THE EMBO JOURNAL

AKT2 reduces IFNβ1 production to modulate anti-viral responses and systemic lupus erythematosus

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DOI: 10.15252/embj.2021108016

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Review Timeline:

Submission Date:	13th Feb 21
Editorial Decision:	6th Apr 21
Revision Received:	4th Aug 21
Editorial Decision:	14th Oct 21
Revision Received:	2nd Dec 21
Accepted:	23rd Dec 21

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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Dear Dr. Wang,

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and I am afraid that the overall decision is not a positive one.

While the referees appreciate that the analysis reports new findings, I am also afraid that they bring up issues that preclude publication here. The referees raise concerns with the conclusiveness of the key findings, find that it is not clear if the effects of Akt2 on IFN signaling are direct or indirect and that many of the effects observed are modest. I realise that referee #3 is more positive, but given the overall assessment from the referees, I am afraid that we can't offer to consider publication here.

I thank you for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but I hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Referee #1:

Zheng et al propose in this manuscript that Akt2 inhibits the expression of type I IFN by phosphorylating IRF3 at Ser207, leading to impairment of nuclear translocation, which itself is mediated by 14-3-3 protein. The manuscript uses co-expression, KO of Akt2, IF of IRF3, co-IP and reporter gene expression in cells, zebrafish and Akt2 KO mice to make their point about Akt2 and IRF3.

After reading through this manuscript multiple times, this reviewer comes away feeling the data is selectively presented, overall conclusions are forced and the effects of Akt2 on IRF3 are minimal - often a 2-fold difference in expression levels. The effects of Akt2 on IFN signaling may be an indirect, not direct, consequence of inhibition.

- As an example the data on Akt-IRF3 association rely on overexpression and this is not satisfactory manner to demonstrate interaction. The Akt-IRF interactions may well be transient (enzyme-substrate). Perhaps mass spec analysis of endogenous proteins would provide a more clear answer.

- Does Ser207A interfere with nuclear localization sequence of IRF3 or is it in the region of the IAD, itself a closed hydrophobic structure that is not easily accessible.

-Other examples - Data on 14-3-3 involvement was not convincing.
-IRF3 nuclear translocation was not apparent.

-The KO studies in mice were more about Akt2 than IRF3-IFN and one may question that modification of a growth regulatory pathway could affect a range of unrelated pathways by 2-fold.

-huge amount of effort placed on this study - one wonders why the authors did not make a Ser207 phospho-specific Ab to determine more precisely the phosphorylation status of IRF3.

Referee #2:

The paper by Zheng et al describes how Akt 2 can phosphorylate Ser207 at IRF3, which leads to a modest reduction in IFNb expression. They further show that the absence of Akt2 I a mouse model confer increased susceptibility to VSV infection and decreased severity of SLE. While the amount of different data is impressive, and merit publication in EMBO, the writing is often compressed to the degree where it is difficult to follow the logic of the experiments. The figure legends and methods are also lacking in several cases. Some key control experiments to ensure the specificity of the proposed model is needed (listed below). Finally I find that the author exaggerate their result in a number of ways, properly they think the use of wording like Akt2 is crucial for IFNb ..... is needed to get accepted in EMBO, however I find this quite disturbing and also unnecessary, and therefore quite irritating when reading the paper.

Major comment
End of page 7: I do not find the following conclusion merited by the data: "Together, we have demonstrated that AKT2 kinase activity is critical for Ifnb1 production and virus clearance in macrophages ex vivo and in zebrafish in vivo." At best their data show that Akt2 has a modest effect on IFNb expression. In connection with this I will argue that the data presentation is biased, normally IFNb expression (with and without inducer) is shown on a logarithmical scale, whereas the authors consequently use a linear scale. It is unclear to me how relative IFNb expression is defined? And using the linear scale it is impossible to judge the IFNb expression in the mock samples. The authors should show all IFNb expression data as IFNb mRNA expression relative to the chosen house keeping gene, on a logarithmical scale. The figure legend should clearly state how the data was obtained. Importantly, they should avoid for further normalization.

