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Accessibility
Recoding of the Vesicular Stomatitis Virus L Gene by Computer-Aided Design Provides a Live, Attenuated Vaccine Candidate

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ABSTRACT Codon pair bias (CPB), which has been observed in all organisms, is a neglected genomic phenomenon that affects gene expression. CPB results from synonymous codons that are paired more or less frequently in ORFeomes regardless of codon bias. The effect of an individual codon pair change is usually small, but when it is amplified by large-scale genome recoding, strikingly altered biological phenotypes are observed. The utility of codon pair bias in the development of live attenuated vaccines was recently demonstrated by recodings of poliovirus (a positive-strand RNA virus) and influenza virus (a negative-strand segmented RNA virus). Here, the L gene of vesicular stomatitis virus (VSV), a nonsegmented negative-sense RNA virus, was partially recoded based on codon pair bias. Totals of 858 and 623 silent mutations were introduced into a 5’-terminal segment of the viral L gene (designated L1) to create sequences containing either overrepresented or underrepresented codon pairs, designated L1sdmax and L1min, respectively. Analysis revealed that recombinant VSV containing the L1min sequence could not be recovered, whereas the virus with the sdmax sequence showed a modest level of attenuation in cell culture. More strikingly, in mice the L1sdmax virus was almost as immunogenic as the parental strain but highly attenuated. Taken together, these results open a new road to attain a balance between VSV virulence and immunogenicity, which could serve as an example for the attenuation of other negative-strand, nonsegmented RNA viruses.

IMPORTANCE Vesicular stomatitis virus (VSV) is the prototypic rhabdovirus in the order Mononegavirales. A wide range of human pathogens belong to this family. Using a unique computer algorithm and large-scale genome synthesis, we attempted to develop a live attenuated vaccine strain for VSV, which could be used as an antigen delivery platform for humans. Recombinant VSVs with distinct codon pair biases were rationally designed, constructed, and analyzed in both cell culture and an animal model. One such recombinant virus, L1sdmax, contained extra overrepresented codon pairs in its L gene open reading frame (ORF) and showed promise as an effective vaccine candidate because of a favorable balance between virulence and immunogenicity. Our study not only contributes to the understanding of the underlying mechanism of codon pair bias but also may facilitate the development of live attenuated vaccines for other viruses in the order Mononegavirales.

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Nonsegmented, negative-sense (NNS) RNA viruses include a wide range of established and emerging mammalian pathogens. For most of them, neither vaccines nor antiviral therapeutics with proven efficacy are available (1–3). Vesicular stomatitis virus, an NNS virus of the Rhabdoviridae, is an arthropod-borne pathogen that primarily causes acute vesicular disease in livestock (4). However, rare cases of human infection have been reported. Individuals infected with VSV usually develop mild flu-like symptoms, but a single case of encephalitis in a 3-year-old child has been related to VSV infection (5). Nevertheless, VSV serves as an attractive model to study a large number of mammalian pathogens of the Rhabdoviridae, including rabies virus (6).

Except for the members of the Orthomyxoviridae (e.g., influenza viruses), whose RNA polymerase is a tripartite complex (7), all the negative-strand RNA viruses encode a single large polymerase protein, L, which contains all the enzymatic activities involved in nucleotide polymerization (8) and mRNA modification (9–11). Amino acid sequence comparison of L proteins from multiple NNS viruses revealed six conserved regions (CR I to VI), which led to the notion that the large single L protein might be organized into a series of domains that retain independent catalytic activities (12).

VSV has been used extensively during the last four decades as a laboratory tool to study molecular virology and cell biology. Its relatively innocuous phenotype in human infection makes this virus attractive for clinical application, a potential realized only recently. However, VSV is an infectious agent with neurotoxicity in experimental animals, which poses serious safety concerns for direct human use (13, 14). Here, we report attempts to develop a live attenuated VSV strain, making use of a genetic phenomenon...
known as codon pair bias (CPB) (15, 16). CPB, which has been observed in ORFeomes of all organisms studied so far, describes an unexpected irregularity of synonymous codon pairing in genomes of a specific phylum, independent of its genome-wide codon usage. We previously reported that codon pairs are either overrepresented or underrepresented in the genome of a particular organism (17). The nature of a codon pair can be defined by calculating its codon pair bias score (CPS), which is the natural logarithm of the ratio of the observed frequency of the codon pair to the expected frequency. The average of the sum of all CPSs in an ORF indicates the codon pair bias of the entire gene. In our previous experiments involving recoding of viral genomes, we have shown that the viral gene expression can be intentionally altered by a rational design of a coding sequence, based on codon pair bias (17–20). More importantly, the alteration of gene expression is likely a result of the numerous synonymous substitutions, which makes phenotypic reversion nearly impossible (21). This specific property of virus attenuation caused by changing codon pair bias is critical for the development of vaccine strains against RNA viruses, like VSV, described here, whose spontaneous mutation rates are much higher than those of DNA viruses and of other microorganisms (22).

