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ORIGINAL ARTICLE

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RIG-I-mediated innate immune signaling in tumors reduces the therapeutic effect of oncolytic vesicular stomatitis virus

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Abstract

Background: Oncolytic viral therapy is a promising method for tumor treatment. Currently, several oncolytic viruses (OVs) have been used as tumor therapy at different phases of research and clinical trials. OVs not only directly lyse tumor cells due to viral replication but also initiate host antitumor immune responses. Previous studies have primarily focused on how OVs activate adaptive immune responses in immune cells. However, the role of innate immune responses in tumors induced by OVs remains unclear.

Methods: To determine the innate immune responses induced by vesicular stomatitis virus (VSV), the mutant VSV$^{\Delta M51}$ strain was used for the infection and quantitative polymerase chain reaction (qPCR) was employed to measure the transcriptional levels of antiviral genes. The knockdown efficiency of RIG-I was examined by qPCR. Viral titers were measured by plaque assays. Tumor models were established by intradermally implanting RIG-I-knockdown and control LLC cells into the flank of wild type C57BL/6J mice. When the tumors reached approximately 50mm^3, they were infected with VSV$^{\Delta M51}$ via intratumoral injections to examine its therapeutic effect.

Results: Infection with VSV$^{\Delta M51}$ triggered remarkable innate immune responses in several tumor cell lines through the cytoplasmic RIG-I sensing pathway. Moreover, we found that intratumoral injection of VSV$^{\Delta M51}$ effectively reduced tumor growth in murine LCC lung cancer model. Importantly, VSV$^{\Delta M51}$-induced antitumor therapy was more effective in murine LLC tumor model established using Rig-I-knockdown cells compared with the tumor model established using control cells.

Conclusion: RIG-I-mediated innate immune signaling in tumor cells plays a negative role in regulating antitumor therapy with VSV$^{\Delta M51}$ virus.

KEYWORDS
cancer therapy, innate immune, oncolytic vesicular stomatitis virus, RIG-I

INTRODUCTION

Oncolytic viral therapy has become a promising method for tumor treatment. Oncolytic viruses (OVs) are distinct agents that specifically replicate and target tumor cells. OVs not only directly kill tumor cells but also induce antitumor immune responses. OVs are generally divided into two types on the basis of their genetic material: DNA or RNA viruses. Oncolytic DNA viruses include herpes simplex virus 1 (HSV-1), oncolytic adenovirus (AdV), vaccinia virus (VACV), parvovirus, and fowlpox virus, whereas RNA viruses contain Newcastle disease virus (NDV), reovirus, vesicular stomatitis virus (VSV), coxsackie virus, Maraba virus, and measles virus. Oncolytic DNA viruses have high genome stability and larger genomes, which is beneficial for the genetic modification of OVs. In contrast, oncolytic RNA viruses have smaller genomes and limited genome packaging ability, but replicate rapidly; moreover, some are more immunogenic.[1] However, whether oncolytic DNA or RNA viruses have greater therapeutic effect for different tumors remains unclear.

Pengfei Zhang and Xinyu Han contributed equally to this work.

Nucleic acids derived from OVs are detected by pattern recognition receptors (PRRs), which consequently initiate innate immune responses that result in the production of type I interferons (IFNs) and proinflammatory cytokines. Different PRRs recognize RNA and DNA viruses. TLR9[2] and cytoplasmic DNA sensors such as DAI,[3] DDX41,[4] and cGAS[5] play important roles in DNA virus recognition; while TLR3/7/8,[6] RIG-I and MDA5[7] act as sensors of RNA viruses. Recent studies have shown that PRRs play important roles in antitumor immunity.[8,9] OVs induce innate immune responses in immune cells, which then produce a large number of cytokines and chemokines, that are involved in the activation, recruitment, proliferation and differentiation of immune cells. Meanwhile, normal somatic cells will quickly inhibit the expansion of OVs after activating innate immune responses.[10] This avoids the damage to normal somatic cells caused by OVs. Previous reports have shown that OVs replicate rapidly in tumors due to defects in innate immune responses in tumor cells.[11] However, other studies have shown that the innate immune responses in tumors affect the replication of OVs.[12,13] Thus, whether DNA sensors or RNA sensors in tumor cells can recognize OVs and induce innate immune responses remains controversial. Meanwhile, it is also unclear whether the activated innate immune responses inhibit the proliferation of viruses in tumors and subsequently affecting therapeutic efficacy.

