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Mouse circulating extracellular vesicles contain virus-derived siRNAs active in antiviral immunity

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1st Editorial Decision **8th Nov 2021**

Thank you for submitting your manuscript proposing transfer of vsiRNA via extracellular vesicles (EVs) to The EMBO Journal. We have now received three referee reports on your study, which are included below for your information. In light of these comments, we would like to invite you to prepare and submit a revised manuscript.

As you will see, the reviewers appreciate the interest in the proposed model, but also raise several major concerns that must be resolved before the study can be considered further for publication. In particular, all referees find that further support for the proposed role of RNAi in the protective effect of NoV deltaB2 is needed (ref #1- major point 1, 2, minor point 1; ref #2- point 3(4); ref #3- major point 2, 3). Please address this issue by providing further experimental evidence. In addition, all referees point out instances where appropriate controls are missing in their view, or where analyses should be revised or described in more detail. These points should all be resolved in the revised manuscript (ref #1- major point 3; ref #2- point 2, 5; ref#3- major point 1, minor points). Please also carefully consider all other referee comments and revise the manuscript and figures as appropriate, as well as providing a detailed response to each comment when submitting the revised version.

Referee #1:

Recent studies demonstrated that antiviral RNA interference (RNAi) is active in mammals. The hallmarks of this antiviral pathway is the cleavage of the viral replication intermediates by Dicer into virus-derived small interfering RNAs (vsiRNAs), which then guide the sequence-specific cleavage of cognate target viral RNAs.

The study of Zhang et al. provide evidence that vsiRNAs are not only present within the infected cells, but are also found in extracellular vesicles (EVs) that can confer homology-dependent protection against virus infection.

The same lab previously uncovered that infection of newborn mice with a viral suppressor of RNAi (VSR)-defective Nodamuravirus (NoV) mutant (NoV deltaB2) lead to the accumulation of canonical vsiRNAs and the induction of an antiviral RNAi response. In this study, the authors first showed that an infection of newborn mice with NoV deltaB2 confer protection towards a subsequent infection 2 days later with a wild-type NoV. The authors further examined the underlying mechanism responsible for the protection in NoV deltaB2-immunised mice. They found that vsiRNAs were detected in muscle tissue up to 27 days post-infection (dpi) despite the virus being cleared from the infected mice after 3 dpi. They then detected vsiRNAs in whole blood and exosome-enriched EVs. These vsiRNAs were of 21-23-nucleotides (nts) in length and were derived from both viral strands, i.e. displaying the same canonical features than the vsiRNAs accumulating in hindlimb muscles. The authors then verified that Nov vsiRNAS were present in EVs not only from Balb/c - infected mice but also from another strain (C57BL/6) and also detected vsiRNAs in EVs after infection with Sindbis virus (SINV) and Zika virus (ZIKV). The authors then demonstrated the antiviral activity of EV-vsiRNAs by showing that BHK-21 cells or infant mice pre-treated with EVs purified from NoV deltaB2-infected mice were more resistant to subsequent NoV infection. Finally, the authors demonstrated that EVs from NoV deltaB2-infected mice were able to mediate an antiviral response in a sequence-specific manner. For this, they constructed a recombinant SINV bearing either a fragment of NoV previously found to be complementary to many NoV vsiRNAs (SINV_NoV) or a fragment from GFP as a control (SINV_GFP). They showed that mice pre-inoculated with EV from NoV deltaB2-infected mice reduced the level of replication of subsequent infection with SINV_NoV compared to SINV-GFP.

The findings that vsiRNAs are transferred into EVs and can confer antiviral activities against incoming virus infection constitute an important step in the fundamental understanding of antiviral RNAi in mammals. The results are also exciting conceptually as it provides evidence for a sequence-specific memory mediated by a RNA-mediated innate immune response in addition to the immune memory displayed by the adaptive immunity. The study from Zhang et al., is well-written and the experiments are well conducted. There are, however, few points that the authors should addressed.

Major comments:

• In figure 1, the authors inoculated six-day-old mice with NoV deltaB2 viruses and then 2 days later mice were challenged with wild-type (WT) NoV. The experiments conclusively show that vaccination with NoV deltaB2 provides protection against subsequent infection with NoV WT since none of the immunized mice exhibited any signs of disease and NoV viral proteins and genomic RNAs failed to accumulate (Figure 1 A-C). The protective effect caused by the pre-inoculation of NoV delta B2 is likely multi-pronged, i.e derived from various innate immune mechanisms. The protective effect is observed 4 days post-inoculation of Nov deltaB2 (2 dpi with NoV WT, which is then 4 days post vaccination). One would expect the adaptive immunity to have an

impact at later time points, yet its involvement in the antiviral effect observed at 4 days post-vaccination cannot be excluded. This reviewer appreciates that the full exploration of the mechanisms underlying this protective effect is beyond the scope of this study. However, it would be important to address to which extend this protective effect is caused by RNAi. The authors should test whether some of this protective effect is sequence-dependent, which is one of the hallmarks of RNAi. For this, they could first inoculate mice with NoV deltaB2 and two days later infect the vaccinated mice with either SINV-GFP or SINV-Nov (viruses that the authors already generated in their study, figure 5E). The viral accumulation should then be measured in the hindlimb muscles 2dpi. On would predict that SINV-NoV would be more restricted than SINV-GFP through an RNAi-dependent antiviral effect. The authors could consider using Ifnar-deficient mice as well for this experiment to alleviate the potential additional contribution of the interferon system.

• In figure 4 A-C, the authors incubated BHK-21 cells with EVs purified from NoV deltaB2-infected mice and then examine the impact on NoV infection. They found that NoV was restricted when cells were treated with EVs from NoV-deltaB2 compared to EVs from mock-infected or SINV-infected mice (Figure 4B and 4C). The authors should address whether the observed antiviral effect is mediated by RNAi. the authors could repeat the same experiment but instead of using BHK-21 cells, they could use mouse embryonic fibroblasts (MEFs) WT and MEFs that harbour a catalytic mutation in Ago2 (Ago2-CD MEFs) previously used in the authors' lab previous publication (Han et al., Mbio, 2020). The impact of EVs derived from Nov-deltaB2 infected mice could then be tested on NoV infection of MEFs WT and AGO2-CD MEFs and determine whether the antiviral effect of EVs is abolished or reduced in AGO2-CD MEFs that are RNAi-deficient.

• The figure 4D provides evidence that the inoculation of mice with EVs derived from NoV-infected mice or infected with NoV deltaB2 confer antiviral activity against subsequent NoV WT infection. This antiviral activity was not observed if mice were inoculated with EVs derived from mock-infected mice. The authors should include in this experiment mice inoculated with EV derived from SINV-infected mice (EVs_SINV) as performed in figure 4C. The EVs_SINV control will provide information about how much of the antiviral effect is sequence-specific and if there is any sequence-unspecific antiviral activity caused by, for instance, viral RNAs or other potential pathogen associated molecular patterns (PAMPs) that might be present in the EVs derived from infected mice.

Minor comments:

• Figure 1: in the current format, it is unclear how the results presented in figure 1 relate to the remaining experiments of the study. The authors showed that that the vsiRNA are detectable many days after infection with Nov deltaB2 in hind limb tissues and suggest that the vsiRNAs remain stable in vivo after clearing of the virus through their association to the RISC complex within the muscle cells. This suggests that the protective effect observed in NoV deltaB2-immunized mice against a secondary infection with NoV WT might be, at least partly, caused by a cell-autonomous antiviral activity that is maintained in the infected muscle cells in which vsiRNAs remain stable. The authors should therefore clarify/discuss in their manuscript that the protective effect might be caused, among other immune mechanisms, by a cell-autonomous antiviral activity due to vsiRNAs remaining stable within the infected cells as well as to non-cell autonomous antiviral activity mediated by vsiRNAs found in EVs.

• Page 10, line 212: "The 22-nt vsiRNAs of SINV founds in EVs were highly enriched for canonical siRNA duplexes with 2-nt 3'overhangs...". The data presented in figure 3C does not include an analysis of the duplexes' extremities. The authors needs to include an analysis of the total counts of pairs of complementary vsiRNAs in each distance category between 5'and 3'ends such as performed in Figure 1F. This should also be included in Figure 2F and figure 3A-D to address whether the vsiRNAs found in EVs have canonical features.

• Page 10, lines 216-219. The authors mentioned that the vsiRNAs of Zika virus (ZIKV) found in EVs are equivalent to 42.3% of the total EVs miRNAs. However, EV table 1 shows that the value of 42.3% corresponds to the percentage of virus reads of 18-nt to 28-nt in length relative to the total miRNA reads found in EVs. The reads of 18-nt to 28-nt are not representative of vsiRNAs, which are typically of 21-23-nt in length. The percentage of vsiRNAs should rather be determined by taking into account the number of ZIKV-derived reads of 21-23-nt in length (157,656) out of the total number of EV miRNA (1,059,961), which means that the vsiRNAs from ZIKV in EVs are instead equivalent to 14.9% of total EVs miRNAs. The value should be changed in the discussion as well (page 15 line 326).

