Characterization of a third generation lentiviral vector pseudotyped with Nipah virus envelope proteins for endothelial cell transduction

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INTRODUCTION

Gene therapy holds the appeal of a ‘one-shot’ cure for human disease (see 1 for review). This is particularly true for the treatment of monogenic diseases. As permanent gene correction is often necessary for maximal therapeutic benefit, the use of retroviral vectors has steadily expanded. In contrast with other viral vectors and plasmid DNA, gamma retroviruses efficiently integrate into the cellular genome. However, the ability to integrate into the genome brings with it a significant risk of insertional oncogenesis. Reducing this risk has led to several improvements in retroviral vector design, including self-inactivating long terminal repeats, insulator sequences, and the use of endogenous promoters. Nevertheless, lentiviral vectors are also garnering increasing attention for their use in clinical trials. These vectors have several key advantages over gamma retrovirus vectors, including the ability to transduce nondividing cells and less risk of insertional oncogenesis.

Targeting of lentiviral vectors to specific cell types can be accomplished in a number of ways. Controlling the route of administration may be the simplest means; however, this method is largely limited to ex vivo approaches. Using cell-specific promoters is an effective method for limiting transgene expression, but still does not prevent genome integration effects in off-target cells. In contrast, altering the viral cell attachment protein to limit or target vectors to specific cell types is a highly effective technique. Often referred to as ‘pseudotyping’, attachment proteins from a wide variety of viruses have successfully replaced the gp160 envelope protein in HIV-1 based lentiviral vectors for gene therapy use (see 2 for a comprehensive list of examples). The glycoprotein from the vesicular stomatitis virus (VSV-G) is the most widely used due to its broad cellular tropism and high titer production. The broad tropism of VSV-G serves as a disadvantage in some gene therapy applications where transduction of only a specific cell type is desired. The VSV-G pseudotype may also enhance peripheral dendritic cell activation to lentiviral vectors in vivo. Consequently, alternative lentiviral pseudotypes are continually being developed. Membrane-anchored glycoproteins from the Paramyxoviridae family are excellent candidates to pseudotype lentiviruses. For example, glycoproteins from measles, Sendai, and more recently, tupaia viruses have all pseudotyped lentiviruses successfully. Therefore, it is feasible that glycoproteins from Nipah virus, a Paramyxoviridae family member, may also incorporate into lentiviral particles. Indeed, one laboratory reported it is possible to pseudotype with Nipah virus proteins using 1st generation HIV vectors.

Nipah virus is a deadly pathogen, which is transmitted by bodily fluids from pteropid fruit bats and livestock to humans. The Nipah virus envelope contains two surface glycoproteins required for cellular entry: the attachment protein (NIV-G) and fusion protein (NIV-F). NIV-G allows for attachment of the virion to cellular receptors; whereas NIV-F mediates the direct union of the virus and cellular membranes. Unlike most attachment proteins of the Paramyxoviridae family, Niv-G does not contain hemagglutinin nor neuraminidase activities. The cellular receptors for Nipah virus are ephrin-B217,18 and, at a 10-fold lower affinity, ephrin-B3.19 Ephrin-B2 expression is strongest in the arterial endothelial cells, but is also expressed in the vascular smooth muscle cells and pericytes.20,21 As a result, using Nipah envelope proteins may be an effective solution for targeted gene therapy vectors to the vasculature where permanent gene integration is desirable.
For example, several methods are available to expose the coronary vasculature to gene therapy vectors.\textsuperscript{22} The use of a Nipah-based vector may improve upon low endothelial transduction rates observed with adenoviruses\textsuperscript{23} and the lack of persistence of plasmid vectors.\textsuperscript{24} Ephrin-B2 is also highly expressed in tumor endothelium.\textsuperscript{25,26} In co-ordination with endothelial-specific promoter sequences,\textsuperscript{27} tumor vasculature could be targeted in a highly specific way with a Nipah-based vector. Additionally, Nipah virus is exceedingly rare, so existing humoral immunity is highly unlikely to be present in humans and laboratory animals to interfere with gene delivery.

In the present study, we describe the production and optimization of a third generation, HIV-based, lentiviral vector pseudotyped with Nipah virus envelope glycoproteins for potential use in gene therapy applications. Critical to our success was truncation of NiV-G. Combined with previous findings on the importance of NiV-F truncation,\textsuperscript{14} high titers can be obtained.

