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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 (VSVind.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).[1] 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.[2,3] 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[4] and purified using chromatography processes.[5]

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,[4] LV producer cell lines with inducible VSVind.G expression have been constructed.[6,7] 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.[8–11] Although a producer cell clone for clinical use has been developed, this has not progressed to a clinical trial.[12]

The RD114 envelope is suited to the transduction of lymphocytes and bone marrow stem cells.[13,14] Also, when cells are transduced *in vitro*, the RD114 LV can be captured by retronectin to purify and concentrate the vectors.[15] However, LV particles with RD114-derived envelopes are less infectious than those with VSVind.G in most cell lines tested.[8] Also, RD114-enveloped LVs tend to shed the envelope during purification, resulting in loss of vector titer.[16] 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,[17] whereas Chandipura virus G (CHAV.G) and PIRYV.G
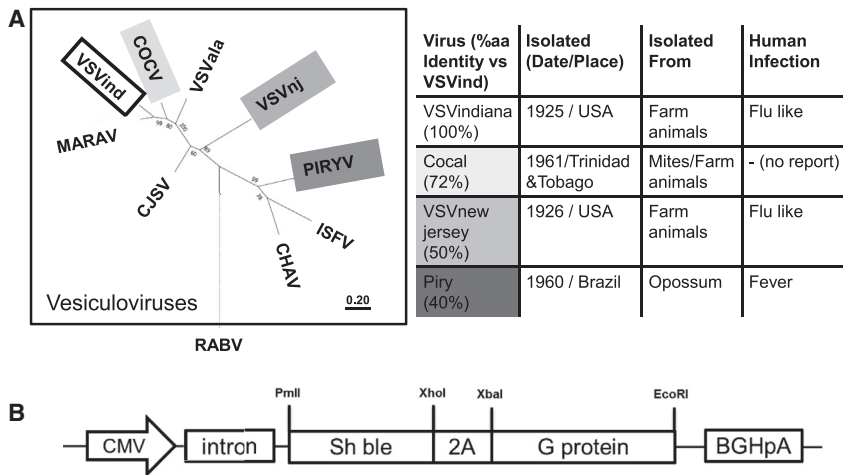
Figure 1. Construction of VesG Envelope Expression Plasmids

(A) G proteins of the major vesiculoviruses as well as the G protein of the rabies virus (RABV) were analyzed with regards to their phylogenetic relationship. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site, depicted in the linear scale. VSVind, vesicular stomatitis virus Indiana strain; COCV, Cocal virus; VSVnj, vesicular stomatitis virus New Jersey strain; PIRYV, Piry virus; CJSV, Carajas virus; ISFV, Isfahan virus; MARAV, Maraba virus; VSVala, vesicular stomatitis virus Alagoas strain. The vesiculoviruses we investigated are highlighted in boxes, and information regarding their isolation and hosts is summarized in the table on the right.[46–48] (B) Vesiculovirus G proteins were subcloned into the pMD2 plasmid backbone in which the marker gene, Sh ble, and the G protein are linked by the FMDV 2A self-cleaving peptide and, therefore, are controlled by a single CMV promoter.

Virus (%aa Identity vs VSVind)	Isolated (Date/Place)	Isolated From	Human Infection
VSVindiana (100%)	1925 / USA	Farm animals	Flu like
Cocal (72%)	1961/Trinidad &Tobago	Mites/Farm animals	- (no report)
VSVnew jersey (50%)	1926 / USA	Farm animals	Flu like
Piry (40%)	1960 / Brazil	Opossum	Fever

pseudotyped LVs are suboptimal in transducing non-adherent cells, including lymphoid and hematopoietic stem cells.[18] Recently, the 3D structure of the interaction between VSVind.G and its major receptor, low-density lipoprotein receptor (LDLR), has been elucidated.[19,20] However, the identified 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.[19] Furthermore, it has been postulated that all VesG go through similar structural and conformational changes during pH-dependent fusion.[21] However, studies of wild-type virus infectivity and pre and post-fusion structures highlighted several differences regarding fusogenicity at acidic pH levels[22] and the nature of pH-sensitive switches.[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