VSV is a negative-strand RNA virus that encodes five viral proteins (N, P, M, G, and L). M protein inhibits host innate immune responses by inhibiting host expression of antiviral genes, such as type I IFNs. $VSV^{\Delta M51}$ is a VSV mutant strain carrying a single amino acid deletion (Met51) in the matrix protein, and thus has lost the inhibition of M protein on antiviral gene products.[14] $VSV^{\Delta M51}$ has been reported to function as an OV with preferential replication in colon cancer[15] and pancreatic cancer.[16] A previous study showed that $VSV^{\Delta M51}$ is recognized by TLR7 and can mediate the maturation and activation of plasmacytoid dendritic cells (pDCs).[17] Mice with a deficiency of MyD88, an important adaptor in TLR7 signaling, show significantly reduced therapeutic effect of OVs in tumors.[18] However, whether and how $VSV^{\Delta M51}$ induces innate immune responses in tumor cells and whether the activity of innate immune responses by $VSV^{\Delta M51}$ in tumor cells has effects on treatment efficacy remain unclear. Here, we demonstrate that RIG-I plays critical roles in recognizing $VSV^{\Delta M51}$ and initiating innate immune responses to inhibit $VSV^{\Delta M51}$ replication in tumor cell lines. More importantly, we found that RIG-I-mediated innate immune signaling in tumor cells reduced the therapeutic effects of $VSV^{\Delta M51}$.

METHODS

Ethics statements

All animal studies were carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China. The protocols for animal studies were approved by the Committee on the Ethics of Animal Experiments of the Institute of Zoology, Chinese Academy of Sciences (Beijing, China) (approval number: IOZ15001).

TABLE 1 The sequences of primers used for quantitative polymerase chain reaction

Gene	Forward	Reverse
Mouse Gapdh	AACTTTGGCATTGTGGAAGG	ACACATTGGGGGTAGGAACA
Mouse Rig-i	ATATGTGCCCCTACTGGT	TTGGTTAGCGAAGAAGAC
Mouse Ifnb1	ATGGTGGTCCGAGCAGAGAT	CCACCACTCATTCTGAGGCA
Mouse Il6	TCGGAGGCTTAATTACACATGTTCT	TGCCATTGCACAACTCTTTTCT
Mouse Isg15	TCCATGACGGTGTCAGAACT	GACCCAGACTGGAAAGGGTA
Mouse Ifit1	CAAGGCAGGTTTCTGAGGAG	GACCTGGTCACCATCAGCAT
Mouse Oas1	TGACCTGGTGGTGTTCCT	CAAACTTGACTCCAAAACGT
Mouse Viperin	ATAGTGAGCAATGGCAGCCT	AACCTGCTCATCGAAGCTGT
Human GAPDH	ATGACATCAAGAAGGTGGTG	CATACCAGGAAATGAGCTTG
Human IFNB1	AGGACAGGATGAACTTTGAC	TGATAGACATTAGCCAGGAG
Human RIG-I	GAAGAGTACCACTTAAACCCAG	TTGCCACGTCCAGTCAAT
Human IL6	GCCGCATCGCCGTCTCCTAC	GCCGCATCGCCGTCTCCTAC
Human ISG15	TCCTGGTGAGGAATAACAAGGG	GTCAGCCAGAACAGGTCGTC
Human IFIT1	TCAGGTCAAGGATAGTCTGGAG	AGGTTGTGTATTCCCACACTGTA
Human MX1	GTTTCCGAAGTGGACATCGCA	CTGCACAGGTTGTTCTCAGC
Human Viperin	TGGGTGCTTACACCTGCTG	GAAGTGATAGTTGACGCTGGTT
Human OAS1	GTGCTGCCTGCCTTTGAT	AACTCGCCCTCTTTCTGC
Human PKR	GCAAAATGGGACAGAAAG	TAGCAAAAGAACCAGAGGAC

Cell lines and viruses

A549, HeLa, LLC, A375, 293T, and Vero cells were cultured in DMEM supplemented with 10% FBS and 1% streptomycin and penicillin. H1299, MC38, HCT116, and B16-F10 cells were cultured in RPMI 1640 culture medium containing 10% FBS and 1% streptomycin and penicillin. Cells were infected with VSV$^{\Delta M51}$ at 1 MOI or HSV-1 at 10 MOI for 9 h. VSV$^{\Delta M51}$ or HSV-1 virus was propagated and titered by plaque assay in Vero cells.

