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Evaluation of attenuated VSVs with mutated M or/and G proteins as vaccine vectors

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ABSTRACT

Vesicular stomatitis virus (VSV) is a promising vector for vaccine and oncolysis, but it can also produce acute diseases in cattle, horses, and swine characterized by vesiculation and ulceration of the tongue, oral tissues, feet, and teats. In experimental animals (primates, rats, and mice), VSV has been shown to lead to neurotoxicities, such as hind limb paralysis. The virus matrix protein (M) and glycoprotein (G) are both major pathogenic determinants of wild-type VSV and have been the major targets for the production of attenuated strains. Existing strategies for attenuation included: (1) deletion or M51R substitution in the M protein (VSVΔM51 or VSVM51R, respectively); (2) truncation of the C-terminus of the G protein (GΔ28). Despite these mutations, recombinant VSV with mutated M protein is only moderately attenuated in animals, whereas there are no detailed reports to determine the pathogenicity of recombinant VSV with truncated G protein at high dose. Thus, a novel recombinant VSV (VSVΔM51-GΔ28) as well as other attenuated VSVs (VSVΔM51, VSV-GΔ28) were produced to determine their efficacy as vaccine vectors with low pathogenicity. *In vitro* studies indicated that truncated G protein (GΔ28) could play a more important role than deletion of M51 (ΔM51) for attenuation of recombinant VSV. VSVΔM51-GΔ28 was determined to be the most attenuated virus with low pathogenicity in mice, with VSV-GΔ28 also showing relatively reduced pathogenicity. Further, neutralizing antibodies stimulated by VSV-GΔ28 proved to be significantly higher than in mice treated with VSVΔM51-GΔ28. In conclusion, among different attenuated VSVs with mutated M and/or G proteins, recombinant VSV with only truncated G protein (VSV-GΔ28) demonstrated ideal balance between pathogenesis and stimulating a protective immune response. These properties make VSV-GΔ28 a promising vaccine vector and vaccine candidate for preventing vesicular stomatitis disease.

1. Introduction

Vesicular stomatitis virus (VSV) is a single-strained negative-sense RNA virus that has been widely used as a vector for vaccine development [1,2]. Recombinant VSV can accommodate large and multiple foreign genes in its genome that are expressed at high levels [3] and confer advantages over other RNA viral vectors. Due to its potent capabilities in triggering cellular, humoral, and mucosal immunities in animals, even after a single administration, recombinant VSV has been studied as a vaccine vector not only for preventing vesicular stomatitis disease in livestock [4], but a number of human pathogens including: Influenza virus, Ebola virus, Marburg virus, Human immunodeficiency (HIV) virus, Severe Acute Respiratory Syndrome (SARS) virus, and Hepatitis C virus [5–9]. However, VSV is a notoriously infectious agent that not only produces acute disease, such as vesicular lesions in cattle, swine and horses, but neurotoxicity in experimental animals, including primates and mice [10–12]. Therefore, modifications are needed to improve the safety of VSV before it can be applied clinically as a replication competent vector.

VSV genomic RNA is transcribed into five capped and polyadenylated mRNAs by the viral RNA-dependent RNA polymerase. The mRNAs encode five structural proteins: nucleocapsid protein (N); phosphoprotein (P), which is a cofactor of the RNA-dependent RNA polymerase (L); matrix protein (M); and attachment glycoprotein (G) [12]. M and G proteins are both primary pathogenic determinants of VSV [13,14]. The M protein is a multi-functional protein involved in virus assembly, budding and pathogenesis [15,16], and capable of inhibiting the transport of host mRNAs out of the nucleus significantly inhibiting type I interferon (IFN) signaling [17]. The G protein is responsible for viral binding to the host receptor and entry of VSV into host cells and its cytoplasmic domain is

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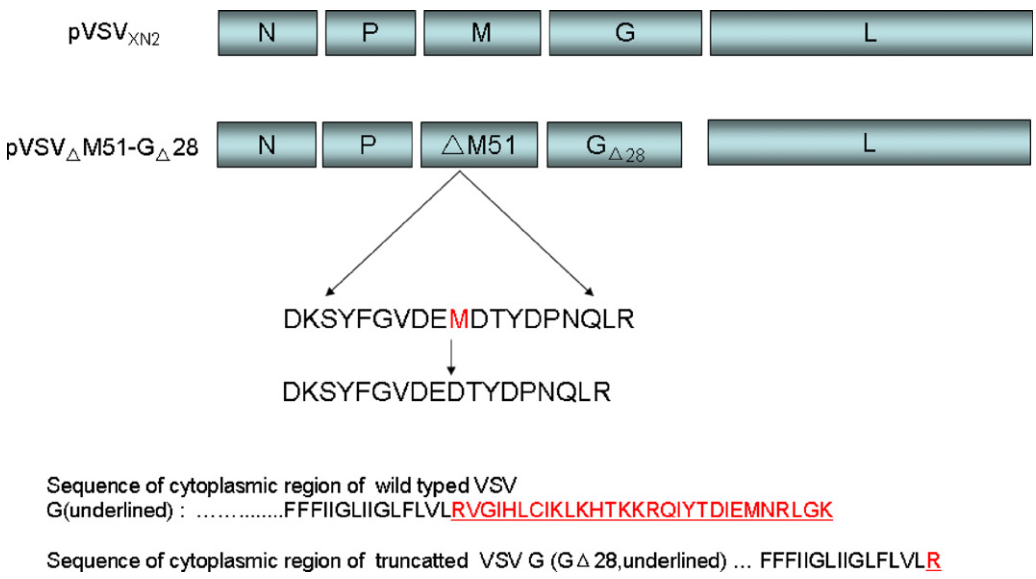

Fig. 1. Genomic structure of pVSV△M51-G△28. Methionine residue 51 in the M protein and 28 amino acids in the C-terminal region of the G protein were deleted. Original wt VSV G with 29 amino acids in the cytoplasmic domain (underlined sequence) containing only one arginine in G△28.

considered to play an important role in viral budding and packaging [18–20].

To date, several strategies have focused on the M or G proteins for the generation of attenuated VSV. It was reported that deletion of methionine residue 51 (VSV△M51) or M51R substitution (VSVM51R) within the M protein can lead to attenuation due to the potent induction of type I IFN [17,21]. Another attenuation strategy is dependent upon truncation within the C-terminal region of the G protein (VSV-G△28) that significantly impacts viral budding and packaging efficiency [22]. *In vivo*, however, VSV M protein mutant proved to be only moderately attenuated in experimental infections [16,21], whereas there is currently no information available if recombinant VSV with truncated G protein is safe or not when animals challenged with high dose of the mutant virus.