### Investigation of the Fusogenic Cytotoxicity of VesGs

To examine the cytotoxicity of vesiculovirus envelope glycoproteins (G proteins), we constructed vectors with the *Streptoalloteichus hindustanus* phleomycin inhibitor expressed as a fusion protein with the G proteins, separated by a foot-and-mouth disease virus (FMDV) 2A self-cleaving peptide[24,25] (Figure 1). As a control envelope, we used that from the feline endogenous gammaretrovirus RD114 with a modified cytoplasmic tail (RDpro),[8] which can be readily expressed on stable packaging cells.[8,10]

We transfected 293T cells with each vector and then plated different numbers of cells in the presence of phleomycin. Interestingly, we found that each envelope yielded a similar number of phleomycin-resistant colonies, with approximately one colony per 100 transfected cells (Figure 2). This suggested that there is no difference in toxicity between the various VesGs and the RDpro envelope.

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.[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 10^6 transduction units (TUs)/mL obtained with VSVind.G and COCV.G, with approximately 5×10^5 TUs/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.[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
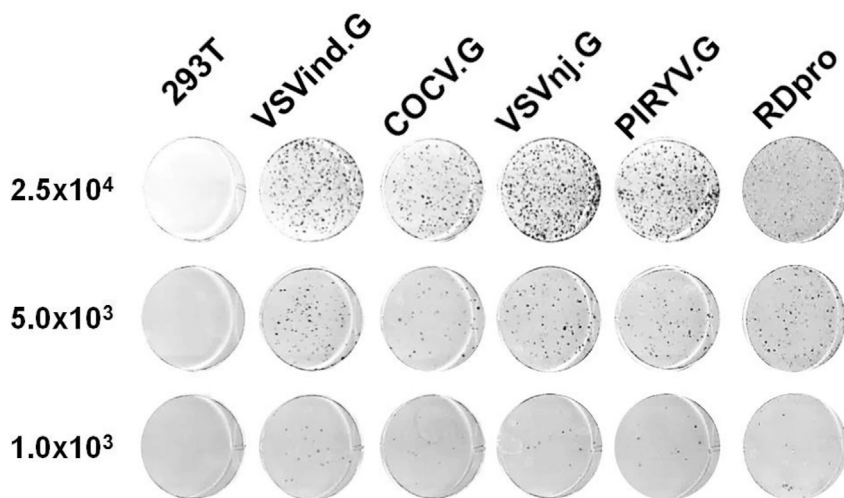
Figure 2. Colony Formation in the Presence of the Selection Marker Reveals No Difference in Toxicity of the G Proteins

To compare the cytotoxicity in various VesGs, HEK293T cells were transfected with pMD2-2A-VSVind.G, pMD2-2A-COCV.G, pMD2-2A-VSVnj.G, pMD2-2A-PIRYV.G, and pRDpro-LF.[8] 48 hr post-transfection, cells were seeded in different dilutions (the seeding density is indicated), selected with phleomycin for 1 week, and then fixed and stained with Giemsa. Similar numbers of colonies were formed for each construct, suggesting that all of these G proteins have similar levels of cytotoxicity. The data shown represent one of the two different repeats performed.

incubation at 4°C, as was the RDpro envelope used as a control (Figure 4B).

COCV.G and PIRYV.G Are More Resistant to Complement-Mediated Inactivation by Mammalian Sera