Lentivirus-mediated knockdown of **RIG-I**

To generate RIG-I-knockdown cells, we used pLKO.1--puro-based lentiviruses expressing specific short hairpin RNAs (shRNAs) against *RIG-I*. A549, LLC, A375 or B16-F10 cells were infected with lentivirus targeting *RIG-I* (shRIG-I) or one control sequence (shControl) for 48 h. The cells were either untreated or infected with VSV$^{\Delta M51}$, followed by subsequent experiments. The knockdown efficiency was determined by quantitative polymerase chain reaction (qPCR). The shRNA sequences against the Control sequence, human or mouse *RIG-I* are as follows (5′–3′):

shControl: AACGTACGCGGAATACTTCGA;
human shRIG-I: AGCACTTGTGGACGCTTTAAA;
mouse shRig-I: ACTGGAACAGGTCGTTTATAA.

Quantitative **PCR** (**qPCR**)

Total RNA was isolated from cells utilizing TRIzol reagent. cDNA was synthesized using a HiScript III 1st Strand cDNA Synthesis Kit (Vazyme). qPCR was conducted in triplicate using SYBR Green Master Mix (Thermo Fisher) on a Bio-Rad CFX connect system. Relative levels of mRNA were normalized to the levels of GAPDH in each sample. The gene-specific primer sequences are shown in Table 1.

Tumor cell implantation and intratumoral injection with viruses

1×10^6 LLC cells in 0.1 ml 1× PBS were injected into the right flank of 6–8-week-old C57BL/6J female mice. When the tumors reached approximately 50 mm^3, the mice were treated with VSV$^{\Delta M51}$ via intratumoral injections. Mice were then treated on Day 0, 1, 3 either with 50 μl VSV$^{\Delta M51}$ (1×10^7 PFU) or PBS. Tumor sizes were

