Review │ Received 15 December 2023; Accepted 27 May 2024; Published 7 June 2024
https://doi.org/10.55092/exrna20240008

Virus-derived small interfering RNAs serve as new intercellular immune modulators against viral infection in mammals

Shengkai Zhou[1,†], Ye Xu[1,†], Yu Zhou[2] and Fangfang Jin[3,*]

[1] State Key Laboratory of Pharmaceutical Biotechnology and Department of Physiology, Jiangsu Engineering Research Center for MicroRNA Biology and Biotechnology, NJU Advanced Institute of Life Sciences (NAILS), School of Life Sciences, Nanjing University, 163 Xianlin Avenue, Nanjing 210023, China

[2] Oriental Fortune Capital Post-Doctoral Innovation Center, Chinese Academy of Sciences, Shenzhen 518055, China

[3] School of Medicine & Holistic Integrative Medicine, Nanjing University of Chinese Medicine, Nanjing 210023, China

[†] These authors contributed equally to this work and should be considered co-first authors.

* Correspondence author; E-mail: ffjin@njucm.edu.cn.

Abstract: RNA silencing serves as the primary antiviral immune system in plants, fungi, and invertebrates. Upon virus invasion, its replication intermediates act as pathogen-associated molecular patterns (PAMPs), promptly recognized and processed by Dicer into siRNAs. These virus-derived small interfering RNAs (vsiRNAs) then guide specific cleavage of the viral genome. In mammalian cells, the presence of vsiRNAs has been difficult to detect. However, recent studies indicate that vsiRNA expression can be detected when viruses infect undifferentiated mammalian cells. These findings complement new antiviral mechanisms in mammalian cells, but also face several controversies. Therefore, we will briefly discuss the current research status of vsiRNAs in mammals and analyze the controversies existing in this field.

Keywords: virus-derived small interfering RNAs; antiviral immunity; mammal

1. Introduction

Viruses are non-cellular pathogens that engage in intracellular parasitism. Different hosts have evolved unique mechanisms to inhibit viral infection and spread. For mammals, innate immunity plays a central role in defense against invading viruses [1,2]. Within innate immunity, a limited set of germline-encoded immune receptors surveil the invasion of foreign pathogens. Upon viral disintegration, its constituents, acting as pathogen-associated

Copyright©2024 by the authors. Published by ELSP. This work is licensed under Creative Commons Attribution 4.0 International License, which permits unrestricted use, distribution, and reproduction in any medium provided the original work is properly cited.

molecular patterns (PAMPs), are rapidly recognized by the host's pattern recognition receptors (PRRs), triggering the production and release of type I interferon (IFN-I). Subsequently, IFN molecules activate multiple signaling cascades and initiate the transcription of immune-related effector genes, thereby exerting systemic antiviral immunity [3]. Currently, the pattern recognition receptors (PRRs) that are well- characterized primarily include Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs). In plants and invertebrates, the RNA interference (RNAi) system serves as a key defense mechanism against viral infections [4,5]. Following viral infiltration, these hosts undergo a significant increase in the production of virus-derived small interfering RNAs (vsiRNAs), characterized by distinct Dicer-mediated processing patterns. These vsiRNAs effectively target viral genomes, hindering the viral replication process. Furthermore, it's noteworthy that plant and invertebrate viruses often exhibit a preference for infecting hosts lacking a functional RNAi system, facilitating higher levels of replication [6,7].

In fact, it has been long recognized that a fully functional RNAi system exists in mammals. However, current understanding mainly revolves around its role in regulating mammalian gene expression and its potential therapeutic applications for targeting aberrant gene expression in disease treatment [8–10]. Early inquiries into whether the RNAi system elicits antiviral effects in mammals faced skepticism due to the difficulty in detecting functional virus-derived small RNA fragments. Nevertheless, recent investigations have unveiled the expression of vsiRNAs in mammals, indicating the potential existence of RNAi-mediated antiviral mechanisms [11,12]. In this article, we aim to comprehensively review the latest insights in this area, focusing on the discovery and immune function of vsiRNAs in mammals. Additionally, we will delve into the cell-intrinsic antiviral defense triggered by vsiRNAs and explore how vsiRNAs released into circulation act as immune molecules, triggering systemic antiviral immunity.

2. VsiRNAs-based antiviral immunity in plants and invertebrates

RNA silencing functions as a primary antiviral immune system in plants, fungi, and invertebrates. Hamilton *et al.* [13] first described this antiviral response in plants, and intensive studies have entered this field and gradually uncovered the mechanism of RNAi-based antiviral immunity [14–18]. When host cells are infected by a virus, viral components are disassembled and genomic nucleic acids are released. Episomal DNA/RNA genome turns on transcription or replication to synthesize other viral materials. RNA virus replication via forming long double-stranded RNA (dsRNA) replication intermediates which are immediately recognized and sliced by endoribonuclease (RNase) III Dicer into small RNAs (Figure 1). In plants, the model plant *Arabidopsis thaliana* encodes four Dicer-like proteins (DCL1 to DCL4), DCL2–4 processes long dsRNA into siRNAs with 22–24 nucleotides in length, separately [19]. whereas *Drosophila* has two distinct Dicers, Dicer-1(Dcr1) and Dicer-2(Dcr2) which engage in miRNAs and siRNAs biogenesis respectively [20], and vertebrates only encode one dicer. New-born viral dsRNAs were recognized by cellular dsRNA-binding proteins(DRB) such as DRB4 (in *Arabidopsis*) [21] or R2D2 (in *Drosophila*) [20]. DsRNA-

DRB complex determines the affinity of Dicer with dsRNAs and further facilitates Dicer processing of viral dsRNAs into small RNAs. These small RNAs are 21 to 23 nucleotides in length and share canonical siRNA features which are seen as vsiRNAs. Upon small RNAs generation including vsiRNAs, specific proteins will add some modifications to prevent its degradation. For example, *Arabidopsis* HEN1 methylates the 2' hydroxy group at the 3'-end of small RNAs [22]. Deep sequencing shows that AGO-bounded vsiRNAs in *Drosophila* are methylated at the 3'-end [23]. When generated by infected cells, vsiRNAs are subsequently loaded into the RNA-induced silencing complex (RISC) and guided specific cleave of the viral genome via base complementary pairing. The Argonaute (AGO) protein family are core components of RISC which exhibit high binding affinity to Dicer-processed small RNAs and are the effector molecules of the RNA silencing pathway which mediate directly catalytic cleaving of target sequence recognized by small RNA.

