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The herpesviral antagonist m152 reveals differential activation of STING-dependent IRF and NF-κB signaling and STING's dual role during MCMV infection

Markus Stempel, Baca Chan, Vanda Juranić Lisnić, Astrid Krmpotić, Josephine Hartung, Søren R. Paludan, Nadia Füllbrunn, Niels A. W. Lemmermann, Melanie M. Brinkmann

Review timeline:

Editor: Karin Dumstrei

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision   19th Nov 2018

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been reviewed by two referees and their comments are provided below.

As you can see the referees appreciate that the analysis adds important new insight and support publication here. They raise some issues that I anticipate that you should be able to sort out in a good way. Given the concerns raised I would like to invite you to submit a suitably revised manuscript.

REFEREE REPORTS:

Referee #1:

In the presented work, Brinkmann and colleagues reveal m152, a murine CMV expressed protein, as a natural antagonist of STING.The screening of a collection of MCMV ORFs led to the identification of m152, which upon overexpression, counteracted the STING-induced type I IFN response, but not the one induced by other pattern recognition receptors (e.g., RIG-I, TLR9). Further analysis shows that m152 interacts with STING at the endoplasmic reticulum and that this interaction is mediated by the N-terminal part of m152 and luminal regions of mouse but not human STING. As an effect, the translocation of STING to the Golgi - a prerequisite for type I IFN induction - is delayed concomitant with a delayed activation of the downstream signaling molecules TBK1 and IRF3, respectively. Interestingly, studying the role of m152 during MCMV infection, the investigators observed a selective impairment of m152 on STING-mediated type I IFN induction, but not on IL6 expression. This led to the hypothesis that STING may differentially regulate type I/IRF3 induction versus IL6/NF-kB activation. Assessing several STING mutants for their effect on

IRF3 versus NF-kB activity, the authors indeed identify one mutant construct (K288R) that failed to translocate and to trigger IFNb expression, but still exerted NF-kB activity. Finally, it is shown that the selective induction of NF-kB by the STING K288R mutant led to increased viral replication, which may explain why STING exerting both antiviral type I IFNs and proviral NF-kB cannot confer resistance to viral replication in the context of MCMV.

Overall, this is a very interesting study, which uncovers a new means of viral antagonism targeting the cGAS-STING pathway. The observation that m152 differentially suppresses type I IFNs from proinflammatory cytokines is in particular intriguing. There are only minor comments that I would have.

1) Using a STING mutant construct, the authors show that NF-kB signaling downstream of the STING pathway can be initiated from the ER. Does the NF-kB activation remain intact if blocking ER-to-Golgi trafficking of STING (e.g., using compounds such as Brefeldin A or Nocodazole)?

2) Along these lines, is there evidence that NF-kB activation occurs earlier upon STING activation than IRF-3?

3) The species-specific effect of m152 is interesting. Could one speculate about a similar mechanisms acting an human STING, which may be encoded by a human pathogenic herpes virus?

Referee #2:

EMBOJ-2018-100983
Stempel et al.

The multifaceted viral antagonist m152 reveals a dual role for STING during MCMV infection

Summary
Authors reveal that the murine cytomegalovirus (MCMV) m152 gene product, which has previously been shown to be a potent inhibitor of NK and T-cell by down regulation of RAE-1 and MHC class 1, is a direct inhibitor of the innate immune signaling molecule STING. They show that m152 binds to and inhibits murine, but not human, STING via its ER luminal domains to delay the translocation of STING to the golgi compartment where it drives the production of type I interferon. It does so independently of STING activation or dimerization. Due to this unique mechanism of inhibition, STING-dependent NFkB activation remains intact to augment initial viral transcription, while type I interferon signaling is prevented.

Impressions
The major claim of this manuscript is that m152 is a multifunctional protein that targets STING during the early phases of infection. The authors demonstrate a very novel mechanism of action for m152, and provide compelling data to support their claims. For the most part, the work is high quality.