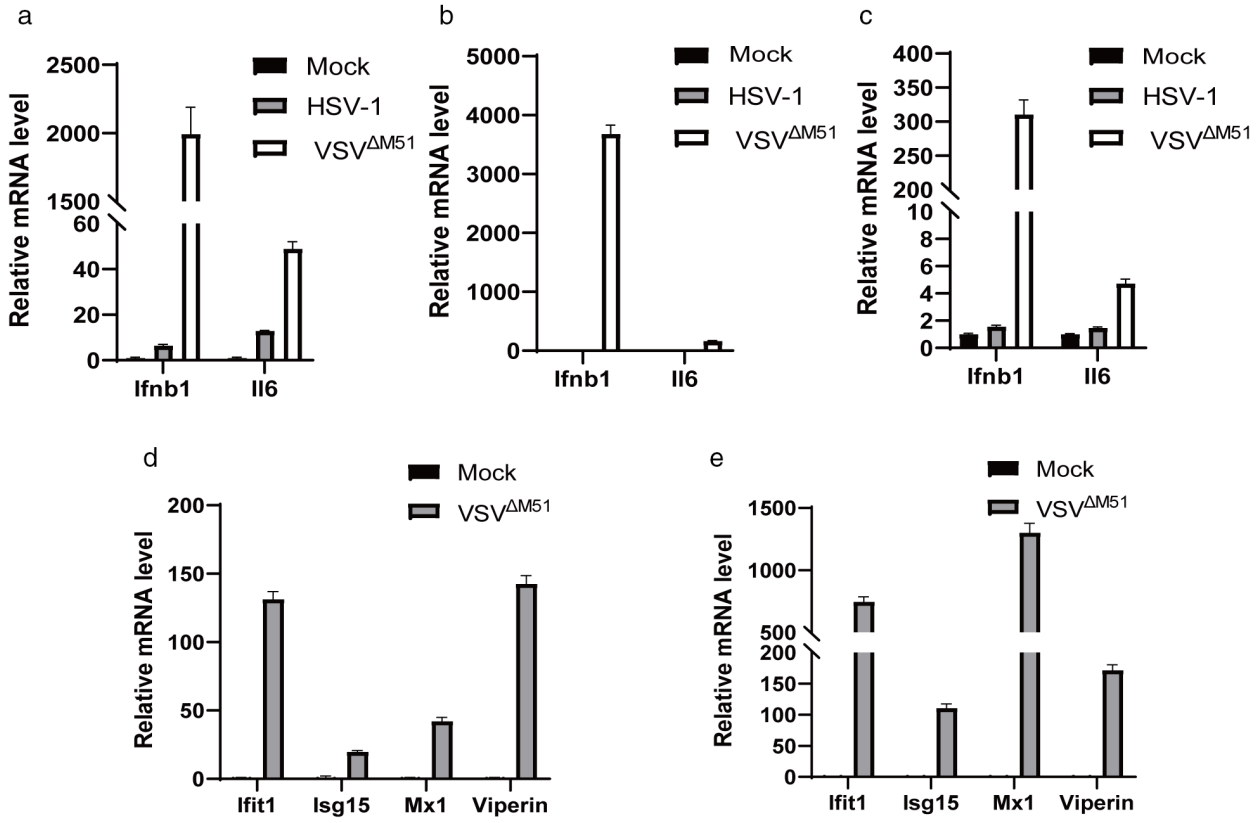

FIGURE 1 VSV$^{\Delta M51}$ induces innate immune responses in a variety of tumor cell lines. (a–c) LLC (a), B16-F10 (b), and MC38 (c) cells were infected with HSV-1 (10 MOI) or VSV$^{\Delta M51}$ (1 MOI) for 9 hours, respectively, followed by quantitative polymerase chain reaction (qPCR) to measure mRNA levels of *Ifnb1* and *Il6*. (d, e) LLC (d) and B16-F10 (e) cells were infected with VSV$^{\Delta M51}$(1 MOI) for 9 hours, followed by qPCR to measure mRNA levels of *Ifnt1*, *Isg15*, *Mx1*, and *Viperin*.

measured every other day after the start of treatment. The long (D) and the short diameter (d) were measured with a digital caliper. Tumor volume (mm^3) was determined as $V = d^2 \times D \times 0.5$.

Statistical analysis

All statistical data are presented as means ± SD, and the Student's t-test was used for all statistical analyses. For all tests, a p-value of less than 0.05 was considered statistically significant.

RESULTS

VSV$^{\Delta M51}$ induces innate immune responses in a variety of tumor cell lines

Because both VSV$^{\Delta M51}$ and HSV-1 viruses have been reported to function as OVs, we used both to determine whether they could induce innate immune responses in tumor cell lines. First, we infected murine Lewis lung cancer (LLC), B16-F10 melanoma, and MC38 colon adenocarcinoma cells with VSV$^{\Delta M51}$ or HSV-1, respectively, and then performed qPCR to measure the transcriptional levels of antiviral genes, such as *Ifnb1* and *Il6*. qPCR results showed that these two genes were remarkably induced by infection with VSV$^{\Delta M51}$ but not HSV-1 (Figure 1a–c). We also observed that the several IFN-stimulated genes (ISGs), such as *Ifit1*, *Isg15*, *Mx*1, and *Viperin*, were induced by VSV$^{\Delta M51}$ in LLC (Figure 1d), and B16-F10 cells (Figure 1e). Consistently, VSV$^{\Delta M51}$ induced higher expression of antiviral genes in several other human tumor cell lines, including lung cancer A549 (Figure S1A,F), melanoma A375 (Figure S1B,G), non-small cell lung cancer H1299 (Figure S1C), colon cancer HCT116 (Figure S1D) and cervical cancer HeLa (Figure S1E) cells. Together, these results show that OV VSV$^{\Delta M51}$ can induce innate immune responses in several tumor cell lines.

RIG-I is required for the innate immune responses induced by VSV$^{\Delta M51}$ infection in tumor cells

RIG-I functions as an RNA virus sensor and plays an important role in initiating innate immune responses induced by RNA viruses.[19,20] To determine whether RIG-I is required for VSV$^{\Delta M51}$-induced innate immune responses in tumor cells, we infected LCC (Figure 2a–c),