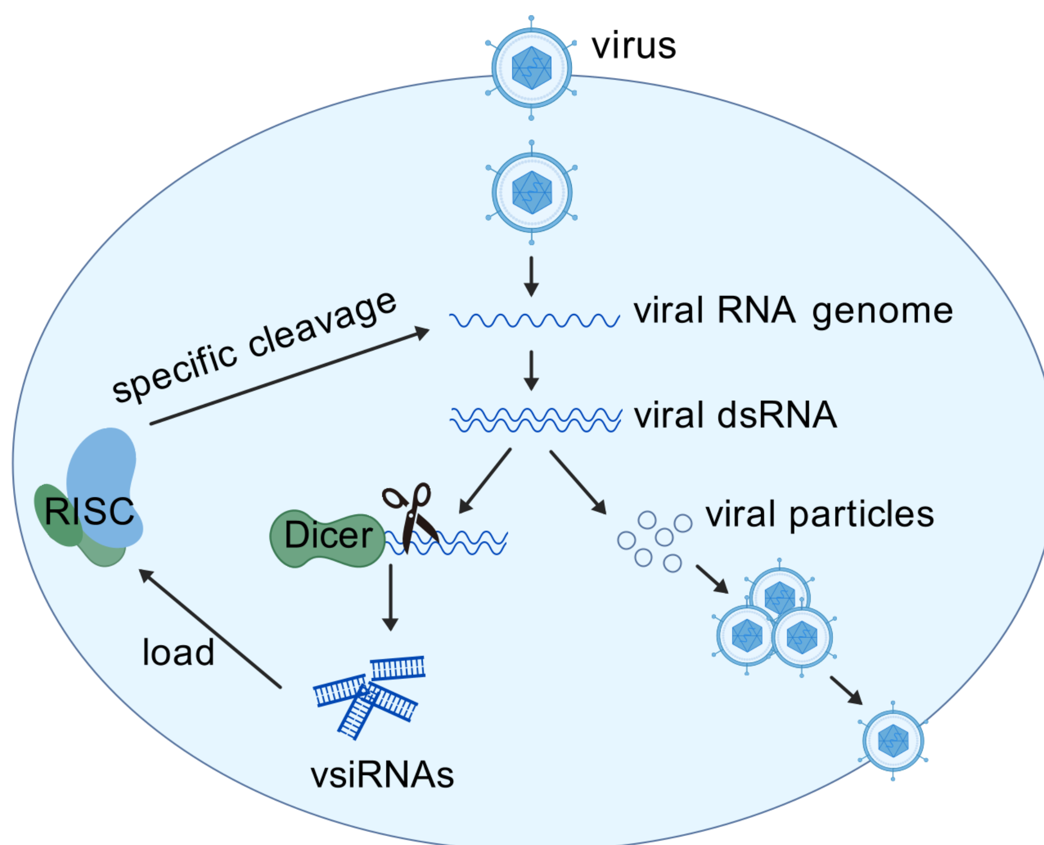

Figure 1. The mechanism of RNAi against viral infection.

RNA viruses invade cells and release their genomic nucleic acids for initial transcription, forming long double-stranded RNA replication intermediates (dsRNAs), which are promptly recognized and cleaved by dicer into vsiRNAs. These vsiRNAs load into RISC and guide specific cleavage of the viral genome via base complementary pairing to prevent virus replication.

To counteract this RNAi-based antiviral immunity, viruses have evolved some strategies to resist it. A main protection mechanism is to generate viral suppressors of RNA silencing (VSRs) which effectively suppress the RNAi pathway by inhibiting RNA sensing and slicing [24–26] or

preventing RISC assembly through binding to its components [27]. Until now, over 35 distinct VSR families have been found in plant virus types [28]. The P38 protein, a VSR of *Turnip crinkle virus* (TCV), could bind viral dsRNAs in a size-independent manner to protect them from DCLs processing [29]. A subsequent study showed that P38 can also interfere with AGO1-related networks to further disrupt DCLs activities in *Arabidopsis* [30]. The P19 protein, derived from *tombusviruses,* specifically prevents siRNA loading into RISC by forming a tail-to-tail homodimer [31,32]. VSR expression in invertebrates has also been reported, the B2 protein of FHV functions to prevent RNA silencing by binding to dsRNA and is essential for virus infection [16].

3. The discovery of vsiRNAs in mammals

While it's well-established that vsiRNAs play a crucial role in antiviral defense mechanisms in plants and invertebrates, a comparable phenomenon has long eluded observation in mammals. The major reason was that it's hard to detect virus-derived small RNAs (vsRNAs) with siRNA features during viral infection in mammalian cells. In 2008, Zhang *et al.* employed sequencing techniques and successfully identified several virus-derived miRNAs in infected mammalian cells, yet vsRNAs with low abundance remained elusive across various viruses [33]. Similarly, Parameswaran *et al.* [34] utilized six different viruses to infect diverse mammalian cell types but failed to identify a group of vsRNAs with specific sizes、distribution patterns or biochemical features like plant and invertebrate. Instead, vsRNAs in mammals predominantly arise as breakdown products of viral RNAs with no size preference [34–36].