VSVind.G is known to be inactivated by complement,[27–31] making it less suitable for *in vivo* gene therapy application, where exposure to the bloodstream will occur. When we tested LVs with other vesiculovirus envelopes, we found that VSVnj.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[32,33] (Figure 5A). This differential sensitivity allowed us to map the region of the envelope responsible by constructing chimeras between VSVind.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 VSVind.G following transient transfection).[34] LVs expressing the chimeric G proteins 4A, 1B, and 2B were resistant to human serum inactivation, suggesting that the region of VSVind.G between amino acids 136 and 370 confers complement sensitivity (Figure 5C). On the crystal structure of VSVind.G, this region corresponds to most of the pleckstrin homology domain and parts of the fusion, trimerization, and lateral domains (Figure S1).[35,36]
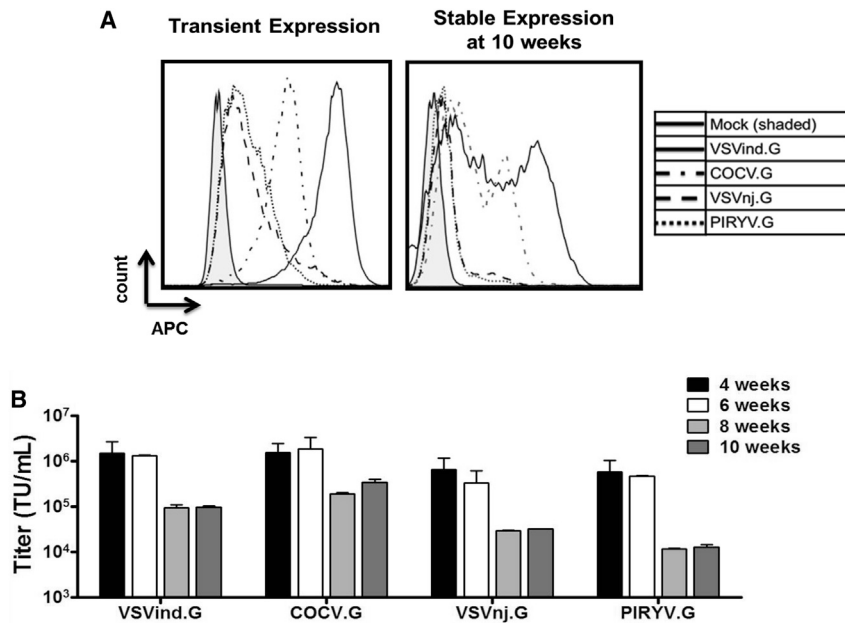
WinPac-COCV.G Packaging Cell Line: Establishment and Identification of Superinfection

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,[10] 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 transfec-

tion of a lentivector construct containing the *gfp* gene; data from 13 of these clones are shown in Figure S2B. Clone H4 was shown to generate LV titers of approximately 10[6] TUs/mL, only 2–3 times lower than that obtained 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 VSVind.G were not blocked for superinfection by VSVind.[37] In contrast, expression of gammaretrovirus (GRV) envelopes blocks superinfection by viruses that use the same receptor.[38] 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 VesG 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

A

Transient Expression   Stable Expression at 10 weeks

count

APC

Mock (shaded)	
VSVind.G	
COCV.G	
VSVnj.G	
PIRYV.G	

B

Titer (TU/mL)

- 4 weeks
- 6 weeks
- 8 weeks
- 10 weeks

VSVind.G   COCV.G   VSVnj.G   PIRYV.G

Figure 3. Development of Stable Envelope-Expressing Cell Lines

(A and B) HEK293T cell lines were transfected with pMD2-2A-VSVind.G, pMD2-2A-COCV.G, pMD2-2A-VSVnj.G, and pMD2-2A-PIRYV.G. VesG-expressing HEK293T cells were cultured under phleomycin selection starting at 48 hr post-transfection. Throughout a 10-week period, these stable envelope-expressing cells were analyzed for their (A) envelope expression and (B) LV production by transient transfection of *gag-pol*, *rev*, and vector expression plasmids. The data shown represent the mean titers ± SD for the titrations performed in triplicate.