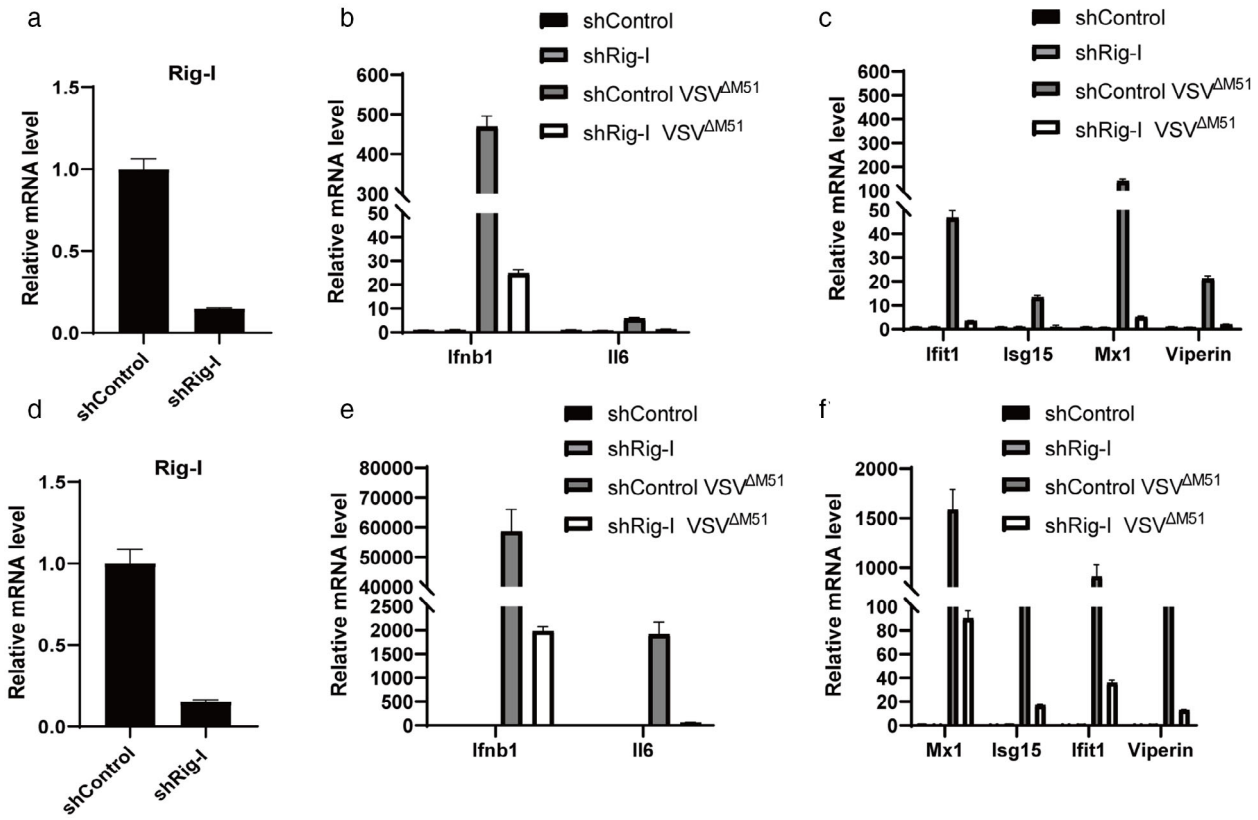

FIGURE 2 RIG-I is required for the innate immune responses induced by VSV$^{\Delta M51}$ infection in tumor cells. (a–c) LLC cells were transduced with shRNA targeting *Rig-I* or control sequence, and then infected with VSV$^{\Delta M51}$ (1 MOI) for 9 hours. The cells were harvested for qPCR analysis to measure the mRNA levels of *Rig-I* (a); *Ifnb1* and *Il6* (b); and *Ifit1*, *Isg15*, *Mx1 and Viperin* (c). (d–f) Similar to (a–c), except B16-F10 cells were used.