Up until 2013, a pivotal breakthrough emerged from two studies, which documented, for the first time, the presence of vsiRNAs in mammals. These two studies revealed that dysfunction of VSR proteins from *Nodamura virus* (NoV) [11]and *Encephalomyocarditis virus* (EMCV) [12]induced the production of vsiRNAs in infected mouse embryonic stem cells (mESCs), baby hamster kidney 21 cells (BHK-21), and newborn mice. Deep sequencing unveiled that these vsiRNAs typically ranged from 21-bp to 23-bp, with a predominant 22-nt peak on both strands. These vsiRNAs exhibited characteristic Dicer-dependent processing features, including an approximately 20-nt perfectly base-paired region and a 2-nt 3' overhang. These seminal studies illuminated the RNAi-based antiviral immunity in mammals.

Subsequently, Benitez *et al.* engineered the *Influenza A virus* (IAV) by incorporating a siRNA sequence from the viral NS segment and successfully detected siRNA expression in infected A549 cells via Northern blotting [37]. Concurrently, Li *et al.* demonstrated that a VSR-deficient mutant of IAV triggered Dicer-dependent production of vsiRNAs in differentiated mammalian somatic cells [38]. The abundance of these vsiRNAs, equivalent to the total mature miRNA content (0.34% *vs.* 0.81% of total sequenced reads), was sufficient for cells to exert RNAi function. Notably, other vsiRNAs derived from various viruses have also been identified in mammalian cells. Qiu *et al.* identified a VSR protein 3A of *Human enterovirus 71* (HEV71) and observed that using a 3A-impaired mutant HEV71 readily induced the production of vsiRNAs in primary murine lung fibroblasts (MLFs), human rhabdomyosarcoma RD cells, and newborn mice [39]. In 2020, Qiu *et al.* discovered that

Dengue virus (DENV), a mosquito-borne *flavivirus*, could also induce vsiRNAs production in human somatic cells when its VSR was disabled [40]. Additionally, three independent studies detected vsiRNAs in human neural progenitors, neural stem cells, and mice during *Zika virus* (ZIKV) infection [41–43].

NoV, EMCV, HEV71, ZIKV, and DENV are all positive-strand RNA viruses from different families. Specifically, NoV belongs to the *Nodaviridae*, while EMCV and HEV71 belong to the *Picornaviridae*. On the other hand, ZIKV and DENV are classified under the *Flaviviridae*. IAV, in contrast, is a negative-strand RNA virus belonging to the *Orthomyxoviridae* (Table 1). Despite their taxonomic diversity, the vsiRNAs induced by these distinct viruses were readily detected in infected mammalian cells through deep sequencing or Northern blot hybridization. Remarkably, the relative abundance of these vsiRNAs bears similarity between insects and mammals. While, why were previous studies unable to detect vsiRNAs? There are several possible reasons. Firstly, most recent studies utilize virus strains lacking VSR proteins, enabling the successful detection of abundant vsiRNAs. Previous experiments might have employed wild-type virus strains, thereby suppressing vsiRNAs production [44,45]. Secondly, insufficient sequencing depth in previous experiments may have hindered the detection of sufficient vsiRNAs levels. Lastly, research suggests that products of RNase-L may mask the presence of vsiRNAs. For instance, Girardi *et al.* detected numerous vsRNAs lacking distribution patterns (mostly RNase-L products) upon Sindbis virus (SINV) infection in 293T cells [46]. Li *et al.* reanalyzed Girardi's data after optimizing algorithms to exclude RNase-L products and uncovered abundant 22-nt vsiRNAs. Furthermore, they found that knocking down RNase-L facilitated the detection of vsiRNAs [47]. It's worth noting that previous studies were conducted in differentiated mammalian cells, where *in vitro* experiments may activate RNase-L, leading to the coverage of vsiRNAs by abundant RNase L products, rendering them undetectable.

Table 1. Validated virus to induce vsiRNAs generation in mammals.

Viral genome	Virus name	Family	VSR	Refs
(+)RNA	Nodamura virus	*Nodaviridae*	B2	[11]
	Encephalomyocarditis virus	*Picornaviridae*	ND	[12]
	Enterovirus-A71	*Picornaviridae*	3A	[39]
	Zika virus	*Flaviviridae*	ND	[41–43]
	Dengue virus 2	*Flaviviridae*	NS2A	[40]
(-)RNA	Influenza A virus	*Orthomyxoviridae*	NS1	[37]

(+)RNA, positive-strand RNA; (-)RNA, negative-strand RNA; ND, not determined

While the generation of vsiRNAs in mammals remains a topic of debate, an increasing number of studies have indeed successfully detected their expression, a non-random phenomenon.These findings imply that the production of vsiRNAs is not exclusive to particular viruses but rather linked to the formation of long dsRNA structures by the infecting virus. Moreover, the substantial abundance of vsiRNAs underscores their potential significance in mediating RNAi-based silencing pathways within mammalian cells. Taken together, these observations suggest that the Dicer-dependent processing of viral dsRNA into vsiRNAs may represent a conserved mechanism employed by mammalian cells in response to viral infection.

4. Cell-intrinsic immune function of vsiRNAs and their relationship with the IFN pathway

With the discovery of vsiRNAs in mammals, various groups started to investigate whether these vsiRNAs can effectively inhibit viral replication in mammals. Li *et al.* respectively infected BHK-21 cells and suckling mice with wild-type NoV strain and NoV-B2 protein mutant strain (NoVΔB2) [11]. The results demonstrated a significant reduction in infection activity both *in vitro* and *in vivo* for NoVΔB2. Suckling mice exhibited resistance to NoVΔB2, surviving for up to 4 weeks post-infection with a 100% mortality rate observed in the wild-type NoV infection, suggesting an enhanced immunity against the mutant strain. Meanwhile, Maillard *et al.* infected mESCs with EMCV and subsequently utilized immunoprecipitation to enrich small RNAs bound to AGO2, revealing the capture of vsiRNAs [41]. These findings collectively imply the involvement of vsiRNAs in mammals' RNAi pathway, potentially serving as an effective mechanism to combat viral replication.