### RESULTS

**Construction and characterization of L recoded VSV variants in tissue culture.** We recoded a large N-terminal segment of the L gene ORF, L1, by computer algorithm-aided design (Fig. 1A). Without alteration of codon usage or amino acid sequence, we rearranged the positions of existing codons to generate either underrepresented or overrepresented codon pairs. The L1sdmax and L1min sequences were synthesized de novo and incorporated into the parental plasmid pVSV1(+) to replace the WT sequence, using HpaI and AvaI sites. (B) Codon pair bias of human genes and the L1 derivatives plotted against their amino acid lengths.

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**FIG 1** Genome organization of VSV and construction of L variants. (A) The VSV genome consists of 2 untranslated regions (3’ leader and 5’ trailer; red boxes) and 5 viral genes (N, P, M, G, and L). Like the other four viral genes, the VSV L ORF is flanked by the untranslated regulatory sequences (white boxes) that are essential for transcription initiation and termination. The L1 region (blue box) is between nt 4961 and nt 7534, corresponding to amino acids 76 to 934 of the L protein. L1sdmax and L1min sequences were synthesized de novo and incorporated into the parental plasmid pVSV1(+) to replace the WT sequence, using HpaI and AvaI sites. (B) Codon pair bias of human genes and the L1 derivatives plotted against their amino acid lengths.
derrepresented or overrepresented codon pairs, resulting in hundreds of synonymous nucleotide substitutions (see Table S1 in the supplemental material). Compared to its WT counterpart, \( L^{1\text{min}} \) has a much lower codon pair score, whereas \( L^{1\text{sdmax}} \) has a significantly higher one (Fig. 1B). We took into account a possible increase in the frequency of XXCpGXX, a dinucleotide formed between two codons that may play a role in inducing an innate immune response (23), and of XXUpAXX, a dinucleotide that at high frequency has been proposed to reduce mRNA stability (24). We particularly avoided generating extra AAAUU (AU-rich) sequences, which are signals for mRNA degradation (25). The distribution of overrepresented and underrepresented codon pairs in these designs is graphed in Fig. S1 in the supplemental material.

A recombinant VSV bearing a “deoptimized” L gene (\( L^{1\text{min}} \)) is expected to be highly attenuated and possibly nonviable. On the other hand, based on our previous experience in “maximizing” the poliovirus P1 region, we anticipated that sdmax might not affect L gene expression negatively (17). We initially included the sdmax design in our study in order to rule out the possibility that certain unidentified RNA sequences critical for virus replication were accidentally modified during the large-scale recoding (26). As shown in Table S1, about 33% of the nucleotides of the L1 region have been mutated in the \( L^{1\text{sdmax}} \) design; therefore, theoretically, any important RNA signal or highly structured element longer than 3 or 4 nucleotides (nt) would have been inactivated.

cDNAs corresponding to these recoded regions were chemically synthesized de novo and cloned into the parental antigenomic plasmid pVSV1(H11001) to replace the WT sequence. The reverse genetics of these recombinant viruses was carried out using the vaccinia virus-mediated system (27). The \( L^{1\text{sdmax}} \) design yielded infectious virus after one blind passage, whereas \( L^{1\text{min}} \) failed to generate a viable virus.