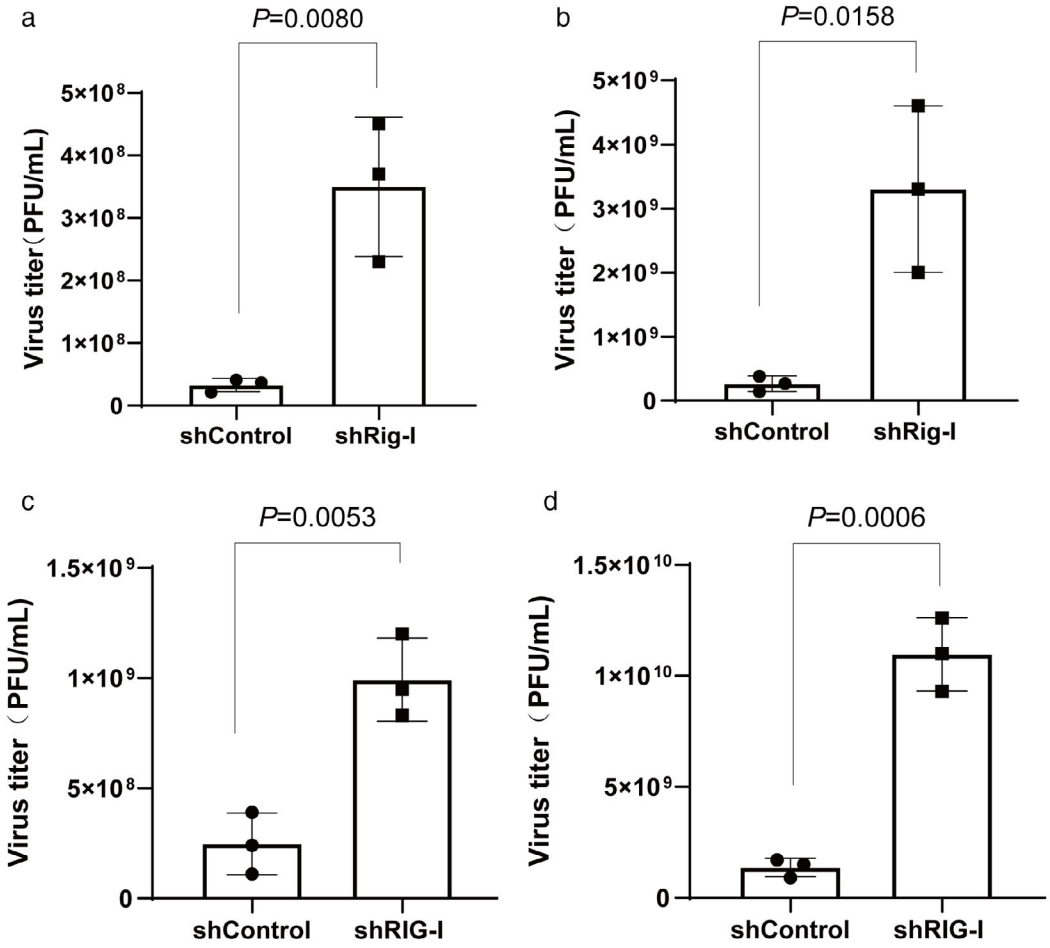

FIGURE 3 Knockdown of RIG-I enhances the replication of $VSV^{\Delta M51}$. (a–d) LLC (a), B16-F10 (b), A549 (c), and A375 (d) cells stably expressing shRNA targeting *Rig-I* or Control sequence were infected with $VSV^{\Delta M51}$ (0.0001 MOI) for 36 hours. The culture supernatants were harvested to measure the viral titer by plaque assays.

and B16-F10 (Figure 2d–f) with lentivirus expressing short hairpin RNAs (shRNAs) against murine *Rig-I* (shRig-I), or nontargeting control sequence (shControl). qPCR results showed that mRNA levels of *Rig-I* were significantly reduced in *Rig-I*-knockdown cells, compared with control cells (Figure 2a,d) and knockdown of *Rig-I* significantly reduced the mRNA levels of *Ifnb1*, *Il6*, *Ifit1*, *Isg15*, *Mx1* and *Viperin* induced by $VSV^{\Delta M51}$ in LLC (Figure 2b,c) and B16-F10 (Figure 2e,f) cells. Similar results were obtained in human A549 and A375 cells (Figure S2A–F). These results suggest that RIG-I is critical for triggering the innate immune responses induced by $VSV^{\Delta M51}$in tumor cells.

Knockdown of **RIG-I** enhances the replication of **$VSV^{\Delta M51}$**

The experiments described above indicated that $VSV^{\Delta M51}$ activated innate immune responses in tumor cells through RIG-I. However, previous reports have shown that innate immune responses cannot effectively inhibit OVs replication due to the abnormalities of innate immune responses in tumor cells.[21] Thus, we next compared the replication of $VSV^{\Delta M51}$ in RIG-I-knockdown and control tumor cell lines. Plaque assays showed that knockdown of RIG-I significantly increased $VSV^{\Delta M51}$ viral amplification in LLC (Figure 3a), B16-F10 (Figure 3b), A549 (Figure 3c), and A375 (Figure 3d) cells. These results indicate that RIG-I plays an important role in inducing antiviral immunity and inhibiting the replication of $VSV^{\Delta M51}$ in tumor cells.