Although the studies mentioned above establish the effectiveness of vsiRNAs in inhibiting viral replication in mammals, these findings were predominantly derived from undifferentiated cells lacking a mature IFN response pathway. In mature mammalian cells, the IFN response has long been recognized as a direct antiviral mechanism. Therefore, the interplay between the IFN response and RNAi-based antiviral mechanisms has become a focal point of interest.

In fact, Maillard *et al.*'s early work revealed a significant reduction in EMCV-derived siRNAs as mESCs differentiated, suggesting a shift in their antiviral mechanisms during mammalian cell differentiation [12]. Flemr *et al.*'s study identified an isoform of Dicer in mouse oocytes lacking the N-terminal helicase domain present in the full-length Dicer [48]. This isoform of Dicer exhibits enhanced cleavage efficiency of long dsRNA compared to the full-length Dicer. Bryan Cullen *et al.*'s work further confirmed this finding by constructing a human Dicer protein lacking the N-terminal helicase domain, demonstrating a 3.39-fold increase in cleavage efficiency of long dsRNA compared to full-length Dicer [49]. This partially explains the reduction in vsiRNAs production observed in differentiating mammalian cells. Similarly, in somatic cells, viral disassembly products appear to be more readily recognized by the RIG-I family, leading to rapid activation of downstream interferon-stimulated genes（ISGs）expression and exerting antiviral effects. In studies on ISGs, Seo *et al.* discovered that increased expression of certain ISGs may suppress RISC

activity [50]. Additionally, Veen *et al.* suggested that mammalian somatic cells can express IFN-inducible proteins such as LGP2, which inhibit Dicer slicing and consequently hinder the RNAi-based antiviral response in the presence of an intact IFN system [51]. This suggests that the RNAi-based antiviral response seems to serve as an early defense mechanism employed by mammals against viral invasion. However, as the organism matures, the IFN response gradually takes precedence over RNAi in antiviral defense.

To investigate whether RNAi-based antiviral response in somatic cells is replaced or masked by the IFN response, Maillar *et al.* constructed an IFN-deficient cell line by deleting Mitochondrial antiviral-signaling protein (MAVS) [52]. MAVS is a key protein that triggers downstream production and secretion of IFN-α and β by binding with PRRs, thereby initiating IFN signaling cascade. By transfecting long dsRNA into Mavs-/- MEFs to simulate virus invasion, Maillar *et al.* found that these "vaccinated" mammalian somatic cells show a comparable antiviral resistance in response to homologous virus infection. This suggests that in somatic cells, when the IFN response is absent, they can still employ RNAi-based antiviral mechanisms to combat virus invasion, whereas under normal conditions, RNAi-based antiviral response is masked by the IFN response. Meanwhile, Li *et al.* devised a novel approach to confirm the presence of vsiRNAs in mammalian somatic cells [38]. It is established that vsiRNAs generation relies on Dicer cleavage of viral RNA intermediates, with AGOs selectively associating with Dicer products. Through Argonaute co-immunoprecipitation (co-IP) from Influenza A virus (IAV) infected mammalian somatic cells, Li *et al.* successfully identified vsiRNAs expression via deep sequencing. Further investigations utilized Ago2 catalytic-deficient primary mouse embryonic fibroblasts (Ago2D597A), revealing a significant elevation in virus levels in Ago2D597A cells compared to wild-type cells post-IAV infection. Subsequent analyses indicated comparable levels of type I IFN and ISG expression in wild-type and Ago2D597A MEFs following IAV infection, suggesting an IFN-independent antiviral response mediated by vsiRNAs in somatic cells. Subsequent studies have also demonstrated the phenomenon of RNAi-mediated antiviral activity in mature cells by pre-inoculating VSR-deficient virus *in vivo* and assess the rescue of viral replication after infecting recombinant virus containing virus fragments [39,42,53].

5. Secreted vsiRNAs for immune function

When cells undergo infection or pathological changes, they often release various factors to prompt the organism to counteract adverse effects. Extracellular vesicles serve as pivotal mediators for intercellular material exchange and communication in this process [54]. These vesicles, characterized by small membrane structures, encapsulate a diverse cargo of proteins, lipids, and nucleic acids [55]. Extracellular vesicles can be secreted by a variety of cell types and remain stably present in various circulating body fluids. During viral infections, the organism can also utilize extracellular vesicles to transport viral materials to other cells, triggering an immune response [56, 57]. Kouwaki *et al.* conducted a comprehensive review on how virus-derived miRNAs (v-miRs) are encapsulated into exosomes and recognized by immune cells, leading to the induction of innate immune responses [58]. For instance,

HCV can associate with exosomes via CD81 and release its RNA into them [59]. These RNA-containing exosomes are then internalized by macrophages and dendritic cells (DCs), resulting in the production of type I interferons (IFNs). Knocking down ESCRT-I or -III complex, key components of exosome biogenesis, in infected cells attenuates IFN production. Similarly, Naqvi *et al.* reviewed the immune function of human herpesvirus-encoded miRNAs in mammals [60]. These v-miRs not only target immune-associated genes in host cells but also exploit host exosome pathways to facilitate viral immune evasion. These findings confirm that vsRNAs can be delivered by exosomes to exert cell-extrinsic immunomodulatory functions in mammals.

RNAi-mediated antiviral immunity has been observed to spread from cell to cell or through cross-species interactions, as reported in plants and worms. Tassetto *et al.* demonstrated a similar phenomenon in their study, where macrophage-like haemocytes were observed to uptake virus-derived double-stranded RNA (dsRNA) and secrete vsiRNAs in exosome-like vesicles (ELVs), initiating systemic antiviral immunity [61]. In brief, haemocytes take up viral RNA released by infected cells and synthesize viral cDNA via endogenous reverse transcriptases. These viral cDNAs act as templates for the production of new vsiRNAs, which are subsequently encapsulated and secreted into ELVs, facilitating the spread of virus-specific immunity to other healthy cells.

