Expert Opinion on Biological Therapy

ISSN: (Print) (Online) Journal homepage: https://www.tandfonline.com/loi/iebt20

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To cite this article: Altar M. Munis , Emma M. Bentley & Yasuhiro Takeuchi (2020): A tool with many applications: vesicular stomatitis virus in research and medicine, Expert Opinion on Biological Therapy, DOI: 10.1080/14712598.2020.1787981

To link to this article: https://doi.org/10.1080/14712598.2020.1787981

Accepted author version posted online: 30 Jun 2020.
Published online: 09 Jul 2020.

EXPERT OPINION ON BIOLOGICAL THERAPY
https://doi.org/10.1080/14712598.2020.1787981

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REVIEW

A tool with many applications: vesicular stomatitis virus in research and medicine

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ABSTRACT

Introduction: Vesicular stomatitis virus (VSV) has long been a useful research tool in virology and recently become an essential part of medicinal products. Vesiculovirus research is growing quickly following its adaptation to clinical gene and cell therapy and oncolytic virotherapy.

Areas covered: This article reviews the versatility of VSV as a research tool and biological reagent, its use as a viral and vaccine vector delivering therapeutic and immunogenic transgenes and an oncolytic virus aiding cancer treatment. Challenges such as the immune response against such advanced therapeutic medicinal products and manufacturing constraints are also discussed.

Expert opinion: The field of *in vivo* gene and cell therapy is advancing rapidly with VSV used in many ways. Comparison of VSV's use as a versatile therapeutic reagent unveils further prospects and problems for each application. Overcoming immunological challenges to aid repeated administration of viral vectors and minimizing harmful host–vector interactions remains one of the major challenges. In the future, exploitation of reverse genetic tools may assist the creation of recombinant viral variants that have improved onco-selectivity and more efficient vaccine vector activity. This will add to the preferential features of VSV as an excellent advanced therapy medicinal product (ATMP) platform.

ARTICLE HISTORY
Received 27 March 2020
Accepted 23 June 2020

KEYWORDS
Gene therapy; immune response; lentivirus; oncolytic virotherapy; pseudotype; vaccine; viral vector; VSV

1. Introduction

Vesicular stomatitis virus Indiana strain (hereafter simply VSV as commonly called) came under the spotlight in the past few decades following the advent and success of lentiviral gene therapy in research and the clinic [1]. Utilized almost exclusively, VSV glycoprotein (VSV.G) pseudotyped vectors, have an untapped potential for gene therapy and editing applications. In addition, owing to its versatility, VSV has been utilized ubiquitously in several other fields of research and in the clinic as a potent oncolytic virus, vaccine vector, and a gene delivery tool to study heterologous viral envelope proteins. Several universal challenges remain to be overcome to maximize the potential of such vectors. Improving targeting to enhance therapeutic effect and overcoming adaptive and innate immune responses curtailing treatment efficacy remains one of the biggest hurdles [2]. In this review, we discuss the translational potential of VSV and other members of vesiculoviruses in expressing therapeutic transgenes. We also look at the progress made in targeting cancerous tissue, and effectively promoting immunity against other pathogens with recombinant vesiculoviruses and review the challenges and directions for the future.

2. Vesicular stomatitis virus

VSV is a species of the genus vesiculovirus which belongs to the rhabdovirus family [3]. Vesiculovirus is the first described virus of the 16 rhabdovirus genera, it naturally targets farm animals and causes lesions in the mouth and udders [4]. VSV is considered to be the prototype virus of the genus while other major serotypes include Cocal, Vesicular stomatitis virus New Jersey strain, Chandipura, Maraba, and Piry viruses [5–7].

The single-stranded, negative-sense RNA genome of VSV encodes five structural proteins: nucleoprotein, phosphoprotein, matrix protein, glycoprotein, and the viral polymerase [8,9] (Figure 1(a)). The matrix protein is responsible for the formation of the viral core and anchoring of the glycoprotein to the viral membrane enabling the formation of glycoprotein homotrimers [10]. The glycoprotein dictates receptor recognition, cell entry, and viral fusion; thus, it is the major target for the humoral immune response [11]. The RNA-dependent RNA polymerase activity for viral replication takes place in the target cell cytosol and is driven by the complex containing the nucleoprotein, viral polymerase, and phosphoprotein [12]. The viral genes are expressed in a single non-segmented negative strand RNA in order [13]. As the transcriptional activity of the 3′ promoter is attenuated at each gene junction, 3′ genes of the viral genome are transcribed more abundantly [14] (Figure 1(a)).

2.1. Use of VSV in viral vector pseudotypes

Virus-based vectors have been utilized in research for several decades. Most vectors based on enveloped viruses are pseudotyped viruses, that is, their envelopes are not encoded by their genome (coined/defined by Rubin in

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Article highlights

- *Vesicular stomatitis virus (VSV) has long been a useful research tool and more recently become an essential part of medicinal products.*
- *The understanding of VSV's biology including structural, molecular, and immunological aspects has been progressing in concert with its adaptation to clinical gene and cell therapy, vaccines and oncolytic virotherapy.*
- *The use of VSV.G as a pseudotyping envelope for lentiviral vectors has cultivated gene therapy research and spearheaded lentivector-based gene and cell therapy clinical trials.*
- *Recombinant VSV vectors have been developed into potent vaccines most notably against Ebola in the recent years.*
- *Although there are still some limitations around use of VSV as an oncolytic virus, it is emerging as a potential alternative to oncotherapy in the future.*

This box summarizes key points contained in the article.

1965 [15]), and can be derived from various related viruses. The cell tropism of retroviral vectors including ubiquitously used lentiviral vectors can be modified by the pseudotyping approach. One of the first and currently most widely used viral envelopes for this belongs to VSV owing to its broad tropism, thermal, and physical stability. Lentiviral vectors pseudotyped with VSV.G are currently regarded as the 'gold-standard' in gene therapy applications (Figure 1(b)).