• In each individual experiments presented in figure 4 and 5, the impact of EVs derived from NoV-infected mice are compared to the effect induced by EV derived from SINV or mock-infected controls. It is important for these experiments to ensure that the same number of EV particles are used within an experiment to then be able to compare their respective impact on subsequent viral infections. How were the various preparations of EVs quantified? Were they all quantified by Nanoparticle Tracking Analysis and then an equal amount of EV particles derived from infected mice versus mock-infected mice used for each experiment?

• Some references within the text includes both the first and the second author. Please change to mention in the text only the first author according to the journal's reference format.

• Page 4, Line 82: "Sousa and colleagues" should be changed to "Reis e Sousa and colleagues".

• Figure 1 B and C: the authors should include in the legend which tissue was used for the western blot and the RT-qPCR.

• Figure 1D, E: the authors should specify in the legend how many mice were used for each time points.

• Page 15, line 332: the authors mentioned "Pierre et al." , but then refer to Maillard et al., 2016. Please change for "Maillard et al. have demonstrated...".

• Page 5, line 92: typo, please change "wok" for "work".

Referee #2:

This is an interesting study that adds to a growing body of evidence suggesting a role for RNAi in mammalian antiviral immunity. The authors suggest that such protection can spread systemically to uninfected cells via EV-mediated transfer of viral siRNAs. This is not easy to prove but the authors have managed to generate some intriguing data to support their claims. A few points remain to be addressed to strengthen the work.

1) The authors try to provide evidence for the sequence-specificity of the NoV-restriction mechanism they describe. This includes EVs generated from SINV-infected mice as a negative control (Fig 4C), as well as an elegant experiment with SINV encoding part of NoV sequence (Fig 5D-E). As much rests on these experiments, it would be good to generate additional data to strengthen the notion of sequence specificity. For example, comparing the left and right panels of Fig. 5E, it appears that EVs(deltaB2) already restrict SINV-GFP. Why were the panels separated and are there other virus combinations that could be tried to nail down specificity?

2) Most of the experiments rely on the purification of EVs, which can be contaminated with virions, especially in the case of SINV infection. From the authors' own data (Fig 5B), it seems that the EVs(ΔB2) preparation contains residual infectious virus. To exclude a contribution of the latter, we suggest that the authors repeat the experiments in Fig. 4A-C but extract RNA from the EVs, followed by size exclusion to obtain small RNAs (and eliminate full-length infectious viral RNA). This EV-derived small RNA can then be transfected into BHKs before challenge with NoV.

3) The evidence linking the EV-mediated antiviral effect to RNAi is rather thin. We suggest that the authors perform additional experiments to bolster this notion. For example, they could knockout or knockdown Ago2 in BHK cells to show that EV-mediated protection is lost (Fig 4).

4) The authors mention systemic antiviral RNAi in insects. In this pathway, DNA reverse transcribed from viral RNA is transcribed to generate siRNAs. This is an amplification mechanism that makes possible large-scale protection of uninfected cells when only a minority of cells are infected. The authors should discuss whether amplification happens in mammals or, otherwise, suggest how you generate enough siRNAs for systemic transfer of protection. Also, the work of the Saleh lab (Institut Pasteur) should be cited in addition to Andino.

5) Excessive normalisation is detrimental to interpretation of the data. The authors should plot their RT-qPCR results for NoV RNA1 using only one step of normalisation on a housekeeping gene rather than arbitrary fold difference over one of the experimental groups.

Referee #3:

In this study, Zhang et al, evaluated the protective role of vaccination of mice with virus- a live attenuated Nodamura virus (NoV) or derived extracellular vesicles (EV) from this virus in a subsequent model of lethal infection with the wt virus. Also, authors analyze the presence of vsiRNAs in extracellular vesicles present in the blood, and analyze the dependency of IFN and the potential contribution of vsiRNA included in the EVs protective effect of their vaccination protocol. The data presented are certainly interesting and in line with previous studies supporting the role of EV/exosomes transferring antiviral properties/immunity in different models. Also, the concept of the vsiRNA as potential protective agents included in the EVs is interesting. However, the authors have not sufficiently demonstrated that the protective role of the attenuated virus or the derived EVs is exclusively mediated by vsiRNAs. Moreover, the low number of repeats or animals used as well as the low statistical significance or the lack of controls in some experiments, are major concerns of the study and make difficult the interpretation of the results. Therefore, a number of significant improvements on the missing mechanistic data as well as the data presentation should be improved before publication.

Major points:
-Data shown in figure 1B-C seem to have been collected from n=3 mice per group, and despite obvious differences no statistical differences are highlighted. Therefore, a higher number of animals from independent experiments should be included to confirm the presented data. Similarly, for figures 1D-1E, authors mentioned that small RNA analysis is performed from two independent sets of mice; could authors clarify how many mice were pooled in each independent analysis since it is not specified in the methods section or the figure legends?
-Authors provide proof of concept of the protective effect of exposure to live NoVΔB2 NoV or extracellular vesicles derived from this mutant variant. However, it is unclear whether the protective effect is mediated by the vsiRNA contained in EVs produced by infected cells. For example, the level of detection of vsiRNA in circulating cells or circulating EVs is extremely low (between 1-3% of detected small RNAs, as shown in Fig 2) which makes difficult to support the observed suppression of viral replication and marked survival of vaccinated mice (Fig 1, Fig 4). Although authors try to support their claims by using the interferon-deficient AG6 model (Fig 5), it is well known that alternative host factors with antiviral properties such as RNA helicases can act independently of IFN induction and could be present in the EVs or induced after exposure to the live mutant virus. In addition, although authors try to address the dependency of vsiRNA in the protective effect of EVs in the AG6 mice using the SINVNoV system, the impact on the levels of the target RNA1 that justify the reduced viral replication are not shown. Instead, levels of viral replication in a particular site are shown (Fig 5E). Therefore, a substantial amount of additional experiments should be included to address these questions and demonstrate the link between the protective effect of live NoVΔB2 or derived EVs and vsiRNAs.
-No information regarding potential immunological effect of the exposure of mice to mutant NoV are included, so the authors assume it is mainly mediated by the vsiRNAs or vesicles. Analysis of innate immune subsets exhibiting signs of trained immunity or increased levels of activation on myeloid cells, NK cells should be studied to rule out other significant cell-mediated mechanisms controlling viral replication after exposure to NoVΔB2 NoV. In this regard, to claim that NoV B2 can be used as a live-attenuated vaccine, which indeed may be an interesting vaccine candidate, the NoV B2 should confer long-term protection and not just short-term as authors show in Fig. 1A. Therefore, the authors should test whether immunization with the mutant virus confers protection against a challenge with the WT virus leaving a time gap of at least 3 weeks between immunization and challenge. In this line, it would be of great interest to include not only immunization with NoV B2, but also with EVs(mock) and EVs(B2) to see if they can also be used as a long-term immunization tool.

Minor points:

- No definition of vsiRNAs abbreviation in Introduction, in addition to abstract.
-Introduction describing background on the topic is a bit vague and the objective of the study is not clearly stated.
- The western blot characterization of expression of CD9 and CD63 on EVs shown in Figure 2D is insufficient to determine whether they represent exosomes, a more rigorous analysis of additional markers previously described enriched in exosomes such as TSG101, HSP70, etc should be included.
- Grammatical errors throughout the manuscript should be corrected. Expressions such as People hypothesized...(line 78); cells that secrete IFN have pattern-recognition receptors (PRR) (line 99), should be avoided.
- The title is not very descriptive of the main findings of the article and gives the idea of the article being rather a review. Hence, I would suggest to reformulate as a summary sentence of the main finding.
- The the referencing style should be kept homogeneous throughout the text (e.g. attention in line 44 or 46).
- Line 57-58: I would introduce the sentence with a connector such as "...2020). Therefore, the role..."
- Line 89: "a recent study" rather than "recent study"
- References should be carefully checked (e.g. Maillard et al., 2019)

Referee #1:

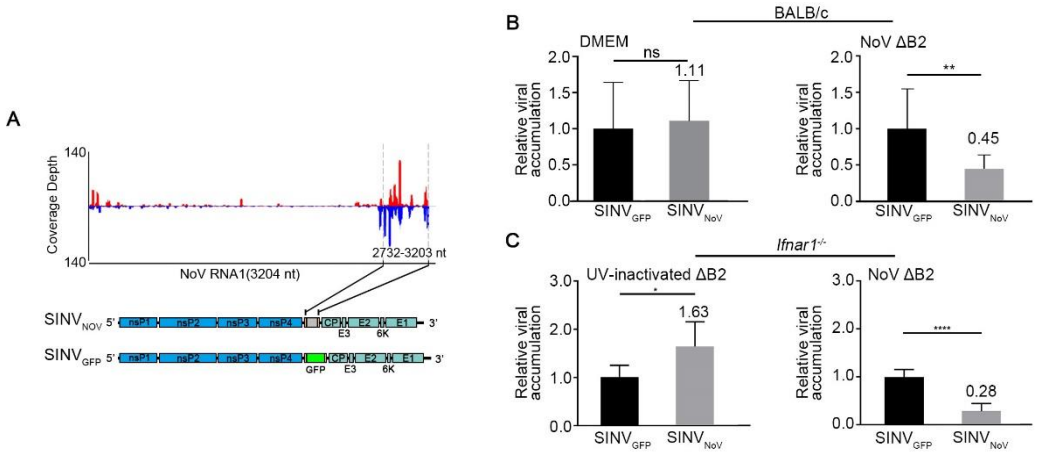
Recent studies demonstrated that antiviral RNA interference (RNAi) is active in mammals. The hallmarks of this antiviral pathway is the cleavage of the viral replication intermediates by Dicer into virus-derived small interfering RNAs (vsiRNAs), which then guide the sequence-specific cleavage of cognate target viral RNAs. The study of Zhang et al. provide evidence that vsiRNAs are not only present within the infected cells, but are also found in extracellular vesicles (EVs) that can confer homology-dependent protection against virus infection. The same lab previously uncovered that infection of newborn mice with a viral suppressor of RNAi (VSR)-defective Nodamuravirus (NoV) mutant (NoV deltaB2) lead to the accumulation of canonical vsiRNAs and the induction of an antiviral RNAi response. In this study, the authors first showed that an infection of newborn mice with NoV deltaB2 confer protection towards a subsequent infection 2 days later with a wild-type NoV. The authors further examined the underlying mechanism responsible for the protection in NoV deltaB2-immunised mice. They found that vsiRNAs were detected in muscle tissue up to 27 days post-infection (dpi) despite the virus being cleared from the infected mice after 3 dpi. They then detected vsiRNAs in whole blood and exosome-enriched EVs. These vsiRNAs were of 21-23-nucleotides (nts) in length and were derived from both viral strands, i.e. displaying the same canonical features than the vsiRNAs accumulating in hindlimb muscles. The authors then verified that Nov vsiRNAS were present in EVs not only from Balb/c - infected mice but also from another strain (C57BL/6) and also detected vsiRNAs in EVs after infection with Sindbis virus (SINV) and Zika virus (ZIKV). The authors then demonstrated the antiviral activity of EV-vsiRNAs by showing that BHK-21 cells or infant mice pre-treated with EVs purified from NoV deltaB2-infected mice were more resistant to subsequent NoV infection. Finally, the authors demonstrated that EVs from NoV deltaB2-infected mice were able to mediate an antiviral response in a sequence-specific manner. For this, they constructed a recombinant SINV bearing either a fragment of NoV previously found to be complementary to many NoV vsiRNAs (SINV_NoV) or a fragment from GFP as a control (SINV_GFP). They showed that mice pre-inoculated with EV from NoV deltaB2-infected mice reduced the level of replication of subsequent infection with SINV_NoV compared to SINV-GFP. The findings that vsiRNAs are transferred into EVs and can confer antiviral activities against incoming virus infection constitute an important step in the fundamental understanding of antiviral RNAi in mammals. The results are also exciting conceptually as it provides evidence for a sequence-specific memory mediated by a RNA-mediated innate immune response in addition to the immune memory displayed by the adaptive immunity. The study from Zhang et al., is well-written and the experiments are well conducted. There are, however, few points that the authors should addressed.

Authors' response: Thank you for supporting our main conclusions.

Major comments:

• In figure 1, the authors inoculated six-day-old mice with NoV deltaB2 viruses and then 2 days later mice were challenged with wild-type (WT) NoV. The experiments conclusively show that vaccination with NoV deltaB2 provides protection against subsequent infection with NoV WT since none of the immunized mice exhibited any signs of disease and NoV viral proteins and genomic RNAs failed to accumulate (Figure 1 A-C). The protective effect caused by the pre-inoculation of NoV delta B2 is likely multi-pronged, i.e derived from various innate immune mechanisms. The protective effect is observed 4 days post-inoculation of Nov deltaB2 (2 dpi with NoV WT, which is then 4 days post vaccination). One would expect the adaptive immunity to have an impact at later time points, yet its involvement in the antiviral effect observed at 4 days post-vaccination cannot be excluded. This reviewer appreciates that the full exploration of the mechanisms underlying this protective effect is beyond the scope of this study. However, it would be important to address to which extend this protective effect is caused by RNAi. The authors should test whether some of this protective effect is sequence-dependent, which is one of the hallmarks of RNAi. For this, they could first inoculate mice with NoV deltaB2 and two days later infect the vaccinated mice with either SINV-GFP or SINV-Nov (viruses that the authors already generated in their study, figure 5E). The viral accumulation should then be measured in the hindlimb muscles 2dpi. On would predict that SINV-NoV would be more restricted than SINV-GFP through an RNAi-dependent antiviral effect. The authors could consider using Ifnar-deficient mice as well for this experiment to alleviate the potential additional contribution of the interferon system.

Authors' response: Thank you for your suggestions. We agree that other immune mechanisms such as adaptive immunity may also play a role in this process. We have recently shown that this protective effect is indeed sequence-dependent using the suggested experiments described in our recent publication (please see PMID: 34343211 – Fig. 5A-5C, and below). In these experiments, we did not monitor the survival of the challenged mice. Nevertheless, we found that $SINV_{NoV}$ accumulated to significantly lower levels than $SINV_{GFP}$ in mice pre-inoculated with NoVΔB2, but not with buffer DMEM (Fig B). Infection with live NoVΔB2 also induced significant inhibition on the accumulation of $SINV_{NoV}$ compared to that of $SINV_{GFP}$ in type I IFN receptor knockout mice (*Ifnar1*$^{-/-}$, Fig C), which are defective in the signaling by type I IFNs. We have added a sentence to describe this finding in the text (see Lines 148-151).



The viral siRNAs of NoV inducing homology-dependent viral RNA degradation.

A The genomic structure of a recombinant SINV (SINV$_{NoV}$) carrying a segment of NoV RNA1 known to be targeted by high densities of vsiRNAs in NoVΔB2-infected mice shown at the top. Also shown below was the genomic structure of SINV$_{GFP}$ introduced above.

B *In vivo* genomic RNA levels of SINV$_{NoV}$ and SINV$_{GFP}$ one day after challenge inoculation of NoVΔB2-infected (right) or DMEM mock-infected (left) BALB/c suckling mice (n=7 per DMEM group, n=10 per NoVΔB2 group). The viral genomic RNA accumulation level was determined by RT-qPCR amplification of the viral nsP2 coding region and normalized by endogenous actin mRNA with SINV$_{GFP}$ level set as 1. Error bars represent SD. ** indicates $p<0.01$ (Student's t-test).

C *In vivo* genomic RNA levels of SINV$_{NoV}$ and SINV$_{GFP}$ measured as in (B) one day after challenge inoculation of *Ifnar1*$^{-/-}$ suckling mice (n=5~7 per group) inoculated two days earlier with live (right) or UV-inactivated (left) NoVΔB2. Error bars represent SD. * indicates <0.05, **** indicates $p<0.0001$ (Student's t-test). The viral RNA accumulation of SINV$_{GFP}$ was set as 1.

• In figure 4 A-C, the authors incubated BHK-21 cells with EVs purified from NoV deltaB2-infected mice and then examine the impact on NoV infection. They found that NoV was restricted when cells were treated with EVs from NoV-deltaB2 compared to EVs from mock-infected or SINV-infected mice (Figure 4B and 4C). The authors should address whether the observed antiviral effect is mediated by RNAi. the authors could repeat the same experiment but instead of using BHK-21 cells, they could use mouse embryonic fibroblasts (MEFs) WT and MEFs that harbour a catalytic mutation in Ago2 (Ago2-CD MEFs) previously used in the authors' lab previous publication (Han et al., Mbio, 2020). The impact of EVs derived from Nov-deltaB2 infected mice could then be tested on NoV infection of MEFs WT and

AGO2-CD MEFs and determine whether the antiviral effect of EVs is abolished or reduced in AGO2-CD MEFs that are RNAi-deficient.

Authors' response: Thanks for your insightful comments. Due to the COVID-19 pandemic, we were unable to ship the cells from USA (Ago2-CD MEFs are kept in Ding lab at UC Riverside) to China in time to complete the experiments as suggested. As an alternative, we added a similar set of experiments to demonstrate that EVs from NoVΔB2-immunized mice mediate Ago2-dependent RNAi in 293T cells (Please see Fig 4 D, E, F in the revised MS, or below).

We performed another set of experiments to examine the RNAi activity of EV-associated vsiRNAs in human 293T cells using a dual luciferase reporter system. A fragment of NoV RNA1 in sense orientation known to be targeted by high densities of EV-associated vsiRNAs from NoVΔB2-infected infant mice was inserted into the 3′ UTR of a dual luciferase reporter plasmid and the same length of GFP mRNA sequence was used in a control construct (Fig 4D). Four hours after transfection with one of the reporter constructs, the human 293T cells were treated by the EVs purified from NoVΔB2-infected infant mice or mock-inoculated mice. We observed significant suppression of the luciferase reporter containing the NoV fragment treated by the EVs purified from NoVΔB2-infected infant mice but not from mock-inoculated mice (Fig 4E). In contrast, treatment with the two different pools of EVs did not cause significant difference in the expression of the luciferase reporter containing the GFP fragment (Fig 4E). We further generated an Argonaute-2 knockout (Ago2 KO) line of 293T cells (Fig EV4) and performed the same luciferase reporter experiment using these cells. We found that the specific suppression of the luciferase reporter containing the NoV fragment by the EVs purified from NoVΔB2-infected infant mice was abolished in 293T-Ago2 KO cells (Fig 4F). These findings suggest that the specific antiviral effect of EVs from NoVΔB2-immunized infant mice is mediated by RNAi.