Recently, a similar phenomenon has been reported in mammal. Zhang *et al.* infected suckling mice with a B2-deficient mutant of NoV, as they had done previously, and successfully detected vsiRNAs in mouse serum [62]. Interestingly, they observed that vsiRNAs were approximately 60-fold enriched in exosomes compared to serum (2.8% *vs.* 0.05% of the total). Furthermore, they tested other viruses known to induce vsiRNAs production in mammalian cells, such as SINV and ZIKV, by infecting C57BL/6 or BALB/c infant mice. Similar results were obtained, indicating a high accumulation of vsiRNAs in exosomes. This suggests that abundant vsiRNAs can be secreted into exosomes and transported through the bloodstream in mice following viral infection. To investigate whether secreted vsiRNAs enriched in exosomes can confer antiviral protection, the authors pretreated cells with purified vsiRNAs-exosomes from virus-infected infant mice and observed a reduction in virus abundance. Additionally, luciferase reporter experiments, in which the NoV genome frame targeted by vsiRNAs from NoVΔB2-infected infant mice was inserted into the 3’ untranslated region (UTR) of the luciferase reporter gene, revealed that exosomes purified from NoVΔB2-infected infant mice significantly suppressed luciferase expression. This effect was not observed with mock exosomes or in an Ago2 knockout cell line. Finally, *in vivo* experiments demonstrated that injection of vsiRNAs-containing exosomes significantly reduced viral RNA accumulation in both wild-type and interferon (IFN)-deficient mice.

Previous studies suggested that cells infected by viruses are in a hijacked state, with their secreted factors potentially favoring the spread of the virus itself [63–65]. The host often maintains homeostasis by eliminating infected cells. However, Zhang's research has revealed that infected cells can also transmit vsiRNAs to healthy cells via exosomes (Figure 2). This transfer equips healthy cells with antiviral capabilities in advance, thus impeding viral

dissemination. This discovery significantly advances our comprehension of the antiviral immune mechanisms in mammals.

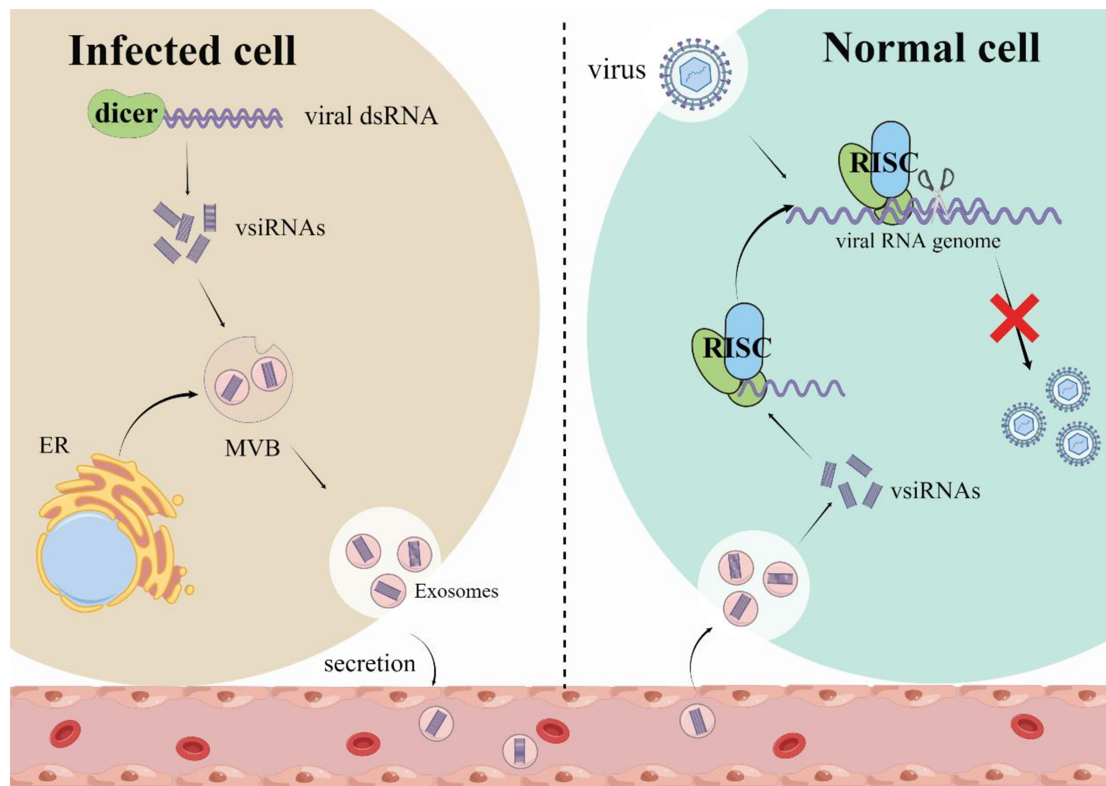

Figure 2. Secreted vsiRNAs for cell-extrinsic immune function.

VsiRNAs produced by infected cells are secreted into the bloodstream encapsulated within exosomes. These exosomes, containing vsiRNAs, disseminate and are taken up by neighboring cells. Upon uptake, the vsiRNAs are released into the cytosol, where they exert their antiviral immune function through RISC-mediated specific cleavage of invading viral genomes.

6. Conclusions

RNAi-based antiviral immunity is highly conserved across eukaryotes. Upon virus invasion of host cells, double-stranded RNA (dsRNA) replication intermediates act as PAMPs, triggering recognition by Dicer. Dicer promptly processes these dsRNAs into small interfering RNAs (siRNAs), which are then incorporated into the AGO effector complex. Subsequently, these vsiRNAs guide the specific cleavage of the viral genome. In mammals, the interferon (IFN) response is considered the primary antiviral mechanism, but recent research has revealed that RNAi-based antiviral mechanisms also exist in mammals. The relationship between the IFN response and RNAi-mediated antiviral mechanisms is not yet fully understood. Some studies suggest that RNAi-based antiviral mechanisms serve as a supplementary system to the IFN response, playing a predominant role during early mammalian development. However, as the IFN system matures, the antiviral processes mediated by RNAi are suppressed.