The growth phenotypes of the recombinant \( L^{1\text{sdmax}} \) virus was then analyzed in tissue culture. Compared to the WT virus, the plaque phenotypes revealed slightly smaller sizes in BHK-21 cells and A549 cells (Fig. 2A and B). Multistep growth kinetics, analyzed in BHK-21 or A549 cells by inoculation at a low multiplicity of infection (MOI) of 0.1 or 0.001, showed maximal titers of \( L^{1\text{sdmax}} \) between 24 and 48 hpi that were comparable to that of the WT virus. Viral replication of \( L^{1\text{sdmax}} \), however, was slightly delayed, independent of the cell type it infected. A quantitative reverse transcription-PCR (RT-PCR) assay that measured the level of viral full-length RNA in infected BHK-21 cells supported the delay of genome synthesis (Fig. 2C). To measure the yield of viral proteins and expand the range of cell types, we then determined the synthesis of N protein by immunocytochemistry in HeLa cells.

**FIG 2** Multistep growth kinetics and plaque phenotypes of WT and \( L^{1\text{sdmax}} \) virus in BHK-21 cells (A) and A549 cells (B) at low MOIs. (C) Quantitative RT-PCR was conducted to quantitate the full-length viral RNA in WT- and \( L^{1\text{sdmax}} \)-infected BHK-21 cells (MOI = 3).
In L1sdmax-infected cells, at 6 hpi, much less viral N protein was detected than in WT virus-infected cells (see Fig. S2 in the supplemental material).

**Reduced expression of the L protein encoded by the L1sdmax sequence.** During the early stages of virus infection, the slower growth of the L1sdmax virus led to a reduced yield of all the viral products (see Fig. S3 in the supplemental material). We were unable to explain the decreased level of L protein synthesis by simply comparing the yields of viral protein to those of mRNA. To circumvent this problem, we constructed two L expression plasmids (designated pL1sdmax and pL1min) (see Fig. S4 in the supplemental material) by replacing the cognate WT L open reading frames of pL with the synthetic L derivatives (L1sdmax and L1min). We then examined the production of full-length L protein after transiently transfecting BHK-21 cells that were previously infected with vTF7-3. In this experimental setting, the entire L gene coding sequences are transcribed in the cytoplasm by T7 polymerase.

To increase the stability of the large polymerase polyepitope, we cotransfected into BHK-21 cells a plasmid expressing the VSV P protein together with the plasmid harboring the modified L sequence (28). In comparison to translation of pLWT, translation of the L1sdmax gene produced only a weak signal (Fig. 3A), even though equal amounts of L transcripts were detected in pLWT- and pL1sdmax-transfected cells (Fig. 3B). This result was unexpected given that codon pairing in L1sdmax has been designed to yield mRNA favorable for translation (17). The synthesis of L1min was even more inefficient than that of L1sdmax (see Fig. S4B in the supplemental material). This was not totally unexpected, although we do not know as yet whether the poor L1min synthesis is due to an inhibition of translation per se or a rapid degradation of mRNA prior to or during translation.

Another interesting observation is that the L1sdmax virus showed an exquisite sensitivity to the pharmaceutical deprivation of Hsp90 (see Fig. S5 in the supplemental material), a host chaperone molecule that has been identified to be essential for the proper folding of the newly synthesized VSV L protein (29). It raises the possibility that an enhanced chaperone engagement may be required for the L1sdmax virus.

**Diminished virulence of the L1sdmax virus after intranasal inoculation of mice.** As mentioned above, the poliovirus variant P1max in which the capsid coding region was codon pair optimized, was as lethal in CD155 transgenic mice as WT poliovirus (17). We therefore tested the pathogenesis of L1sdmax virus in a mouse model. Five- to six-week-old male BALB/c mice were infected with different doses of WT or L1sdmax viruses intranasally. WT-infected mice lost weight rapidly and exhibited typical symptoms of infection starting around 3 dpi. Under our experimental conditions, the 50% lethal dose (LD50) for WT VSV was 105 PFU for intranasal infection. In contrast, neither mortality nor severe morbidity was observed in any mice infected with the L1sdmax virus, even at a very high infection titer (2 × 108 PFU) (see Fig. S6B in the supplemental material). Taking these data together, we conclude that L1sdmax virus is highly attenuated in BALB/c mice. This was a surprise, but it matched our unpublished results obtained with recoded dengue virus variants that phenotypes observed in tissue culture with a codon pair-recoded virus do not necessarily predict phenotypes of the same recoded virus when it is tested in experimental animals.