some time. To confirm this, early-passage stable WinPac-COCV.G producer cells were cultured in the presence of nevirapine, a nonnucleoside 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.[39] 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 4.0×10^5 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 titers 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.[40] However, an M mutant virus retains some cytotoxicity because of the G protein.[41] The assumption that VSVind.G alone cannot be stably expressed seems to come from brief observations reported by Burns et al. in 1993.[4] Interestingly, Humbert et al.[42] 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,[42] which we did not observe (Figure 3). Similarly to Humbert et al.,[42] we proceeded to develop packaging cells based on COCV.G because it is resistant to complement inactivation (Figure 5A)[17] 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.[42] Here we report the construction of a COCV.G *gfp* LV producer cell clone that could generate 1.3×10^5 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.,[42] may constitute a problem for clinical LV production from COCV.G producer cells. First, the extent of cell expansion we or Humbert et al.[42] 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,[8] the infidelity of HIV reverse transcriptase (approximately 1 substitution per 10,000 bases[43]) 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
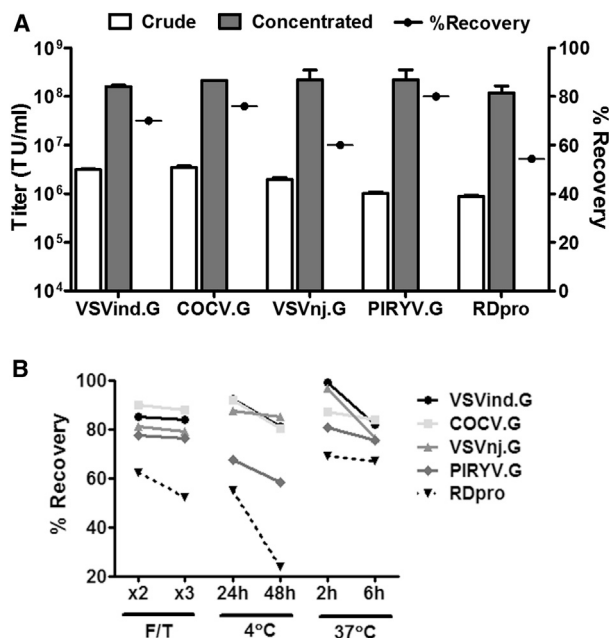
Figure 4. All Envelopes Demonstrated High Physical and Thermal Stability

(A) Vectors were concentrated by high-speed ultracentrifugation. The titers before and after concentration indicate successful concentration of these vectors. For all pseudotypes, the percentage of recovery was higher than 50%. The graph represents three repeats from two separate transient LV productions; error bars indicate SEM. (B) pMD2-2A-VesG LVs were divided into 3 groups: three cycles of freeze-thawing, incubation at 37°C for 2 and 6 hr, and incubation at 4°C for 24 and 48 hr. The functional titers were then determined via flow cytometric analysis, and the percentage of recovery was calculated. Data represent the mean of three repeats performed in duplicates from two separate batches of transient LV productions.

option is mixing unenveloped viral particles with G proteins produced from stably transfected cells. This avoids plasmid transfection, which would reduce cost, increase ease of scale-up and reproducibility, and also remove the risk of plasmid DNA contamination in the LV preparation.[44] However, the production 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[20] or the additional receptor Lgr4.[45] 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.[46–48]

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% CO_2. 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.

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: F8SPF4; 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: Q8JXF6) were included in the analysis. The amino acid sequences were aligned using the ClustalOmega online multiple sequence alignment tool (EMBL-EPI). The evolutionary analyses were conducted in MEGA7.[49] The evolutionary history was inferred by using the maximum likelihood method based on the Jones-Taylor-Thornton matrix-based model.[50] 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.

pMD2-2A-VesG Construction and Generation of Stable Envelope-Expressing Cells

To successfully co-express the different envelopes with the reporter gene, we designed a vector in which the marker gene (Sh ble) and the envelope were linked by a 2A self-cleaving peptide sequence (−LLNFDLLKLAGDVESNPG↓P−) and both controlled by the cytomegalovirus (CMV) promoter. The 2A peptide flanked in the desired restriction sites was ordered from Genewiz (South Plainfield, NJ). This sequence was then sub-cloned into the pMD2 backbone, originated from pMD2.COCV.G (a kind gift from Dr. Hans Peter-Kiem[42]) using the restriction enzymes PmlI and EcoRI to generate the pMD2.2A plasmid. Following this, the phleomycin resistance gene and VSVind.G, COCV.G, VSVnj.G, and PIRYV.G proteins (codon-optimized G protein sequences for VSVnj.G and PIRYV.G were purchased from Genewiz) were introduced by PmlI/XhoI and XbaI/EcoRI sites, respectively. To generate stable envelope-expressing cell lines, 3×10^6 HEK293T cells were transfected with 3 μg of the respective pMD2.2A.VesG plasmid using FuGene6 and put under phleomycin selection 48 hr later. The envelope expression levels of the cell lines, as well as their transient LV titers were monitored weekly.