Furthermore, the RNAi-based antiviral effects offer a new therapeutic approach for early-stage viral infections, making "siRNA vaccines" a promising possibility. Compared to antibody proteins, siRNA vaccines offer faster and higher specificity, theoretically capable of targeting all viral genomes to inhibit their replication and exerting comprehensive antiviral effects in the early stages of infection. However, the challenges of delivery difficulties and off-target effects associated with siRNA drugs need to be carefully considered. Additionally, further research is needed to address how to mitigate the inhibitory effects of the IFN system on RNAi-mediated antiviral responses in somatic cells. Further assessment is also required to evaluate the timing of its efficacy.

Although studies have successfully revealed the antiviral role of vsiRNAs in mammals, debates and challenges persist in this field [66–68]. Firstly, this antiviral response appears to be inconsistent. Tsal *et al.* found that Influenza A virus-derived siRNAs failed to inhibit virus replication in human 293T cell lines, despite previous effectiveness [69]. Similarly, Schuster *et al.* observed no enhanced viral susceptibility in AGO2-knockout MEFs compared to controls, across three different viruses [70]. Additionally, questions remain regarding the adequacy of vsiRNA abundance secreted into non-infected cells for defense against virus invasion. While studies indicate that vsiRNAs can be secreted into the bloodstream to aid non-infected cells in viral defense, these findings rely on artificially purified and enriched vsiRNAs. Further investigation is warranted to determine whether secreted vsiRNAs can effectively mount an antiviral response under natural conditions, and whether insect-like vsiRNA amplification mechanisms exist in mammals. Moreover, the impact of the IFN pathway on vsiRNA production, as well as how mammalian cells balance the RNAi and IFN antiviral mechanisms, remain unclear. There is an urgent need for improved infection models to comprehensively explore the molecular characteristics and biological function mechanisms involved.

In summary, the discovery of RNAi-based antiviral immunity has contributed to a better understanding of the mammal immune system in response to viral infection. It may act as an alternative antiviral mechanism in mammals when IFN pathways are immature or impaired.

Acknowledgments

The authors sincerely appreciate all the people who provided assistance and support for this project. The figures were created using Figdraw and Generic Diagramming Platform (www.figdraw.com/gdp.renlab.cn).

Conflicts of interests

The authors declare no conflicts of interest.

Authors' contribution

Conception and design: F.J.; Writing and revision of the manuscript: S.Z.; validation: Y.X.; Editing: Y.Z. All authors have read and agreed to the published version of the manuscript.

References

[1] Takeuchi O, Akira S. Innate immunity to virus infection. *Immunol. Rev*. 2009, 227(1):75–86.

[2] Hu MM, Shu HB. Cytoplasmic Mechanisms of Recognition and Defense of Microbial Nucleic Acids. *Annu. Rev. Cell Dev. Biol.* 2018, 34:357–379.

[3] Schneider WM, Chevillotte MD, Rice CM. Interferon-stimulated genes: a complex web of host defenses. *Annu. Rev. Immunol.* 2014, 32:513–545.

[4] Ding SW, Voinnet O. Antiviral immunity directed by small RNAs. *Cell* 2007, 130(3):413–426.

[5] Bronkhorst AW, van Rij RP. The long and short of antiviral defense: small RNA-based immunity in insects. *Curr. Opin. Virol*. 2014, 7:19–28.

[6] Ding SW. RNA-based antiviral immunity. *Nat. Rev. Immunol.* 2010, 10(9):632–644.

[7] Samuel GH, Adelman ZN, Myles KM. Antiviral Immunity and Virus-Mediated Antagonism in Disease Vector Mosquitoes. *Trends Microbiol*. 2018, 26(5):447–461.

[8] Sparmann A, Vogel J. RNA-based medicine: from molecular mechanisms to therapy. *EMBO J.* 2023, 42(21):e114760.

[9] Setten RL, Rossi JJ, Han SP. The current state and future directions of RNAi-based therapeutics. *Nat. Rev. Drug Discov.* 2019;18(6):421–446.

[10] Jadhav V, Vaishnaw A, Fitzgerald K, Maier MA. RNA interference in the era of nucleic acid therapeutics. *Nat. Biotechnol*. 2024, 42(3):394–405.

[11] Li Y, Lu J, Han Y, Fan X, Ding SW. RNA interference functions as an antiviral immunity mechanism in mammals. *Science* 2013, 342(6155):231–234.

[12] Maillard PV, Ciaudo C, Marchais A, Li Y, Jay F, *et al.* Antiviral RNA interference in mammalian cells. *Science* 2013, 342(6155):235–238.

[13] Hamilton AJ, Baulcombe DC. A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* 1999, 286(5441):950–952.

[14] Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* 2000, 404(6775):293–296.

[15] Li H, Li WX, Ding SW. Induction and suppression of RNA silencing by an animal virus. *Science* 2002, 296(5571):1319–1321.

[16] Wang XH, Aliyari R, Li WX, Li HW, Kim K, *et al.* RNA interference directs innate immunity against viruses in adult Drosophila. *Science* 2006, 312(5772):452–454.

[17] Wang XB, Wu Q, Ito T, Cillo F, Li WX, *et al.* RNAi-mediated viral immunity requires amplification of virus-derived siRNAs in *Arabidopsis thaliana. Proc. Natl. Acad. Sci. U. S. A*. 2010, 107(1):484–489.

[18] Zhang X, Segers GC, Sun Q, Deng F, Nuss DL. Characterization of hypovirus-derived small RNAs generated in the chestnut blight fungus by an inducible DCL-2-dependent pathway. *J. Virol*. 2008, 82(6):2613–2619.