Knockdown of **RIG-I** improves the therapeutic effect of **$VSV^{\Delta M51}$** in **LLC** tumor implantation models

To examine the effects of RIG-I-mediated innate immune responses in tumor cells on treatment with $VSV^{\Delta M51}$, we implanted Rig-I-knockdown and control LLC cells into C57BL/6J mice. When the tumor grew to approximately 50 mm^3, they were injected with 1×10^7 PFU $VSV^{\Delta M51}$, or the same volume of PBS as the control. First, we tested whether viral replication in tumor cells was accelerated after knocking down *Rig-I*. The viruses were injected into the

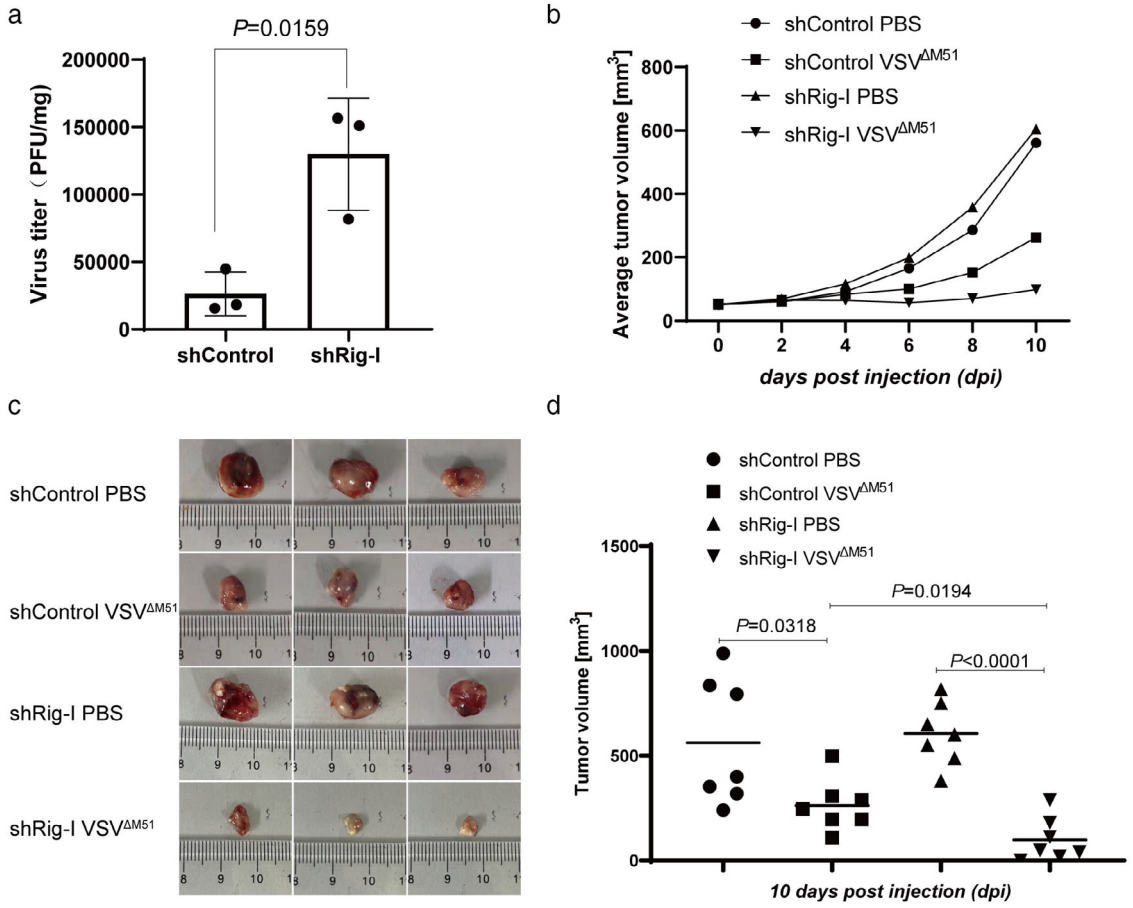

FIGURE 4 Knockdown of RIG-I improves the therapeutic effect of VSV$^{\Delta M51}$ in LLC tumor implantation models. (a) Rig-I-knockdown and control LLC cells were intradermally implanted into the shaved skin on the flank of wild-type C57BL/6J mice. When the tumor reached approximately 50 mm^3, they were infected with VSV$^{\Delta M51}$ (1×10^7 PFU) via intratumoral injection for 48 hours. The tumors were harvested to measure viral titer. (b) Similar to (a), tumors were implanted and injected with VSV$^{\Delta M51}$. Tumor volume was measured every 2 days, and the average tumor volume in mice ($n = 7$) is shown for the indicated times. (c, d) Similar to (b), tumors were harvested at on day 10 after injecting VSV$^{\Delta M51}$ (c), and tumor sizes were measured (d).