To verify the model the authors put forward in vivo please conduct the following exp: In figure 5 where they characterize a Akt2 KO mouse, would they please measure both IFNb and IFNa expression post infection with VSV AND Influenza virus (nasal infection for influenza). According to their model IFNb expression should be altered by the absence of Akt2 but not IFNa (which is driven by IRF7)

General for all luciferase assay: Please show a western blow which verify expression of all relevant constructs. Also, please specify how data are reproduced. Normally I would do 3 side by side replicate using the same DNA prep for transfection and label them as technical replicate, than repeating the experiment 3 times using different DNA preparations, which would give biological replicate. Would the authors please specify how the experiment is carried out !

Figure 3A, while interesting the author should expand the data collected in the HEK293 system, TRIF is notorious difficult in the HEK system due to cell toxicity and TBK1 overexpression is a very general way of activating the IFNb reporter. Could the authors please use MAVS overexpression in an additional exp just to confirm the observation in figure 3A

Figure 3D. This is the entire figure legend to figure 3D: "Immunoblot analysis of the interaction between AKT2 and IRF3 in PEMs infected with VSV for 6 hours." it difficult to decipher the data based upon this. Please be much clearer about how experiments are conducted throughout the paper!

Figure 3D. I am not sure why they are looking at Ser396-P as several recent papers show that phosphorylation at this site has little impact on IRF3 function. Same for figure 4D

Figure 3I, I am not sure there is a significant difference between AKT2 alone and the combination of AKT2 and 14-3-3e, was this tested statistically?

Figure 4A, the difference in nuclear IRF3 are small land hard to make out from a single WB, the authors should show the duplicated experiments as supplementary data.
Based upon the description , I cannot understand (yet reproduce) figure 4B, what is a phosphate-gel? How does this work, no information is given.

Minor comment
This statement in the introduction is incorrect and do not take the newest literature into consideration "Phosphorylation of Ser396 and the five serine or threonine residues in the C-terminal of IRF3 (Ser396, Ser398, Ser402, Thr404, and Ser405) has been demonstrated as the major sites that represent the activated IRF3 via enhancing IRF3 dimerization(Fitzgerald et al, 2003; Lin et al, 1998; Lin et al, 1999)." Phosphorylation of Ser386 is clearly the defining event. Please site the following papers PMID: 32826280; 33205822

There is a data not shown in the discussion, do EMBO allow this?

The authors should distinguish between zebrafish larvae and adult zebrafish.

Referee #3:

Manuscript # EMBOJ-2021-108016
Title AKT2 inhibits type I IFN responses to regulate viral infections and SLE
Reviewer:
The authors describe the role of the AKT serine/threonine kinase 2 (AKT2) in the regulation of interferon (IFN) and IFN induced transcription upon pathogen infection and IFN-driven pathogenic autoimmune disorders driven by IRF3 activation. While the role of PI(3)K-AKT pathway in the regulation of innate immunity is controversial, this is a very interesting work that demonstrated a novel negative regulation of IRF3 activation and type I IFN production by the specific isoform AKT2 isoform. AKTs are a critical kinase family that can phosphorylate different target proteins and control a variety of cellular functions. Here, they showed that upon infection with various pathogens, induction of IFN and IFN signaling decreases AKT2 expression (knockdown of negative