In addition, the VSV core serves in the development of effective pseudotyped vectors. This was made possible through the establishment of VSV reverse genetics enabling the production of recombinant VSV [16]. Pseudotyped vectors, bearing the surface protein of a foreign virus, enable the study of virus-target cell interactions, including viral entry mechanisms and the inhibition of viral entry by different biologicals. In these VSV-based recombinant vectors, the viral genome is edited where VSV.G gene is swapped for a reporter gene allowing for easy and high-throughput analysis of viral infectivity (Figure 1(c)). Similarly, researchers have also utilized the VSV core to generate replication-competent chimeric viruses engineered to express heterologous glycoproteins. In this approach, gene encoding VSV.G is replaced with another viral glycoprotein (Figure 1(d)).

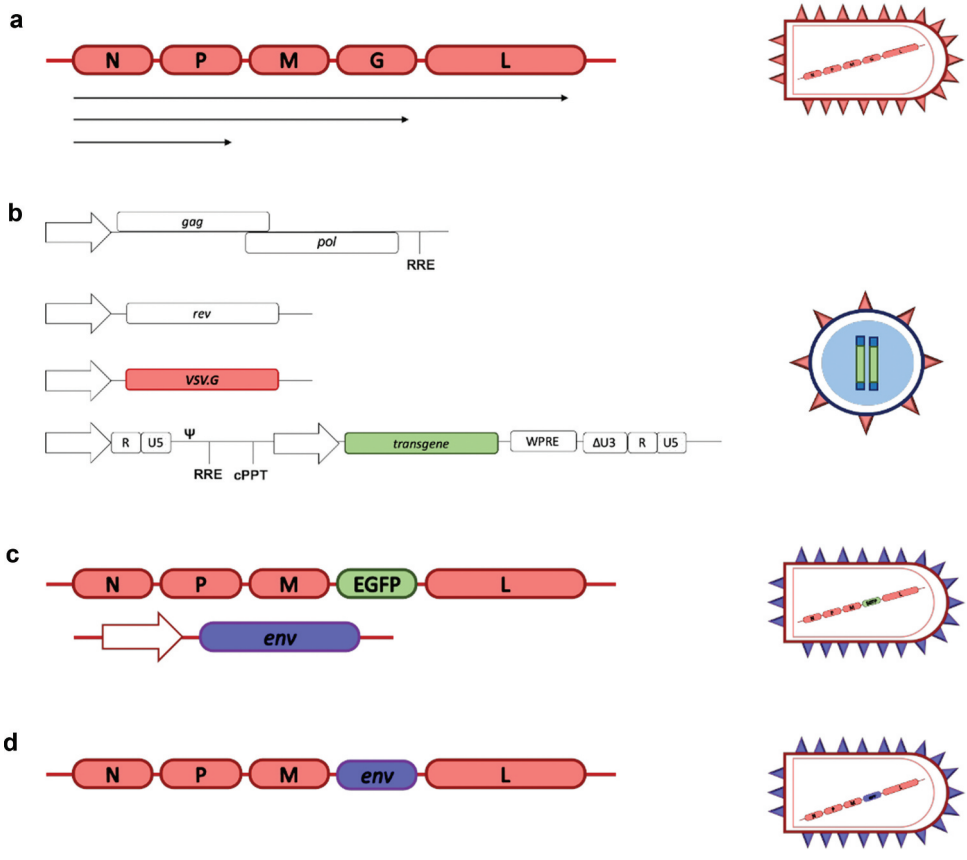

Figure 1. Schematic of wild-type VSV and VSV-based Vectors. (A) VSV genome encodes five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and the viral polymerase (L). This produces the characteristic 'bullet-shaped' virion with VSV.G protruding from its surface (right). Black arrows represent the transcriptional activity of the 3' viral promoter using the negative strand genome RNA as the template. (B) Summary of the four-plasmid approach to the third-generation lentiviral vector system to produce VSV.G pseudotyped vectors with a lentivirus core (right). U3: LTR element derived from sequences unique to 3' end of the RNA genome; R: LTR element derived from sequences repeated in both LTRs; U5: LTR element derived from sequences unique to 5' end of the RNA genome; Ψ: packaging signal; cPPT: central polypurine tract; RRE: rev response element; WPRE: Woodchuck hepatitis virus posttranscriptional regulatory element. Arrows denote locations of the heterologous promoters. (C) Generation of rVSV-ΔG pseudotyped with a heterologous viral envelope. (left) Schematic of the recombinant VSV (rVSV) genome in which the gene encoding VSV.G is replaced with a reporter transgene (e.g. EGFP; enhanced green fluorescent protein). The pseudotyping viral envelope protein is provided in trans via an expression plasmid, prior to infection of cells with rVSV-ΔG. (right) This enables the production of rVSV-ΔG virions encoding EGFP complemented with a heterologous viral envelope. (D) Generation of a replication-competent rVSV pseudotyped with a heterologous viral envelope. In the rVSV genome gene encoding VSV.G is replaced with another viral envelope protein in order to produce a replication-competent non-neurotropic oncolytic virus or vaccine.

2.2. VSV glycoprotein structure

During infection, viral membrane proteins mediate virus-cell and cell-cell fusion [17]. These proteins are generally divided into three different groups based on their structure and fusion mechanisms: class I, II, and III [18]. While vesiculoviruses employ a single glycoprotein on their surface, other viruses, such as human parainfluenza and measles, use multiple surface glycoproteins for viral attachment and fusion [17].

All vesiculovirus glycoproteins, VSV.G being the most studied, belong to the most recently defined group of class III fusion proteins typified by their reversible pH-dependent fusion kinetics [19–21]. They are made up of a combination of α-helices and β-sheets [11]. During infection, both receptor recognition and membrane fusion are controlled by the single glycoprotein. Following endocytosis of the virus, the gradually decreasing pH in early endosome compartments serves as the environmental cue for pH-dependent membrane fusion which is optimal around pH 6 [22,23]. Identified fusion kinetics of VSV.G suggest that the glycoprotein assumes two key conformational states: pre-fusion (native) and post-fusion (inactive) with several monomeric and multimeric intermediates in between each having their own unique biochemical characteristics [11,24,25]. A pH-dependent equilibrium controls the structural changes between these states which shifts toward the post-fusion conformation at low pH [26]. The reversibility of these structural changes, unique to vesiculoviruses (at large to the rhabdovirus family), allows the transportation of the viral glycoprotein, during viral replication, through acidic cellular compartments in its inactive form on to the cell surface where it assumes its active state [27,28].