tumors for 48 h, after which the tumors were harvested to measure viral titers. The results showed that knockdown of *Rig-I* increased VSV$^{\Delta M51}$ replication in tumors (Figure 4a). Next, we injected the viruses into tumors (tumor size approximately 50 mm^3) on days 0, 1, and 3, and then measured tumor sizes every 2 days to compare the therapeutic effects of VSV$^{\Delta M51}$. As shown in (Figure 4b), VSV$^{\Delta M51}$ had therapeutic effects in both the implantation tumor models of control cells and Rig-I-knockdown cells. More importantly, we found that the tumor growth was remarkably inhibited in Rig-I-knockdown tumor models compared with in control models (Figure 4c,d). These results suggest that RIG-I-dependent signaling in tumor cells inhibits VSV$^{\Delta M51}$ replication during OV treatment and further inhibits the killing effect of VSV$^{\Delta M51}$ on tumors.

DISCUSSION

Because OVs can specifically attack tumor cells without damaging normal somatic cells, they have become one of the most promising methods for cancer therapy. Given that innate immune responses play important roles in tumor treatment with OVs, we first examined whether OVs HSV-1 and VSV$^{\Delta M51}$ induced innate immune responses in several tumor cell lines. qPCR results showed that innate immune responses were effectively induced by VSV$^{\Delta M51}$, but not HSV-1 in tumor cells, which was consistent with a previous study, which showed that human epithelial cancer cells preferentially respond to RNA viruses but not DNA viruses.[22] We also found that knockdown of RIG-I significantly reduced the expression of antiviral proteins, and subsequently accelerated VSV$^{\Delta M51}$ replication in tumor cell lines. These findings suggested that the innate immune responses in tumor cells inhibited the replication of OVs and potentially attenuating the efficacy of VSV$^{\Delta M51}$ therapy.

Previous studies have shown that RIG-I agonists play important roles in tumor treatment.[23] The combination of a RIG-I agonist with an immune checkpoint inhibitor can improve therapeutic efficacy.[24] Moreover, it has been reported that VSV virus with IFNβ can improve antitumor efficacy.[25] However, in this study, we established an LLC

tumor implantation model, treated it with $VSV^{\Delta M51}$, and found that $VSV^{\Delta M51}$ inhibited the growth of both control and Rig-I-knockdown LLC tumors. However, the reduction in tumor growth was much higher in the Rig-I-knockdown model compared with in the control model. We also found that the viral titer was much higher in the Rig-I-knockdown LLC tumor model than in the control model. These findings suggested that rapid replication of $VSV^{\Delta M51}$ in tumors is a critical step in its therapeutic effects. Additionally, we noted that different tumor cell lines had drastic differences in $VSV^{\Delta M51}$-induced innate immune responses. In this study, we found that RIG-I played a critical role in triggering the innate immune responses induced by $VSV^{\Delta M51}$ in tumor cells and reduced its therapeutic effect for tumor treatment. Previous studies have shown that TLRs, such as TLR7,[26] are also involved in regulating innate immune response against VSV infection. Whether TLRs are required for the innate immune responses induced by $VSV^{\Delta M51}$ in tumor cells, and whether TLR-mediated signaling influences the therapeutic effect of $VSV^{\Delta M51}$ need further investigation. Of note, we found that unlike $VSV^{\Delta M51}$, the DNA virus HSV-1 induced much weaker innate immune responses in our tested tumor cell lines. Whether HSV-1 triggers innate immune responses in other tumor cell lines remain unclear. Given that cGAS functions as an important sensor of DNA virus and is a double-edged sword for cancer therapy, it would be interesting to explore whether cGAS-mediated innate immunity in tumor cells impairs the antitumor effects of oncolytic DNA viruses.

In summary, RIG-I-mediated innate immune signaling in tumor cells plays a negative role in regulating tumor therapy with $VSV^{\Delta M51}$ virus. These results suggest that inhibition of innate immune responses in tumors could enhance antitumor treatment with OVs by augmenting OV replication.

AUTHOR CONTRIBUTIONS

Q.S. and D.C conceived, designed, and supervised the project. P.Z, X.H carried out experiments and analyzed data. D.C, Q.S and P.Z. wrote the manuscript, and W.T edited the manuscript.

CONFLICT OF INTEREST

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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