Major criticisms

1) The mutant virus constructed, as well as the virus designated as 'parental' are also deficient for m157 (fig 6). While technically controlled throughout the manuscript, it's kind of disingenuous to be doing in vivo experiments in B6J mice and chalking the phenotype entirely up to m152. I can entirely appreciate that the cell culture experiments and transfections are supported by the data, but at best authors should be prefacing their conclusions with "in the absence of m157....." Moreover, it's also insufficient to suggest that loss of m157 influences MCMV replication (e.g. line 365-6 "Defective replication of MCMV lacking m152 in vivo is mediated by STING-dependent signaling") when the data provided is only transcriptional analyses. If you say replication, measure replication. You've already biased the in vivo infections in B6J mice by using an m157-null mutant, so there should be plenty of replication to measure.

2) What kinetic class is m152? Authors show expression at 2hrs post-infection. Is this an immediate-early gene? Pinning that down would REALLY help the model.

Minor Criticisms
1) Figure 1 B, C, D, E, J, K are all negative data, and could be more appropriate as supplementary data. Also, Fig 1 F&G could be combined.
2) Figure 3D - also could be supplementary.
3) Figure 4F - there's still a pretty decent amount of human STING co-IPing with m152, enough that it makes it tough to make really definitive statements about the amount being pulled down. Can the three independent experiments be quantitated and graphed to bolster the conclusion?
4) Figure 6 -Infection of m152-V5 transduced cells would be expected to rescue the defects seen with the knockout virus? This might help get at Major point #1.
5) Line 437 - looks like a space missing in "IRFarms." Maybe just my copy.

1st Revision - authors' response   2nd Dec 2018

Please see next page.

Response to reviewers

Referee #1:

In the presented work, Brinkmann and colleagues reveal m152, a murine CMV expressed protein, as a natural antagonist of STING.The screening of a collection of MCMV ORFs led to the identification of m152, which upon overexpression, counteracted the STING-induced type I IFN response, but not the one induced by other pattern recognition receptors (e.g., RIG-I, TLR9). Further analysis shows that m152 interacts with STING at the endoplasmic reticulum and that this interaction is mediated by the N-terminal part of m152 and luminal regions of mouse but not human STING. As an effect, the translocation of STING to the Golgi - a prerequisite for type I IFN induction - is delayed concomitant with a delayed activation of the downstream signaling molecules TBK1 and IRF3, respectively. Interestingly, studying the role of m152 during MCMV infection, the investigators observed a selective impairment of m152 on STING-mediated type I IFN induction, but not on IL6 expression. This led to the hypothesis that STING may differentially regulate type I/IRF3 induction versus IL6/NF-kB activation. Assessing several STING mutants for their effect on IRF3 versus NF-kB activity, the authors indeed identify one mutant construct (K288R) that failed to translocate and to trigger IFNb expression, but still exerted NF-kB activity. Finally, it is shown that the selective induction of NF-kB by the STING K288R mutant led to increased viral replication, which may explain why STING exerting both antiviral type I IFNs and proviral NF-kB cannot confer resistance to viral replication in the context of MCMV.

Overall, this is a very interesting study, which uncovers a new means of viral antagonism targeting the cGAS-STING pathway. The observation that m152 differentially suppresses type I IFNs from proinflammatory cytokines is in particular intriguing. There are only minor comments that I would have.

We thank the reviewer for these very nice words of appreciation.

1) Using a STING mutant construct, the authors show that NF-kB signaling downstream of the STING pathway can be initiated from the ER. Does the NF-kB activation remain intact if blocking ER-to-Golgi trafficking of STING (e.g., using compounds such as Brefeldin A or Nocodazole)?

Thank you for this insightful comment. Indeed, we have performed this experiment and used Brefeldin A to block ER to Golgi transport. However, in the presence of Brefeldin A, we did not observe STING-dependent activation of the NF-κB pathway (and, as expected and shown in **Figure EV3B**, of the IRF pathway). This result was not completely surprising for us, since Brefeldin A leads to a collapse of the Golgi compartment, and results in accumulation of proteins in the ER, which may negatively affect their function. This result therefore neither supported nor contradicted our hypothesis that STING may activate NF-κB signaling already from the ER. Since the Brefeldin A approach did not give us a conclusive result, we performed the screen for a STING mutant that is unable to leave the ER and can therefore not induce the interferon response. With the K288R mutation we could identify such a mutant and test if it was capable of inducing the NF-κB pathway, which it could. This approach allowed us to draw the conclusion that STING indeed activates the NF-κB pathway from the ER.