RESULTS

A number of other previously published studies where truncation of envelope protein cytoplasmic domains was used as a strategy to successfully pseudotype both murine retroviruses\textsuperscript{28,29} and paramyxoviridae family member, measles virus, observed that a truncation leaving 10\textsuperscript{19} or 16\textsuperscript{11} amino acids in the cytoplasmic domain of the attachment glycoprotein H led to optimal titers. Therefore, we designed a series of five NiV-G cytoplasmic domain truncation mutants with between 10 and 14 amino acids expressed in HEK293T producer cells, co-transfection was performed with wild-type NiV-G, all NiV-F proteins were expressed at similar levels (Figure 2a). As would be expected, removal of the endocytosis motif reduced the levels of mature F\textsubscript{1} protein in FA25 (Figure 2b).

Next, we examined the ability of the modified Nipah proteins to create functional lentiviral particles. Combinations of each NiV-G and NiV-F mutant were co-transfected with a standard third generation lentiviral packaging system, which expresses enhanced green fluorescent protein (EGFP).\textsuperscript{2} Lentivirus packaged with VSV-G envelope (VpL) was used as a positive control and a basis of comparison. Filtered supernatants were incubated with HEK293 cells, which are commonly used to titer Vpl but also endogenously express the Nipah receptor ephrin-B2.\textsuperscript{18} Figure 3 shows the combined results of two independent experiments. The NiV-G truncations G\textsubscript{33} and G\textsubscript{34} appeared to be the most consistent at producing relatively high titers, and wild-type NiV-G was not able to produce a measureable titer with any NiV-F combination (Figure 3). The NiV-F truncations, in contrast, had only a modest effect on titer with FA25 having the best performance (Figure 3).

![Figure 1. Nipah G cytoplasmic domain truncation mutants and their expression in HEK293T cells.](image)

**Figure 1.** Nipah G cytoplasmic domain truncation mutants and their expression in HEK293T cells. (a) Schematic of the Nipah G mutants compared with the wild-type sequence. Only the cytoplasmic domain is shown. (b) Equal amounts of plasmid expressing the Nipah G proteins from (a) with wild-type Nipah G were transfected into HEK293T cells (lentiviral plasmids were excluded). Untransfected cells were loaded in the lane marked ‘con’. A representative western blot is shown (n = 2). Densitometry readings are displayed in parentheses and correspond to the Nipah G signal divided by the tubulin signal. Densitometry readings were normalized to the wild-type value.

![Figure 2.](image)

**Figure 2.** Nipah F cytoplasmic domain truncation mutants and their expression in HEK293T cells. (a) Schematic of the Nipah F mutants compared with the wild-type sequence. Only the cytoplasmic domain is shown. (b) Equal amounts of plasmid expressing the Nipah F proteins from (a) with wild-type Nipah G were transfected into HEK293T cells (lentiviral plasmids were excluded). A representative western blot is shown (n = 2). Densitometry readings are displayed in parentheses and correspond to the Nipah F signal divided by the tubulin signal (the upper densitometry value corresponds to NiV-F\textsubscript{0} and the lower to NiV-F\textsubscript{1}). Densitometry readings were normalized to the wild-type values.
Figure 3. Titers of lentiviral productions using different combinations of Nipah envelope proteins compared with the vesicular stomatitis virus (VSV-G) envelope. Lentivirus was produced by transient transfection as described in the methods section. Titers were measured by exposing HEK293 cells to viral supernatants for 16 h and represented as transducing units per ml (TU ml⁻¹). The displayed results are the average of two independent experiments. Gray bar = VSV-G. Black bars = Nipah envelopes. # denotes below assay detection limit.

Figure 4. Titers of NpL and VpL after preincubation with soluble ephrin-B2 receptor. Aliquots of unconcentrated virus were incubated with 1 μg ml⁻¹ human ephrin-B2/Fc chimera or buffer control for 30 min before titering on HEK293 cells. The percent remaining titer was calculated by dividing the ephrin-B2/Fc chimera treated values by buffer control values. A representative experiment is shown (n = 2). * denotes significant difference compared to identically treated VpL (P < 0.05 by two-tailed t-test).