**Virus burdens in the infected organs.** The efficacy of live attenuated vaccines depends on reduced viral replication in vivo but a successful stimulation of immunological memory (18). Moreover, virulence and virus load in infected organs are usually positively correlated (19). Therefore, we examined virus burden in the infected animals by both plaque-forming assays and quantitative RT-PCR. At 3 dpi, the virus load in the L1sdmax-infected organs was at least two orders of magnitude less than that in their WT-infected counterparts. On day 6, the infectious L1sdmax virus could not be recovered in any of the mice lungs that we examined and could be detected in only one brain sample (n = 3) (Fig. 4A). In contrast, in WT-virus-infected animals, a virus burden of approximately 104 PFU/g was observed in brain tissues up to 6 dpi, which ultimately resulted in the death of all the remaining animals by 9 dpi (data not shown). We also examined the viral RNA load in mouse brains using quantitative RT-PCR (Fig. 4B). Total RNA was extracted from infected brains, and a 150-bp fragment corresponding to the viral N gene was amplified. Viral RNA was present in every brain of the infected animals, but the viral RNA load of L1sdmax-infected animals was lower than that of WT virus-infected ones.

**Disparate histopathology of WT- and L1sdmax-infected brains.** Even the limited replication of L1sdmax in the brains of infected animals raises the question of whether such viral variants could serve as vaccine candidates. We therefore carried out histological studies. BALB/c mice were infected intranasally with 107 PFU of viruses, and brains were harvested for hematoxylin-and-eosin (H&E) staining after 5 days. Brain tissues infected with...
WT VSV exhibited severe immunopathological characteristics, evidenced by multifocal perivascular cuffing, mononuclear cell infiltration in the parenchyma, meningeal mononuclear cell infiltration, and hemorrhage (Fig. 5), consistent with previous studies (14, 30). Tissues collected from the L1sdmax group exhibit only mild, if any, inflammatory changes upon virus infection.

VSV infection compromises the integrity of the blood-brain barrier (BBB) (31). The destruction of BBB accompanied by the intensive immunological infiltration causes severe immunopathogenesis that leads directly to the death of experimental animals. Therefore, we used the well-established Evans blue assay to assess the integrity of the BBB after virus infection, a method providing an additional indicator of virus pathogenesis (32). As shown in Fig. S6C in the supplemental material, at 6 dpi the brain of the animal infected with L1sdmax showed no sign of BBB damage, whereas a widespread, intensive parenchyma staining was apparent in the WT-infected brain, which is a clear sign of diminished BBB integrity.

The immune response in L1sdmax-infected mice. We next determined whether the restricted replication of this viral variant provides effective immunological protection against challenge with WT virus. We vaccinated 5- to 6-week-old BALB/c mice with different doses of L1sdmax virus intranasally. After 3 weeks, all mice received 10^7 PFU of WT virus intranasally, representing an approximately 100-fold LD50 of WT VSV. None of the L1sdmax-immunized mice exhibited signs of disease, and their body weights increased steadily, whereas age-matched naive mice succumbed to challenge after 6 to 8 days (Fig. 6A).

We then asked whether the L1sdmax immunization elicited a B cell-mediated humoral immunity. Sera were collected 21 days postvaccination from tail vein blood, and titers of virus-specific neutralizing antibodies (NAb) were measured. The L1sdmax virus was capable of inducing a robust protective antibody response (Fig. 6B). A similar level of NAb was detected in mice that were immunized with the same amount of WT or L1sdmax virus, whereas the control group did not develop a detectable humoral response against VSV infection. Taken together, these data indicate that a single-dose vaccination of the L1sdmax virus can elicit a potent immune response that fully protects mice from a subsequent virulent challenge.

Prolonged induction of proinflammatory cytokines and chemokines in WT- but not in L1sdmax-infected brains. During VSV infection, mouse susceptibility correlated positively with extensive cytokine/chemokine expression in the central nervous system (CNS) (14). To determine the nature of the inflammatory response during L1sdmax infection, we profiled the transcription levels of several proinflammatory cytokines and chemokines in virus-infected brains. Compared to their WT-infected counterparts, MCP-1 and RANTES were induced to a lesser extent in L1sdmax-infected mice (Fig. 7A). Additionally, at 3 dpi, L1sdmax-infected mice showed levels of tumor necrosis factor alpha (TNF-α) and interleukin 6 (IL-6) similar to those of WT-infected animals (Fig. 7B). WT-infected mice retained a high level of cytokine production until 6 dpi, while L1sdmax-infected mice showed a significant decline of cytokine synthesis by then. This suggests that in contrast to the highly attenuated L1sdmax virus, WT virus infection in the CNS cannot be controlled without a sustained inflammation, which eventually leads to immunopathogenesis and irreversible neuron damage.