2) Along these lines, is there evidence that NF-kB activation occurs earlier upon STING activation than IRF-3?

To our knowledge, no data exists in the literature about the precise kinetics of NF-κB and IRF3 activation upon STING signaling. We have not performed a time course experiment (using immunofluorescence or cellular fractionation and immunoblotting as a readout) to determine NF-κB and IRF3 nuclear translocation upon activation of the cGAS-STING pathway. The reasons are the following: We do not know how much of the total NF-κB pool in the cell is activated by ER-residing STING, however, we see that whatever gets activated suffices to fully activate NF-κB mediated transcription (**Figure 8D**). Since NF-κB is a well expressed protein, nuclear translocation of small amounts of NF-κB are difficult to be visualized - a faint signal in the nucleus may be masked by the strong signal in the cytosol, and we do not know how much NF-κB actually needs to be present in the nucleus to see transcriptional activation. In addition, antibodies have very different specificities and sensitivities, and just because we detect an endogenous protein by immunoblotting or immunofluorescence at a specific timepoint, it does not mean that it may not be expressed or present in a certain compartment already at an earlier timepoint. This is why we think that it would be difficult to draw conclusions from a time course experiment using immunofluorescence or cellular fractionation and compare NF-κB with IRF3. Even looking at phosphorylated versions of p65 or IRF3 by immunoblotting in a time-resolved manner may not be conclusive due to different sensitivities of the respective antibodies.

When looking into the literature regarding STING-dependent signaling, not much has been shown regarding STING-dependent NF-κB activation. However, there is evidence that STING activates NF-κB via a different mechanism than the activation of IRF3 by the kinase TBK1, including the publication from the Barber group showing that the human STING K224R mutant, which is not able to activate IRF signaling, can activate NF-κB signaling (Ni et al., 2017). Our study with the murine STING K288R mutant shows that NF-κB can be activated by ER-resident STING upon stimulation prior to STING trafficking (which is a prerequisite for IRF3 activation). Therefore we conclude that NF-κB activation occurs at an earlier time point than IRF3 activation.

Along these lines, in the revised version of the manuscript we now include data showing that STING WT and STING K288R both still activated the NF-κB reporter in TBK1 knockdown cells, but not the IRF3 reporter. We have now included this result as our new **Appendix Figure 3**. Taken together, we are convinced that STING-dependent NF-κB activation occurs prior to trafficking of STING to the Golgi compartment, and consequently prior to activation of IRF3, and that this NF-κB activation is independent of the kinase TBK1. However, how STING exactly activates the NF-κB pathway from the ER still merits further investigation.

3) The species-specific effect of m152 is interesting. Could one speculate about a similar mechanisms acting an human STING, which may be encoded by a human pathogenic herpes virus?

This is indeed an interesting thought. To our knowledge, no such mechanism has been reported about for a human herpesvirus. Since herpesviruses have evolved with their respective hosts, a similar or comparable mechanism can, but not necessarily has to, be present in a human beta-herpesvirus. For example, human cytomegalovirus encodes the UL82 protein, which targets trafficking of STING, but by a different mechanism than m152 (Fu et al., 2017, Cell Host & Microbe). The interaction of UL82 and STING disrupts both IRF3- and NF-κB dependent responses, while m152 interacts with STING via its luminal loop regions and does not disturb the cytosolic signaling platform, and thus NF-κB signaling can still occur. While HCMV and MCMV show a high degree of similarity, they may have different requirements at different stages of their respective life cycle. Another possibility is that HCMV has evolved other means to activate the NF-κB response to promote viral replication.

Referee #2:

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Summary
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Impressions
The major claim of this manuscript is that m152 is a multifunctional protein that targets STING during the early phases of infection. The authors demonstrate a very novel mechanism of action for m152, and provide compelling data to support their claims. For the most part, the work is high quality.

Thank you very much for taking the time to carefully assess our manuscript and your very constructive criticism. We are very pleased that you consider our study novel and compelling.