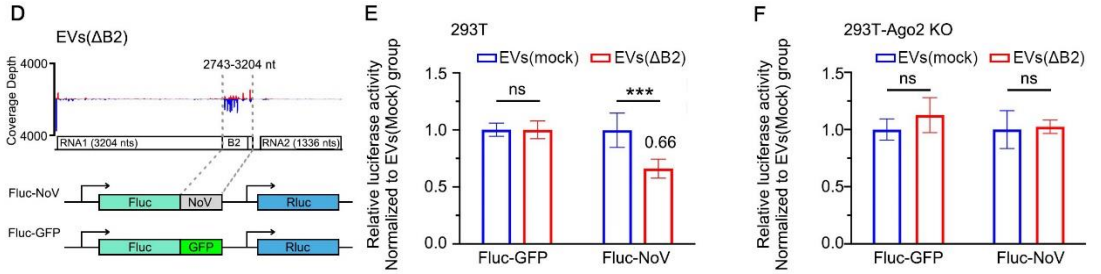
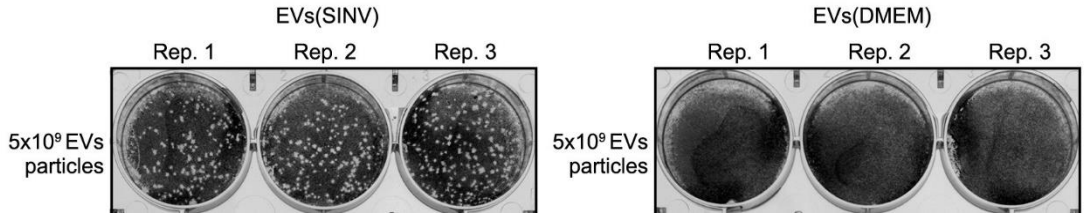


Fig 4 D, Diagram showing the 462-nt sequence in length from NoVΔB2 RNA1 was inserted into the 3' untranslated region (UTR) of firefly luciferase reporter mRNA, to be targeted by antisense vsiRNAs with EVs from NoVΔB2-immunized BALB/c suckling mice. A translation initiation codon-deleted GFP sequence of the same length was inserted into the same site as control. **E, F**, Relative luciferase activity of the two reporter constructs after transfection of 293T cells (**E**) and 293T-Ago2 KO cells (**F**) and treatment by different pools of EVs, EVs (ΔB2) or EVs (Mock). Error bars indicate standard deviation of three independent replicates. *** indicates $p<0.001$, ns indicates no significant difference by student's t-test.

• The figure 4D provides evidence that the inoculation of mice with EVs derived from NoV-infected mice or infected with NoV deltaB2 confer antiviral activity against subsequent NoV WT infection. This antiviral activity was not observed if mice were inoculated with EVs derived from mock-infected mice. The authors should include in this experiment mice inoculated with EV derived from SINV-infected mice (EVs_SINV) as performed in figure 4C. The EVs_SINV control will provide information about how much of the antiviral effect is sequence-specific and if there is any sequence-unspecific antiviral activity caused by, for instance, viral RNAs or other potential pathogen associated molecular patterns (PAMPs) that might be present in the EVs derived from infected mice.

Authors' response: Thank you for your suggestions. As we mentioned in the discussion part (lines: 334-337), we failed to purify the EVs free of SINV virions. We detected an amount of SINV particles in the purified EVs using our isolation protocol (please see below). When infant mice were previously inoculated with EVs from SINV-infected infant mice, we detected high levels of SINV replication, and the mice even became symptomatic. Thus, the EVs purified from SINV-infected infant mice would not serve as a negative control since the mice will be infected by SINV present in EVs before challenge with WT NoV. Nevertheless, demonstrating Ago2-dependent RNAi in 293T cells by EVs from NoVΔB2-immunized mice should serve to rule out activities of other components in the EVs.



Representative plaque assay images of the EVs purified from SINV-infected infant mice and the EVs from mock-inoculated mice on BHK cells.

Minor comments:

• Figure 1: in the current format, it is unclear how the results presented in figure 1 relate to the remaining experiments of the study. The authors showed that that the vsiRNA are detectable many days after infection with Nov deltaB2 in hind limb tissues and suggest that the vsiRNAs remain stable in vivo after clearing of the virus through their association to the RISC complex within the muscle cells. This suggests that the protective effect observed in NoV deltaB2-immunized mice against a secondary infection with NoV WT might be, at least partly, caused by a cell-autonomous antiviral activity that is maintained in the infected muscle cells in which vsiRNAs remain stable. The authors should therefore clarify/discuss in their manuscript that the protective effect might be caused, among other immune

mechanisms, by a cell-autonomous antiviral activity due to vsiRNAs remaining stable within the infected cells as well as to non-cell autonomous antiviral activity mediated by vsiRNAs found in EVs.

Authors' response: We have modified the introduction section to link the NoVΔB2 immunization experiments with the subsequent studies on EVs (see Lines 110-118). We agree that the protective effect may come from both cell-autonomous and non-cell autonomous antiviral activity mediated by vsiRNAs. As suggested, we have included it in the revised manuscript "We propose that the cell-autonomous and systemic antiviral RNAi are both active in mammals" (please see lines 122-124).

• Page 10, line 212: "The 22-nt vsiRNAs of SINV founds in EVs were highly enriched for canonical siRNA duplexes with 2-nt 3'overhangs...". The data presented in figure 3C does not include an analysis of the duplexes' extremities. The authors needs to include an analysis of the total counts of pairs of complementary vsiRNAs in each distance category between 5'and 3'ends such as performed in Figure 1F. This should also be included in Figure 2F and figure 3A-D to address whether the vsiRNAs found in EVs have canonical features.

Authors' response: Thank you for your suggestion. We have added the requested analysis of the total counts of pairs of complementary vsiRNAs in each distance category between 5'and 3'ends in the updated Fig 3 and a supplementary Fig EV3 (please see also below).

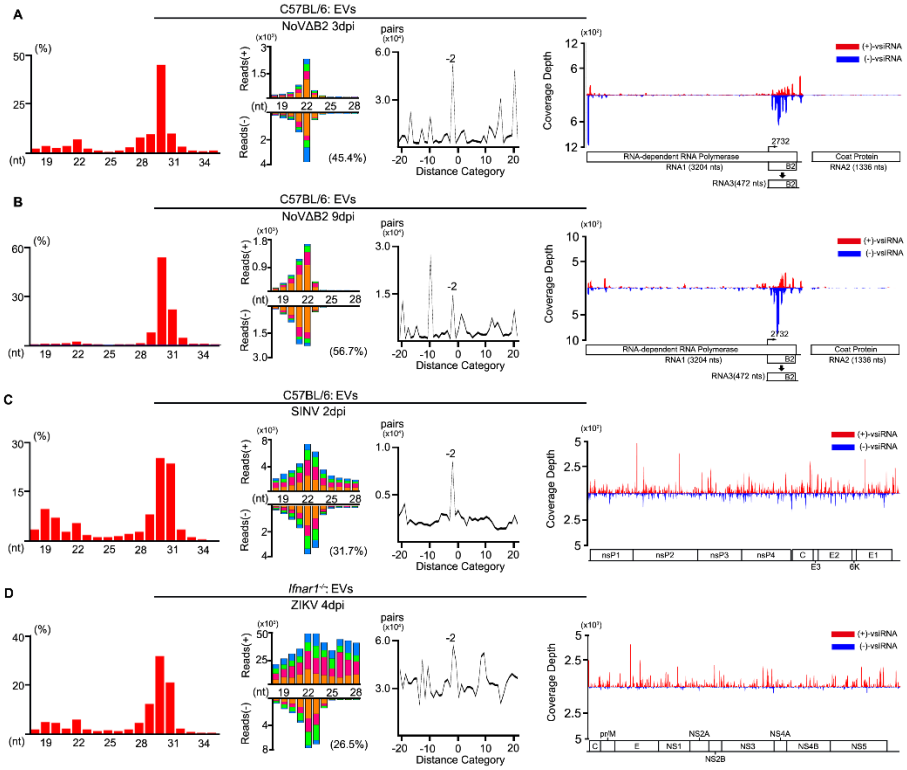
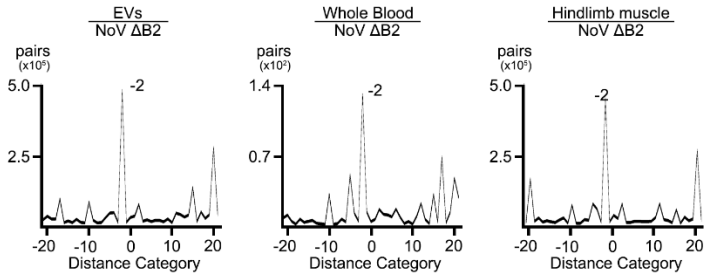


Figure 3 | Characterization of EV-vsiRNAs with distinct virus infections. Reads are shown as per million total mature miRNAs. 5' terminal nucleotide and the percentage of 1U vsiRNAs and duplex pattern of the 22-nt vsiRNAs are indicated.