DISCUSSION

Impact of codon pair deoptimization on gene expression. In this study, we evaluated the consequences of large-scale L gene recoding upon VSV replication and pathogenesis. The L1 region comprises the coding sequences of domains I to III and part of domain IV, which primarily forms the core ring structure of the VSV RNA-dependent RNA polymerase (RdRp) and catalyzes RNA polymerization (33). The L1min sequence yielded only trace amounts of full-length L protein after transient expression in tissue culture cells, which is reminiscent of data obtained with variants of codon pair-deoptimized poliovirus and influenza virus (17–19).

Codon pair deoptimization appears to affect several mecha-
nisms that interfere with gene expression in a context-dependent manner. For example, it might be difficult for the ribosome to read through a string of “rare” codon pairs, a predicament leading to less protein production per mRNA. In addition, it is known that the dinucleotide frequency of CpG and UpA in eukaryotic RNA viruses, as well as in mammalian mRNA in general, is lower than expected (34–36), a phenomenon that must be advantageous for viruses. Generating rare codon pairs by rearranging existing synonymous codons inadvertently leads to an enrichment of UpA and CpG dinucleotides in the mRNA sequences. For instance, the L1min but not the L1sdmax design contains significantly more XXUpAXX and XXCpGXX dinucleotides than the WT sequence (see Table S1 in the supplemental material), with the increase mapping to between codons, as shown, since codon use remained unchanged. Recent studies suggest that the dinucleotide composition in RNA virus genomes influences RNA virus replication, possibly because of an unclear host-pathogen interaction (37) and/or increased instability of the mRNA (23). Although we cannot exclude the possibility that strengthened host recognition of UpA/CpG dinucleotides contributed to the attenuation phenotype of our codon pair-deoptimized viruses, the reverse genetics of L1min was conducted in BHK-21 cells, a cell line well known for its deficiency in innate immune response (38). Hence, the nonviable phenotype of the L1min design cannot be simply attributed to an enhanced host response.

Impact of codon pair “scramble-max” on protein synthesis. The most striking phenotype that we observed with L1sdmax is its attenuation that results from an enrichment of overrepresented codon pairs. Our previous experiments in which we “maximized” the encoding of the poliovirus P1 region suggested that such genetic alteration enhanced the expression of the corresponding viral ORF without affecting virulence (17). This appears to be contrary to the observation in this study. The attenuation of L1sdmax is not host range restricted, because the virus forms smaller plaques in all cell lines tested. Considering that the transcriptional regulatory sequences in the untranslated region of the L gene were never altered, it becomes less likely that the L1sdmax sequence is inadequately transcribed by the viral polymerase. Indeed, the poor expression of the L1sdmax sequence does not seem to occur at the transcription level, because (i) a high level of L mRNA was de-

FIG 5 Histopathology after VSV infection. Mice were inoculated with PBS (A and B), 10⁷ PFU WT (C and D), or L1sdmax (E and F) virus intranasally, and the horizontal brain sections containing cerebral cortex were stained with H&E at 5 dpi. Representative brain histology is shown for each group (magnification, ×40). In panel C, solid yellow arrows indicate the perivascular cuffing, and dashed yellow arrows indicate the mononuclear cell infiltration in the brain parenchyma. In panel D, solid black arrows indicate meningeal hemorrhage, and dashed black arrows indicate the meningeal mononuclear cell infiltration.
tected when the L1sdmax sequence was placed under the control of the T7 promoter and transfected into cells expressing T7 polymerase (Fig. 3B) and (ii) during L1sdmax virus infection, the same kinetics of mRNA accumulation were found for the L and N genes (see Fig. S3 in the supplemental material) (ranging from 1 hpi to 6 hpi).