Major criticisms

1) The mutant virus constructed, as well as the virus designated as 'parental' are also deficient for m157 (fig 6). While technically controlled throughout the manuscript, it's kind of disingenuous to be doing in vivo experiments in B6J mice and chalking the phenotype entirely up to m152. I can entirely appreciate that the cell culture experiments and transfections are supported by the data, but at best authors should be prefacing their conclusions with "in the absence of m157....." Moreover, it's also insufficient to suggest that loss of m157 influences MCMV replication (e.g. line 365-6 "Defective replication of MCMV lacking m152 in vivo is mediated by STING-dependent signaling") when the data provided is only transcriptional analyses. If you say replication, measure replication. You've already biased the in vivo infections in B6J mice by using an m157-null mutant, so there should be plenty of replication to measure.

Thank you for this valuable comment.
Regarding the term "replication": we agree and have modified the text accordingly. As pointed out by the reviewer, we have only measured transcription of viral genes and not replication by standard plaque assay. We could not measure replication because we look at very early time points (6 and 16h) post infection. At this point, the virus has not replicated enough to measure viral titers. It was not possible to infect the mice with higher viral doses, they would have succumbed to it. We cannot look at later time points because m152 also has an effect on the NK cell response and CD8 T cell response at later stages of infection. Moreover, the type I IFN response at this early phase of infection (6-16h) is STING-dependent - at later time points (e.g. 36h post infection) TLRs are responsible for the type I IFN response and not STING. Since m152 only affects STING-mediated type I IFN induction we therefore have to look at those early time points.

Regarding the MCMV m157 protein: we kindly but firmly disagree. We think that we can draw the conclusion that the phenotype we observe is solely due to the m152 protein, and not to the m157 protein. As shown in Figure S6 (**now Figure EV5**), we observe the same phenotype as reported by us for B6J mice also in Balb/c mice infected with WT MCMV, which expresses both the m152 and the m157 protein, and the Δm152 mutant, which does not express m152 but still expresses m157. We agree with the reviewer that performing the *in vivo* experiments in B6J mice with the Δm157 virus was not optimal, but since STING$^{-/-}$ mice are not available on the Balb/c background, this was the only option. To convince ourselves that m157 does not contribute to the observed phenotype, we performed the Balb/c experiment shown in Figure S6 of our original submission (**now Figure EV5**). To make this more clear, we could consider moving current Figure EV5 into the main figures, but would leave this decision to the editor.

2) What kinetic class is m152? Authors show expression at 2hrs post-infection. Is this an immediate-early gene? Pinning that down would REALLY help the model.

Thank you for pointing this out. Indeed, we see very early expression of m152 in the context of MCMV infection by qPCR and immunoblotting (**Figure EV4**). Previous studies on m152 have proposed that m152 is expressed with early kinetics. However, to our knowledge, no study has investigated the m152 expression pattern according to the classical definition of the herpesviral expression stages.

We now performed the experiment that formally addresses the question if m152 expression occurs with immediate-early or early kinetics:

iMEF were infected with parental MCMV (MCMV Δm157) at an MOI of 0.1 with centrifugal enhancement. Kinetic phases of viral gene expression, referred to as immediate-early (IE), early (E) and late (L) were determined as follows:
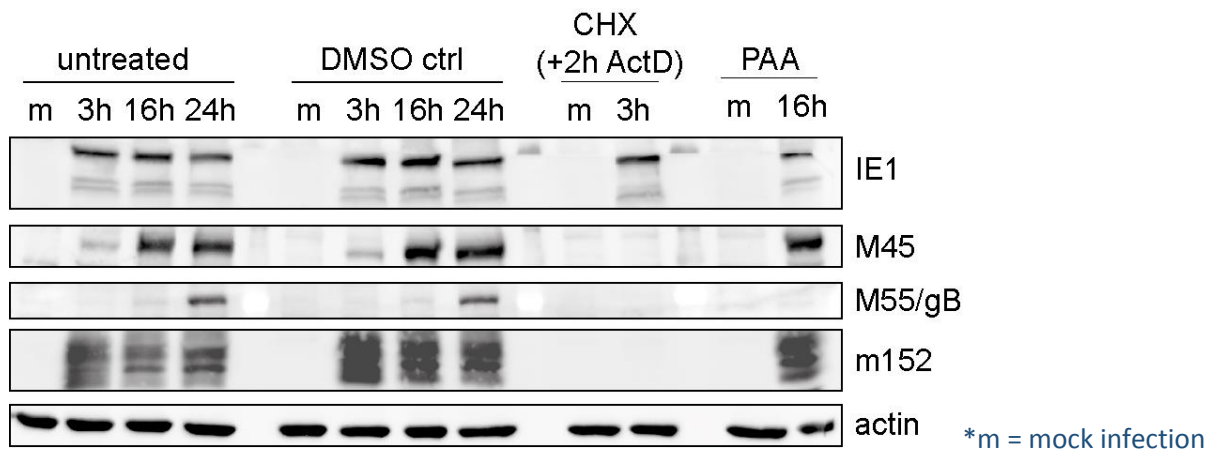
(i) immediate-early phase: 15 minutes prior to MCMV infection, culture medium was replaced with fresh medium containing 50 µg/ml Cycloheximide (CHX, stock solution diluted in DMSO) to block *de novo* protein synthesis. Infection was performed in the presence of CHX. 3 hours post infection, cells were washed with medium containing 5 µg/ml Actinomycin D and were incubated for 2 hours to allow IE gene expression and block further E-phase transcription.