[19] Brodersen P, Voinnet O. The diversity of RNA silencing pathways in plants. *Trends Genet*. 2006, 22(5):268–280.

[20] Hammond SM. Dicing and slicing: the core machinery of the RNA interference pathway. *FEBS Lett.* 2005, 579(26):5822–5829.

[21] Vaucheret H. Post-transcriptional small RNA pathways in plants: mechanisms and regulations. *Genes Dev*. 2006, 20(7):759–771.

[22] Chen X. A marked end. *Nat. Struct. Mol. Biol*. 2007,14(4):259–260.

[23] Aliyari R, Wu Q, Li HW, Wang XH, Li F, *et al.* Mechanism of induction and suppression of antiviral immunity directed by virus-derived small RNAs in Drosophila. *Cell Host Microbe* 2008, 4(4):387–397.

[24] Mérai Z, Kerényi Z, Molnár A, Barta E, Válóczi A, *et al.* Aureusvirus P14 is an efficient RNA silencing suppressor that binds double-stranded RNAs without size specificity. *J. Virol*. 2005, 79(11):7217–7226.

[25] Love AJ, Laird J, Holt J, Hamilton AJ, Sadanandom A, *et al.* Cauliflower mosaic virus protein P6 is a suppressor of RNA silencing. *J. Gen. Virol*. 2007, 88(Pt 12):3439–3444.

[26] Mérai Z, Kerényi Z, Kertész S, Magna M, Lakatos L, *et al.* Double-stranded RNA binding may be a general plant RNA viral strategy to suppress RNA silencing. *J. Virol.* 2006, 80(12):5747–5756.

[27] Giner A, Lakatos L, García-Chapa M, López-Moya JJ, Burgyán J. Viral protein inhibits RISC activity by argonaute binding through conserved WG/GW motifs. *PLoS Pathog*. 2010, 6(7):e1000996.

[28] Li F, Ding SW. Virus counterdefense: diverse strategies for evading the RNA-silencing immunity. *Annu. Rev. Microbiol*. 2006, 60:503–531.

[29] Deleris A, Gallego-Bartolome J, Bao J, Kasschau KD, Carrington JC, *et al.* Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense. *Science* 2006, 313(5783):68–71.

[30] Azevedo J, Garcia D, Pontier D, Ohnesorge S, Yu A, *et al.* Argonaute quenching and global changes in Dicer homeostasis caused by a pathogen-encoded GW repeat protein. *Genes Dev*. 2010, 24(9):904–915.

[31] Vargason JM, Szittya G, Burgyán J, Hall TM. Size selective recognition of siRNA by an RNA silencing suppressor. *Cell*. 2003;115(7):799-811.

[32] Ye K, Malinina L, Patel DJ. Recognition of small interfering RNA by a viral suppressor of RNA silencing. *Nature* 2003, 426(6968):874–878.

[33] Zhang X, Segers GC, Sun Q, Deng F, Nuss DL. Characterization of hypovirus-derived small RNAs generated in the chestnut blight fungus by an inducible DCL-2-dependent pathway. *J. Virol.* 2008, 82(6):2613–2619.

[34] Parameswaran P, Sklan E, Wilkins C, Burgon T, Samuel MA, *et al.* Six RNA viruses and forty-one hosts: viral small RNAs and modulation of small RNA repertoires in vertebrate and invertebrate systems. *PLoS Pathog*. 2010, 6(2):e1000764.

[35] Grundhoff A, Sullivan CS. Virus-encoded microRNAs. *Virology* 2011, 411(2):325–343.

[36] Weng KF, Hung CT, Hsieh PT, Li ML, Chen GW, *et al.* A cytoplasmic RNA virus generates functional viral small RNAs and regulates viral IRES activity in mammalian cells. *Nucleic Acids Res*. 2014, 42(20):12789–12805.

[37] Benitez AA, Spanko LA, Bouhaddou M, Sachs D, tenOever BR. Engineered Mammalian RNAi Can Elicit Antiviral Protection that Negates the Requirement for the Interferon Response. *Cell Rep*. 2015, 13(7):1456–1466.

[38] Li Y, Basavappa M, Lu J, Dong S, Cronkite DA, *et al.* Induction and suppression of antiviral RNA interference by influenza A virus in mammalian cells. *Nat. Microbiol*. 2016, 2:16250.

[39] Qiu Y, Xu Y, Zhang Y, Zhou H, Deng YQ, *et al.* Human Virus-Derived Small RNAs Can Confer Antiviral Immunity in Mammals. *Immunity* 2018;49(4):780–781.

[40] Qiu Y, Xu YP, Wang M, Miao M, Zhou H, *et al.* Flavivirus induces and antagonizes antiviral RNA interference in both mammals and mosquitoes. *Sci. Adv*. 2020, 6(6):eaax7989.

[41] Zeng J, Luo Z, Dong S, Xie X, Liang X, *et al.* Functional Mapping of AGO-Associated Zika Virus-Derived Small Interfering RNAs in Neural Stem Cells. *Front. Cell. Infect. Microbiol*. 2021, 11:628887.

[42] Zhang Y, Li Z, Ye Z, Xu Y, Wang B, *et al.* The antiviral RNA interference not only exists in neural progenitor cells but also in somatic cells in mammals. *Emerg. Microbes Infect*. 2020, 9(1):1580–1589.

[43] Xu YP, Qiu Y, Zhang B, Chen G, Chen Q, *et al.* Zika virus infection induces RNAi-mediated antiviral immunity in human neural progenitors and brain organoids. *Cell Res*. 2019, 29(4):265–273.

[44] Díaz-Pendón JA, Ding SW. Direct and indirect roles of viral suppressors of RNA silencing in pathogenesis. *Annu. Rev. Phytopathol*. 2008, 46:303–326.