regulator of IFN signaling pathway SOCS1/SOCS3 further reduce AKT2 expression). Mechanistically, they reported that AKT2, through the assist of 14-3-3ε, binds IRF3 and phosphorylates IRF3 at the position Thr207, a newly identified phosphorylation site, to attenuate IRF3 nuclear translocation and type I IFN production without affecting IRF3 dimerization. Overexpression of the AKT2 kinase-dead mutant or IRF3-T207A in zebrafish improved protection against infection. Akt2 deficient mice increased type I IFN induction and reduced mortality in response to virus infection, but aggravated severity of systemic lupus erythematosus (SLE). The treatment of mice with AKT2 selective inhibitor CCT128930 enhanced the survival rates in response to VSV infection. Adoptive transfer experiments of Akt2 KO BMs into irradiated CD45.1 recipient mice followed by VSV challenge showed higher survival rates, increased concentrations of serum Ifnb1, reduced VSV copies and titers from livers, spleens or lungs confirming that deletion or blocking AKT2 activity could be an effective strategy to enhance I-IFN production and facilitate the host against viral infection. Heightened type I IFN is a prominently hallmark in SLE patients and correlate with disease severity. TMPD-treated Akt2 KO mice (and adoptive transfer of Akt2 KO BM) developed a more serious diffuse pulmonary hemorrhage (DPH) that occur in some SLE patients with the increased mortality. They further confirmed the significantly reduced expression of AKT2 in PBMCs isolated from SLE patients, when compared that from healthy donors, strongly suggesting that AKT2 plays a protective role in the pathology of SLE. The study identifies AKT2 kinase as a novel target to either promote innate immune responses upon pathogen infection (by inhibition) and to reduce pathogenic type I IFN production upon autoimmune disorders as SLE (by promoting AKT2 kinase activity and phosphorylation of T207-IRF3).

Specific comments

IRF3 is a master transcription factor for I-IFN production, and IRF3 transcriptional activity and biological function are precisely regulated via its phosphorylation. This is an interesting perspective into IRF3 activation as it is generally thought that phosphorylation is a positive post-transcriptional modification that mediates the progression of antiviral signaling. Such inactivation via phosphorylation is relevant to the reported study describing phosphorylation of IRF3 at Ser97 (Thr75/Thr253) that is release by PTEN to activate IRF3 and the subsequent nuclear import [Li S, et al. PMID: 26692175]. Here, the authors provide strong evidence for the requirement of the kinase activity of AKT2 and phosphorylation of residue T207 of IRF3 to attenuate IRF3 nuclear translocation and to negatively regulate type I IFN production upon various PRR-mediated activation. This novel regulatory mechanism is strongly supported by the experimental evidence in this manuscript, and it adds relevant and highly significant information to our understanding of IRF3 activation and antiviral signaling pathways. Overall, the data clearly support the author's conclusions and ultimately the model provided. Nonetheless, several points remain to be addressed to fully appreciate the significance and generality of the findings.

Antiviral signalling pathways is differentiated from one another based on their recognition of specific pathogen associated molecular patterns (PAMP) or their spatial distribution inside the cell by various pathogen recognition receptors (PRR) leading to IFN production. Despite that upstream divergence, it can be observed that all the antiviral pathways converge on a limited set of downstream transcription factors, namely IRF3/7 and NF-κB (p65/p50). Once activated, theses transcription factors will mediate the first wave of antiviral defences by initiating the transcription of type I IFN (α/β) and pro-inflammatory cytokines (early phase). Then, in an autocrine and paracrine fashion, these IFNs will pledge to stimulate the second wave of antiviral defences through the cytoplasmic receptors and downstream effectors of the JAK/STAT mediated IFN amplification loop (IFNAR1/2-JAK-STAT1/2-IRF9 pathway) (late phase). For the clarity of the manuscript, the authors may wish to add a few sentences to make this explicit.