X-ray crystallography studies have elucidated that VSV.G is made up of four distinct domains, dubbed I, II, III, and IV [23,25] (Figure 2(a-c)). The β-sheet rich domain I is called the lateral domain. Domain II, formed of six α-helices, is responsible for homotrimerization of VSV.G through hydrophobic interactions in both pre- and post-fusion conformations. Domain III which is formed of a combination of α-helices and β-sheets contains the fold of pleckstrin homology (PH) domain. PH domain plays a crucial role in the membrane localization of VSV.G. Domain III, alongside the lateral domain, is the most exposed of the VSV.G protein. Several neutralizing epitopes have been located on these two domains [29,30] (Figure 2(d-e)) and it has been shown that they both are involved in receptor recognition [31] (Figure 2(f)). Domain IV contains the two fusion loops which are extended toward the target membrane during viral fusion.

During structural changes from the pre-fusion to post-fusion conformations, the VSV.G homotrimer dissociates and goes through major structural rearrangements [33]. However, it has been demonstrated that domains I, III, and IV retain their tertiary structures. The changes occur in the relative location and orientation of the domains and hinge regions between domains III and IV followed by

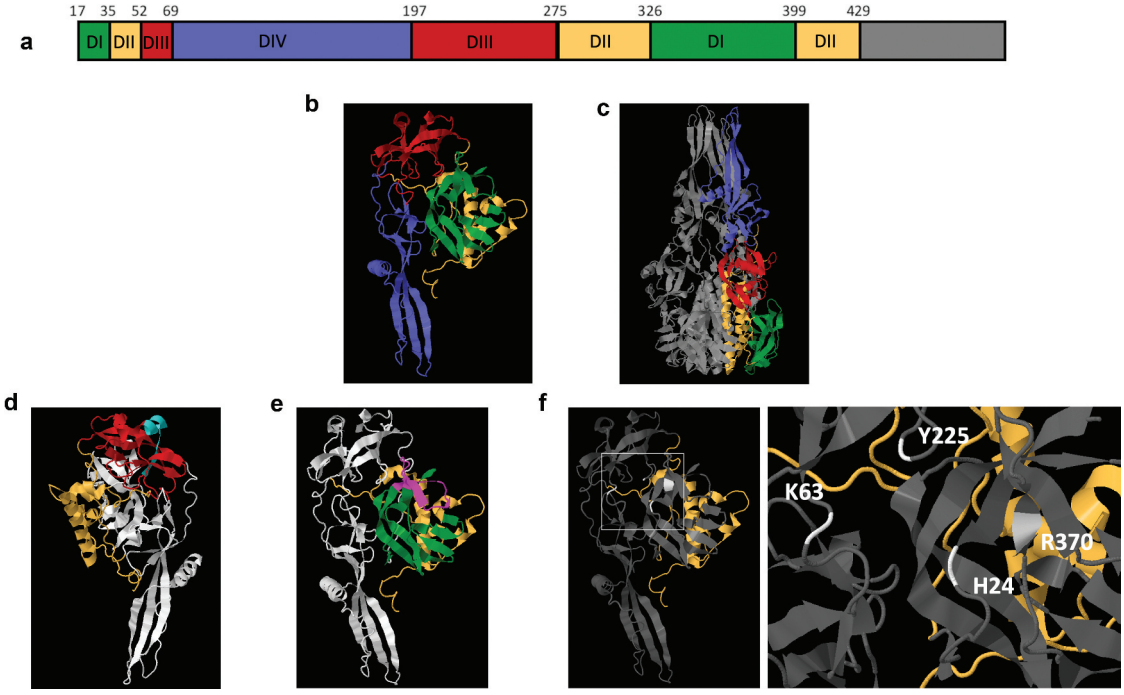

Figure 2. Structure and Domain Organization of VSV Glycoprotein. (a) Linear diagram depicting domain architecture of VSV.G. Domain boundaries are labeled based on the precursor protein numbering including the signal peptide. Green: DI, lateral domain; yellow: DII, trimerization domain; red: DIII, pleckstrin homology domain; blue: DIV, fusion domain; gray: transmembrane and cytoplasmic domains. X-ray crystallography images of mature VSV.G produced by thermolysin-mediated cleavage of virions in (b) pre-fusion (PBD ID 5I2 S) and (c) post-fusion (PBD ID 5I2 M) structures, with VSV.G monomers colored by domains. (d-e) Epitopes recognized by strongly neutralizing anti-VSV.G antibodies 8G5F11 and IE9F9 [32]. Residues that dictate antibody binding are colored cyan and magenta respectively. The trimerization domain and the respective domains where the epitopes are located in are colored for reference. (f) Residues that are involved in receptor binding mapped onto the pre-fusion structure of VSV.G (left panel). H24, K63, Y225, and R370 create a receptor binding pocket which interacts with CR2 and CR3 domains of LDLR (right panel). 3D structures were retrieved from RCSB Protein Data Bank, visualized and colored via JalView software.

refolding of domain II [23]. Therefore, the post-fusion structure of VSV.G resembles a flipped version of the pre-fusion conformation [11,25] (Figure 2(b-c)).

2.3. VSV glycoprotein function

Binding of vesiculovirus glycoproteins to receptors guides the virus-receptor complex to cellular compartments where viral fusion takes place. Low-density lipoprotein receptor (LDLR) and its family members have been identified as the cellular receptors for VSV cell entry [34].

LDLR is a type I transmembrane protein that is involved in the uptake of cholesterol molecules bound to lipoproteins (reviewed in [35]). Following binding to its ligand, LDLR molecules are endocytosed and the low-pH of the endosomal environment leads to the release of the ligand. After ligand release, the receptor molecules are recycled back to the cell surface [36,37]. LDLR is the prototype member of a family of proteins typified by the similarity of their structural domains. These receptors, involved in many cellular functions including lipoprotein transport, intercellular signaling, and protease inhibitor clearance (reviewed in [38]), all are made up of cysteine-rich, epidermal growth factor-like, and YWTD (named after conserved tyrosine, tryptophan, threonine, and aspartate residues) domains arranged in various different patterns. LDLR, specifically, is made up of seven continuous cysteine-rich domains (responsible for ligand recognition and binding), followed by three EGF-like and the YWTD domain [39–41]. It is anchored to the lipid membrane via a highly glycosylated stalk [42].