The current study developed a novel recombinant VSV (VSV△M51-G△28) with mutations in both M and G proteins, including deletion of methionine 51 in the M protein and a truncation of 28 amino acids in C-terminal region of the G protein. It was hypothesized that VSV△M51-G△28 could combine the advantages observed for the △M51 and G△28 mutations to produce a safer and more effective vaccine vector. Furthermore, for the first time, different attenuated VSVs with mutated M and/or G proteins were comprehensively compared *in vitro* and *in vivo*. Based on pathogenicity and capabilities to stimulate potent immune responses, we aimed to identify a suitable recombinant VSV vaccine vector and vaccine candidate for preventing vesicular stomatitis disease.

2. Materials and methods

2.1. Plasmid construction and rescue of recombinant VSVs

The recombinant VSVs rescued in our study were based on the infectious clone, VSV$_{Indiana}$ [1]. The plasmid pVSV△M51 was kindly provided by Prof. John Bell (Univ. of Ottawa, Canada). The pVSV$_{XN2}$ and helper plasmids, pBS-N, P, L, were provided by Prof. Glen Barber's Lab (Univ. of Miami, USA). The pVSV-G△28 and pVSV△M51-G△28 were constructed based on pVSV$_{XN2}$ and pVSV△M51, respectively. In the constructs, the wild-type VSV G protein cytoplasmic tail (29 aa) was truncated with only one arginine residue remaining in the tail (G△28) (Fig. 1). The forward primer used for polymerase chain reaction (PCR) amplification of G△28 was: 5′-CCGGAGCGCTATGAAGTGCCTTTTGTACTTA-3′ (underlined indicates the MluI restriction site), and the reverse primer was: 5′-CCGGCTCGAGCGTGATATCTGTTAGTTTTTTTCATACCTAGCAGGATTTGAG**TCATTA**TCGGGAGAACCAAGAATAG-3′ (underlined indicates the XhoI restriction site and bold residues indicate the two tandem repeat stop codons). To construct pVSV-G△28 or pVSV△M51-G△28, VSV *G* gene of pVSV$_{XN2}$ or pVSV△M51 was substituted with *G△28* by restriction digestion with MluI and XhoI. All the start/stop signals for viral gene transcription were preserved. A schematic representation of pVSV△M51-G△28 is shown in Fig. 1.

Recovery of recombinant VSVs from the infectious clones was performed as previously described [3]. Briefly, co-transfection of VSV constructs (pVSV△M51, pVSV-G△28, pVSV△M51-G△28) with helper plasmids, pBS-N, P, and L, was performed into BHK21 cells infected with a recombinant vaccinia virus (vTF7-3) expressing T7 RNA polymerase. At 48 h post-transfection, culture supernatants were collected and filtered through a 0.2 μM filter into fresh BHK21 cells. Cells were checked daily. If typical cytopathic effect (CPE) was observed 2–3 days after VSV infection, supernatants were collected and viruses were plaque-purified in Vero cells. Individual plaques were isolated and seed stocks were amplified in BHK21 cells. Recombinant viruses were concentrated by ultracentrifugation at 30,000 rpm/min for 2 h and frozen at −70 °C. Viral titer was determined by plaque assay performed in Vero cells. VSV$_{XN2}$ with wild-type G (wt G) and M proteins was prepared from the stock kept in our laboratory.

2.2. Analysis of proteins synthesis

To identify rescued recombinant VSVs, western blot was used to identify typical bands for L, G, N/P, and M proteins using specific convalescent sera from VSV$_{XN2}$-infected mice. In short, confluent BHK21 cells were infected with VSV△M51, VSV G△28 or VSV△M51-G△28 at a multiplicity of infection (MOI) of 1. VSV$_{XN2}$ was used as a control. At 24 h post-infection (p.i.), cells were lysed in buffer containing 5% β-mercaptoethanol, 0.01% NP-40, and 2% sodium dodecyl sulfate (SDS). Proteins were separated by 12% SDS polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membrane (Thermo Scientific,

Rockford, IL, USA) in a Mini Trans-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA, USA). Sera from a VSV_{XN2} infected mouse was used as the primary antibody at a dilution of 1:2000 with horseradish peroxidase-conjugated goat anti-mouse IgG as the secondary antibody at a dilution of 1:5000 (Santa Cruz, CA, USA). Target bands were observed using West Pico Chemiluminescent Substrate (Thermo Scientific) and exposed to Kodak BioMax MR film (Kodak, Rochester, NY, USA).

2.3. Plaque forming characterization of recombinant VSVs

Attenuation of virus could be characterized by analyzing plaque formation sizes in cells [16]. The A549 cell line was used as a type I IFN signaling competent cell line [23], whereas Vero cells were used as an incompetent cell line [24,25]. Plaque formation experiments were set up in these cells to identify the role by type I IFNs in viral attenuation. Briefly, 90% confluent A549 or Vero cells in 12-well plates were infected with optimally diluted VSVΔM51-GΔ28,VSVΔM51, VSV GΔ28, or VSV_{XN2} and then covered with low melting temperature agar (Invitrogen, Carlsbad, CA, USA) after rinsing with phosphate buffered saline (PBS). At 24 h p.i., 1% crystal violet was used to stain Vero cells, whereas A549 cells were stained 48 h p.i. Plaques were first scanned with Gel Imager (Tanon-1600R, Tanon, Shanghai, China) under bright light and then individual plaques were viewed and photographed under 4× magnification using a microscope (Nikon, Tokyo, Japan) equipped with a digital camera. The mean plaque size was determined by measuring the area of each plaque in each group using Nikon NIS-Elements BR software (Nikon).

2.4. Multi-step growth curves by different VSVs and IFN-β assay

The human prostate cancer cell line, PC3, was used as another cell line with competent type I IFN signaling [17]. At 70% confluency, PC3 cells in 24-well plates were infected with viruses at a low MOI of 0.01. After 1 h of absorption, the inoculum was removed and cells were washed three times with PBS, fresh Dulbecco's modified Eagle's medium (DMEM supplemented with 2% fetal bovine serum) was added, and the infected cells were incubated at 37 °C. Aliquots of cell culture supernatants were removed at 24, 48, and 72 h p.i. Viral titers in supernatants were detected by plaque assays in Vero cells. The harvests at 24 h p.i. were also assayed for IFN-β level using the Human Interferon-β ELISA Kit (R&D Systems, Minneapolis, MN, USA).