(ii) early phase: The culture medium was replaced with fresh medium containing 250 µg/ml phosphonoacetic acid (PAA) 15 minutes before infection to block DNA synthesis and thereby MCMV replication, allowing the expression of IE and E genes, but not L proteins. MCMV infection was performed in the presence of PAA and cells were harvested at 16 hours post infection.

(iii) late phase: The culture medium was replaced with fresh medium without the addition of metabolic inhibitors 15 minutes before infection. Cells were harvested at 24 hours post infection.

Samples were analyzed using immunoblotting analysis to determine the protein expression of immediate-early (MCMV IE1), early (MCMV M45) and late (MCMV M55) proteins.

Representative result of two independent experiments:

CHX	
untreated	DMSO ctrl	(+2h ActD)	PAA
m 3h 16h 24h	m 3h 16h 24h	m 3h	m 16h

IE1

M45

M55/gB

m152

actin   *m = mock infection

Based on our results, m152 would be classified as an early gene according to the classical definition of herpesviral expression kinetics. However, this classical definition is dependent on the aforementioned experimental setup, which may or may not reflect the real picture. In 2014, Weekes et al. published quantitative temporal viromics for HCMV and observed that the IE/E/L classification is not descriptive enough and proposed a model of five temporal classes for gene expression. This may also apply to MCMV.

Supporting our results, Marcinowski et al. (2012) reported transcriptomic profiling during MCMV infection and showed that newly synthesized m152 mRNA reaches its peak already at 1-2 hours post MCMV infection. We also see this in our experimental setups.

Taken together, we conclude that although MCMV m152 falls into the classical category of an early gene, the very early transcription of the m152 gene upon infection enables MCMV to express the protein at very early time points post MCMV infection.

Minor Criticisms
1) Figure 1 B, C, D, E, J, K are all negative data, and could be more appropriate as supplementary data. Also, Fig 1 F&G could be combined.

Thank you for this comment. However, we would prefer to leave these figures in the main figure. Although they are "negative" results, they are important to demonstrate the specificity of m152. But we leave the final decision to the editor.

2) Figure 3D - also could be supplementary.

We agree; we moved Figure 3D now to **EV Figure 2A**.

3) Figure 4F - there's still a pretty decent amount of human STING co-IPing with m152, enough that it makes it tough to make really definitive statements about the amount being pulled down. Can the three independent experiments be quantitated and graphed to bolster the conclusion?

We now quantified the three independent experiments and graphed the results as suggested. The results are presented as new **Appendix Figure 2B** and support our conclusions.

4) Figure 6 -Infection of m152-V5 transduced cells would be expected to rescue the defects seen with the knockout virus? This might help get at Major point #1.

Thank you for this valuable comment. Indeed, when infecting cells stably expressing m152-V5 with MCMV m152stop, we would expect a rescue of the phenotype. However, as pointed out above, since we performed and thoroughly controlled our *in vitro* and *in vivo* experiments, we can clearly pinpoint the phenotype down to the m152 protein.

5) Line 437 - looks like a space missing in "IRFarms." Maybe just my copy.

Thank you, we have corrected this.

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been re-reviewed by the referees and their comments are provided below. As you can see both referees appreciate the introduced changes and support publication here. I am therefore very pleased to accept the manuscript for publication in The EMBO Journal.