It has been demonstrated that VSV.G can interact with LDLR and the other family members using the highly conserved cysteine-rich (CR) domains [31,34]. Several basic residues located in VSV.G domain I and III are responsible for interacting with CR2 and CR3 domains on LDLR resulting in a calcium-cage-mediated protein–protein binding. Residues H24 and K63 on VSV.G interact with D69/73/79, E80 on CR2 or D108/112/118, E119 on CR3. These interactions are required for the docking of the calcium ion which coordinates structural changes in LDLR to ensure the binding of the glycoprotein. On the other hand, R370 in VSV.G is responsible for initiating interactions with both CR2 and CR3 domains.

However, the use of LDLR and its family members is not universal to all vesiculoviruses. While the close phylogenetic relatives to VSV, Cocal, and Maraba viruses also interact with LDLR, it has been shown that Piry and Chandipura viruses do not [31,43]. This is thought to be due to the key residues on the glycoprotein which dictate the interaction with CR2 and CR3 domains not being conserved on all vesiculoviruses.

The interaction between VSV.G and LDLR results in rapid endocytosis of the virus. This enables the viral antigens to be present on the cell surface for a reduced time period aiding the virus in immune evasion. In addition, viral fusion from endocytic vesicles limits membrane damage and bolsters viral replication (reviewed in [44]). Different clusters of amino acid residues on VSV.G facilitate the conformational changes from pre-fusion structure to the post-fusion and back [45]. The protonation of three histidine residues is responsible for the destabilization for the pre-fusion structure which is followed by the fusion peptides to move away from the viral membrane and protract toward the target membrane [23,46]. On the other hand, deprotonation of four aspartic acid residues brings them together. The cluster of these acidic residues then acts as the molecular switch for the transition back to the pre-fusion structure [45]. Comparison of amino acid sequences of vesiculovirus G proteins reveals that these amino acid clusters are not conserved amongst all of them. Recently elucidated 3D structure of Chandipura G illuminated the substantial divergence in the location of pH sensing residues compared to VSV.G [6,47].

Membrane fusion is one of the most critical steps in the viral life cycle. It enables the transfer of the viral genome into the target cell cytosol [48]. However, while some vesiculoviruses have lower pH thresholds (e.g. VSV New Jersey and Chandipura), physiological pH (pH 7.2) is optimal for VSV to retain its infectivity and fusogenicity [49]. Even mildly acidic pHs (e.g. pH 6.8) results in several logs of drop in VSV titers [7]. Around pH7-8 most VSV G proteins can be found in their pre-fusion homotrimer structures on the virion surface. However, some monomeric intermediates with exposed fusion loops have also been observed [21,24]. This is thought to be the reason why VSV.G expression on the cell surface results in syncytia formation through spontaneous fusion. More and more fusion loops are exposed in the endosomal compartments with a drop to pH ~6.6. pH 6.2, acting as the threshold ensuring adequate numbers of monomeric intermediates are present on the viral surface, following which membrane fusion occurs through hemifusion [23,50].

2.4. Pseudotypes bearing a VSV envelope glycoprotein

While VSV has been successfully used as a vaccine and gene delivery vector, the use of lentiviral vectors for gene therapy and gene editing purposes is one of the most popular systems. In this setting, the VSV.G has been most widely utilized as the 'gold-standard' viral envelope to pseudotype lentiviral vectors (LVs) in routine molecular biology and then in both preclinical and clinical gene therapy.

Gene therapy was born with the use of Moloney murine leukemia virus (MoMLV) based oncoretroviral vectors to deliver genetic payloads into target cells [51]. LVs were later designed by genetically modifying the HIV-1 genome and providing the necessary structural proteins *in trans* (reviewed in [52]). Overall, LV is currently the vector of choice for many gene therapy applications, in particular *ex vivo* cell therapies, due to their high carrying capacity, ability to be pseudotyped with heterologous envelopes, increased biosafety owing to the generation of self-inactivating vectors, integration into the host genome allowing for sustained transgene expression, and arguably lower immunogenicity of viral proteins. The choice of envelope used to pseudotype LVs dictates various properties of the vector including cell tropism, serum sensitivity, physical, and thermostability.

Prior to its ubiquitous use for lentiviral pseudotyping, VSV.G was initially developed as a heterologous envelope for avian sarcoma virus [53] and MoMLV retrovirus-based gene therapy vectors [53,54]. Currently, VSV.G is widely regarded as the model envelope to pseudotype LVs owing to its broad tropism, robust stability, and high vector titers [55]. However,

several aspects of VSV.G still need to be improved upon. It is suboptimal in transducing resting lymphocytes since its main receptor LDLR is not highly expressed [56]. In addition, several groups have reported that VSV.G is hypersensitive to fresh human serum and sera from other mammalians, curtailing its efficacy *in vivo* [57,58]. To this extent, several other vesiculovirus envelopes (e.g. Cocal, Piry, Chandipura) have been proposed as resistant replacements [58–60]. Moreover, high VSV.G expression-related cytotoxicity has been reported in LV producer cells. This has been a major hurdle in the generation of VSV.G-based packaging and producer cell lines. Yet, stable envelope expressing cells [58,61] and producer cells with inducible promoters have been reported [62–64]. In addition, a cell-free *in trans* pseudotyping method which enables stable production of LV has been proposed as an alternative. It has been known that purified G particles from virion surfaces can be readily incorporated into synthetic liposomes to create virus-like particles [65,66]. Abe and colleagues later demonstrated that when expressed on cell surface VSV.G 'buds out' from the cell surface into the media. They could isolate and sediment these G containing particles as well as use them to pseudotype otherwise envelope-free gammaretroviral particles [67]. In Tijani *et al*. we demonstrated that this admixing method can be utilized for functional LV preparation using envelope-less LVs and envelopes separately produced in stable, constitutive producer cell lines potentially with reduced cost and increased reproducibility [58]. Lastly, it has been reported that VSV.G pseudotyped LVs (VSVG.LVs) induce a robust immune response following intravenous administration *in vivo*, resulting in the production of neutralizing antibodies and clearance in transgene expression due to transduction of 'unwanted' immune cells [43,52,68].