2.5. Pathogenesis of mutated VSVs

Specific pathogen free (SPF) female BALB/c mice (∼20 g body weight) were purchased from Shanghai SLAC Experimental Animal Company (Chinese Academy of Sciences, China) and divided into five groups (10 per group) inoculated intranasally with VSVΔM51-GΔ28, VSV-GΔ28, VSVΔM51, VSV_{XN2}, or PBS under light anesthesia with ketamine–xylazine. The inoculated dose of different VSVs was 10^7 plaque-forming units (PFU) in 20 μL of PBS; the highest dose that could be prepared by ultracentrifugation for VSV-GΔ28 or VSVΔM51-GΔ28. Animal body weight losses and survival were monitored every day until 14 days p.i. All animal studies were performed in accordance with a protocol approved by the Shanghai JiaoTong University Experimental Animal Center.

2.6. Recovery of recombinant VSVs in brain

Since wild-type VSV possesses neurotropism that could lead to serious neurotoxicities in animals, recovery of VSV in brain tissues was performed to identify the associations of pathogenesis with viral replications in host. In short, SPF mice were divided into five groups and were challenged intranasally with VSV_{XN2}, VSVΔM51, VSVGΔ28 or VSVΔM51-GΔ28 at a dose of 10^7 PFU, and the control group with PBS. In each group, two mice were sacrificed at 2 and 5 days post-inoculation and their brains were removed for viral detection. Tissues were weighed in 2 mL cell frozen vials, quickly frozen in liquid nitrogen and then kept under −70 °C. For viral assay, tissues were thawed and suspended in 1 mL PBS and then completely homogenized using a Dounce homogenizer. The homogenized tissues were centrifuged at 10,000 rpm for 5 min and the suspensions were serially diluted in PBS. Viral titers were determined by standard plaque assays in Vero cells, as described above. Plaques were observed and counted after 24 h incubation.

2.7. Neutralizing antibody after immunization with mutated VSVs

Immune responses induced by VSVΔM51-GΔ28 or VSV-GΔ28 were evaluated in BALB/c mice (∼20 g each). Female SPF BALB/c mice were divided into three groups and immunized with VSVΔM51-GΔ28, VSV-GΔ28 or PBS as the control. Animals in VSVΔM51-GΔ28 or VSV-GΔ28 treated groups were further divided into two subgroups (five per subgroup) that were housed in isolation hutches and intranasally inoculated once with viruses at doses of 10^3 PFU or 10^4 PFU in 20 μL PBS. Blood was collected from anesthetized mice by retro-orbital bleeds before and 21 days after vaccinations. The vaccinated mice were then challenged with VSV_{XN2}. Blood was allowed to clot at room temperature and sera were collected after centrifugation, and stored at −80 °C for neutralization assays.

Sera from animals were heat inactivated at 56 °C for 30 min. Those from VSV vaccinated animals were serially diluted two-fold in DMEM starting at 1:5. For determination of neutralizing antibody titer, 50 μL of the diluted sera were then mixed with 100 PFU VSV_{XN2} in 50 μL DMEM. The mixtures were incubated for 1 h at 37 °C before being added to 60–70% confluent BHK21 cells. Cells were incubated at 37 °C for at least 2–3 days and checked for CPE. Additionally, 50 μL sera from PBS treated animals were directly combined with 100 PFU/50 μL VSV_{XN2} and treated as for other sera. For titer determination, the reciprocal of the dilution giving a 100% inhibition of CPE was recorded.

2.8. Virus challenge

At 21 days after immunization, all mice were intranasally administered 10^7 PFU VSV_{XN2} in 20 μL of PBS. Animals were observed every day to calculate survival curves and body weight loss, as previously described.

2.9. Statistical analysis

Plaque areas, IFN-β concentrations, and body weight loss among different groups were compared by Student's *t*-test, with a two-tailed distribution using the statistical features in Microsoft Excel (Microsoft, Redmond, WA, USA).

3. Results

3.1. Construction of recombinant VSVs with mutated M or/and G proteins

All viruses rescued in the current study were based on the $VSV_{Indiana}$ infectious clone [1]. To construct safer recombinant VSV, VSVΔM51-GΔ28 was successfully generated that, for the first time, incorporated double mutations in M and G proteins. The novel virus and other attenuated VSV (VSVΔM51, VSV-GΔ28) were identified through western blotting with convalescent serum from mice infected with VSV_{XN2} was used as the wild-type VSV control. Bands

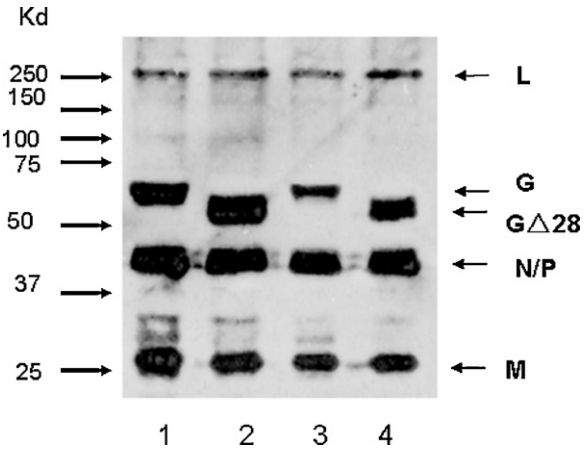

Fig. 2. Identification of recombinant VSV with mutated M and/or G protein by western blotting. Anti-VSV serum was used as the primary antibody, and HRP conjugated goat anti-mouse IgG was used as the secondary antibody. Lane 1: VSV_{XN2}; lane 2: VSV-G△28; lane 3: VSV△M51; lane 4:VSV△M51-G△28. Compared with wtVSV G (lanes 1 and3), G△28 displayed a lower molecular than wild-type G protain due to the truncation of 28 amino acids (lanes 2 and 4).

representing VSV structural proteins were identified (Fig. 2). It was proved that although length of G protein cytoplasmic domain was critical for viral budding and packaging [19], we did find that with only one arginine residue remaining in the G protein cytoplasmic domain, VSV could still be rescued. In comparison with wild-type VSV G protein, a low molecular weight band presumed to be the G△28 was detected (Fig. 2). Viral RNA of the different recombinant VSVs (VSV△M51-G△28, VSV△M51, and VSV-G△28) were extracted and reverse-transcription (RT)-PCR was used to amplify viral *M* or *G* genes. The PCR products were sequenced for mutations occurring in *M* and *G* genes (data not shown).