Colony Formation

3×10^6 HEK293T cells were seeded in 10-cm plates. 24 hr thereafter, they were transfected with VesG expression plasmids and pRDpro-LF.[8] 48 hr post-transfection, cells were counted and seeded in three different dilutions of 2.5×10^4, 5×10^3, and 10^3 cells/well in 6-well plates. After a week of phleomycin selection, the medium was
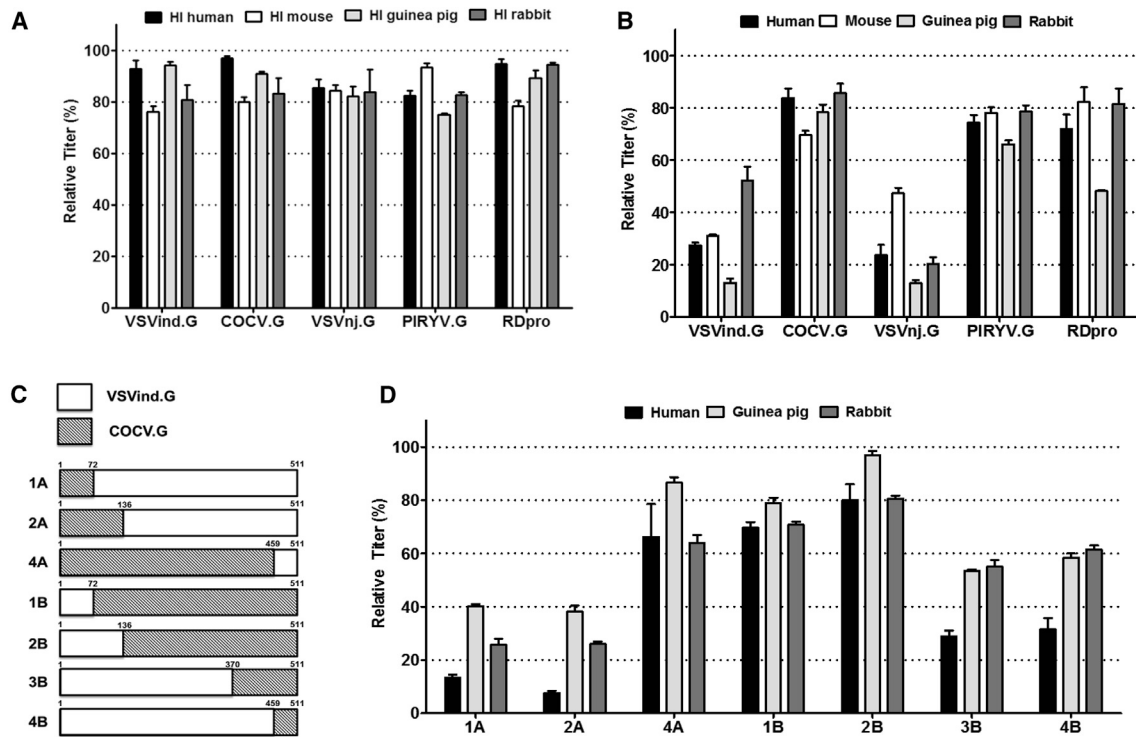
Figure 5. LVs Bearing COCV.G and PIRYV.G Envelope Are Resistant to Inactivation by Complement

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 titers were normalized to those of serum-free samples. (A) The reduction of titers 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 titers ± 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 titers by HI sera were less than 30% for all LVs (data not shown). The data shown represent relative titers ± SD for four experiments performed in duplicate.