Despite drawbacks outlined above, VSVG.LV demonstrated great promise in preclinical *in vivo* studies as well as having been successfully used *ex vivo* in the clinic. Currently, there are more than 150 active lentiviral vector-based clinical trials in the US most of which exploiting VSV.G's advantageous characteristics [69]. Furthermore, VSVG.LV's unprecedented transduction ability ushered in the era of cell therapies leading to the advent of chimeric antigen receptor T (CAR-T) cell therapies [70]. These genetically modified T cells offered curative therapies for several cancers and two, Kymriah and Yescarta, have been approved by the U.S. Food and Drug Administration (FDA) for use in B-cell malignancies in the recent years [71,72].

VSVG.LV's success thus far has led to the establishment of good manufacturing practices (GMP) to ensure consistent production of efficient and safe vectors with high quality and minimal contaminants. Under the current GMP, the production of LVs is performed through production cell lines (usually HEK 293 or derivatives) named packaging cell lines [73]. However, current processes, both in academia and industry have been designed based on LVs' *ex vivo* use and success. For these manufacturing processes to be able to support the volume of viral vector required in *in vivo* applications, substantial optimization and advancement are necessary [74]. Each batch of LV produced for clinical use is put through stringent quality control tests confirming purity, functionality, and the lack of replication-competent viruses [75–77]. Recent successes in *ex vivo* gene and cell therapy approaches in immune-oncology (e.g. CAR-T therapies) have highlighted the burden of using the traditional transient transfection approaches to produce viral vectors on production time and costs [78]. In order to advance these early successes expanding to *in vivo* gene therapies and support translational development of advanced therapy medicinal products, substantial improvements are necessary regarding efficient and large-scale vector manufacturing.

3. VSV-based vectors

VSV-based vectors are extremely versatile examples of such recombinant vectors. Studies during the 1970s showed utilizing wild-type VSV could generate heterogenous pseudotypes; virions with multiple different envelope glycoproteins on their surface [79–82]. These phenotypically mixed viruses were obtained by simultaneous infection of cells with VSV and other viruses (e.g. SV5, MLV, and Sendai viruses) [83]. This co-infection of cells yielded both homogenous pseudotyped viruses as well as viral progeny bearing heterogeneous envelope glycoprotein on its surface of different ratios, and thus susceptible to dual neutralization [84]. Neutralization of VSV.G via incubation of mixed pseudotype viruses in anti-VSV antisera allowed the detection and measurement of infection mediated by the heterologous envelope. Alternatively, this method also enabled the investigation of the host-range specificity of the viral envelope protein. In the absence of the pantropic VSV.G activity host-range of the heterologous envelope could be tested. The use of thermosensitive or production defective VSV mutants, such as the VSV *t*IB_{17} mutant glycoprotein which is inactivated by heating to 45°C for 60 minutes [79,85,86] or VSV *ts*045 which is subject to temperature-sensitive maturation from the endoplasmic reticulum [87–89], allowed scientists to select for the homogenous pseudotypes to undertake further studies on the foreign envelope protein [83,90]. At the time, these phenotypically mixed viruses were considered valuable tools in the study of latent, defective, or partially expressed viruses. However, there remained a need to improve their method of production and to gain a greater understanding of their structural formation [79].

The early interpretation that rhabdovirus assembly of virion envelope glycoproteins is nonspecific [91] was enhanced by advances in recombinant genetics [92]. Using a VSV-ΔG it was determined that rhabdoviruses assemble and bud independent of the envelope protein, producing bald particles, yet at a 10–30 fold lower efficiency than wild-type VSV [93,94]. This higher efficiency of virus budding in the presence of envelope protein is in contrast to retroviruses, which are another commonly used viral vector system, where bald particles bud at the same rate as those encompassing envelope in a process primarily driven by the capsid protein [95,96]. The nonspecific nature of this envelope incorporation has further been demonstrated by using diverse sequences, varying in length, with truncated and extended cytoplasmic domains [97,98]. The use of the VSV core to produce pseudotyped viruses has remained popular, with replication-defective recombinant VSV (rVSV) produced with the virus glycoprotein gene replaced by that encoding a reporter, such as fluorescent protein or

luciferase. The use of this system was first described in a study investigating the entry of Ebola virus [99]. Accessibility to the system was enhanced upon publication of detailed methods for the production and recovery of rVSV-ΔG pseudotypes [100], together with the availability to purchase the vector commercially.

A major application of rVSV-ΔG pseudotypes has been enabling wide accessibility to study properties of high containment level 3 or 4 viruses at a low containment level [101–103]. It is particularly valuable in the study of novel emerging viruses, where a pseudotype can be rapidly produced once the sequence of the envelope glycoprotein is known. This foregoes the need to establish cell culture systems. The system is also conducive to high-throughput screening, with the acquisition of reporter gene expression from transduced cells possible after 24 hours, in contrast to several days of incubation in the case of wild-type virus infectivity experiments. This has provided researchers with a powerful tool, with rVSV-ΔG pseudotypes being applied to the study of virus entry, determining tropism and elucidating cell receptors, screening of antivirals, and serological investigation, and also seen their development as gene therapy and vaccine vectors (Table 1).

Table 1. Recombinant VSV-based Pseudotypes Used for Various Applications.

Application	Viral Envelope	Reference
Virus Entry	Ebola virus	[100,104–106]
	Herpes virus	[107]
	MERS-CoV	[108]
	SARS-CoV	[109–111]
	Hepatitis C virus	[112,113]
	Measles virus	[114]
	Influenza A virus	[115]
	Influenza C virus	[116]
	Human T-cell leukemia virus type 1	[117,118]
	Borna disease virus	[119]
	Hantaan virus	[120]
	Crimean-congo hemorrhagic fever virus	[121]
	Lassa virus	[122,123]
Cellular Tropism	Bas-Congo virus	[124]
	Chikungunya virus	[125]
	Ebola virus	[105,126]
	Hepatitis B virus	[127]
	SARS-CoV	[128]
	HIV-1	[129]
Antiviral Screening	Hepatitis C virus	[130]
	Nipah virus	[131,132]
	SARS-CoV	[110]
	Hendra virus	[132]
	Lassa virus	[133]
Serology	Hantaan virus	[134]
	SARS-CoV	[135]
	Nipah virus	[136,137]
	Ebola virus	[105,138]
	Influenza A virus	[139]
	Lassa virus	[140]
	Morbillivirus	[141,142]
	Puumala virus	[143]
	Human T-cell leukemia virus type 1	[144]
Gene Therapy	Baculovirus	[233]
	Sindbis virus	[205]
	Simian Parainfluenza virus	[145]
	Lymphocytic Choriomeningitis virus	[210]
	Measles virus	[209]
	Lassa virus	[208]
Vaccine	Measles virus	[164]
	Influenza A virus	[162,165]
	Lassa virus	[146,147]
	Ebola virus	[171]
	Crimean-Congo hemorrhagic fever virus	[148]
	Nipah virus	[149,150]
	SARS-CoV	[151]
	MERS-CoV	[152]
	Zika virus	[153–155]
	HIV-1	[180–183]