Figure 5. Analysis of cell fusion abilities of wild-type Nipah F and FΔ25 with Nipah G proteins. HEK293T cells were transfected with equal amounts of Nipah F and Nipah G plasmids (lentiviral plasmids were excluded). Plasmids expressing VSV-G and EGFP served as controls. (a) The average percent area of cytopathic effect (CPE) from cell fusion per 100x field is defined as the ‘fusion index’. * denotes significant difference compared with GFP only cells (P ≤ 0.05 by two-tailed t-test). # denotes significant difference compared with F(wt)/G(wt) cells (P ≤ 0.001 by two-tailed t-test). (b) Representative images of fusion experiment. White arrows label areas of CPEs/cell fusion. Note the lack of CPEs/cell fusion in the ‘GFP only’ image. The remaining experimental groups not shown appear similar to FΔ25/GΔ33.
3'-azido-3'-deoxythymidine in the titer assay prevented ~92% of the GFP-positive reading in both VpL and FΔ25/GΔ33 transduced HEK293 cells (data not shown). Titer assays of VpL and FΔ25/GΔ33 were also performed after preincubation with soluble human ephrin-B2 to confirm specificity to ephrin-B2. The data in Figure 4 demonstrates that the transduction of permissive cells by FΔ25/GΔ33 is dependent on binding ephrin-B2. Furthermore, Chinese hamster ovary cells, which are not permissive to Nipah virus infection, were not transduced by FΔ25/GΔ33 (data not shown). Our results indicate that several combinations, particularly FΔ25/GΔ33, can successfully pseudotype lentiviral vectors at titers within two to three fold of VpL, and display the predicted specificity to ephrin-B2.

It was interesting how NiV-G truncation had a more profound overall effect on titer when compared with NiV-F truncation alone (Figure 3). As a result, we further investigated the mechanism behind the titer differences. It is known that co-transfection of wild-type NiV-G and NiV-F in ephrin-B2-positive cells leads to cell fusion.34,36 Therefore, extensive producer cell fusion may be a limiting factor when producing lentivirus. To explore this possibility, HEK293T cells were transfected with combinations of plasmids expressing wild-type or truncated NiPah G with FΔ25 or wild-type NiV-F. As shown in Figure 5a, coexpression of wild-type NiV-G with either FΔ25 or wild-type NiV-F led to extensive cytopathic effect (CPE) due to cell fusion. Likely because of the reduction of mature F1 protein produced by FΔ25/GΔ33 dependent on binding ephrin-B2. Furthermore, Chinese hamster ovary cells, which are not permissive to Nipah virus infection, were not transduced by FΔ25/GΔ33 (data not shown). Our results indicate that several combinations, particularly FΔ25/GΔ33, can successfully pseudotype lentiviral vectors at titers within two to three fold of VpL, and display the predicted specificity to ephrin-B2.

As in vivo studies require large quantities of a relatively stable lentivirus, we investigated ways to maximize titer and tested the serum stability of FΔ25/GΔ33. For clarity, from this point on we will refer to FΔ25/GΔ33 as Nipah pseudotyped lentivirus (NpL). The measles lentiviral pseudotype required adjustment of the ratio of envelope plasmids to maximize titer.1 A modest 1.5-fold gain in titer was obtained by lowering the amount of FΔ25 plasmid; however, no other alterations to the envelope plasmid ratios had any significant effect (data not shown). The final optimized plasmid ratios are reported in the 'Materials and Methods' section. We also examined the ability of NpL to be concentrated by low-speed centrifugation and ultracentrifugation. NpL could be concentrated by at least 72-fold, but appeared sensitive to the ultracentrifugation method (Table 1). To compare the serum stabilities of NpL and VpL, we incubated unconcentrated viral supernatant with an equal amount of mouse or human serum for 1h and then compared the titer to supernatant incubated with D10 media (contains 10% FBS). Both pseudotypes are inactivated by mouse serum to a similar extent (Figure 8). However, NpL is less sensitive to human serum inactivation (Figure 8). Taken together, NpL can be effectively concentrated to high titer and may have a stability advantage compared with VpL in gene therapy applications, where exposure to human serum is necessary.