1. The study provides strong evidence of the transcriptional regulation of AKT2 upon the late phase of IFN signaling. It is expected to take place following IFN production in infected cells (autocrine) and in bystander cells of infection site (paracrine) when exposed to IFN-α/β. The authors should provide evidence or at least discuss the contribution of AKT2 in both phases, and specifically upon the antiviral priming of bystander cells and the impact on propagation of viral infection. Second, as IRF3 is responsible for the early phase of the induction of IFN-α/β, whereas IRF7 is involved in the late phase, whether AKT2 expression regulates IRF7-mediated activation IFN-α/β (PTEN was reported to regulate only IRF3-mediated activation of Ifnb1).
2. Li S et al., 2016 (PMID: 26692175) described a mechanism of negative regulation by phosphorylation of IRF3 at Ser97 in prevention of IRF3 dimerization and subsequent nuclear import and regulation by PTEN. This is briefly indicated in the discussion. PTEN was shown to promote antiviral response and IFN production by acting on IRF3 (dephosphorylation Ser97) but not on IRF7 induced in the late IFN amplification phase. PTEN is also known to antagonize the PI(3)P/AKT pathway which could contribute to a release of IRF3 activation via inactivation of AKT2 and reduced phosphorylation of T207-IRF3. The author should address this aspect. Author should mention this study in the introduction of the manuscript.
3. AKT2 mRNA is almost undetectable in PBMCs (Fig.S1A). Is this consistent with the main findings of the study?
4. The authors may wish to discuss a role of the cytoplasmic phosphorylated T207-IRF3 in the process of RIG-I-like receptor-induced IRF3 mediated pathway of apoptosis (RIPA) (Chattopadhyay S, PMID: 27815826; PMID: 27178468).
5. The main functions of IFN-Stimulated Genes (ISGs) are to restrict viral replication, balance viral recognition by PRRs, balance downstream activation of transcription factors, such as IRF3, and balance the induction of the IFN amplification loop. A subset of ISGs are themselves components of the IFN pathway or promote it signaling. However, IFN also induces several negative regulators which can target PRR, IRFs, or JAK/STAT to dampen the response. The authors may discuss a time frame and existence of a diverse set of genes for the novel regulatory mechanism by AKT2 (in early and late phase of antiviral responses) using a similar approach than Goulet et al. (PMID: 23633948) for analysis of the genes induced early (6 hrs.) and late (24 hrs.) following the activation of the RLR pathway by 5'pppRNA as a viral surrogate.
6. Does AKT2 also regulates PRR-mediated type III IFN production and requires IFN-λ receptor.
7. While this paper focused mainly on phosphorylation, it cannot be ignored that many other PTM, such as ubiquitination, methylation, acetylation, SUMOylation, ADP-ribosylation and glutamylation, oversee the activation of IRF3, the authors should discuss any potential relationship.
8. TBK1 kinase phosphorylate the adaptor proteins (MAVS, TRIF STING) that in turn recruit IRF3, thereby licensing IRF3 for

phosphorylation by TBK1. Phosphorylated IRF3 dissociates from the adaptor proteins, dimerizes, and then enters the nucleus to induce IFNs. As the phosphorylated Thr207-IRF3 is still able to dimerize it must not affect the licensing of IRF3. This should be discussed.
9. Mutation of the 5ST sites constitutively activates IRF3 following phosphomimic (aspartic acid) substitution. Phosphorylation of 5ST residues enable transition from an inactive to an active conformation. The authors demonstrated that IRF3-5D driven IFNB1 production is not regulated by AKT2. The authors may discuss if active conformation of IRF3 cannot be phosphorylated by AKT2.
10. How zebrafish (Danio rerio) IRF3 (DrIRF3) amino acid sequence related to IRF3 in relation to the Thr207.
Minor comments
Intro Page 3 - Phosphorylation of Ser396 and the five serine or threonine residues in the C-terminal of IRF3 (Ser396, Ser398, Ser402, Thr404, and Ser405) has been demonstrated as the major sites that represent the activated IRF3 via enhancing IRF3 dimerization (Fitzgerald et al, 2003; Lin et al, 1998; Lin et al, 1999). The authors probably mean: Phosphorylation of Ser385/Ser386 and the five .....
Fig. 1D PEM - At 12h post VSV infection, both AKT2 and IFNB1 mRNA are reduced. What does it mean?
Fig. S1B and C (lung) - Relative AKT2 mRNA level of PBS control is different than 1.
Fig. 2E and F - Legends should include more details of the transfection. In Fig. 2H What is the significance of the arrows (in other panels as well).
Fig. 3 2F and 2G (right panel) - Explain why the data of VSV and ISD (WT and KO) are similar in panel 2G while quite different with analysis of IB:pIRF3 and dimer detection (2F).

Rebuttal letter (#EMBOJ-2021-108016)
(AKT2 inhibits type I IFN responses to regulate viral infection and SLE)

TO: The EMBO Journal

Aug. 4th, 2021

Dear Editors,

We appreciate you and the reviewers' efforts to evaluate our manuscript. Although our manuscript was initially rejected in April, some reviewers acknowledged the importance and novelty of our findings. We have communicated with the editor, and performed a substantial amount of experiments to address the concerns (highlighted with red in the revised figures & manuscript). A point to point response letter is included.