Considering the possibility that an enhanced chaperone engagement might be required for the L1sdmax virus (see Fig. S5 in the supplemental material), we hypothesized that the alteration of the translation elongation rate, presumably caused by the accumulation of overrepresented codon pairs, might interfere with the coordination between translation and other events closely related to translation rate, such as protein folding. Recent reports have shown that clustered rare codons are frequently distributed between defined protein domains, especially for large multidomain proteins. This distribution pattern of “slow” codons might collectively reduce the read-through activity of the ribosome, which turns out to be beneficial for proper protein folding (39, 40). An early study conducted in a bacterial system also suggests that codon pair utilization influences protein folding by altering translational efficiency, although only a small number of codon pairs was included and analyzed at that time (41). Herein we speculate that the folding efficiency of a multidomain protein, like the VSV L protein described in this paper, might be perturbed by the largescale genome recoding. The relatively low codon pair bias in the WT L1 region (Fig. 1B) might be a compromise between translation accuracy and folding efficiency.

L1sdmax serves as a candidate for VSV-based vaccines and vectors. VSV has been studied as an agent with unique oncolytic properties aided by its broad tissue tropism and exquisite sensitivity to innate immunity, which result in its preferential replication in tumors. In addition, VSV might serve as an excellent vaccine delivery platform, because of its easily manipulated genome and the capacity to stimulate a robust cytokine response via mucosal delivery. In this study, we generated and identified a recombinant virus, L1sdmax, which is highly attenuated (its LD50 is at least 2,000-fold more than that of the WT). Our results showed that L1sdmax underwent a limited replication and dissemination in vivo that barely caused any morbidity, but this self-restricted infection was highly immunogenic. The L1sdmax virus appears to be an attractive live vaccine candidate applicable to other viruses in the order of Mononegavirales that encode very large polymerase polypeptides. In the development of vaccines, conventional strategies usually lead to a limited number of mutations in viral genomes, whereas L1sdmax carries in its L-encoding segment 858 point mutations. We have passaged the L1sdmax virus 5 times in BHK-21 cells at low MOIs, and we did not observe any phenotypic reversion (data not shown), but long-term passaging is required to confirm genetic stability. Of course, the current construct may have to be further attenuated to avoid all proliferation in the CNS.

The recent Ebola outbreak in West Africa and the sporadic cases in the Western Hemisphere have elicited international concern. One of the most promising vaccine candidates against Ebola infection, VSV-ZEBOV, is a VSV chimera that expresses Ebola genetic information (42). It will be of interest to further evaluate the safety and efficacy of L1sdmax as a platform to deliver foreign antigens.

In summary, the present study describes the first large-scale recoding of VSV, a rhabdovirus that belongs to a large family of human pathogens. The outcome of the experiments is surprising but nevertheless may herald a unique approach to develop live attenuated strains for VSV and possibly other NNS viruses.

MATERIALS AND METHODS

Cells and viruses. BHK-21, Vero, A549, and HeLa cells were maintained in Dulbecco’s modified Eagle medium (DMEM) plus 10% fetal bovine serum at 37°C. WT VSV is recovered from the cDNA clone pVSV1(+), using the well-established vaccinia virus-mediated system (27). Briefly, to

![FIG 6 Immunogenicity of the L1sdmax virus. (A) Dynamics of L1sdmax-immunized mice body weights after a lethal WT challenge at day 21. (B) Titters of virus-specific neutralizing antibody (NAb) in WT- and L1sdmax-immunized mice 21 days after the primary exposure. For this experiment, n was 5 per group. A larger number of animals (n = 10) for the groups infected with 10^7 PFU of the WT were initially immunized, because the infection titer is close to the LD50 of WT virus. The sera from the survivors (4/10) were subsequently collected at 21 dpi, and the titers of virus-specific neutralizing antibody were determined as described in Materials and Methods.}

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construct the cDNA clone of L1sdmax or L1min virus, the HpaI-to-AvaI fragment of pVSV1(+) was replaced with the corresponding synthetic fragment L1sdmax or L1min (GenScript, NJ, USA) in which overrepresented or underrepresented codon pairs were substantially enriched. The recovered viruses were plaque purified, and their full-length sequences were confirmed by RT-PCR.