Extended Data Figure 3 | Duplex pattern of 22-nt vsiRNAs in EVs, whole blood and hindlimb muscle tissue from NoVΔB2 infected suckling mice. Duplex pattern of 22-nt vsiRNAs in EVs, whole blood and hindlimb muscle tissue from NoVΔB2 infected suckling mice. Peaks at -2 nt and 20 nt indicated a typical duplex model by cleavage of endoribonuclease Dicer.

• Page 10, lines 216-219. The authors mentioned that the vsiRNAs of Zika virus (ZIKV) found in EVs are equivalent to 42.3% of the total EVs miRNAs. However, EV table 1 shows that the value of 42.3% corresponds to the percentage of virus reads of 18-nt to 28-nt in length relative to the total miRNA reads found in EVs. The reads of 18-nt to 28-nt are not representative of vsiRNAs, which are typically of 21-23-nt in length. The percentage of vsiRNAs should rather be determined by taking into account the number of ZIKV-derived reads of 21-23-nt in length (157,656) out of the total number of EV miRNA (1,059,961), which means that the vsiRNAs from ZIKV in EVs are instead equivalent to 14.9% of total EVs miRNAs. The value should be changed in the discussion as well (page 15 line 326).

Authors' response: Thank you for pointing it out. We have modified it in the revised manuscript (please see lines: 219-221).

• In each individual experiments presented in figure 4 and 5, the impact of EVs derived from NoV-infected mice are compared to the effect induced by EV derived from SINV or mock-infected controls. It is important for these experiments to ensure that the same number of EV particles are used within an experiment to then be able to compare their respective impact on subsequent viral infections. How were the various preparations of EVs quantified? Were they all quantified by Nanoparticle Tracking Analysis and then an equal amount of EV particles derived from infected mice versus mock-infected mice used for each experiment?

Authors' response: Yes, EVs from infected and mock-infected mice were all quantified in this study. In brief, after isolation of EVs by ExoQuick Exosome Precipitation Solution, EVs were suspended in 1xPBS (usually 1/3 volume of initial serum) for quantification by Nanoparticle Tracking Analysis. The concentration of EVs varied from 1.1 $x10^{12}$ to 4.6 $x10^{12}$ particles/ml. The EVs were diluted to an equal amount of $8x10^{11}$ particles/mL for all experiments.

• Some references within the text includes both the first and the second author. Please change to mention in the text only the first author according to the journal's reference format.
Authors' response: We have fixed this problem. Thanks.

• Page 4, Line 82: "Sousa and colleagues" should be changed to "Reis e Sousa and colleagues".
Authors' response: Thank you for pointing it out. We have corrected it. Thanks

• Figure 1 B and C: the authors should include in the legend which tissue was used for the western blot and the RT-qPCR.
Authors' response: We used hindlimb muscle tissue for the western blot and the RT-qPCR. We have added it in the updated legend.

• Figure 1D, E: the authors should specify in the legend how many mice were used for each time points.
Authors' response: Usually, we use three mice in each independent analysis, and we had added this information in our updated legend.

• Page 15, line 332: the authors mentioned "Pierre et al." , but then refer to Maillard et al., 2016. Please change for "Maillard et al. have demonstrated...".
Authors' response: We have fixed this problem. Thanks.

• Page 5, line 92: typo, please change "wok" for "work".
Authors' response: We have corrected it. Thanks.

Referee #2:

This is an interesting study that adds to a growing body of evidence suggesting a role for RNAi in mammalian antiviral immunity. The authors suggest that such protection can spread systemically to uninfected cells via EV-mediated transfer of viral siRNAs. This is not easy to prove but the authors have managed to generate some intriguing data to support their claims. A few points remain to be addressed to strengthen the work.

Authors' response: Thanks for your positive comments.

1) The authors try to provide evidence for the sequence-specificity of the NoV-restriction mechanism they describe. This includes EVs generated from SINV-infected mice as a negative control (Fig 4C), as well as an elegant experiment with SINV encoding part of NoV sequence (Fig 5D-E). As much rests on these experiments, it would be good to generate additional data to strengthen the notion of sequence specificity. For example, comparing the left and right panels of Fig. 5E, it appears that EVs(deltaB2) already restrict SINV-GFP. Why were the panels separated and are there other virus combinations that could be tried to nail down specificity?

Authors' response: As suggested, we have combined the left and right panels of Fig 5E (left) and also included the measurement of the viral genomic RNA accumulation in the updated Fig 5E (right) (please see below). Indeed, EVs (ΔB2) have some influence on the replication of SINV-GFP since compared to EVs (Mock), treatment with EVs (ΔB2) led to some reduction in the virus titer, but not the viral RNA accumulation levels (Fig 5E). This is in contrast to the suppression of SINV-NoV in both the virus titer and the viral RNA levels by treatment with EVs (ΔB2). We have now added another set of experiments to suggest that the specific antiviral effect of EVs from NoVΔB2-immunized infant mice is mediated by Ago2-dependent RNAi (please see Fig 4D described below, which is not yet known for EV-associated vsiRNAs from the infected fruit flies). At present, we are unclear whether the non-specific effect of EVs (ΔB2) on SINV-GFP is mediated by another component of EVs.

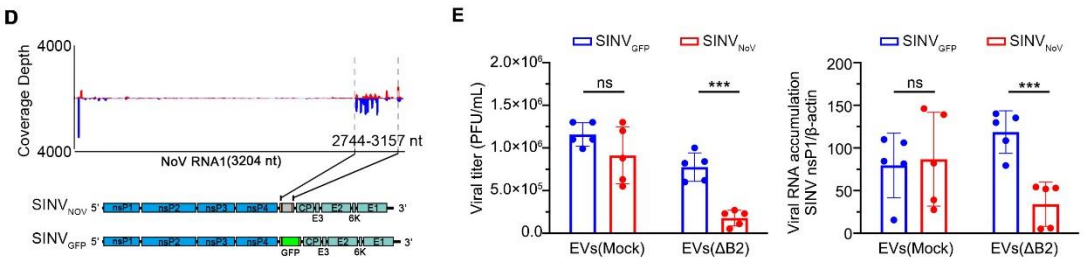


Figure 5 E, AG6 suckling mice were challenged with EVs from naïve or NoVΔB2 -infected BALB/c suckling mice and then inoculated by $SINV_{NoV}$ and $SINV_{GFP}$ respectively. Viral titer (PFU/ml) in the hindlimb muscle of infected mice was measured by a standard plaque assay and normalized by tissue mass, and the viral genomic RNA accumulation was determined by RT-qPCR amplification of the viral nsP1 coding region. Normalization was done by β-actin mRNA levels. *** indicates $p<0.001$, ns indicates no significant difference by student's t-test, n=5 per group.

2) Most of the experiments rely on the purification of EVs, which can be contaminated with virions, especially in the case of SINV infection. From the authors' own data (Fig 5B), it seems that the EVs(ΔB2) preparation contains residual infectious virus. To exclude a contribution of the latter, we suggest that the authors repeat the experiments in Fig. 4A-C but extract RNA from the EVs, followed by size exclusion to obtain small RNAs (and eliminate full-length infectious viral RNA). This EV-derived small RNA can then be transfected into BHKs before challenge with NoV.
Authors' response: To address whether EVs-associated vsiRNAs mediate specific RNAi, we have added another set of experiments using human 293T and Ago2-knockout 293T cells (please see below).

3) The evidence linking the EV-mediated antiviral effect to RNAi is rather thin. We suggest that the authors perform additional experiments to bolster this notion. For example, they could knockout or knockdown Ago2 in BHK cells to show that EV-mediated protection is lost (Fig 4).
Authors' response: We thank you for your insightful suggestion. Due to the low efficiency of genetic manipulation of BHK cells, we have added another set of experiments using human 293T and Ago2-knockout 293T cells to show that EVs-associated vsiRNAs direct RNAi/Ago2-dependent gene silencing in 293T cells (Fig 4 D, E, F in the revised MS, or below).

We performed another set of experiments to examine the RNAi activity of EV-associated vsiRNAs in human 293T cells using a dual luciferase reporter system. A fragment of NoV RNA1 in sense orientation known to be targeted by high densities of EV-associated vsiRNAs from NoVΔB2-infected infant mice was inserted into the 3′ UTR of a dual luciferase reporter plasmid and the same length of GFP mRNA sequence was used in a control construct (Fig 4D). Four hours after transfection with one of the reporter constructs, the human 293T cells were treated by the EVs purified from NoVΔB2-infected infant mice or mock-inoculated mice. We observed significant suppression of the luciferase reporter containing the NoV fragment treated by the EVs purified from NoVΔB2-infected infant mice but not from mock-inoculated mice (Fig 4E). In contrast, treatment with the two different pools of EVs did not cause significant difference in the expression of the luciferase reporter containing the GFP

fragment (Fig 4E). We further generated an Argonaute-2 knockout (Ago2 KO) line of 293T cells (Fig EV4) and performed the same luciferase reporter experiment using these cells. We found that the specific suppression of the luciferase reporter containing the NoV fragment by the EVs purified from NoVΔB2-infected infant mice was abolished in 293T-Ago2 KO cells (Fig 4F). These findings suggest that the specific antiviral effect of EVs from NoVΔB2-immunized infant mice is mediated by RNAi.

