably. For transient production, HEK293T cells were co-transfected with p8.91 and SIN pHV-GFP or VesG expression plasmids using FuGene6.

Supernatant from cells expressing VesG proteins constitutively and transiently were harvested 24 hr after cells were taken off of antibiotic selection or 48 hr after transfection, respectively. In a similar fashion, unenveloped GFP-encoding LVs were harvested from the bald WinPac-GFP packaging cell line or HEK293T cells transiently transfected. Bald LVs and soluble exogenous G proteins were mixed at a 1:3 v/v ratio, incubated at 37°C for 1 hr, and plated onto HEK293T cells. The percentages of GFP-positive cells were measured 48 hr thereafter via flow cytometry, and titers were calculated as described previously.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes four figures and four tables and can be found with this article online at https://doi.org/10.1016/j.omtm.2018.07.013.
performed in duplicate.

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CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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AUTHOR CONTRIBUTIONS

M. Tijani, A.M.M., and C.P. performed experiments to obtain the presented data and wrote the paper. K.S. and M.F. performed experiments and obtained initial data on COCV.G-bearing WinPac cells and vectors. T.M., M. Themis, J.N., and G.M. helped with designing experiments and interpreting data. M.K.C. and Y.T. supervised the study and wrote the paper.

REFERENCES

1. Collins, M., and Thrasher, A. (2015). Gene therapy: progress and predictions. Proc. Biol. Sci. 282, 20143003.

2. Naldini, L., Blömer, U., Gallay, P., Ory, D., Mulligan, R., Gage, F.H., Verma, I.M., and Trono, D. (1996). In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272, 263–267.

3. Zufferey, R., Nagy, D., Mandel, R.J., Naldini, L., and Trono, D. (1997). Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat. Biotechnol. 15, 871–875.

4. Burns, J.C., Friedmann, T., Driever, W., Burrascano, M., and Yee, J.K. (1993). Vesicular stomatitis virus G glycoprotein pseudotyped retroviral vectors: concentration to very high titer and efficient gene transfer into mammalian and nonmammalian cells. Proc. Natl. Acad. Sci. USA 90, 8033–8037.

5. Yamada, K., McCarty, D.M., Madden, V.J., and Walsh, C.E. (2003). Lentivirus vector purification using anion exchange HPLC leads to improved gene transfer. Biotechniques 34, 1074–1078, 1080.

6. Farson, D., Witt, R., McGuinness, R., Dull, T., Kelly, M., Song, J., Radeke, R., Bukovsky, A., Consiglio, A., and Naldini, L. (2001). A new generation stable inducible packaging cell line for lentiviral vectors. Hum. Gene Ther. 12, 981–997.

7. Broussau, S., Jabbour, N., Lachapelle, G., Durocher, Y., Tom, R., Transfiguracion, J., Gilbert, R., and Massie, B. (2008). Inducible packaging cells for large-scale production of lentiviral vectors in serum-free suspension culture. Mol. Ther. 16, 500–507.

8. Ikeda, Y., Takeuchi, Y., Martin, F., Cosset, F.L., Mitrophous, K., and Collins, M. (2003). Continuous high-titer HIV-1 vector production. Nat. Biotechnol. 21, 569–572.

9. Throm, R.E., Ouma, A.A., Zhou, S., Chandrasekaran, A., Lockey, T., Greene, M., De Ravin, S.S., Moayeri, M., Malech, H.L., Sorentino, R.P., and Gray, J.T. (2009). Efficient construction of producer cell lines for a SIN lentiviral vector for SCID-X1 gene therapy by concatemer array transfection. Blood 113, 5104–5110.

10. Sanber, K.S., Knight, S.B., Stephen, S.L., Bailey, R., Escors, D., Minshull, J., Sanelli, G., Thrasher, A.J., Collins, M.K., and Takeuchi, Y. (2015). Construction of stable packaging cell lines for clinical lentiviral vector production. Sci. Rep. 5, 9021.

11. Marin, V., Stornaiuolo, A., Piovani, C., Corna, S., Bossi, P., Perna, M., Giuliani, E., Scavullo, C., Zucchelli, E., Bordignon, C., et al. (2016). RD-MolPack technology for the constitutive production of self-inactivating lentiviral vectors pseudotyped with the nontoxic RD114-TR envelope. Mol. Ther. Methods Clin. Dev. 3, 16033.

12. Wielgosz, M.M., Kim, Y.S., Carney, G.G., Zhan, J., Reddivari, M., Coop, T., Heath, R.J., Brown, S.A., and Nienhuis, A.W. (2015). Generation of a lentiviral vector producing cell clone for human Wiskott-Aldrich syndrome gene therapy. Mol. Ther. Methods Clin. Dev. 2, 14063.

13. Porter, C.D., Collins, M.K., Tailor, C.S., Parkar, M.H., Cosset, F.L., Weiss, R.A., and Takeuchi, Y. (1996). Comparison of efficiency of infection of human gene therapy target cells via four different retroviral receptors. Hum. Gene Ther. 7, 913–919.