Referee #1:

Zheng et al propose in this manuscript that Akt2 inhibits the expression of type I IFN by phosphorylating IRF3 at Ser207, leading to impairment of nuclear translocation, which itself is mediated by 14-3-3 protein. The manuscript uses co-expression, KO of Akt2, IF of IRF3, co-IP and reporter gene expression in cells, zebrafish and Akt2 KO mice to make their point about Akt2 and IRF3.

After reading through this manuscript multiple times, this reviewer comes away feeling the data is selectively presented, overall conclusions are forced and the effects of Akt2 on IRF3 are minimal - often a 2-fold difference in expression levels.

1. We have carefully considered the Reviewer's criticism, and cited the data from others' studies that also show "about a 2-fold difference in Ifnb1 mRNA expression levels" (see below). Furthermore, our and others' findings noticed that a large number of macrophages were accumulated in the infected organs and viral-infected macrophages could enhance I-IFN levels over thousands folds compared to resting macrophages. *Akt2* deficiency further enhanced expression of I-IFN as well as a series of IFN-stimulated genes (ISGs) about 2-folds; together with the accumulation of macrophages in the infected organs, this could indeed significantly affect the pathological process of infection.

Figure 2G: HDAC6 regulates cellular viral RNA sensing by deacetylation of RIG-I. ***EMBO J.*** 2016 Feb 15;35(4):429-42.

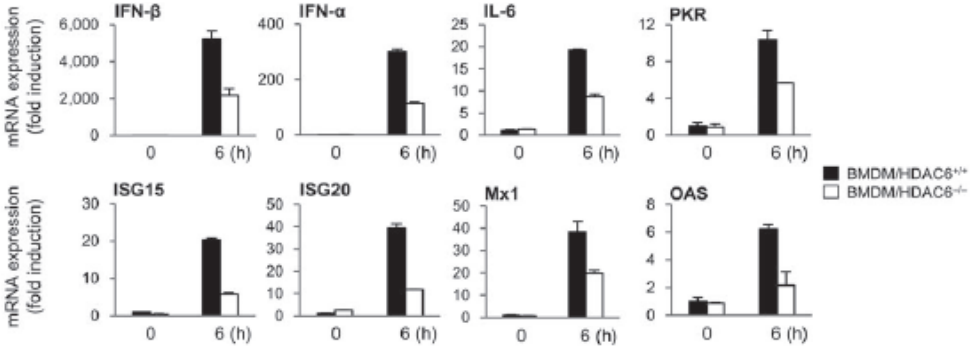


Figure 2A: The Zinc-Finger Protein ZCCHC3 Binds RNA and Facilitates Viral RNA Sensing and Activation of the RIG-I-like Receptors. ***Immunity.*** 2018 Sep 18;49(3):438-448.e5.

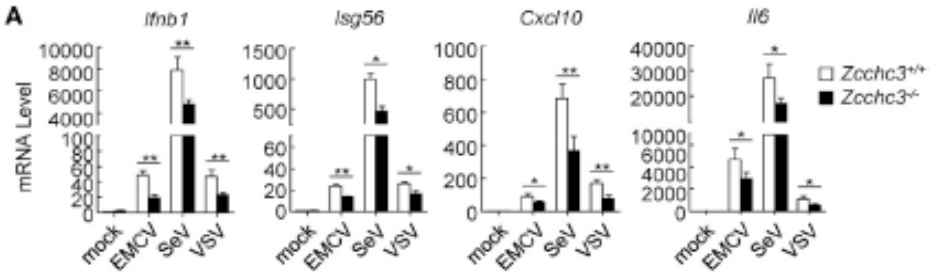
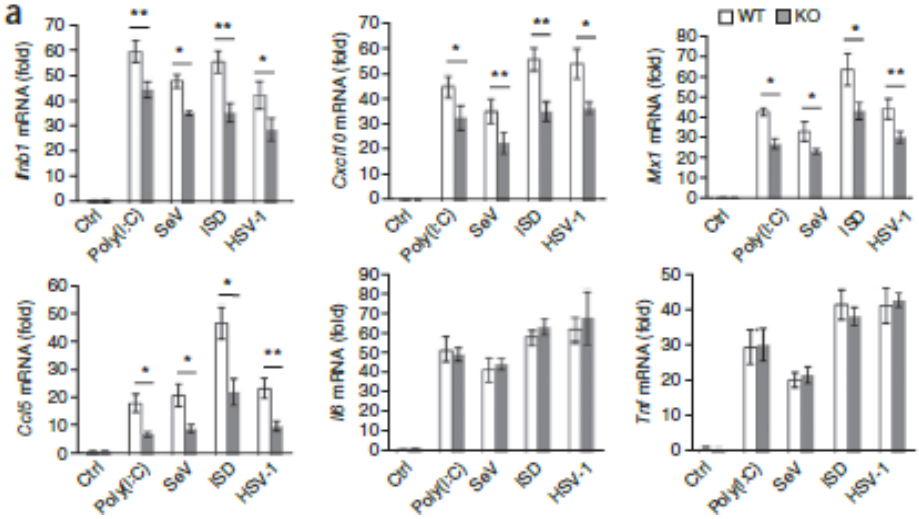