4. VSV as a vaccine vector

In recent years considerable advances have been made in tackling infectious diseases using vaccine vectors. These vectors aim to stimulate a strong and specific immune response against proteins expressed by the vectors. They achieve this by exploiting the inflammatory properties of the viruses. Vaccine vectors can be administered via multiple delivery routes including intravenous and intranasal.

Most vaccines to date have been live-attenuated versions of the pathogens (e.g. measles, polio) which have proven to grant long-lasting protection [156,157]. However, for many infectious diseases, the attenuation of the pathogen is not viable or like in the case of HIV-1, several safety concerns remain despite attenuation. Modified recombinant viral vaccine vectors represent a promising alternative strategy to tackle such diseases. The ability to induce a strong immune response, lack of preexisting immunity to the vector, and ability to be repeatedly administered are the major hurdles in the generation of vaccine vectors [158]. Successful vaccine vectors created to date, especially the ones based on adenoviruses, allow for heterologous prime-boost regimens but cannot be repeatedly administered [159–161].

VSV aligns with the qualities of a good recombinant vaccine vector: capacity and genetic stability for insertion of transgenes, non-integrating viral life cycle with low toxicity, and ability to be produced in high-titers. In addition to the general lack of preexisting VSV immunity, the virus is relatively safe as it replicates in the host cell cytosol and does not integrate into the genome. Lastly, in preclinical studies, it has been demonstrated that VSV-based vaccines can induce strong humoral and cellular immune responses following administration [162,163]. For example, a single dose of recombinant VSV encoding hemagglutinin of influenza A and measles viruses can protect rodents against lethal challenges [164,165]. In addition to its use in tackling other infectious diseases such as hepatitis C [166], the most recent VSV success was the recombinant replication-competent VSV-based vaccine vector pseudotyped with the glycoprotein of Ebola virus (reviewed in [167]). After demonstrating full protection in preclinical non-human primate studies [168], the vector (rVSV-ZEBOV) was used during the outbreak in West Africa in 2013–2016 [169]. In this phase III trial, it was demonstrated that the vaccine was safe for use in human [170] and early data on its protective efficacy are very encouraging [171,172]. There is thus potential, with studies ongoing, to apply this recombinant VSV vaccine platform to other emerging viruses [173]. rVSV-ZEBOV vaccine, sold under brand name Erbevo, was approved for use in the US and EU in 2019 [174,175].

The use of a replication-competent neurotropic virus came with several safety concerns. Neurovirulence of wild-type (wt) VSV and administration of a replicating viral vector to immunocompromised patients were the two main topics of

contention. While the vaccine lacked VSV.G, the main contributors to the virus' neurotropism, several non-human primate studies were performed to demonstrate the safety of the vaccine. First, the lack of neurovirulence (i.e. lack of neurological disease symptoms and lesions) was determined via intrathalamic inoculation [176]. In addition, NOD-SCID mice and rhesus macaques infected with simian-immunodeficiency virus were immunized with rVSV-ZEBOV. No vaccine-associated disease was observed in all of the animals demonstrating the vaccine's safety in immunocompromised individuals [177,178]. Lastly, wt VSV causes widespread disease in livestock and is an OIE (World Organization for Animal Health)-listed virus. Therefore, the pathogenicity of rVSV-ZEBOV was assessed in porcine studies [179]. No signs of disease were observed in animals and viral shedding was detected for only one out of six animals involved in the study.

On the back of its success with the Ebola outbreak, recombinant VSV is now being engineered as an anti-HIV-1 vaccine (reviewed in [180]). Generated via an identical method, HIV-1 vaccine was able to generate antibodies against the HIV-1 envelope and induce a modest cell-mediated immune response in several animal models [181,182]. However, the humoral response was not neutralizing, a problem which is thought to be related to the low expression of HIV-1 env on rVSV surface [183].

Furthermore, the exploitation of the transcriptional activity of the viral VSV promoter has also been a viable strategy to create attenuated vaccine vectors. Studies have shown that translocation of viral genes essential for viral replication (e.g. nucleocapsid) to the 5′ end of the viral genome reduces replication rate while not compromising immunogenicity [184]. Similarly, rVSV viruses with their glycoprotein gene in the 3′ location were able to elicit stronger immune responses in mice [185].

Despite these successes outlined, there are two major drawbacks associated with VSV-based vaccine vectors. Vaccine vectors expressing the VSV.G cannot be re-administered due to the potent neutralizing antibody response generated [162]. However, heterologous prime-boost regimens have been used with varying degrees of success [43,186]. Second, VSV, naturally, is a neurotropic virus as discussed above for rVSV-ZEBOV. Therefore, the use of the live replication-competent virus raises concern with regards to neurotoxicity-related diseases including encephalitis [187].

5. Oncolytic activity of VSV

Oncolytic activity of certain viruses has been explored for the controlled killing of cancerous tissue. It was first discovered through the 'bystander effect' when cancer regression was observed in patients with unrelated viral infections [188]. This sparked rigorous research on the use of viruses for oncotargeting by exploiting the lack of anti-viral machinery in cancer cells [189]. This enables oncolytic viruses to preferentially infect and replicate in cancer cells, spreading to other tumor tissues and killing them in the process. Since then, many viruses from diverse families (e.g. herpes, adeno, and rhabdoviruses) have been evaluated as oncolytic vectors, with the first oncolytic virus therapy, based on herpes simplex virus, being approved in 2015 in the western hemisphere [190].