Before I can send you the formal acceptance letter there are just a few things that we have to sort out.

- Please add 3-5 keywords to the manuscript

- Please add a running title to the manuscript

- We are also missing an AC (Author contributions) section.

- The figures should be removed from main MS file but their legends should stay in. The EV figure legends need to be added to main MS.

- You have at the moment 7 EV figures, but we can only have 5. Is there a way to combine some of them or alternatively you can add the 2 extra ones to the appendix.

- Figure callouts: Fig 3D is not called out; Fig 3F is called out but there is no Fig 3F.

- The M&M should be placed before References.

- The appendix file needs a Table of content

- Our publisher has done a pre-publication check on the manuscript and has some comments (see figure legends). Please address their comments and incorporate their suggestions. You should be able to see the file when you log in. The file is called Wiley Pre-acceptance Check - please check the word document as it is this file where you can see the marked changes

- We now encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. It would be great if you could provide me with a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figure? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files.

- We include a synopsis of the paper that is visible on the html file (see http://emboj.embopress.org/). Could you provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper?

- It would also be good if you could provide me with a summary figure that I can place in the synopsis. The size should be 550 wide by 400 high (pixels).

That should be it - you can use the link below to upload the revised version. Let me know if you have any further questions

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REFEREE REPORTS:

Referee #1:

The authors`s reply to my concerns are convincing. I do not have any additional comments.

Referee #2:

The authors have sufficiently addressed the concerns of this reviewer. Incorporation of additional data strengthens the conclusions and enhances the manuscript. While we may 'kindly, but firmly disagree' on the execution of the viral genetics, the authors' position is on this point is widely accepted in the field. This reviewer is ultimately comfortable with the authors conclusions.

2nd Revision - authors' response   16th Dec 2018

The authors made all editorial changes.

Referee #2:

EMBO PRESS

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Melanie M. Brinkmann
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2018-100983

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- 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.
➜ figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
➜ graphs 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 and any statistical test employed should be justified
➜ Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship 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.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html	Biosecurity Documents from NIH
http://www.selectagents.gov/	List of Select Agents