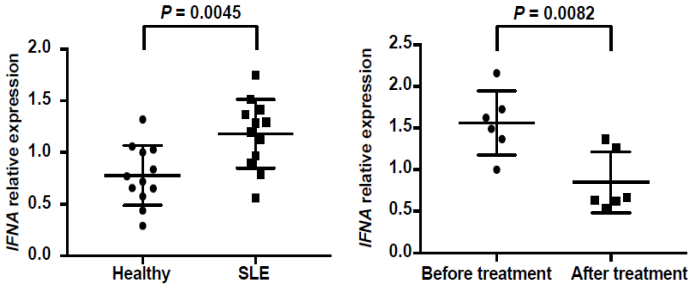
Figure 2a: E3 ubiquitin ligase RNF128 promotes innate antiviral immunity through K63-linked ubiquitination of TBK1. ***Nat Immunol.*** 2016 Dec;17(12):1342-1351.

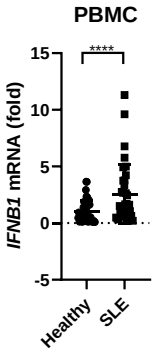


2. Additionally, previous studies showed that IFNα expression levels were enhanced less than 2 folds in PBMCs from SLE patients when compared with the healthy controls, or reduced less than 2 folds after effective treatment (see below). Our data also confirmed the 2-fold enhancement of IFNβ1 levels in PBMCs from SLE patients (see below). These evidences suggest that the differences of I-IFN expression between WT and *Akt2* KO macrophages could play a critical role during viral infection and SLE.

Figure 7A, 7C: LncRNA Malat1 inhibition of TDP43 cleavage suppresses IRF3-initiated antiviral innate immunity. *PNAS*. 2020 Sep 22;117(38):23695-23706.

Our data:





3. We have followed another Reviewer's advice and repeated the experiments about "the effects of Akt2 on IRF3" under the optimized conditions (i.e. using the cells with better status). The qualities of these data have been improved, and included in Figure 3H, S3G, 4A (as below) to replace the old data.