VSV's onco-selectivity stems from its sensitivity to type I interferon dependent cellular immune responses [191]. In healthy cells, a robust IFN-1 response significantly curtails VSV infections; however, VSV infection flourishes in cancer cells where parts of this immune pathway are missing or damaged. Hence, this leads VSV to preferentially replicate in cancerous tissue [192] (Figure 3).

Furthermore, VSV has a short replication time which produces a large amount of daughter virions and, as mentioned above, can be genetically altered. Both of these characteristics make it a prime candidate for an oncolytic vector [193]. Its primary receptor, LDLR, is widely expressed in most tissues conferring the virus broad tropism in mammalian cells and, therefore, VSV infections are not limited by receptor availability. Its cytoplasmic replication makes it a safe choice, while fast *in vitro* growth kinetics enable the production of high titer viral preparation in the laboratory [194]. Onco-selectivity and lytic potential of VSV have been demonstrated against several cancer types including cervical and breast cancers, melanomas, and glioblastomas [195].

Despite its excellent oncolytic activity, VSV has been shown to cause deaths due to neurotoxicity in both murine and non-

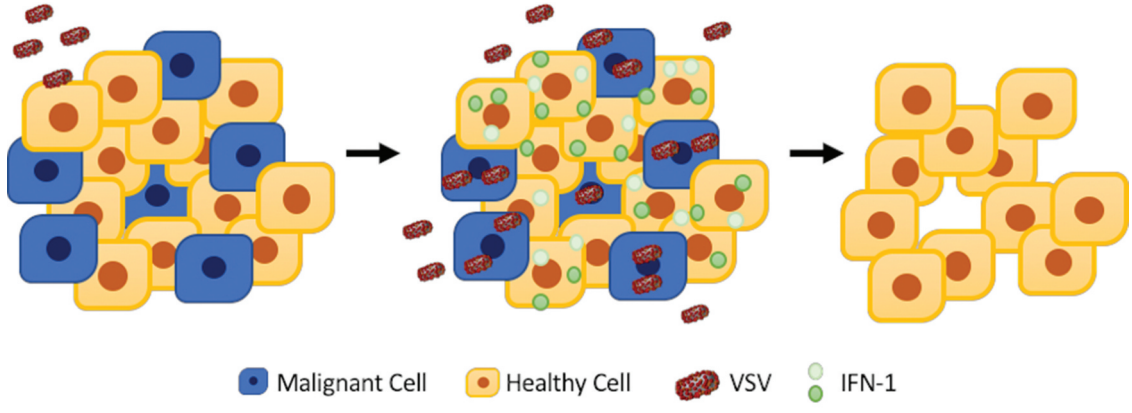

Figure 3. Oncoselectivity of VSV. Infection of healthy cell(s) with VSV results in a strong IFN-1 response limiting the virus' efficacy, protecting neighboring cells, and further cascading the cytokine response. However, cancer cells lacking this immune pathway remain susceptible to VSV infection [198]. Therefore, VSV selectively replicates in malignant cells killing them in the process and clearing the tissue.

human primate models [196,197]. Several strategies to attenuate the virus have been developed to combat this. The most promising and widely used is the ΔM51 mutant [198]. In wt virus, the matrix (M) protein has evolved to combat IFN-1-based immune response in the cells. The M protein can block the host mRNA export preventing host gene expression and ultimately leading to apoptosis (reviewed in [199]). This mutation in the protein compromises its ability to combat the immune response hence rending it more sensitive to IFN-1 signaling, increasing its onco-selectivity and alleviating the toxic effects. In contrast to the wt virus, ΔM51 mutants utilize the death receptor pathways to kill cancer cells [200]. Additionally, there are also reports highlighting that VSV's effectiveness may vary between individual cancer patients. Individuals with upregulated IFN-1 pathways tend to be resistant to VSV-induced oncolysis [201]. In addition, interactions between live VSV-based therapies and human peripheral blood cells following intravenous administration of the virus have been a cause for concern regarding the clinical safety of VSV-based therapies. Several studies have demonstrated that peripheral blood leukocytes differentiate into dendritic cells as a consequence of being exposed to VSV *ex vivo* raising questions about onco-selectivity of VSV-based oncolytic viruses [202,203].

Current research focuses on improving four aspects of oncolytic VSV variants: resistance to host antiviral responses, enhanced onco-selectivity, improved oncolysis, and safer vectors. In addition to the M protein mutant mentioned above, several other M protein variants (e.g. A1 and A2) have been generated in the hope of targeting IFN-1 sensitive tumors [195,204]. In addition, heterologous pseudotyping strategies have been used to try to decrease the neurotropism of the virus (e.g. using envelope proteins from Sindbis virus [205–207], Lassa virus [208], measles virus [209]) and to avoid the humoral immune response generated against VSV.G (e.g. by using the nonimmunogenic LCMV envelope [210]). Another measure taken to increase the safety profile and onco-selectivity of the virus was the incorporation of surface targeting markers (e.g. anti-her2/neu receptor [205,207] and microRNA targets [211]).

In recent years, insertion of immunostimulatory transgenes (e.g. GM-SCF [212,213]) to promote DC maturation and antigen presentation has been one of the strategies explored. Furthermore, enhancement of T cell maturation and differentiation was attempted using immunomodulatory transgenes (e.g. IL-12, IL-15 [214–216]). However, further work is necessary with such approaches to achieve sustainability of the response and minimize off-target effects as such transgenes that can affect both healthy and cancerous cells. Another immunomodulatory approach has been the use of dual transgene VSV oncolytic viruses encoding IFN-β and thyroidal sodium iodide symporter (NIS) [217]. While IFN-β helps with virus-targeting to myelomas, NIS allows real-time noninvasive monitoring of viral activity in animals and patients as well as providing the option to couple the virotherapy with targeted radiotherapy [218]. This approach has proven to provide one-shot curative treatment in mice and currently is used in several phase I clinical trials (ClinicalTrials.gov ID NCT03017820, NCT03120624, and NCT02923466).