B- Statistics and general methods

Please fill out these boxes ⬇ (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Based on previous experience and published literature we calculated a group size of 5-6 mice using the program biomath to ensure a statistical significance in our observations when analyzing the data with unpaired t-test.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	see 1a
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No data were excluded from this study.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Animals were selected randomly but matched for gender and age. Cell lines were controlled regularly for mycoplasm and experiments were performed with cells at different passage stages to ensure consistent phenotypes.
For animal studies, include a statement about randomization even if no randomization was used.	Mice for in vivo experiments were age-matched to 8-9 weeks and divided into experimental groups of the same number. The constellation of groups was randomized. Infection and sample analysis were performed by two different people.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	For in vivo experiments, the constellation of the groups was randomized. The person performing the experiments was blinded regarding the viruses. Moreover, infection experiments were performed at the Helmholtz Centre for Infection Research in Braunschweig, Germany, and the Faculty of Medicine, University of Rijeka, Croatia from two independent persons blinded to the viruses used.
4.b. For animal studies, include a statement about blinding even if no blinding was done	For the in vivo experiments, the person performing the infection of the mice was blinded regarding the viruses used (i.e. WT vs. mutant virus).
5. For every figure, are statistical tests justified as appropriate?	Differences between two data sets were evaluated by Student's t-test (unpaired, two-tailed), in the case of viral transcript levels after log transformation of the data sets, using Graphpad Prism version 5.0 (GraphPad Software, San Diego, CA). P values <0.05 were considered statistically significant. Statistical tests were only performed when biological replicates were used, not in the case of experimental replicates.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Data have a normal distribution. For viral transcript numbers, data was log transformed to obtain normal distribution.
Is there an estimate of variation within each group of data?	Yes. Data is represented with the corresponding standard deviation and the sample number indicated in the respective figure legend.
Is the variance similar between the groups that are being statistically compared?	The variance between groups is similar in all experiments. In vitro experiments were mostly performed three independent times, in the case of in vivo experiments with at least 5 mice per group, to allow statistical relevance. All respective replicates were performed with a standarized protocol, allowing a similar variance between the statistically compared groups.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibodies used in this study: Cell Signaling Technology rabbit anti-STING (#13647, clone D2P2F): dilution 1:1000 (IB) Cell Signaling Technology rabbit anti-phospho-TBK1 (#5483, clone D52C2): dilution 1:1000 (IB) Cell Signaling Technology rabbit anti-phospho-IRF3 (#4947, clone 4D4G): dilution 1:1000 (IB) Cell Signaling Technology rabbit anti-p65 (#4764, clone C22B4): dilution 1:100 (IF) Cell Signaling Technology rabbit anti-TBK1 (#3504, clone D1B4): dilution 1:1000 (IB) Sigma-Aldrich mouse anti-tubulin (T6199, clone DM1A): dilution: 1:1000 (IB) Sigma-Aldrich mouse anti-actin (A5441, clone AC-15): dilution 1:5000 (IB) Invitrogen mouse anti-V5 antibody (R960-25): dilution 1:5000 (IB), 1:100 (IF) Biolegend mouse anti-V5 antibody (#680601): dilution 1:4000 (IB) Invitrogen mouse anti-V5 HRP conjugate (R961-25): dilution 1:2000 (IB) Mouse MCMV IE1 (#HR-MCMV-08, clone CROMA101): dilution 1:8000 (IB) Mouse MCMV m152 (#HR-MCMV-11, clone m152.05): dilution 1:1000 (IB) MCMV IE1 and MCMV m152 antibodies were generated at the Center for Proteomics (CapRi), Faculty of Medicine, University of Rijeka. Validation statements for all antibodies are provided on the respective manufacturer website. Data provided in the manuscript also confirmed specific reactivity with the relevant protein. Antibodies against MCMV IE1, M45, M55 and m152 were generated at the Center for Proteomics (CaPri) and extensively tested in different applications (Immunoblot, Immunofluorescence, Immunoprecipitation) for their specificity.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	M2-10B4 (CRL-1972), HeLa S3 (CCL-2.2) and human embryonic kidney 293-T/17 cells (293T, CRL-11268) were directly purchased from ATCC and authenticated by this organization. Primary B6J and Balb/c wildtype (WT) mouse embryonic fibroblasts (MEF), goldenticket MEF (MEFgt/gt) and primary bone marrow-derived macrophages were generated by standard protocol. The corresponding immortalized cell lines were genereted using SV 40 LT antigen, except the immortalized murine bone marrow-derived macrophage (iBMDM) cell line, which was obtained through BEI Resources, NIAID NIH (NR-9456). Cell lines generated in this study were controlled by the growth rate, morphology and the expression level of respective transduced proteins. No differences in growth rate or morphology compared to the corresponding wildtype cells were observed. All cell lines were tested negative for mycoplasma contamination

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Species: Mus musculus; Strains: C57BL/6J, Balb/c, MPYS-/- (also referred to as STING-/-), C57BL/6J-Tmem173gt (also referred to as goldenticket); gender: all mice used in this study were female; age: mice were age-matched to 8-9 weeks when performing the experiment. Mice were regularly genotyped to confirm their knockout status. Mice used in this study were bred at the animal facility of the Helmholtz Centre for Infection Research in Braunschweig, Germany, and at the Central Animal Facility, Faculty of Medicine, University of Rijeka, Croatia, were bred and maintained under specific pathogen-free conditions.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were performed in compliance with the German animal protection law (TierSchG BGBI S. 1105; 25.05.1998). The mice were handled in accordance with good animal practice as defined by FELASA and GV-SOLAS. All animal experiments were approved by the responsible state office (Lower Saxony State Office of Consumer Protection and Food Safety) under permit numbers #33.9-42502-04-12/0930 and #33.19-42502-04-17/2657. For NK-cell depletion experiments, all animals were housed and bred under specific pathogen–free conditions at the Central Animal Facility, Faculty of Medicine, University of Rijeka, Croatia, in accordance with the guidelines contained in the International Guiding Principles for Biomedical Research Involving Animals. The Ethics Committee at the University of Rijeka and National ethics committee approved all animal experiments.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	We confirm compliance

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	N/A
12. 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.	N/A
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	N/A
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	N/A
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	N/A
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	N/A
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	N/A

F- Data Accessibility

18. Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	N/A
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	N/A