3.2. Attenuation of VSV

Two methods were used to assess attenuation of VSV△M51-G△28, which included plaque formation size and multi-cycle growth curves in type I IFN signaling competent cells (A549, PC3) [17,23] or incompetent cells (Vero) [24,25]. The results were compared to those produced by other recombinant VSVs. Crystal violet stained plaque sizes were determined for Vero or A549 cells infected with different VSVs (Figs. 3a and 4a). In Vero cells, the average plaque sizes ($n = 10$) between VSVs with wt G (VSV_{XN2} and VSV△M51) were similar ($p > 0.05$), but both were significantly larger than those containing the G△28 mutation (VSV-G△28 or VSV△M51-G△28) ($p < 0.01$; Fig. 3a and b). Average plaque sizes for VSV-G△28 and VSV△M51-G△28 were comparable. These data indicated that, in type I IFN signaling incompetent Vero cells, G△28 but not △M51 was involved in VSV attenuation.

In A549 cells, the average plaque areas produced by VSV_{XN2} were much larger than those by VSV△M51, however, areas by VSV△M51 was also larger than VSV-G△28 ($p < 0.01$; Fig. 4a and b). The smallest and turbid plaques were formed by VSV△M51-G△28 in A549 cells and were difficult to detect. Based on these results, both G△28 and △M51 assist the attenuation of VSVs in type I IFN signaling competent A549 cell and the attenuation tendencies as follows: VSV_{XN2} > VSV△M51 > VSV-G△28 > VSV△M51-G△28.

PC3 was also used in the current study as a type I IFN signaling competent cell line [17]. To quantify the relationship between amount of VSV-induced type I IFN and viral replication titers, multi-cycle growth curves were performed with inoculation of viruses at low MOI of 0.01. As shown in Fig. 5a, titers of VSV△M51-G△28 reached the highest levels at 24 h p.i., but only around 5×10^3 PFU/mL and decline thereafter. VSV△M51 also reached the highest titer 24 h p.i. at around 5×10^6 PFU/mL. Interestingly, VSV with wt G reached maximum titer later compared with those containing mutant M protein. VSV_{XN2} and VSV-G△28 reached the highest titer at 48 h p.i. at $\sim 5 \times 10^8$ and $\sim 5 \times 10^4$ PFU/mL, respectively.

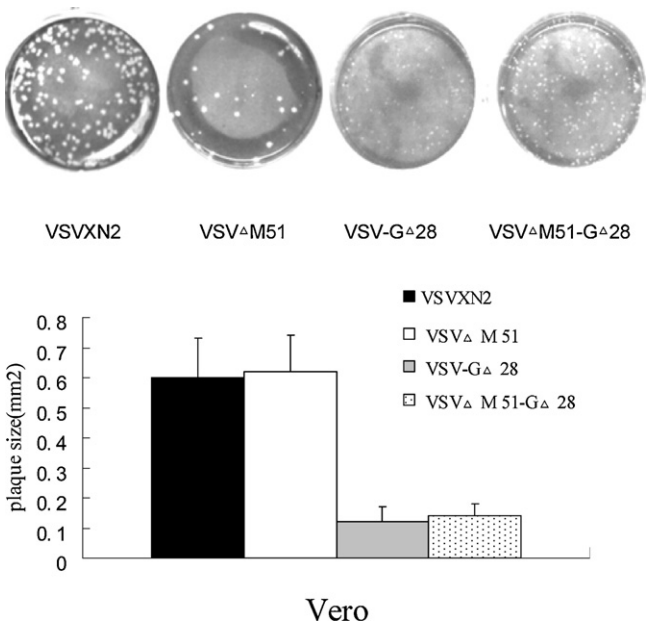

Fig. 3. Plaque size formed by recombinant VSVs in Vero cells. (a) Plaques in Vero cells infected with VSVs; (b) Areas of plaques as means ± SD produced by different VSVs. Ten typical individual plaques were selected randomly to calculate the areas.

IFN-β levels in VSV-treated PC3 cells were quantified at 24 h p.i. to identify the role by type I IFNs. In different recombinant VSV-infected PC3 cells, induction of IFN-β was inversely correlated with viral replication titers. As shown in Fig. 5b, the high levels of IFN β were induced in △M51 VSV-treated cells. In VSV△M51 infected cells, IFN-β concentrations reached ∼2000 pg/mL, but the highest replication titer was ∼2 orders of magnitude lower than for VSV_{XN2}. Although IFN-β production in VSV△M51-G△28

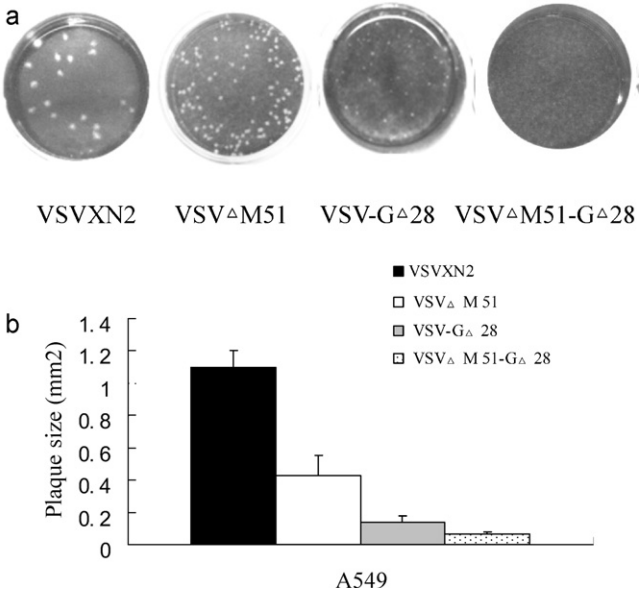

Fig. 4. Plaque size formed by recombinant VSVs in A549 cells. (a) Plaques in Vero cells infected with VSVs; (b) Areas of plaques as means ± SD by different VSVs. Ten typical individual plaques were selected randomly to calculate the areas.