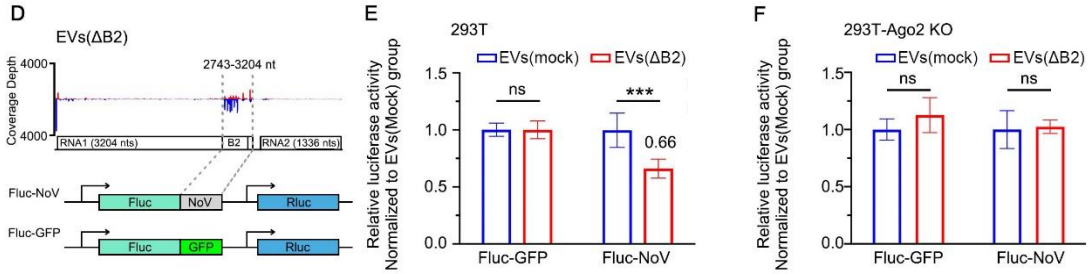


Fig 4 D, Diagram showing the 462-nt sequence in length from NoVΔB2 RNA1 was inserted into the 3' untranslated region (UTR) of firefly luciferase reporter mRNA, to be targeted by antisense vsiRNAs with EVs from NoVΔB2-immunized BALB/c suckling mice. A translation initiation codon-deleted GFP sequence of the same length was inserted into the same site as control. E, F, Relative luciferase activity of the two reporter constructs after transfection of 293T cells (E) and 293T-Ago2 KO cells (F) and treatment by different pools of EVs, EVs (ΔB2) or EVs (Mock). Error bars indicate standard deviation of three independent replicates. *** indicates $p<0.001$, ns indicates no significant difference by student's t-test.

4) The authors mention systemic antiviral RNAi in insects. In this pathway, DNA reverse transcribed from viral RNA is transcribed to generate siRNAs. This is an amplification mechanism that makes possible large-scale protection of uninfected cells when only a minority of cells are infected. The authors should discuss whether amplification happens in mammals or, otherwise, suggest how you generate enough siRNAs for systemic transfer of protection. Also, the work of the Saleh lab (Institut Pasteur) should be cited in addition to Andino.

Authors' response: Thanks for these comments and we have modified our discussion accordingly in the revised manuscript (please see lines: 379-387, or below)

"Similar to antiviral RNAi in fruit flies (Li *et al*, 2002; Wang *et al*, 2006; Galiana-Arnoux *et al*, 2006; van Rij *et al*, 2006; Tassetto et al, 2017), therefore, antiviral RNAi is not only active in the infected cell but also sends immune signals to prevent viral infection in distant non-infected cells in mammals (Fig 6). In addition to the biogenesis of vsiRNAs from Dicer processing of the viral dsRNA replicative intermediates, insect vsiRNAs are further amplified using viral DNA templates reverse-transcribed from the invading RNA viruses (Goic *et al*, 2013; Tassetto *et al*,

2017; Poirier *et al*, 2018). There are reports of viral DNA synthesis from non-retroviral RNA viruses by endogenous reverse transcriptases in mammalian cells (Geuking *et al*, 2009; Klenerman *et al*, 1997; Shimizu *et al*, 2014). Thus, it will be interesting to investigate a possible contribution of an insect-like vsiRNA amplification mechanism to the prolonged accumulation of vsiRNAs and the spread of the induced protection in mice immunized by VSR-deficient mutant of NoV."

5) Excessive normalization is detrimental to interpretation of the data. The authors should plot their RT-qPCR results for NoV RNA1 using only one step of normalisation on a housekeeping gene rather than arbitrary fold difference over one of the experimental groups.

Authors' response: Thank you for your suggestion. We have modified the normalization as advised in the revised manuscript (please see: Fig 1C; Fig 4 B, C, H and I; Fig 5 B, C and E).

Referee #3:

In this study, Zhang et al, evaluated the protective role of vaccination of mice with virus- a live attenuated Nodamura virus (NoV) or derived extracellular vesicles (EV) from this virus in a subsequent model of lethal infection with the wt virus. Also, authors analyze the presence of vsiRNAs in extracellular vesicles present in the blood, and analyze the dependency of IFN and the potential contribution of vsiRNA included in the EVs protective effect of their vaccination protocol. The data presented are certainly interesting and in line with previous studies supporting the role of EV/exosomes transferring antiviral properties/immunity in different models. Also, the concept of the vsiRNA as potential protective agents included in the EVs is interesting. However, the authors have not sufficiently demonstrated that the protective role of the attenuated virus or the derived EVs is exclusively mediated by vsiRNAs. Moreover, the low number of repeats or animals used as well as the low statistical significance or the lack of controls in some experiments, are major concerns of the study and make difficult the interpretation of the results. Therefore, a number of significant improvements on the missing mechanistic data as well as the data presentation should be improved before publication.

Authors' response: Thanks for your support of our main conclusions. We have modified the relevant sections accordingly, which have led to significant improvements of our manuscript. We have also added additional experiment to clarify that the protective role of the derived EVs is mediated by vsiRNAs (please see lines: 241-256).

Major points:

-Data shown in figure 1B-C seem to have been collected from n=3 mice per group, and despite obvious differences no statistical differences are highlighted. Therefore, a higher number of animals from independent experiments should be included to confirm the presented data. Similarly, for figures 1D-1E, authors mentioned that small RNA analysis is performed from two independent sets of mice; could authors clarify how many mice were pooled in each independent analysis since it is not specified in the methods section or the figure legends?

Authors' response: Thank you for pointing these out. We have repeated this experiment with additional suckling mice and presented the results in the revised manuscript (Figure 1 C, or please see below). Usually, we use three mice in each independent analysis as stated in our recent publication (please see PMID: 34343211, in the methods section: Deep sequencing and bioinformatic analysis of small RNAs). We have now included this information in the revised manuscript (line: 535).

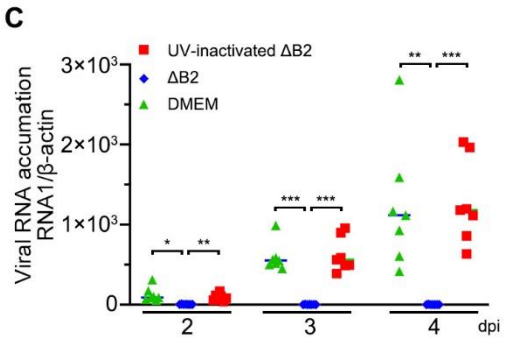


Figure 1 (**C**). n=7 per group. * indicates p<0.05, ** indicates p<0.01, and*** indicates p<0.001 by student's t-test.

-Authors provide proof of concept of the protective effect of exposure to live NoVΔB2 NoV or extracellular vesicles derived from this mutant variant. However, it is unclear whether the protective effect is mediated by the vsiRNA contained in EVs produced by infected cells. For example, the level of detection of vsiRNA in circulating cells or circulating EVs is extremely low (between 1-3% of detected small RNAs, as shown in Fig 2) which makes difficult to support the observed suppression of viral replication and marked survival of vaccinated mice (Fig 1, Fig 4). Although authors try to support their claims by using the interferon-deficient AG6 model (Fig 5), it is well known that alternative host factors with antiviral properties such as RNA helicases can act independently of IFN induction and could be present in the EVs or induced after exposure to the live mutant virus. In addition, although authors try to address the dependency of vsiRNA in the protective effect of EVs in the AG6 mice using the SINVNoV system, the impact on the levels of the target RNA1 that justify the reduced viral replication are not shown. Instead, levels of viral replication in a particular site are shown (Fig 5E). Therefore, a substantial amount of additional experiments should be included to address these questions and demonstrate the link between the protective effect of live NoVΔB2 or derived EVs and vsiRNAs.

Authors' response: As suggested, we have included the levels of the target RNA1 in the updated Fig 5 (please see below).

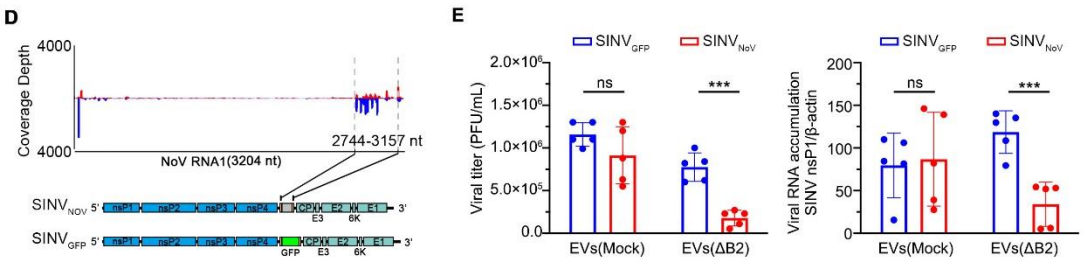


Figure 5 E, AG6 suckling mice were challenged with EVs from naïve or NoVΔB2 -infected BALB/c suckling mice and then inoculated by $SINV_{NoV}$ and $SINV_{GFP}$ respectively. Viral titer (PFU/ml) in the hindlimbs of infected mice was measured by a standard plaque assay and normalized by tissue mass, and the viral genomic RNA accumulation was determined by RT-qPCR amplification of the viral nsP1 coding region. Normalization was done by β-actin mRNA levels. *** indicates p<0.001, ns indicates no significant difference by student's t-test, n=5 per group.