We next compared the transduction ability of NpL with VpL using primary endothelial cells in vitro. Endothelial cells have been shown to be highly permissive to Nipah virus infection in vitro.37,38 Human umbilical vein (HUVECs), human dermal microvascular (HDMECs) and mouse lung microvascular (MLMECs) endothelial cells were transduced over a range of concentrations. Table 2 shows a representative experiment where NpL is compared with VpL in transduction ability. HUVECs, particularly at 25 and 100 multiplicity of infection, are highly transduced by both lentiviruses. However, NpL only modestly transduced HDMECs and MLMECs. We also observed that GFP+ cells of the HDMECs and MLMECs had a drastically reduced median fluorescent intensity.
when transduced with NpL (Table 2). These transduction results are likely the result of HUVECs being a relatively homogenous population of cells expressing ephrin-B2; whereas microvascular endothelial cells comprises of both arterial and venous sources known to be either positive or negative for ephrin-B2, respectively.  

To test this possibility, we performed a western blot analysis of ephrin-B2 expression in human microvascular endothelial cell; HDMECs, mouse lung microvascular endothelial cell; MOI, multiplicity of infection; NpL, Nipah pseudotyped lentivirus; VpL, VSV-G envelope. Endothelial cells were exposed to various concentrations lentivirus for 16 h in the presence of 4 μg ml⁻¹ polybrene. GFP-positive cells were determined by flow cytometry 3 days after virus incubation. The value in parenthesis corresponds to the median fluorescence intensity channel of the GFP-positive cells (min – 1, max = 9910). Untreated cells had median fluorescence intensity channels between 2–4. *Denotes significant difference compared to VpL for identical MOI and cell type (P<0.001 by two-tailed t-test).

**DISCUSSION**

We demonstrated that NpL can be produced at high titer in a third generation lentivirus system, concentrated and has improved stability in human serum when compared with VpL. NpL was also shown to transduce primary endothelial cells but not significantly transduce CD34+ hematopoietic progenitors. Therefore, NpL may be a useful vector for in vivo gene transfer to the vascular system.

As shortened cytoplasmic tails (10–16 amino acids) of the attachment proteins enabled high lentiviral titers of other *Paramyxoviridae* pseudotypes, it is not unexpected that this strategy was successful with the Nipah virus pseudotype. Several factors were examined to determine the mechanism of using truncated Nipah attachment protein (NIV-G) in a lentiviral pseudotype. The relative total cellular expression levels of the NIV-G mutants (Figure 1b) did not appear to influence titers (Figure 3). Likewise, relative cell surface presence of truncated NIV-G did not affect titers (Figure 6). Instead, the key determinant was efficient packaging into lentiviral particles (Figure 7). These findings are consistent with previous work, which demonstrated lentiviral particles primarily assemble in endosomal compartments with some pseudotypes. It has been demonstrated that NIV-F and NIV-G do not affect the cell surface expression of each other. As only wild-type NIV-G was capable of mediating extensive CPE (Figure 5a), truncated NIV-G proteins are likely confined to plasma

### Table 1. Concentration of NpL and VpL using two different methods

| Virus   | Unconcentrated | Low-speed concentration | Ultracentrifuge concentration |
|---------|----------------|-------------------------|-------------------------------|
| VpL     | 3.6            | 220 (61)                | 320 (89)                      |
| NpL     | 1.1            | 77 (72)                 | 41 (38)                       |

Abbreviations: NpL, Nipah pseudotyped lentivirus; VpL, VSV-G envelope. A production of each pseudotype was divided into two fractions that were concentrated using either a low-speed centrifugation (3600 g for 1 h) or an ultracentrifugation (100 000 g for 1 h) method to reduce the volume 100-fold. Aliquots were then titered using HEK293 cells. The values in parenthesis corresponds to the fold-increase from unconcentrated titer. Data are representative of two independent experiments.

| Cells   | Virus   | MOI = 5   | MOI = 25  | MOI = 100 |
|---------|---------|-----------|-----------|-----------|
| HUVEC   | VpL     | 74.0 ± 0.7 (1534) | 98.8 ± 0.1 (6887) | 99.7 ± 0.1 (9910) |
| NpL     | 60.4 ± 0.9 (795)* | 94.5 ± 0.8 (2605)* | 98.7 ± 0.2 (9910)* |
| HDMEC   | VpL     | 86.8 ± 0.1 (522)  | 95.0 ± 1.6 (2238)  | 93.5 ± 1.3 (3023)  |
| NpL     | 5.7 ± 0.6 (30)*   | 38.8 ± 1.7 (404)*   | 62.6 ± 3.5 (594)*   |
| MLMEC   | VpL     | 43.8 ± 1.4 (363)  | 81.4 ± 1.1 (762)   | 91.0 ± 1.4 (2345)  |
| NpL     | 8.9 ± 1.7 (299)*  | 27.9 ± 0.4 (411)*   | 51.2 ± 1.9 (613)*   |