Figure 3H

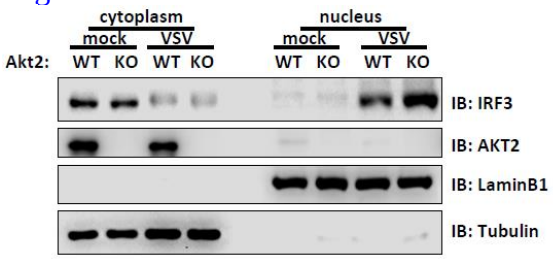


Figure S3G

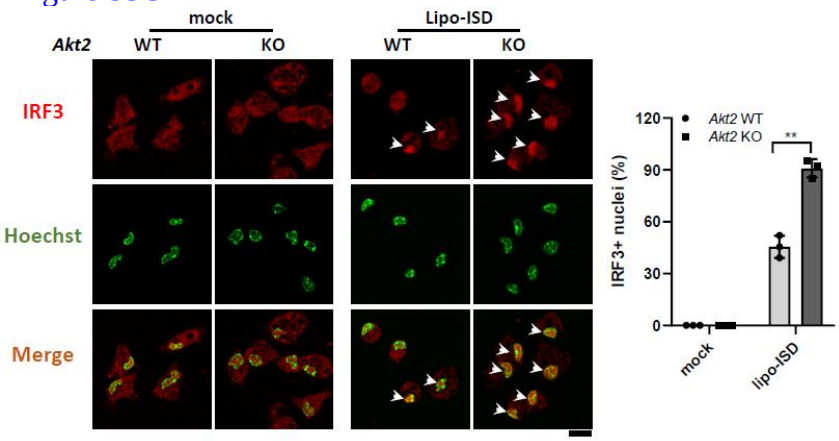
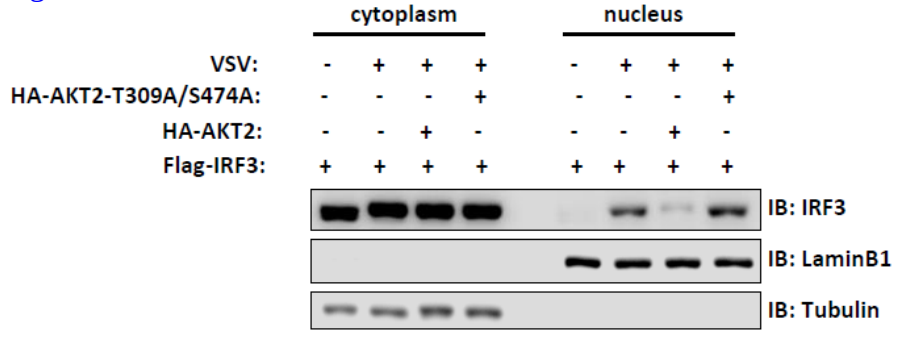


Figure 4A



The effects of Akt2 on IFN signaling may be an indirect, not direct, consequence of inhibition. -As an example the data on Akt-IRF3 association rely on overexpression and this is not satisfactory manner to demonstrate interaction. The Akt-IRF interactions may well be transient (enzyme-substrate). Perhaps mass spec analysis of endogenous proteins would provide a more clear answer.

As suggested, we have performed the mass spectrometry assay and identified the peptides of AKT2 or other binding proteins after immunoprecipitation of Flag-IRF3 (see below). The mass spectrometry analysis usually provides lots of possible binding candidates, which need further validation. The Co-IP assay is a common used approach to detect protein-protein interaction via overexpressing the bait protein. Therefore, we overexpressed WT AKT2 and its truncated domains. The full-length AKT2 and its N-terminal could interact with IRF3, while the AKT2 fragment without N-terminal failed to bind IRF3 (Figure S3E). This experiment contains the positive and negative results, demonstrating a proper approach to show how AKT2 interacting with IRF3.

locus	description
Q14653	Interferon regulatory factor 3 OS=Homo sapiens GN=IRF3 PE=1 SV=1
P63104	14-3-3 protein zeta/delta OS=Homo sapiens GN=YWHAZ PE=1 SV=1
P62258	14-3-3 protein epsilon OS=Homo sapiens GN=YWHAE PE=1 SV=1
P31751	RAC-beta serine/threonine-protein kinase OS=Homo sapiens GN=AKT2 PE=1 SV=2

To endogenously confirm the interaction between AKT2 and IRF3 in macrophages, we performed immunoprecipitation using anti-IgG/anti-AKT2 and anti-IRF3 antibodies (Figure 3D). Furthermore, we purified GST-AKT2 and His-IRF3 from *E.coli*, and performed the GST-pull down assay to demonstrate their direct interaction (Figure 3E). These results together could confirm the direct association between AKT2 and IRF3.