**Virus load and protection assay.** Animal studies were carried out in accordance with a protocol that adhered to the Guide for the Care and Use of Laboratory Animals of the NIH (43) and was reviewed and approved (approval number 354543) by the Stony Brook University Institutional Animal Care and Use Committee (IACUC). Five- to six-week-old male BALB/c mice (Taconic Farms, Inc.) were intranasally infected with $5 \times 10^6$ PFU viruses, and at 3 and 6 dpi, their lungs and brains were harvested for determinations of virus titers by plaque-forming assay. For viral RNA load analysis, total RNA was extracted from brains of mice (TRIzol; Life Technologies) that were infected with $5 \times 10^6$ PFU viruses, and the abundance of viral RNA was subsequently evaluated by real-time RT-PCR using a primer pair that recognizes the viral N gene (VSV N primer) (see Table S2 in the supplemental material). In parallel, qPCR directed against mouse β-actin mRNA was performed to control for any differences in the amount of total RNA inputs (mouse β-actin primer) (Table S2 in the supplemental material). For protection assay, mice were vaccinated intranasally with various doses of L1sdmax virus and challenged with $10^7$ PFU of WT virus at 21 days after vaccination. A group of age-matched virus-naive mice ($n = 3$) were challenged as well, as a negative control. After the virulent challenge, the mice body weights and neuronal disorder symptoms were closely monitored for another 14 days.

**Neutralizing antibody assay.** The mice were immunized with various doses of either WT or L1sdmax virus intranasally. The serum was isolated from tail vein blood 21 days after vaccination, followed by a 2-fold serial dilution in DMEM starting at a dilution of 1:20. Fifty microliters of the diluted serum was incubated with 100 PFU WT VSV in 50 μl DMEM at 37°C for 1 h. One hundred microliters of Vero cell suspension was then combined with the mixture and seeded on 96-well plates in triplicate. Cell morphology was checked daily, and after a 48-h incubation at 37°C, the remaining cells were stained with 1% crystal violet. The reciprocal of the

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**FIG 7** Cytokine/chemokine profile after virus infection. (A) Quantitative RT-PCR to measure chemokine (MCP-1 and RANTES) expression relative to the mouse β-actin after virus infection. (B) Proinflammatory cytokine induction in PBS-, WT-, and L1sdmax-inoculated mice. Data were collected from 4 to 6 mice per group. Statistical significance is indicated by asterisks (*, $P < 0.05$; **, $P < 0.01$ [two-tailed Mann-Whitney U test]).
dilution that gives 100% protection from VSV CPE was recorded as the virus-specific neutralizing-antibody titer.

**Histopathology.** Mice were infected with $10^6$ PFU virus by intranasal inoculation and euthanized at 5 dpi. Mice were transcardially perfused with 10% PFA-PBS, followed by 50% ethanol processing and paraffin embedding. The brain tissues were subsequently sectioned at 5 μm and stained with H&E. The histopathological results were examined independently by two neuropathologists at the School of Medicine, Stony Brook University.

**Quantitative RT-PCR to analyze cytokine/chemokine inductions.** Total RNA was extracted from mice brains inoculated with PBS, WT, or L1sdmax at both 3 and 6 dpi and then treated with RNase-free DNase I (Life Technologies) for 30 min at 37°C before a reverse transcription was conducted with oligo(dT)$_5$-SuperScript III; Life Technologies). Quantitative PCR was subsequently performed to evaluate the induction of cytokines and chemokines using gene-specific primers (see Table S2 in the supplemental material). All reactions were subsequently normalized to the RNA levels of mouse β-actin and reported as increases in induction over that in PBS-inoculated animals.

**Quantitative RT-PCR to measure viral genome replication.** BHK-21 cells were infected with viruses at a MOI of 3. At various time points, total RNA was extracted and reverse transcribed by random hexamer priming. Quantitative PCR was carried out with a primer pair spanning the corresponding region of the WT L gene in the expression plasmid pL (27) was replaced by two recorded derivatives using the XhoI and Xbal digestion sites. For transient expression, $10^6$ BHK-21 cells were infected with vTF7-3 for 1 h and then cotransfected with 1.5 μg pUG and 1.25 μg pL derivatives. Whole-cell lysates and total RNA were harvested at various time points, and the yield of viral products was subsequently determined by Western blotting and quantitative RT-PCR.

**Statistical analysis.** Statistical analysis was performed using GraphPad Prism software version 5.0c (San Diego, CA). Data are presented as means with standard deviations (SDs). Two-tailed Mann-Whitney U tests were used for Fig. 4B and 7.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl doi:10.1128/mBio.00237-15/-/DCSupplemental.

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