As shown in Fig 2H, the vsiRNAs in the hindlimb muscle tissue and EVs of suckling mice immunized with NoVΔB2 are equivalent to 2.4% and 2.8% of the total cellular miRNAs in the same libraries. We wish to point out that our observed abundance of vsiRNAs is similar to or higher than that of vsiRNAs detected in human 293T cells infected with VSR-deficient enterovirus-71 (PMID: 28636969), in fruit flies infected with VSR-deficient Flock house virus (PMID: 21957285) or in the exosome-like vesicles from Sindbis virus -infected fruit flies (PMID: 28388413).

Moreover, we have now added another set of experiments using human 293T and Ago2-knockout 293T cells to show that EVs-associated vsiRNAs direct RNAi/Ago2-dependent gene silencing in 293T cells (Fig 4 D, E, F in the revised MS, or below).

We performed another set of experiments to examine the RNAi activity of EV-associated vsiRNAs in human 293T cells using a dual luciferase reporter system. A fragment of NoV RNA1 in sense orientation known to be targeted by high densities of EV-associated vsiRNAs from NoVΔB2-infected infant mice was inserted into the 3′ UTR of a dual luciferase reporter plasmid and the same length of GFP mRNA sequence was used in a control construct (Fig 4D). Four hours after transfection with one of the reporter constructs, the human 293T cells were treated by the EVs purified from NoVΔB2-infected infant mice or mock-inoculated mice. We observed significant suppression of the luciferase reporter containing the NoV fragment treated by the EVs purified from NoVΔB2-infected infant mice but not from mock-inoculated mice (Fig 4E). In contrast, treatment with the two different pools of EVs did not cause significant difference in the expression of the luciferase reporter containing the GFP fragment (Fig 4E). We further generated an Argonaute-2 knockout (Ago2 KO) line of 293T cells (Fig EV4) and performed the same luciferase reporter experiment using these cells. We found that the specific suppression of the luciferase reporter containing the NoV fragment by the EVs purified from NoVΔB2-infected infant mice was abolished in 293T-Ago2 KO cells (Fig 4F). These findings suggest that the specific antiviral effect of EVs from NoVΔB2-immunized infant mice is mediated by RNAi.

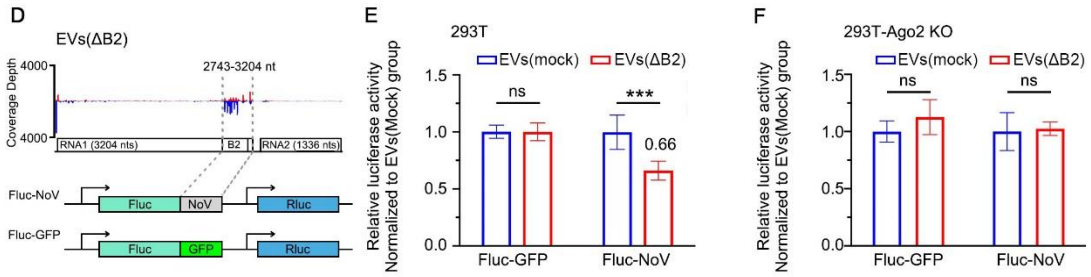


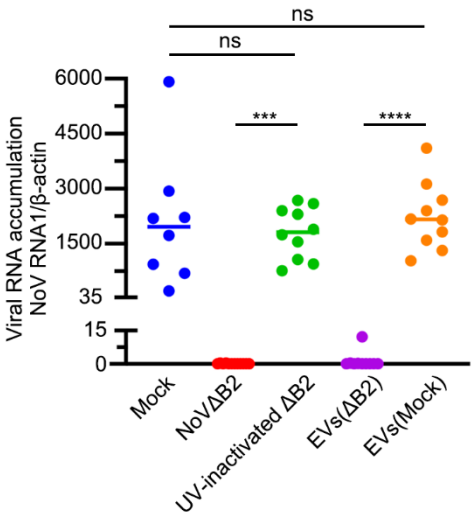
Fig 4 D, Diagram showing the 462-nt sequence in length from NoVΔB2 RNA1 was inserted into the 3' untranslated region (UTR) of firefly luciferase reporter mRNA, to be targeted by antisense vsiRNAs with EVs from NoVΔB2-immunized BALB/c suckling mice. A translation initiation codon-deleted GFP sequence of the same length was inserted into the same site as control. E, F, Relative luciferase activity of the two reporter constructs after transfection of 293T cells (E) and 293T-Ago2 KO cells (F) and treatment by different pools of EVs, EVs (ΔB2) or EVs (Mock). Error bars indicate standard deviation of three independent replicates. *** indicates $p<0.001$, ns indicates no significant difference by student's t-test.

-No information regarding potential immunological effect of the exposure of mice to mutant NoV are included, so the authors assume it is mainly mediated by the vsiRNAs or vesicles. Analysis of innate immune subsets exhibiting signs of trained immunity or increased levels of activation on myeloid cells, NK cells should be studied to rule out other significant cell-mediated mechanisms controlling viral replication after exposure to NoVΔB2 NoV. In this regard, to claim that NoVΔB2 can be used as a live-attenuated vaccine, which indeed may be an interesting vaccine candidate, the NoVΔB2 should confer long-term protection and not just short-term as authors show in Fig. 1A. Therefore, the authors should test whether immunization with the mutant virus confers protection against a challenge with the WT virus leaving a time gap of at least 3 weeks between immunization and challenge. In this line, it would be of great interest to include not only immunization with NoVΔB2, but also with EVs (mock) and EVs (ΔB2) to see if they can also be used as a long-term immunization tool.

Authors' response: We appreciate your insightful comment on the additional immune components that may also contribute to the protection induced by NoVΔB2 immunization. In this work, we hope to combine all of the results, including those from the newly added experiments, to show a novel antiviral role of EVs mediated by the associated vsiRNAs in mice immunized by a VSR-deficient mutant virus. As suggested, we have performed the long-term protection experiments (please see lines 275-277, or below).

We found that mice at 21 days post-immunization with either live NoVΔB2 or EVs from NoVΔB2-immunized mice remained highly resistant to NoV challenge compared those immunized with DMEM, UV-inactivated ΔB2 or EVs from mock-immunized mice (Fig EV5).

Extended Data Figure 5 | Long-term protection by NoVΔB2 and EVs (ΔB2). BABL/c suckling mice were immunized with DMEM, live NoVΔB2, UV-inactivated NoVΔB2, or EVs from mock or NoVΔB2-immunized mice. At 21 days post-immunization, the mice were challenged with WT NoV by intraperitoneal injection. These challenged mice were sacrificed at 3dpi, and NoV RNA1 levels in hindlimb muscle tissue were determined by RT-qPCR. Normalization was done by β-actin mRNA. *** indicates p<0.001, **** indicates p<0.0001, ns indicates no significant difference by student's t-test, n=8-10 per group.



Minor points:

- No definition of vsiRNAs abbreviation in Introduction, in addition to abstract.

Authors' response: We have included the definition of vsiRNAs abbreviation in revised introduction (lines 59-60).-Thanks

-Introduction describing background on the topic is a bit vague and the objective of

the study is not clearly stated.
Authors' response: We have modified the introduction section to clearly state the objective of the study (see Lines 110-118): "We have shown that infection with the VSR-deficient mutant of NoV, NoVΔB2, induces production of abundant vsiRNAs and is rapidly cleared in suckling mice in contrast to lethal infection by wild-type NoV (Li *et al*., 2013). Intriguingly, we subsequently found that infant mice became fully protected against lethal NoV challenge after immunization with NoVΔB2 for only two days, suggesting rapid induction of protective immunity at whole organism level. A recent study has shown that infection of fruit flies with Sindbis virus (SINV) induces secretion of vsiRNAs-containing exosome-like vesicles circulating in the haemolymph sufficient to confer passive protection against SINV challenge in naive flies (Tassetto *et al*, 2017). This prompted us to investigate a potential role of EVs in NoVΔB2-triggered protection in mice."

- The western blot characterization of expression of CD9 and CD63 on EVs shown in Figure 2D is insufficient to determine whether they represent exosomes, a more rigorous analysis of additional markers previously described enriched in exosomes such as TSG101, HSP70, etc should be included.
Authors' response: As suggested, we have included additional markers in the updated Fig 2D (please see below).

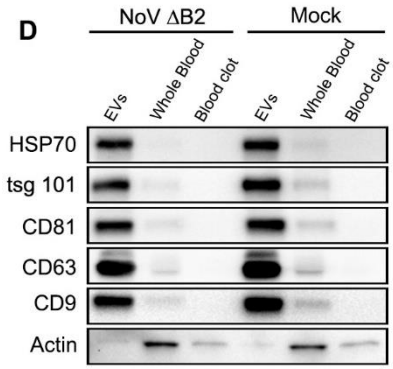


Figure 2 D, Western blotting of HSP70, tsg 101, CD81, CD63, and CD9 in EVs, whole blood, and blood clot derived from suckling mice infected with DMEM or NoVΔB2. Staining of β-actin was used as a loading control.

- Grammatical errors throughout the manuscript should be corrected. Expressions such as People hypothesized...(line 78); cells that secrete IFN have pattern-recognition receptors (PRR) (line 99), should be avoided.
Authors' response: Thank you for pointing these out. We have thoroughly corrected these and other errors in the revised manuscript (please see lines: 79-81, 101-102).