Abbreviations: GFP, green fluorescent protein; HDMEC, human dermal microvascular endothelial cell; HUVEC, Human umbilical vein endothelial cell; MLMEC, mouse lung microvascular endothelial cell; MOI, multiplicity of infection; NpL, Nipah pseudotyped lentivirus; VpL, VSV-G envelope. *Denotes significant difference compared to VpL for identical MOI and cell type (P<0.001 by two-tailed t-test).
membrane regions sequestered from ephrin-B2 on neighboring cells (that is, away from cell–cell junctions). This possibility is highlighted by the fact that GA34 has a strong cell surface presence (Figure 6), causes almost no CPE (Figure 5), and yet is still capable of efficiently binding ephrin-B2 in the context of a lentivirus particle (Figure 3). Taken together, Niv-G truncations, particularly GA33 and GA34, enable more effective incorporation into lentiviral particles.

NIV-F truncation had only a modest effect on titer (Figure 3). This result is in contrast to the measles lentiviral pseudotype, where truncation led to logarithmic increases in titer.\(^{10,11}\) FA25 displayed reduced CPE compared with wild-type NIV-F, but the effect on overall virus release was less profound because the NIV-G truncation alone had a larger effect (Figure 5). Similar to previous work, removing the endocytosis signal in FA25 modestly improved titer—even though less of the mature (F\(_1\)) form was incorporated into lentiviral particles (Figure 7). Potentially, a relatively small amount of NIV-F\(_1\) is an advantage in the context of lentiviral particles. In support of this possibility, both wild-type and FA25 incorporated into lentiviral particles with GA33 equally well (Figure 7): yet FA25 yielded higher titers (Figure 3).

Npl was effective in transducing primary endothelial cells (Table 2). This result is not surprising considering that during infection, Nipah virus antigen is present in endothelial cells and leads to significant vasculature pathology in vivo.\(^{46,47}\) In vivo, ephrin-B2 is typically localized to arterial vessels.\(^{39,40}\) HUVECs also express ephrin-B2 and are permissive to Nipah virus infection.\(^{37,38}\) Npl was able to transduce HUVECs almost as efficiently as Vpl (Table 2). In contrast, the HDMECs and MLMECs were more difficult to transduce with Npl (Table 2); this was likely due to reduced expression of ephrin-B2 (Figure 9). The fact that we observed transduction of mouse endothelial cells (Table 2) was interesting. Mice have been reported to be nonpermissive to Nipah virus infection.\(^{46,47}\) At least in the context of a lentivirus particle, our results show that mouse ephrin-B2 is capable of binding NIV-G and allows for viral entry. Of note, heat-inactivated mouse serum retained most of its neutralizing effects against NpL (Figure 8). This finding suggests that a significant contribution to Nipah virus resistance in mice may be heat-stable, innate immunity serum proteins, such as \(2\)-macroglobulin, that are known to bind viruses.\(^{48,49}\) As with any viral vector, delivery methods will need to be optimized before Npl will be useful in testing gene delivery in mouse models of disease. For example, to avoid dilution effects and promote enhanced contact with target cells, it may necessitate isolating the targeted vasculature with catheter systems similar to those used for cardiac gene therapy.\(^{22}\)

In animal models, Nipah virus envelope proteins have been reported to possess immunogenicity in the context of viral vector\(^{50–52}\) and as recombinant proteins (with adjuvant)\(^{53}\) in vaccine studies. It is unknown whether this immunogenicity would present in humans in the context of a lentivirus vector. For example, lentivirus vectors stimulate mouse dendritic cells in a pseudotype-independent manner;\(^{54}\) in contrast, human dendritic cells appear to only respond to the VSV-G pseudotype.\(^{9}\) Although a strong immune reaction to Npl in humans would complicate gene therapy applications, perhaps it would be functional as a vaccine.