Owing to its success as a vaccine vector, there have been attempts to develop VSV into an oncolytic vaccine. Sometimes used in combination with other reagents (reviewed in [219]), substantial tumor debulking has been achieved using this strategy [220]. Genetically engineered immunogenic VSV variants encoding tumor antigens have become popular based on findings indicating VSV's ability to amplify organisms' anti-tumor response. In addition to melanoma antigens [221,222], recombinant VSV encoding endogenous tumor-specific antigens have been used to successfully activate systemic T cell response leading to tumor lysis [223–225].

6. Immune challenges related to VSV-based therapies

VSV as well as VSV-based vectors and recombinant variants have been developed into effective vectors for their use in medicine [194,226]. The efficacy of many VSV-based oncolytic and vaccine vectors has been established preclinically and their clinical evaluation is underway [162,227–229]. In addition, VSV.G pseudotyped LVs are regarded as the benchmark in gene therapy and they have been successful in the clinic in achieving therapeutic disease correction in several cases.

The most significant immune challenge ahead of VSV vectors is the highly immunogenic envelope glycoprotein. The primary immune response elicited toward VSV.G almost exclusively leads to the production of strongly neutralizing antibodies [230]. This significantly affects the efficacy of secondary doses limiting re-administration of the vectors. In addition, VSV.G's sensitivity to complement mediated inactivation is also a cause for concern. The complement system not only inactivates the VSV.G bearing vectors but also enhances the function of neutralizing antibodies. We have recently demonstrated that the efficacy of VSV.G pseudotyped LV intravenously administered to mice pre-immunized with soluble G (produced via limited thermolysin cleavage from wt VSV [11]) is completely abrogated by neutralizing anti-G antibodies [43]. However, it was possible to circumvent this undesirable anti-vector immunity. Used similarly to a heterologous prime-boost regimen, complete immune evasion was achieved with LV pseudotyped with G from another vesiculovirus, Piry and to a lesser extent with Cocal virus G. We believe that tailoring panels of distinct G will allow for repeated administration of advanced therapy medicinal products for a sustained amount of time. VSV's ability to replicate fast in combination with its highly cytopathic nature results in the presentation of viral proteins to MHC-I pathways. Release of these proteins following cell apoptosis may lead to the uptake of antigens by APCs. These all contribute to the induction of a robust cytotoxic T cell response as well as T cell priming [231].

7. Conclusion

Applications of VSV since the 1970s, as a research tool or in conjunction with other types of viruses, have paved the way for recent developments in the various uses of VSV and its

derivatives in medicine. Alongside this wide range of developments, the understanding of VSV's biology including structural, molecular, and immunological aspects has been progressing and in turn helping further advance and refine its application. Current challenges for future development, such as antiviral immunity and safety in *in vivo* use of VSV derived ATMP, have been identified.

8. Expert opinion

VSV.G has established itself as the default choice of envelope in lentiviral vectors and will continue to play important roles in both basic research and gene therapy applications. For a wide area of basic research VSV.G pseudotyped lentiviral vectors are routinely used to deliver exogenous genetic sequences including various transgenes, shRNA, and cDNA libraries. It is however unclear if they are as widely used for gene editing, where the gene editing machines, such as CRISPR-Cas9, are only transiently required. In the gene therapy field VSV.G, as often called a 'gold standard,' sets a mark for gene delivery performance in both preclinical and clinical studies. Vector developers exploring other envelopes, such as alternative vesiculovirus G proteins [43,58,59], Sendai virus F/HN proteins [232], baculovirus GP64 [233,234], and measles virus H and F proteins [235] as well as those engineered for tissue targeting, would ask what advantages their alternative envelopes have over VSV.G, in terms of gene delivery efficacy, cell specificity, immunity, ease in manufacturing and other characteristics. While the field continues to search for improvement in gene delivery methods, studies in gene therapy research and development will contribute to a better understanding of VSV. G biochemistry and VSV virology. For example, a recent study by Petrillo *et al*. revealed an innate immunity mediated by IFITM3 targeting VSV.G in hematopoietic stem cells in the context of lentiviral vector-mediated gene delivery [236]. This confirmed earlier observations of IFITM3's anti-VSV activity [237,238] and helped dissect the host defense system by IFITM proteins.

Research use of replication-defective rVSV-ΔG vectors has been increasing recently, partly because the system became commercially available. They have been applied to receptor and seroepidemiological studies, as well as vaccine potency testing. For immunization purposes, VSV-based vectors can be used to express immunogens in the host cell and pseudotyped virus particles can be potent immunogens to induce immunity against viral envelope proteins. More innovative applications may arise, as the world continues to encounter emerging viruses. It will be interesting to see what roles this system could play in the current COVID-19 epidemic and we predict more researchers will note its versatile utility, e.g. use at lower containment levels, speed of production, and relevance both in pathogen biology and clinical application.

We predict there will be more effort in developing replication-competent VSV-based viral vaccines following the successful use of rVSV-ZEBOV. The attraction of vectored vaccine approaches is the relative ease of design and large-scale manufacture of recombinant virus constructs incorporating the sequence of target envelope proteins. In particular, rVSV has been shown to elicit strong humoral and cellular responses and has a low seroprevalence in the human population. As a relatively new player in the vectored vaccine field, the completion of recent and ongoing phase I–III human clinical trials will help to realize the full potential of this platform; paving the way for other candidates in preclinical stages of development. In contrast, the application of VSV for oncolytic virotherapy will face challenges before its efficacy is fully demonstrated preclinically and it reaches clinics. VSV seems to have less tight cancer tropism compared to many other oncolytic viruses in clinical use or under development. For both vaccine and oncolytic purposes, the safety and regulatory issues are paramount for the use of replication-competent viruses. For example in the UK vesicular stomatitis is under a tight control by The Department for Environment, Food and Rural Affairs (DEFRA) and is a notifiable animal disease [239] and VSV is covered by Specific Animal Pathogen Order (SAPO) [240].

In conclusion, we hope we have presented compelling evidence suggesting VSV will continue to be important for many types of research tools and medicinal products. In five years from now, we predict with a better understanding of VSV biology we will have seen more novel, innovative applications of VSV to tackle both old and emerging medical challenges.

Acknowledgments

The authors thank Dr Elsa Abranches for the critical reading of the manuscript.

Funding

This article is funded by the National Institute for Biological Standards and Control.

Declaration of interest

The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

Reviewer disclosures

Peer reviewers on this manuscript have no relevant financial relationships or otherwise to disclose.

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