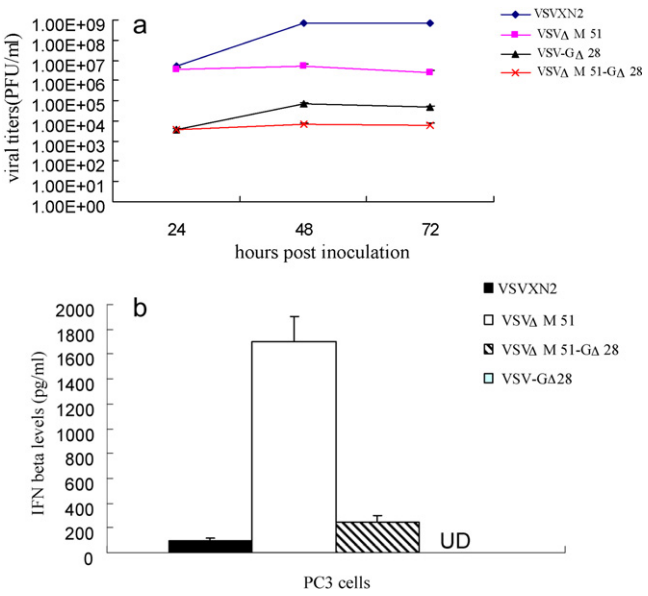

Fig. 5. Multi-step growth curves for recombinant VSVs and induction of IFN-β in PC3 cells. PC3 cells were infected with recombinant VSV at MOI of 0.01. Viruses were adsorbed to cells for 1 h, and the cells were then washed to remove input viruses and returned to DMEM with 2% fetal bovine serum. (a) Supernatant samples were collected at the indicated times post infection, kept on ice, and titrated by plaque assays in Vero cells. Results are shown as means ± SD. (b) Supernatants collected at 24 h p.i. were used to detect IFN-β levels (pg/mL). Results are shown as means ± SD.

treated cells was as low as ~300 pg/mL, this was still significantly higher than VSV_{XN2} ($p < 0.05$). IFN-β detected in the supernatants of VSV-GΔ28 treated cells was below the limit of detection for the assay. Therefore, viral attenuation showed the following trends: VSV_{XN2} > VSVΔM51 > VSV-GΔ28 > VSVΔM51-GΔ28. Importantly, the fact that ΔM51 led to attenuation of VSV through the induction of antiviral IFNs was proved again in our study.

Based on the above data, both GΔ28 and ΔM51 were shown to be involved in the attenuation of VSV, however, GΔ28 could play a more important role than ΔM51. Regardless, the double mutation VSV, VSVΔM51-GΔ28, showed the most significant attenuation *in vitro*.

3.3. VSVΔM51 was not as safe as VSVs containing truncated G proteins in vivo

The current study investigated the pathogenesis of VSVΔM51-GΔ28 in BALB/c mice and compared with other attenuated VSV or VSV_{XN2} as the wild-type virus control. Inoculation of VSV through intranasal has been proven to be the most sensitive way to evaluate pathogenesis by VSV [10,22]. A challenge dose of 10^5–10^6 PFU wild-type VSV has been previously shown to result in significant mortality in BALB/c mice [10]. Therefore, in our studies, SPF BALB/c mice were inoculated intranasally with different recombinant VSVs at a dose of 10^7 PFU/20 μL; the maximum dose that could be prepare for concentrated VSV-GΔ28 or VSVΔM51-GΔ28 in a 20 μL volume. Body weight and survival curve were used as indications of pathogenesis and monitored every day for 14 days p.i.

As shown in Fig. 6a, the body weight of naïve mice treated with VSVΔM51-GΔ28 or VSV-GΔ28 increased steadily post infection without death, similar to the PBS group. However, in the VSV_{XN2} treated group, mice lost significant body weight (~30%) and neurotoxicities were evident in some animals (Fig. 6a and c) with all animals dying at around 6 days p.i. (Fig. 6b). VSVΔM51 showed moderate attenuation compared with VSV_{XN2}, but infected mice still showed significant loss of body weight with 40% mortality.

Wild-type VSV possesses neurotropism in many animals, including primates, rats, and mice that can lead to serious neurological deficits, such as hind limb paralysis. To identify the association of pathogenesis with viral replication, naïve mice were inoculated intranasally with different VSVs at a dose of 10^7 PFU. Viruses isolated from the brains of animals were screened by plaque assay at 2 and 5 days p.i. As shown in Table 1, VSV_{XN2} treated mice showed viral titers up to 4×10^5 PFU/g at 2 days p.i and 1.6×10^3 PFU/g at 5 days p.i. Although much lower than VSV_{XN2}, viral titers detected in brain tissues from VSVΔM51 infected mice were determined to be 3.5×10^2 PFU/g at 2 days p.i., but no virus was detected at 5 days p.i. In VSVΔM51-GΔ28 and VSV-GΔ28 treated mice, no virus could be detected in brain tissues at the two time points, which could explain why animal body weight kept steadily increased over the duration of the study in these groups. Therefore, this study showed that virulence of different VSVs was closely related to replication levels in the target organ, namely the brain. VSVΔM51 was not as safe as those with truncated G proteins *in vivo*. VSVΔM51-GΔ28 and VSV-GΔ28 were attenuated enough as vaccine vectors.

3.4. VSV-GΔ28 induced a more potent protective immune response than VSVΔM51-GΔ28

Recombinant VSVs with truncated G protein (VSVΔM51-GΔ28, VSV-GΔ28) have been proved to be significantly attenuated both *in vitro* and *in vivo*, whereas the attenuation of the viral vector was often associated with the loss of vector immunogenicity. Therefore, an ideal viral vector should retain both essential characteristics to be considered safe and effective.

To evaluate protective immunity stimulated by VSVΔM51-GΔ28 or VSV-GΔ28 *in vivo*, SPF mice were immunized at different doses and then challenged with a lethal dose of VSV_{XN2}. Blood was taken before and 21 days post immunization for neutralization antibody assays. As shown in Fig. 7, the VSVΔM51-GΔ28 immunized group immunized with 10^4 PFU showed survival of all animals after challenge (Fig. 7a), however, mice still lost body weight up to ~20%. At an immunization dose of 10^3 PFU, 40% of animals died at around 6 days post-challenge and all animals suffered serious body weight loss up to 25% and recovered very slowly (Fig. 7b). In mice immunized with VSV-GΔ28 at doses of 10^3 or 10^4 PFU, the body weights of all animals increased steadily after challenge with no death and significant body weight loss. All animals in PBS control group died at 6 days post-challenge (Fig. 7c). Therefore, these data suggested that VSV-GΔ28 could stimulate more potent protective immunities than VSVΔM51-GΔ28.