During the preparation of this manuscript, a study published ahead of print reported the use of a second generation lentiviral vector pseudotyped with NIV-G and NIV-F to transduce a specific population of human CD34\(^+\) hematopoietic stem cells.\(^{55}\) In support of our findings, CD34\(^+\) hematopoietic progenitors were not significantly transduced.\(^{55}\) Potential transduction of hematopoietic stem cells will be important to address from a safety standpoint if the Nipah pseudotype is to be used in human gene therapy. For example, can the Nipah pseudotype transduce hematopoietic stem cells when used intravenously? In contrast to our work, the study by Palomares et al.\(^{55}\) did not find NIV-G truncation beneficial to titer. However, only truncation down to an equivalent to our GA32 was tested. Interestingly, when their Nipah pseudotype was administered intravenously to mice, vector genomes were only detected in the lung and spleen.\(^{46}\) Conceivably, cells in the lung positive for the vector genomes could be endothelial as we found Npl to transduce MLMECs (Table 1). However, the specific transduced cell types were not identified by microscopy.

In sum, Npl is a useful tool for targeting the ephrin-B2 receptor. It is worth noting that preferential arterial expression of ephrin-B2 exists down to the microvascular level,\(^{20}\) it is conceivable Npl could be directed to this specific subset of endothelial cells. Npl could also be used to target ephrin-B2 on particular cancer cells,\(^{56,57}\) or to the areas of neovascularization.\(^{26}\) Vpl infects cells using an undefined cellular binding site present on most cells types.\(^{58}\) Therefore, when compared with Vpl, Npl transduces endothelial cells with greater specificity. Further specificity of Npl could be obtained by vastly reducing the affinity to ephrin-B3 by two point mutations in NIV-G.\(^{59}\) In addition to mice, we anticipate Npl could be utilized in several different animals already known to be permissive to Nipah virus infection including pigs, Syrian golden hamsters and primates.\(^{56}\) Further studies are warranted to identify cell types transduced with in vivo administration of Npl and identifying the optimal means of delivery.

### MATERIALS AND METHODS

#### Cell culture

HEK293 cells were obtained from the American Type Culture Collecti on (ATCC) (Manassas, VA, USA) and HEK293T cells from Cell Genesys (San Francisco, CA, USA); both were cultured in D10 media (Dulbecco’s modified eagle medium containing \(1 \times \) Glutamax, 4.5 gm l\(^{-1}\) glucose, \(5000 \text{ U ml}^{-1}\) penicillin, 5000 \(\text{mg ml}^{-1}\) streptomycin (Invitrogen, Carlsbad, CA, USA), containing 10% fetal bovine serum (Catalog no. SI1550, Atlanta Biologicals, Atlanta, GA, USA). HDMECs were acquired from Lonza (Walkersville, MD, USA). HUVECs were isolated as described\(^{20}\) (the Institutional Review Board at the Indiana University School of Medicine approved all protocols, and informed consent was obtained from the parents of newborns). MLMECs were obtained from Cell Biologicals (Chicago, IL, USA). All endothelial cells were cultured on collagen coated flasks/plates in EGM-2 BulletKit media (Lonza) with 10% fetal bovine serum (Atlanta Biologicals). We did not use the fetal bovine serum supplied with the EGM-2 BulletKit. Endothelial cells were used in experiments between passages 4–7.

#### Table 3. A comparison between Npl and Vpl in the transduction of human CD34+ progenitor cells

| Cells | Virus | MOI = 50 | MOI = 100 |
|-------|-------|----------|-----------|
| CD34+ | Vpl   | 63.6 ± 0.6 (25) | 68.9 ± 0.1 (27) |
|       | Npl   | 2.3 ± 0.3 (14)* | 2.8 ± 0.1 (15)* |

Abbreviations: MOI, multiplicity of infection; Npl, Nipah pseudotyped lentivirus; Vpl, VSV-G envelope. Cord blood CD34+ cells were exposed to lentivirus for 16 h and GFP-positive cells determined by flow-cytometry 3 days after virus incubation. The value in parenthesis corresponds to the median fluorescence intensity channel of the GFP-positive cells (min = 1, max = 9910). Untreated cells had a median fluorescence intensity channel of 2. *denotes significant difference compared with Vpl for indetical MOI (\(p \leq 0.001\) by two-tailed t-test).