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,[26] 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.[3] Briefly, 4×10^6 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[3]), 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, 2×10^5 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
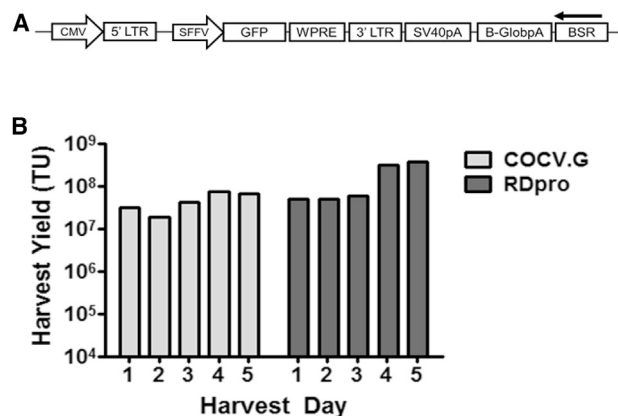
Figure 6. Construction of the WinPac-COCV.G Producer Cell Line
(A) Schematic representation of pSIN-GFP-BSR (promoterless blasticidin vector genome). (B) Total transduction units of COCV.G- and RDpro-enveloped LVs harvested from stable producers in Corning HYPERFlask 2D culture. 560 mL of LV-containing 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 8.2×10^4 and 2.9×10^5 transduction units (TUs)/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. Titers were calculated from virus dilutions, where 1%–20% of the cell population was EGFP-positive, using the following formula:

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 $2.1–2.3 \times 10^5$ 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 (SLS, 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

$$\text{Titer} \left(\frac{\text{transduction units (TU)}}{\text{mL}} \right) = \frac{(\text{no. of cells at transduction}) \times (\% \text{ of GFP} - \text{positive cells} \div 100) \times (\text{dilution factor})}{(\text{the volume of virus preparation added (mL)})}.$$

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 ($87,119 \times g$) 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.

selection and 10 μM nevirapine in the case of WinPac-COCV.G and seeded at a density of 1.16×10^5 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.
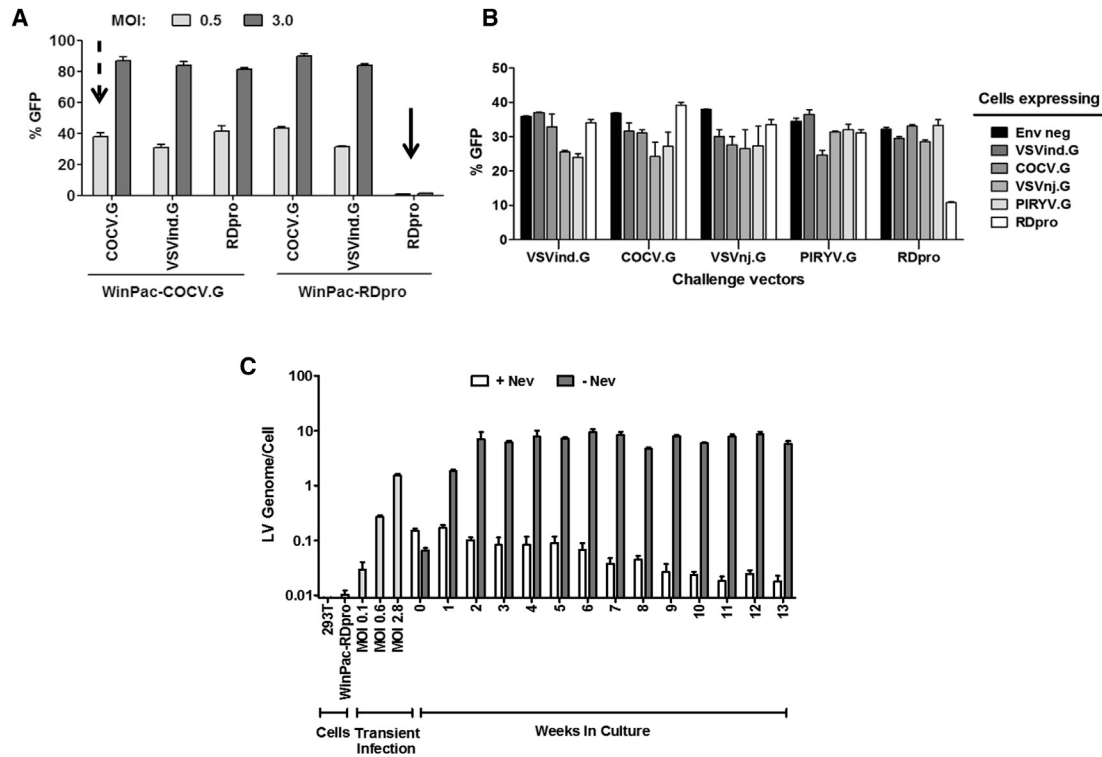
Figure 7. Vector Copy Accumulation in WinPac-COCV.G Vector Producers because of Superinfection