[45] Wu Q, Wang X, Ding SW. Viral suppressors of RNA-based viral immunity: host targets. *Cell Host Microbe* 2010, 8(1):12–15.

[46] Girardi E, Chane-Woon-Ming B, Messmer M, Kaukinen P, Pfeffer S. Identification of RNase L-dependent, 3'-end-modified, viral small RNAs in Sindbis virus-infected mammalian cells. *mBio* 2013, 4(6):e00698-13.

[47] Wang J, Li Y. Current advances in antiviral RNA interference in mammals. *FEBS J*. 2024, 291(2):208–216.

[48] Flemr M, Malik R, Franke V, Nejepinska J, Sedlacek R, *et al.* A retrotransposon-driven dicer isoform directs endogenous small interfering RNA production in mouse oocytes. *Cell* 2013, 155(4):807–816.

[49] Kennedy EM, Whisnant AW, Kornepati AV, Marshall JB, Bogerd HP, *et al.* Production of functional small interfering RNAs by an amino-terminal deletion mutant of human Dicer. *Proc. Natl. Acad. Sci. U. S. A*. 2016, 113(12):E6547.

[50] Seo GJ, Kincaid RP, Phanaksri T, Burke JM, Pare JM, *et al.* Reciprocal inhibition between intracellular antiviral signaling and the RNAi machinery in mammalian cells. *Cell Host Microbe* 2013, 14(4):435–445.

[51] van der Veen AG, Maillard PV, Schmidt JM, Lee SA, Deddouche-Grass S, *et al.* The RIG-I-like receptor LGP2 inhibits Dicer-dependent processing of long double-stranded RNA and blocks RNA interference in mammalian cells. *EMBO J*. 2018, 37(4):e97479.

[52] Maillard PV, Van der Veen AG, Deddouche-Grass S, Rogers NC, Merits A, *et al.* Inactivation of the type I interferon pathway reveals long double-stranded RNA-mediated RNA interference in mammalian cells. *EMBO J*. 2016, 35(23):2505–2518.

[53] Zhang Y, Xu Y, Dai Y, Li Z, Wang J, *et al.* Efficient Dicer processing of virus-derived double-stranded RNAs and its modulation by RIG-I-like receptor LGP2. *PLoS Pathog*. 2021, 17(8):e1009790.

[54] Théry C, Witwer KW, Aikawa E, Alcaraz MJ, Anderson JD, *et al.* Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell. Vesicles* 2018, 7(1):1535750.

[55] Colombo M, Raposo G, Théry C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu. Rev. Cell Dev. Biol*. 2014, 30:255–289.

[56] Kouwaki T, Okamoto M, Tsukamoto H, Fukushima Y, Oshiumi H. Extracellular Vesicles Deliver Host and Virus RNA and Regulate Innate Immune Response. *Int. J. Mol. Sci.* 2017, 18(3):666.

[57] Buzas EI. The roles of extracellular vesicles in the immune system. *Nat. Rev. Immunol.* 2023, 23(4):236–250.

[58] Kouwaki T, Fukushima Y, Daito T, Sanada T, Yamamoto N, *et al.* Extracellular Vesicles Including Exosomes Regulate Innate Immune Responses to Hepatitis B Virus Infection. *Front. Immunol*. 2016, 7:335.

[59] Hsu M, Zhang J, Flint M, Logvinoff C, Cheng-Mayer C, *et al.* Hepatitis C virus glycoproteins mediate pH-dependent cell entry of pseudotyped retroviral particles. *Proc. Natl. Acad. Sci. U. S. A.* 2003, 100(12):7271–7276.

[60] Naqvi AR. Immunomodulatory roles of human herpesvirus-encoded microRNA in host-virus interaction. *Rev. Med. Virol*. 2020, 30(1):e2081.

[61] Tassetto M, Kunitomi M, Andino R. Circulating Immune Cells Mediate a Systemic RNAi-Based Adaptive Antiviral Response in Drosophila. *Cell* 2017, 169(2):314–325.

[62] Zhang Y, Dai Y, Wang J, Xu Y, Li Z, *et al.* Mouse circulating extracellular vesicles contain virus-derived siRNAs active in antiviral immunity. *EMBO J.* 2022, 41(11):e109902.

[63] Lee SR, Roh JY, Ryu J, Shin HJ, Hong EJ. Activation of TCA cycle restrains virus-metabolic hijacking and viral replication in mouse hepatitis virus-infected cells. *Cell Biosci.* 2022, 12(1):7.

[64] Zou L, Wang X, Zhao F, Wu K, Li X, *et al.* Viruses Hijack ERAD to Regulate Their Replication and Propagation. *Int. J. Mol. Sci*. 2022, 23(16):9398.

[65] Racicot K, VanOeveren S, Alberts A. Viral Hijacking of Formins in Neurodevelopmental Pathologies. *Trends Mol. Med.* 2017, 23(9):778–785.

[66] Cullen BR, Cherry S, tenOever BR. Is RNA interference a physiologically relevant innate antiviral immune response in mammals?. *Cell Host Microbe* 2013, 14(4):374–378.

[67] Cullen BR. Viruses and RNA interference: issues and controversies. *J. Virol.* 2014, 88(22):12934–12936.

[68] Gantier MP. Processing of double-stranded RNA in mammalian cells: a direct antiviral role?. *J. Interferon Cytokine Res*. 2014, 34(6):469–477.

[69] Tsai K, Courtney DG, Kennedy EM, Cullen BR. Influenza A virus-derived siRNAs increase in the absence of NS1 yet fail to inhibit virus replication. *RNA* 2018, 24(9):1172–1182.

[70] Schuster S, Tholen LE, Overheul GJ, van Kuppeveld FJM, van Rij RP. Deletion of Cytoplasmic Double-Stranded RNA Sensors Does Not Uncover Viral Small Interfering RNA Production in Human Cells. *mSphere* 2017, 2(4):e00333-17.