To further characterize the protective response, neutralization antibodies developed in the vaccinated animals were assayed. As shown in Table 2, no antibody titer could be detected in animals before vaccination and PBS treated mice. It was determined that the antibody titer generated by immunization with VSV-GΔ28 against the parental, $VSV_{Indiana}$, was significantly higher than those produced by VSVΔM51-GΔ28 immunization. In VSV-GΔ28 groups, antibody titers were 320–640 at a vaccination dose of 10^3 PFU and 640–1280 at 10^4 PFU. However, in the VSVΔM51-GΔ28 group with a dose of 10^3 PFU, antibody titer was only 10–20 and all mice with a titer of 10 died post-challenge (Fig. 7b) indicating that the titer was unable to protect animals. Moreover, even animals vaccinated at 10^4 PFU, antibody titer only reached 40–80. Based on these results, it could be concluded that VSV-GΔ28 could stimulate a more potent protective humoral immune response than VSVΔM51-GΔ28, which might be due to the extreme attenuation of VSVΔM51-GΔ28 *in vivo*. As a result, VSV-GΔ28 may attain an ideal balance between pathogenesis and stimulating a protective immune response and with the potential as a vaccine vector and vaccine candidate for vesicular stomatitis disease.

4. Discussion

Replication-competent viruses with little or no pathogenesis have been important alternatives for developing vaccines or vaccine vectors due to their capabilities in stimulating potent and comprehensive immunity *in vivo*. However, safety and efficacy are equally important criteria. Traditionally, the isolation of spontaneously attenuated virus in host or serial passages of wild-type virus in a non-natural host were important methods to acquire attenuated viruses. With the development of molecular technology, the construction of recombinant viruses based on reverse genetics, have become common for developing novel viral vectors. VSV is a promising vector for both vaccination and oncolysis, but still has not been applied clinically. One of the main reasons lies in its potential pathogenesis in humans and animals. Rearrangement of structural protein genes [26] and mutations in M or G proteins are existing strategies to generate attenuated VSV that are replication competent.

Our study focused on the two pathogenic proteins of VSV, M and G proteins, for the purposes of: (1) making a safer VSV; (2) determining a suitable recombinant VSV as a vaccine vector. The ΔM51 and GΔ28 mutations were based on different mechanisms to generate attenuated VSV. The M protein with ΔM51 impaired the ability of the protein to inhibit the transportation of host mRNA from the nucleus to the cytoplasm resulting in the induction of type I IFNs to protect the neighboring cells from infection. Thus,

Table 1
Recombinant VSVs recovered from brain tissues of mice (PFU/g). Naïve mice were inoculated with recombinant VSVs including VSV_{XN2}, VSVΔM51,VSV-GΔ28,VSVΔM51-GΔ28 or PBS as a control. The brains of two mice were removed at 2 or 5 days post-inoculation. Homogenized brains were assayed for viral titers and the mean values were calculated. ND: not detected.

Inoculum	Viral titers in mice brain (PFU/g)	
	2 d	5 d
VSV-GΔ28	ND	ND
VSVΔM51-GΔ28	ND	ND
VSV_{XN2}	4×10^5	1.6×10^3
VSVΔM51	3.5×10^2	ND
PBS	ND	ND