- The title is not very descriptive of the main findings of the article and gives the idea of the article being rather a review. Hence, I would suggest to reformulate as a

summary sentence of the main finding.
Authors' response: Thank you for your suggestion. We changed the Title to "Mouse circulating extracellular vesicles contain virus-derived siRNAs active in antiviral immunity".

- The referencing style should be kept homogeneous throughout the text (e.g. attention in line 44 or 46).
Authors' response: We have fixed it. Thanks.

- Line 57-58: I would introduce the sentence with a connector such as "...2020). Therefore, the role..."
Authors' response: We have fixed it. Thanks.

- Line 89: "a recent study" rather than "recent study"
Authors' response: We have corrected this error. Thanks.

- References should be carefully checked (e.g. Maillard et al., 2019)
Authors' response: We have corrected this error. Thanks.

1st Revision - Editorial Decision **21st Feb 2022**

Thank you for submitting your revised manuscript. We have received comments from the initial three referees (please see below) and I am pleased to say that they all now support publication. Referee #1 has some remaining minor comments that can be addressed in the final revised version of the manuscript. In addition, in this version, I would ask you to please resolve a number of editorial issues that are listed in detail below. Please feel free to contact me if you have further questions regarding the revision or any of the specific points listed below. Thank you again for giving us the chance to consider your manuscript for The EMBO Journal.

Referee #1:

As mentioned in my previous comments, the findings from Zhang, Dai and colleagues are exciting and constitute an important step in the understanding of antiviral RNAi in mammals. The authors showed the presence of viral-derived small interfering RNAs (vsiRNAs) in extracellular vesicles (EVs) and demonstrated that these EV-associated vsiRNAs were functionally active as they conferred a homology-dependent antiviral activity to both cells or mice. The authors addressed all the concerns that I raised previously, I only have the following minor comments:

• Page 10, line 204-205 : the authors found that the most abundant miRNAs in extracellular vesicles (EVs) from mice infected with NoV deltaB2 are miR-381-3p. The authors showed that these miRNAs are in low amounts in blood samples, whereas they represented the third most abundant miRNA extracted from muscle tissue. To this reviewer, these data suggest that the EVs likely derive from infected muscle tissue and not from blood cells. However, the authors mentioned that "The accumulation patterns of specific cellular miRNAs and vsiRNAs indicate that although an origin from the virus-infected muscle tissues cannot be ruled out, EVs-associated vsiRNAS are unlikely from blood cells". It is unclear why the authors mentioned that the likely origin of these EVs "cannot be ruled out". The authors should consider changing their sentence to clarify their conclusion on the potential origin of the EVs based on their findings.

Page 14, line 296-297: "....EVs purified from NoV deltaB2-infected AG6 mice." According to Figure 5A, in this experiment EVs

•

are purified from BALB/c suckling mice and injected in AG6 mice. Please change "AG6 mice" for "BALB/c suckling mice" in this sentence.

• Page 32, lines 799-802: the figure legend for figure 5C should mention more details on the experiment for clarity, e.g. that the data show the relative viral RNA 1 accumulation levels at 1dpi in Nov WT-infected AG6 mice 12 hours after injection with EVs from NoV deltaB2 or mock-inoculated BALB/c mice.

• Extended Data Figure 4: The labels "si-GAPDH" and "si-NC" are misplaced in the figure. There is no description of what is represented in blue and red in the figure legend. To this reviewer's understanding, the blue histograms represent the level of GAPDH mRNA in cells (293T or Ago2 KO) transfected with si-GAPDH, whereas the red histogram shows GAPDH mRNA levels in cells transfected with siRNA control (si-NC). The "si-GAPDH" label should therefore be placed under or on top of the blue histograms and the "si-NC" should be placed under/on top of the red histograms.

Referee #2:

The authors have addressed all my comments and I am happy to recommend publication of the revised manuscript.

Referee #3:

The authors have addressed most of my previous concerns, including increasing the sample size for the figure 1C experiments, providing proof of implication of vsiRNA present on EVs reducing viral RNA, using the lucierase promoter experiments and by quantifying levels of viral RNA. Finally, authors have also demonstrated that immunization with EVs also confers long term protection to secondary viral challenges. Although the authors did not address the potential immune innate and adaptive mechanisms contributing to the mentioned control, the manuscript has considerably improved from the original version

Referee #1:

As mentioned in my previous comments, the findings from Zhang, Dai and colleagues are exciting and constitute an important step in the understanding of antiviral RNAi in mammals. The authors showed the presence of viral-derived small interfering RNAs (vsiRNAs) in extracellular vesicles (EVs) and demonstrated that these EV-associated vsiRNAs were functionally active as they conferred a homology-dependent antiviral activity to both cells or mice. The authors addressed all the concerns that I raised previously, I only have the following minor comments:

Authors' response: Thanks for your positive comments.

• Page 10, line 204-205 : the authors found that the most abundant miRNAs in extracellular vesicles (EVs) from mice infected with NoV deltaB2 are miR-381-3p. The authors showed that these miRNAs are in low amounts in blood samples, whereas they represented the third most abundant miRNA extracted from muscle tissue. To this reviewer, these data suggest that the EVs likely derive from infected muscle tissue and not from blood cells. However, the authors mentioned that "The accumulation patterns of specific cellular miRNAs and vsiRNAs indicate that although an origin from the virus-infected muscle tissues cannot be ruled out, EVs-associated vsiRNAS are unlikely from blood cells". It is unclear why the authors mentioned that the likely origin of these EVs "cannot be ruled out". The authors should consider changing their sentence to clarify their conclusion on the potential origin of the EVs based on their findings.

Authors' response: Thank you for your suggestions. We have modified the sentence in the revised manuscript "The accumulation patterns of specific cellular miRNAs and vsiRNAs indicate that although additional studies are necessary to conclude an origin from the virus-infected muscle tissues, EVs-associated vsiRNAs are unlikely from blood cells"(please see lines: 199-201).

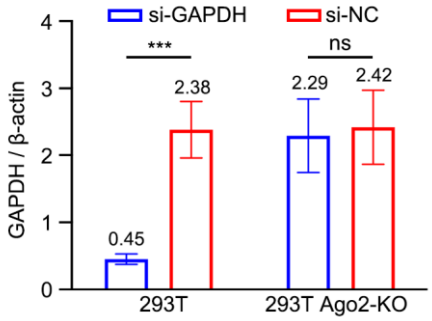
• Page 14, line 296-297: "....EVs purified from NoV deltaB2-infected AG6 mice." According to Figure 5A, in this experiment EVs are purified from BALB/c suckling mice and injected in AG6 mice. Please change "AG6 mice" for "BALB/c suckling mice" in this sentence.

Authors' response: Thank you for pointing it out. We have changed it in the revised manuscript.

• Page 32, lines 799-802: the figure legend for figure 5C should mention more details on the experiment for clarity, e.g. that the data show the relative viral RNA 1 accumulation levels at 1dpi in Nov WT-infected AG6 mice 12 hours after injection with EVs from NoV deltaB2 or mock-inoculated BALB/c mice.

Authors' response: We have modified the figure legend for fig 5C "Relative viral RNA1 accumulation levels at 1dpi in NoV WT-infected AG6 mice 12 hours after injection with EVs from NoVΔB2 or mock-inoculated BALB/c mice were measured by RT-qPCR".-Thanks

• Extended Data Figure 4: The labels "si-GAPDH" and "si-NC" are misplaced in the figure. There is no description of what is represented in blue and red in the figure legend. To this reviewer's understanding, the blue histograms represent the level of GAPDH mRNA in cells (293T or Ago2 KO) transfected with si-GAPDH, whereas the red histogram shows GAPDH mRNA levels in cells transfected with siRNA control (si-NC). The "si-GAPDH" label should therefore be placed under or on top of the blue histograms and the "si-NC" should be placed under/on top of the red histograms.
Authors' response: Thank you for pointing it out. We have updated this Figure.



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The authors have addressed all my comments and I am happy to recommend publication of the revised manuscript.
Authors' response: Thanks for your positive comments.

Referee #3:
The authors have addressed most of my previous concerns, including increasing the sample size for the figure 1C experiments, providing proof of implication of vsiRNA present on EVs reducing viral RNA, using the lucierase promoter experiments and by quantifying levels of viral RNA. Finally, authors have also demonstrated that immunization with EVs also confers long term protection to secondary viral challenges. Although the authors did not address the potential immune innate and adaptive mechanisms contributing to the mentioned control, the manuscript has considerably improved from the original version
Authors' response: Thanks for your positive comments.

ACCEPTED **4th Mar 2022**

Thank you again for submitting the final revised version of your manuscript. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

EMBO Press Author Checklist

Corresponding Author Name: Yang Li
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2021-109902

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Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures
1. Data
The data shown in figures should satisfy the following conditions:
- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:
- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
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Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods

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For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods

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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods, Table EV2

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Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and Methods
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods

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Please detail **housing and husbandry conditions**.	Yes	Materials and Methods

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Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Materials and Methods
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Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods

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In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Materials and Methods, Figure
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Materials and Methods, Figure

Ethics

Ethics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approva**l (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

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Data availability	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
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