14. Relander, T., Johansson, M., Olsson, K., Ikeda, Y., Takeuchi, Y., Collins, M., and Richter, J. (2005). Gene transfer to repopulating human CD34+ cells using amphotropic, GALV-, or RD114 pseudotyped HIV-1-based vectors from stable producer cells. Mol. Ther. 11, 452–459.

15. Hu, J., Kelly, P., Bonifacino, A., Agricola, B., Donahue, R., Yanin, E., and Dunbar, R.E. (2003). Direct comparison of RD114 pseudotyped versus amphotropic pseudotyped retroviral vectors for transduction of rhesus macaque long-term repopulating cells. Mol. Ther. 8, 611–617.

16. Strang, B.L., Ikeda, Y., Cosset, F.L., Collins, M.K., and Takeuchi, Y. (2004). Characterization of HIV-1 vectors with gammaretrovirus envelope glycoproteins produced from stable packaging cells. Gene Ther. 11, 591–598.

17. Trobridge, G.D., Wu, R.A., Hansen, M., Ironside, C., Watts, K.L., Olsen, P., Beard, B.C., and Kiem, H.P. (2010). Cocal-pseudotyped lentiviral vectors resist inactivation by human serum and efficiently transduce primate hematopoietic repopulating cells. Mol. Ther. 18, 725–733.

18. Hu, S., Mohan Kumar, D., Sax, C., Schuler, C., and Akkina, R. (2016). Pseudotyping of lentiviral vector with novel vesiculovirus envelope glycoproteins derived from Chandipura and Piry viruses. Virology 488, 162–168.
19. Nikolic, J., Belot, L., Raux, H., Legrand, P., Gaudin, Y., and Albertini, A. (2018). Structural basis for the recognition of LDL-receptor family members by VSV glycoprotein. Nat. Commun. 9, 1029.

20. Finkelestein, D., Werman, A., Novick, D., Barak, S., and Rubinstein, M. (2013). LDL receptor and its family members serve as the cellular receptors for vesicular stomatitis virus. Proc. Natl. Acad. Sci. USA 110, 7306–7311.

21. Baquero, E., Albertini, A.A., Raux, H., Abou-Hamdan, A., Boeri-Erba, E., Ouldali, M., Buonocore, L., Rose, J.K., Lepault, J., Bressanelli, S., and Gaudin, Y. (2017). Structural intermediates in the fusion-associated transition of vesiculovirus glycoprotein. EMBO J. 36, 679–692.

22. Martinez, I., and Wertz, G.W. (2005). Biological differences between vesicular stomatitis virus Indiana and New Jersey serotype glycoproteins: identification of amino acid residues modulating pH-dependent infectivity. J. Virol. 79, 3578–3585.

23. Baquero, E., Albertini, A.A., Raux, H., Buonocore, L., Rose, J.K., Bressanelli, S., and Gaudin, Y. (2015). Structure of the low pH conformation of Chandipura virus G protein reveals important features in the evolution of the vesiculovirus glycoprotein. PLoS Pathog. 11, e1004756.

24. Palmenberg, A.C., Parks, G.D., Hall, D.J., Ingraham, R.H., Seng, T.W., and Pallai, P.V. (1992). Proteolytic processing of the cardiovascular P2 protein: primary 2A/2B cleavage in clone-derived precursors. Virology 190, 754–762.

25. Szymczak, A.L., and Vignali, D.A. (2005). Development of 2A peptide-based strategies in the design of multistropic vectors. Expert Opin. Biol. Ther. 5, 627–638.

26. Hoshino, H., Nakamura, T., Tanaka, Y., Miyoshi, I., and Yanagihara, R. (1993). Functional conservation of the neutralizing domains on the external envelope glycoprotein of cosmopolitan and melanese strains of human T cell leukemia/lymphoma virus type I. J. Infect. Dis. 168, 1368–1373.

27. Tesfay, M.Z., Kirk, A.C., Hadae, E.M., Griesmann, G.E., Federspiel, M.J., Barber, G.N., Henry, S.M., Peng, K.W., and Russell, S.J. (2013). PEGylation of vesicular stomatitis virus extends virus persistence in blood circulation of passively immunized mice. J. Virol. 87, 3752–3759.

28. Tesfay, M.Z., Ammayappan, A., Federspiel, M.J., Barber, G.N., Stodil, D., Peng, K.W., and Russell, S.J. (2014). Vesiculovirus neutralization by natural IgM and complement. J. Virol. 88, 6148–6157.

29. Beebe, D.P., and Cooper, N.R. (1981). Neutralization of vesicular stomatitis virus (VSV) by human complement requires a natural IgM antibody present in human serum. J. Immunol. 126, 1562–1568.

30. DePolo, N.J., Reed, J.D., Sheridan, P.L., Townsend, K., Sauter, S.L., and Dubensky, T.W., Jr. (2000). VSV-G pseudotyped lentiviral vector particles produced in human cells are inactivated by human serum. Mol. Ther. 2, 218–222.