#### Nipah protein truncations

The plasmid pTM1\(^{43}\) encoding the Nipah fusion protein (F) or glycoprotein (G) were kind gifts from Dr Paul Rota (Center for Disease Control, Atlanta, GA, USA). Truncations were made by using PCR to amplify the Nipah F or G complementary DNAs at the desired lengths. Restriction enzyme cleavage
sequences for PmlI in the 5′ primers and EcoRI (for Nipah F) or Stul (for Nipah G) in the 3′ primers allowed cloning into the lentiviral packaging plasmid pmD2.6 This cloning step also served to remove the complementary DNA sequence for VSVG in pmD2.g. Primer sequences are available upon request.

Production and titer of lentiviruses

We used a third generation lentivirus with components split over several plasmids. A plasmid containing an HIV-based, self-inactivating lentiviral vector, pCSGWG (pCS-CSP-PW-GFP,62) expressing eGFP from a cyto-megalovirus promoter served as the viral backbone. The remaining packaging plasmids included the following: pMDL containing the HIV gag-pol gene; pmD2.g, containing envelope proteins (Nipah G, Nipah F or VSVG); and pRSV-REV, containing the HIV rev gene.63 HEK293T cells were seeded at 6.2 × 10^5 per well (six well plates) or 1.2 × 10^5 cells per T175 flask in D10 media. The following day, the cells were transfected by the calcium phosphate method as previously described.63 The final transfection mixture had a DNA concentration of 25 μg ml^{-1} and the pellet resuspended in one-hundredth of the original volume (120–250 μl). Aliquots of virus were stored at −80 °C. For infectious titer determination, HEK293 cells were plated in six well plates at a concentration of 125 000 cells/well in D10 media. The following day, serial dilutions of lentivirus-containing media with a final concentration of 8 μg ml^{-1} polybrene was added to the cells in a final volume of 1 ml. Each dilution had two corresponding wells as replicates. In some experiments, virus was preincubated with soluble human ephrin-B2/Fc chimera (R&D Systems, Minneapolis, MN, USA) to preserve complement activity: human, Sigma (St Louis, MO, USA). Titers obtained with serum treatments were divided by the calcium phosphate method as previously described.63 The final transfection mixture had two corresponding wells as replicates. In some experiments, virus was preincubated with soluble human ephrin-B2/Fc chimera (R&D Systems, Minneapolis, MN, USA) to preserve complement activity: human, Sigma (St Louis, MO, USA). The authors declare no conflict of interest.

Cell surface biotinylation

HEK293T cells in T25 flasks were transfected with Nipah G plasmids (with or without lentiviral packaging plasmids) using FuGene HD (Promega) according to the manufacturer’s protocol. At 24 h post-transfection, the cells were washed with Hank’s buffered saline and then biotinylated with 0.5 mg/ml EZ-Link Sulfo-NHS-SS-Biotin (Pierce) for 15 min. After quenching with 0.1 μM glycine in Hank’s buffered saline, cells were harvested in radio immunoprecipitation assay buffer and protein concentration determined as described above. Between 125–150 μg of cell protein was incubated with 75 μl of a 1:1 slurry of NeutrAvidin agarose beads (Pierce) for 1 h. Beads were extensively washed and biotinylated proteins eluted by boiling in LDS sample buffer (Invitrogen) containing 75 μg/ml mercaptoethanol. A western blot was performed as described above.

Cell fusion/toxicity assay

HEK293T cells in six well plates were transfected with a 1:1 ratio of NIV-G and F plasmids using FuGene HD (Promega) according to the manufacturer’s protocol. In control wells or wells receiving Nipah G or F alone, pEGFP-C1 (Clontech, Mountain View, CA, USA) was used to replace the missing plasmid in order to equalize the total amount of transfected plasmid. At 24 h post-transfection, the cells were fixed with 4% paraformaldehyde (in PBS) for 1 h and then stained overnight with Giemsa stain at room temperature. Stained cells were washed several times with distilled water and examined under a light microscope. To obtain a semi-quantitative value, two blinded observers examined five, 100x fields per treatment and estimated the percent area of CPEs in each field with the aid of a gridded ocular. These values were averaged together to obtain a ‘fusion index’. Representative digital images were obtained using an Olympus (Center Valley, PA, USA) CKX-41 microscope and DP12 imaging system.

Statistics

Data were analyzed by two-tailed t-test where applicable. P-values under 0.05 were considered significant.

CONFLICT OF INTEREST

The authors declare no conflict of interest.
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