(A) Superinfection is blocked in a WinPac-RDpro cell clone when challenged with RDpro-bearing LVs because of receptor interference (30, solid arrow), whereas there is a lack of superinfection inhibition in WinPac-COCV.G cells (H4, dashed arrow). (B) Bulk populations of HEK293T cells stably expressing VesG and RDpro were challenged with LVs pseudotyped with these G proteins at MOI 0.5. Although RDpro-expressing cells blocked RDpro-LV entry, others were permissive to infection by all LVs. The data presented represent mean ± SEM of two experiments performed in duplicate. (C) qPCR quantification of superinfection in WinPac-COCV.G cultured with and without nevirapine over 13 weeks. Infection was quantified by amplification of the integrated LV genome via qPCR and normalized to HB-actin copy numbers per cell. The data presented represent mean ± SEM of the qPCR assay performed in triplicate. The map of the LV vector with the primer locations, the expected fragments before and after integration, and the overall vector titers measured are shown in Figure S3.

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.
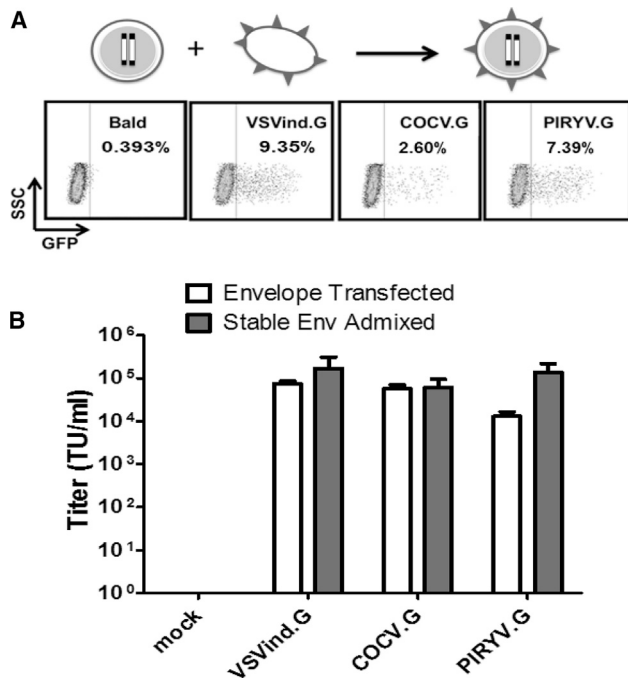
Figure 8. Infectious LV Formation by Admixing Unenveloped LV with Exogenous G

(A) Non-infectious unenveloped LVs encoding GFP were mixed with supernatant collected from cells transiently expressing vesiculovirus G proteins (VSVind, COCV, and PIRYV) at a 1:3 v/v ratio, incubated at 37°C for 1 hr, and plated onto HEK293T cells. Percentages of GFP-positive cells were measured 48 hr thereafter via flow cytometry. Cell supernatant COCV.G and PIRYV.G, albeit slightly less efficiently than VSVind.G, made env-less LV infectious. (B) The same could be achieved using supernatant collected from WinPac-GFP and stable envelope-expressing cells. The titers achieved by admixing were comparable or better than LVs produced via transient transfection of envelope expression plasmids into the bulk WinPac-GFP cell population. The data shown represent mean titers ± SD for three experiments performed in duplicate.

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, I.N., and G.M. helped with designing experiments and interpreting data. M.K.C. and Y.T. supervised the study and wrote the paper.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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