31. Croyte, M.A., Callahan, S.M., Auricchio, A., Schumer, G., Linse, K.D., Wilson, J.M., Brunner, L.J., and Kohberger, G.P. (2004). PEGylation of a vesicular stomatitis virus G protein-pseudotyped lentiviral vector prevents inactivation in serum. J. Virol. 78, 912–921.

32. Sandrin, V., Boson, B., Salmon, P., Gay, W., Négre, D., Le Grand, R., Tronc, D., and Cosset, F.L. (2002). Lentiviral vectors pseudotyped with a modified RD114 envelope glycoprotein show increased stability in sera and augmented transduction of primary lymphocytes and CD34+ cells derived from human and nonhuman primates. Blood 100, 823–832.

33. Cosset, F.L., Takeuchi, Y., Battini, J.L., Weiss, R.A., and Collins, M.K.L. (1995). High-titer packaging cells producing recombinant retroviruses resistant to human serum. J. Virol. 69, 7430–7436.

34. Munis, A.M., Tijani, M., Hassali, M., Mattiuzzo, G., Collins, M.K., and Takeuchi, Y. (2018). Characterisation of Antibody Interactions with the G Protein of Vesicular Stomatitis Virus Indiana Strain and Other Vesiculovirus G Proteins. bioRxiv. https://doi.org/10.1101/330910.

35. Roche, S., Bressanelli, S., Rey, F.A., and Gaudin, Y. (2006). Crystal structure of the low-pH form of the vesicular stomatitis virus glycoprotein G. Science 313, 187–191.

36. Roche, S., Rey, F.A., Gaudin, Y., and Bressanelli, S. (2007). Structure of the prefusion form of the vesicular stomatitis virus glycoprotein G. Science 315, 843–848.

37. Vogt, B., Roscher, S., Abel, B., Hildinger, M., Lamarre, A., Baum, C., and von Laer, D. (2001). Lack of superinfection interference in retroviral vector producer cells. Hum. Gene Ther. 12, 359–365.

38. Sommerfeld, M.A., and Weiss, R.A. (1990). Receptor interference groups of 20 retroviruses plating on human cells. Virology 176, 58–69.

39. Abe, A., Chen, S.T., Miyanoohara, A., and Friedmann, T. (1998). In vitro cell-free conversion of noninfectious Moloney retrovirus particles to an infectious form by the addition of the vesicular stomatitis virus surrogate envelope G protein. J. Virol. 72, 6356–6361.

40. Kopecky, S.A., and Lyles, D.S. (2003). The cell-rounding activity of the vesicular stomatitis virus matrix protein is due to the induction of cell death. J. Virol. 77, 5524–5528.

41. Hoffmann, M., Wu, Y.J., Gerber, M., Berger-Rentsch, M., Heinrich, B., Schwenmme, M., and Zimmer, G. (2010). Fusion-active glycoprotein G mediates the cytotoxicity of vesicular stomatitis virus M mutants lacking host shut-off activity. J. Gen. Virol. 91, 2782–2793.

42. Humbert, O., Gisch, D.W., Kohlbahf, M.E., Adams, A.B., Greenberg, P.D., Schmitt, T.M., Trobridge, G.D., and Kiem, H.P. (2016). Development of Third-generation Cocal Envelope Producer Cell Lines for Robust Lentiviral Gene Transfer into Hematopoietic Stem Cells and T-cells. Mol. Ther. 24, 1237–1246.

43. Roberts, J.D., Bebenek, K., and Kunkel, T.A. (1988). The accuracy of reverse transcriptase from HIV-1. Science 242, 1171–1173.

44. Pichlmair, A., Diebold, S.S., Gschmeissner, S., Takeuchi, Y., Ikeda, Y., Collins, M.K., and Reis e Sousa, C. (2007). Tubulo-endosomal structures within vesicular stomatitis virus G protein-pseudotyped lentiviral vector preparations carry DNA and stimulate antiviral responses via Toll-like receptor 9. J. Virol. 81, 539–547.

45. Zhang, N., Huang, H., Tan, B., Wei, Y., Xiong, Q., Yan, Y., Hou, L., Wu, N., Siwko, S., Gimarelli, A., et al. (2017). Leucine-rich repeat-containing G protein-coupled receptor 4 facilitates vesicular stomatitis virus infection by binding vesicular stomatitis virus glycoprotein. J. Biol. Chem. 292, 16527–16538.

46. Jonkers, R.P. (1952). The natural history of vesicular stomatitis. Bacteriol. Rev. 16, 179–204.

47. Letchworth, G.J., Rodriguez, L.L., and Del barrera, J. (1999). Vesicular stomatitis. Vet. J. 157, 239–260.

48. Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol. Biol. Evol. 33, 1870–1874.

49. Jones, D.T., Taylor, W.R., and Thornton, J.M. (1992). The rapid generation of mutation data matrices from protein sequences. Comput. Appl. Biosci. 8, 275–282.