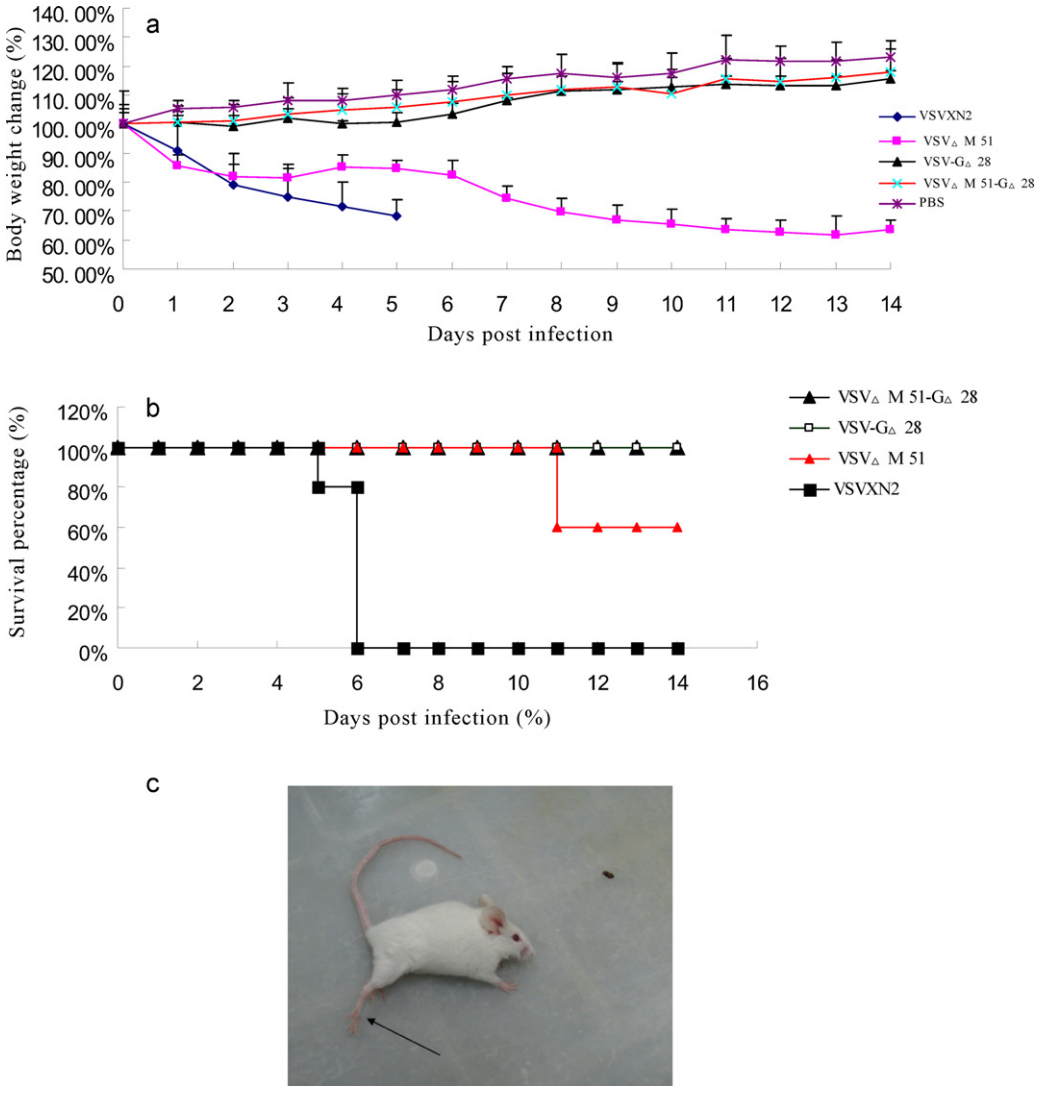

Fig. 6. Evaluation of pathogenesis caused by different recombinant VSVs in BALB/c mice measured by body weight loss and survival curve. Animals were divided into five groups and infected intranasally with 10^7 PFU VSV_{XN2}, VSVΔM51, VSV-GΔ28 or VSVΔM51-GΔ28, with the control group inoculated with PBS. (a) Mice were weighed daily before and after inoculation. The graph represents average body weights changes (%) ±standard error of the mean (SEM) for each group. (b) Survival curves of VSV_{XN2} treated mice indicated suffered serious body weight loss and all died 6 days post-inoculation, mice in VSVΔM51 treated group also lost weight seriously and 40% of animals died, whereas in VSV-GΔ28 or VSVΔM51-GΔ28 treated groups, no pathogenesis such as serious body weight loss or death were observed. (c) Hind limb paralysis of a BALB/c mouse infected with VSV_{XN2} was indicated by the arrow.

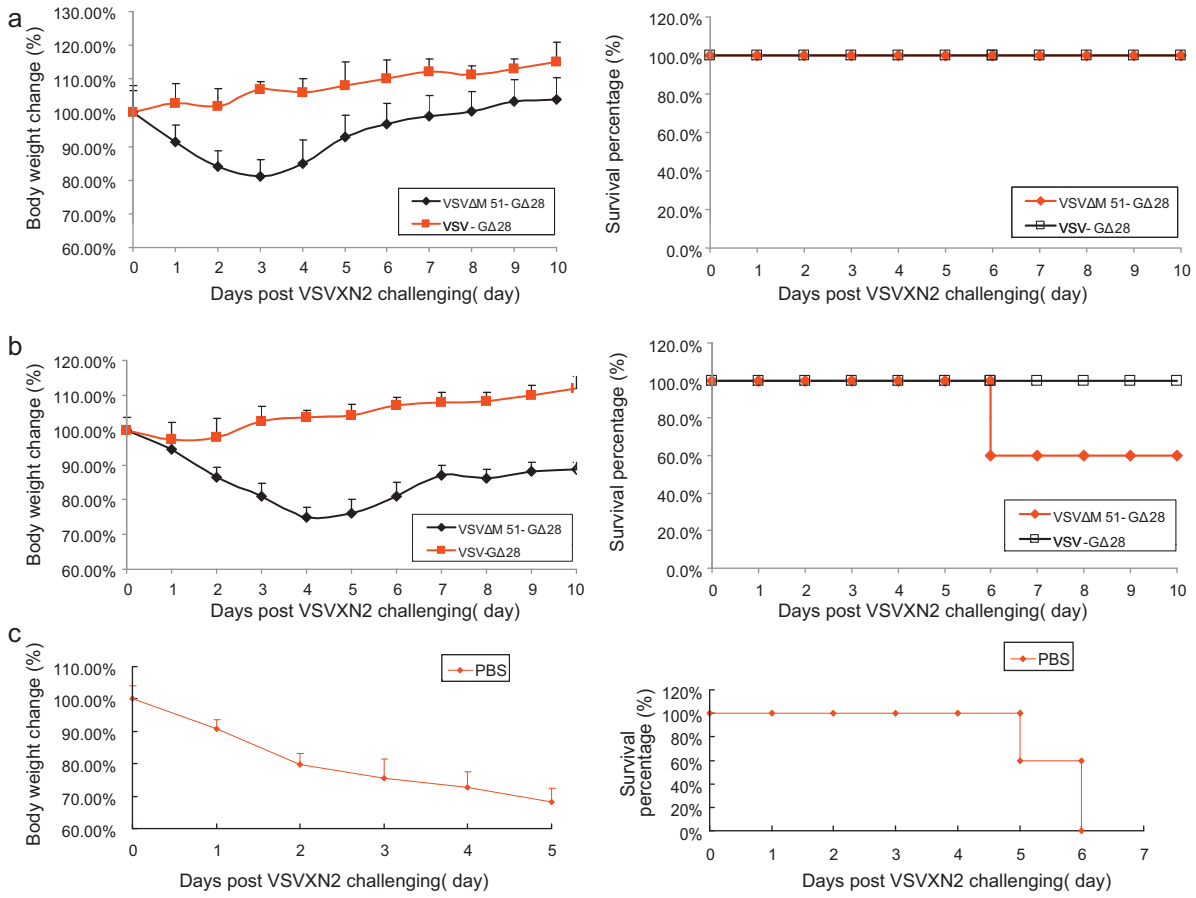

Fig. 7. Body weight loss and survival curves of VSV-GΔ28 or VSVΔM51-GΔ28 immunized mice challenged with VSV_{XN2} at dose of 10^7 PFU. Animals were assigned into three groups and immunized with VSV-GΔ28 or VSVΔM51-GΔ28 at a dose of 10^4 PFU or 10^3 PFU and with PBS as the non-immunized control. (a) 10^4 PFU; (b) 10^3 PFU; (c) PBS. All of the inoculated mice were challenged 21 days after immunization.

VSVΔM51 was attenuated *in vivo*. On the other hand, VSV is an enveloped virus that is released *via* budding from host cell membranes. Previous studies have noted that the length of cytoplasmic domain on VSV G, not the specific sequence, was required for efficient viral budding [19]. The real role of truncated G protein in attenuation of VSV has still not been identified, although it was reported that VSV C-terminal truncated G proteins acquired complex oligosaccharides ~6-fold slower than for wt G protein and displayed a reduced rate of transport from the endoplasmic reticulum to the Golgi apparatus, and presumably to the cell surface [18].

Through *in vitro* assays in type I IFN signaling competent cells/incompetent cells, both ΔM51 and GΔ28 were proven to be important for VSV attenuation. In Vero cells, GΔ28 but not ΔM51, was involved in VSV attenuation. Recombinant VSV with GΔ28 was shown to form significantly smaller plaques than virus with wild-type G, whereas plaque sizes between VSV_{XN2} and VSVΔM51 or those between VSV-GΔ28 and VSVΔM51-GΔ28 were similar, no matter whether M protein was mutated or not. However, in A549 cells with effective type I IFN signaling, attenuation tendencies were demonstrated follow these tendencies: VSV_{XN2} > VSVΔM51 > VSV-GΔ28 > VSVΔM51-GΔ28. This tendency was also proven in PC3 cells through multi-cycle growth curve characterization. The replication titers of different VSVs were shown to be inversely correlated with the expression levels of type I IFNs. VSVΔM51-GΔ28 formed the smallest plaque in A549 cells and lowest titer in PC3 cells among all VSVs tested in the current study due to the double mutations in M and G proteins. This study developed two main conclusions both *in vitro* and *in vivo* studies: (1) Both GΔ28 and ΔM51 mutations assisted attenuation of VSV, and (2) GΔ28 could play a more important role than ΔM51 for attenuation. Therefore, VSVΔM51 was not suitable as a vaccine vector due to its potential pathogenesis in animals and safety is the most important criteria for developing a vaccine or vaccine vector. On the other hand, VSVΔM51 may be suitable for virotherapy, as many studies have attempted [27,28], since, (1) efficacy is a priority as an antitumor drug and (2) many types of tumor cells are type I IFNs signaling defective [29]. Therefore, as an oncolytic virus, VSVΔM51 could replicate selectively and potently in tumor cells, but rarely in normal cells with competent type I IFN signaling.

An ideal replication competent vaccine vector should possess a suitable balance between pathogenesis and capability to stimulate immunity effectively. Both VSV-GΔ28 and VSVΔM51-GΔ28 have demonstrated significant attenuation compared to VSV_{XN2} or VSVΔM51, which are considered safe as vaccine vectors. However, we sought to determine which attenuated virus would work better in stimulating potent protective immunities that are critical for their clinical applications. To evaluate protective immunities stimulated by VSV-GΔ28 and VSVΔM51-GΔ28, animals were immunized with different doses of viruses and then challenged with a lethal dose of VSV_{XN2}, which is the "gold standard" to evaluate efficacy of a VSV vaccine. As shown in the current study, VSV-GΔ28 could stimulate more potent protective immunities than VSVΔM51-GΔ28. Of note, VSV-GΔ28 treated animals inoculated with a low 10^3 PFU dose, no deaths or significant body weight losses were observed post-challenge with VSV_{XN2}. However, animals in VSVΔM51-GΔ28 treated group suffered serious body weight loss even when immunized with 10^4 PFU and some

Table 2
Serum neutralizing antibody titers in mice against $VSV_{Indianna}$ after inoculation with VSV-GΔ28 or VSVΔM51-GΔ28. Animals were inoculated with 10^3 or 10^4 PFU of different VSVs with PBS as a control. Sera were collected before and 21 days after inoculation. Neutralizing antibody titers against $VSV_{Indiana}$ were determined and expressed as the reciprocal of the highest dilution of antibody giving a 100% inhibition of cytopathic effect. '-': not detectable.

Recombinant VSVs	Neutralization titers against VSV_{Ind}			
	Before vaccination		21 d post vaccination	
	10^3 PFU	10^4 PFU	10^3 PFU	10^4 PFU
VSV-GΔ28	-,-,-,-,-	-,-,-,-,-	640, 640, 320, 320, 640	1280, 640, 1280, 640, 640
VSVΔM51-GΔ28	-,-,-,-,-	-,-,-,-,-	10, 10, 20, 20, 20	40, 40, 80, 80, 40
PBS	-,-,-,-,-		-,-,-,-,-	

animals died when the dose was as low as 10^3 PFU. The results correlated with neutralization antibody titers stimulated by different VSVs. Neutralization antibodies titers in animals vaccinated with VSV-GΔ28 were shown to be much higher than those with VSVΔM51-GΔ28. Therefore, although VSVΔM51-GΔ28 contained the double mutated M and G proteins and was a very safe viral vector, it was not as effective as VSV-GΔ28 in stimulating protective immunity, possibly due to its extreme attenuation *in vivo*.

In summary, among different attenuated VSVs with mutated M or/and G proteins, recombinant VSV with only truncated G protein (VSV-GΔ28) indicated ideal balance between pathogenesis and capabilities in stimulating protective immune response and could be a promising vaccine vector. However, for the purpose of developing a vaccine candidate for the prevention of a VSV pandemic, these vaccine candidates would need to be evaluated in swine and cattle, which are the natural host of VSV, before its application in the field.

5. Conclusions

In the current study, a novel recombinant VSV was constructed with mutations in both M (ΔM51) and G (GΔ28) proteins. For the first time, VSV with mutated M and/or G proteins (VSVΔM51, VSV-GΔ28, VSVΔM51-GΔ28) were compared to evaluate their potentials as vaccine vectors. The experimental conclusions included: (1) both GΔ28 and ΔM51 contribute to the attenuation of VSV, however, GΔ28 is likely to play a more important role than ΔM51. VSVΔM51-GΔ28 was determined to show the most significant attenuation *in vitro*. (2) Virulence of recombinant VSVs with truncated G protein (VSV-GΔ28, VSVΔM51-GΔ28) significantly decreased compared with wild-type VSV or VSVΔM51. (3) VSV-GΔ28 could stimulate a more potent protective immune response than VSVΔM51-GΔ28 possibly due to the extreme attenuation of VSVΔM51-GΔ28. Among different attenuated VSVs with mutated M and/or G proteins, recombinant VSV with only truncated G protein (VSV-GΔ28) displayed an ideal balance between pathogenesis and stimulation of a protective immune response that could be used as a promising vaccine vector.

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