Figure 3D

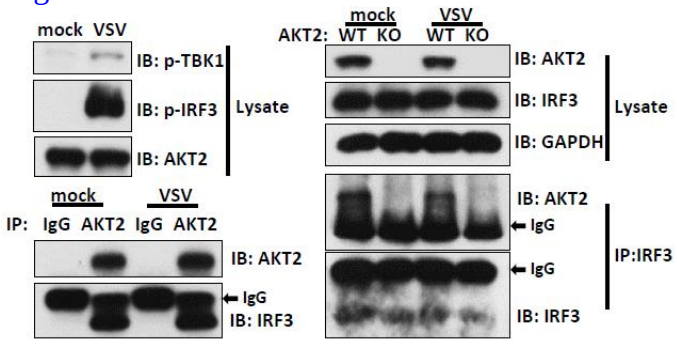
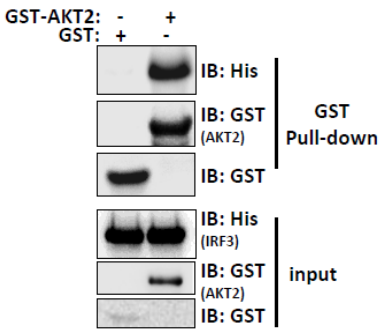


Figure 3E

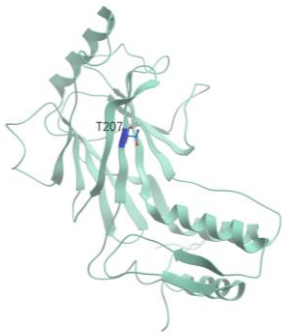


-Does Ser207A interfere with nuclear localization sequence of IRF3 or is it in the region of the IAD, itself a closed hydrophobic structure that is not easily accessible.

We thank the Reviewer's important advice. According to the integrated crystal

structure analysis of IRF3, IRF3 was divided into the N-terminal (1-123, 1-113, 137-148) and the rest part (i.e. no nuclear localization domain is suggested in particular). The related reference (PMID: 25994966) suggests 77KR78/86RK87 as part of the nuclear localization sequences in IRF3. From these studies, it is difficult to speculate that Thr207A interferes with the nuclear localization sequence of IRF3.

Thr207 is in the region of IRF3-IAD (202-383). Even if Thr207 is in the hydrophobic structure of IRF3, IRF3 exists as a monomer in the inactive form, and Thr207 might position at the exposed lateral side of a monomer IRF3 according to its structure (see the model below). Therefore, it is possible that a monomer IRF3 in the cytoplasm could bind and be phosphorylated by AKT2. Then, with the assistance of 14-3-3ε, AKT2 could keep IRF3 in the cytoplasm and inhibit IRF3 entry into the nuclei.



Monomer-IRF3

-Other examples - Data on 14-3-3 involvement was not convincing.

We appreciate the Reviewer's concern and have provided new and different evidences to confirm the involvement of 14-3-3ε. As showed in Page 4, we identified several peptides of the14-3-3 family members in the mass spectrometry assay after immunoprecipitation of Flag-IRF3. In addition, others' publications have indicated the interaction of AKT2 and 14-3-3, which could function together to restrain $p27^{Kip1}$ in the cytoplasm (PMID: 12042314). Based on our MS data and others' work, we further elucidated the involvement of 14-3-3 in details.

Knockdown or overexpression of 14-3-3ζ did not affect *Ifnb1* mRNA levels or IFNβ1 luciferase activity (Figure S3I). In contrast, knockdown of 14-3-3ε could enhance *Ifnb1* production (Figure S3J). This indicates that 14-3-3ε, but not 14-3-3ζ, might work together with AKT2 to inhibit *Ifnb1* transcription. Interestingly, 14-3-3ε also interacted with IRF3 (Figure S3K) and reduced the amount of IRF3 in the nuclei (Figure 3I). Similar to AKT2, 14-3-3ε did not affect IRF3 Ser396 phosphorylation and dimerization (Figure S3L). In addition, in the IFNβ1 luciferase assays, overexpression of 14-3-3ε alone, AKT2 alone, or 14-3-3ε + AKT2 could all significantly suppress IFNβ1 (i.e. readings around 150-250 folds) when compared to the control samples (i.e. readings around 600 folds) (Figure 3J).

Figure 3I

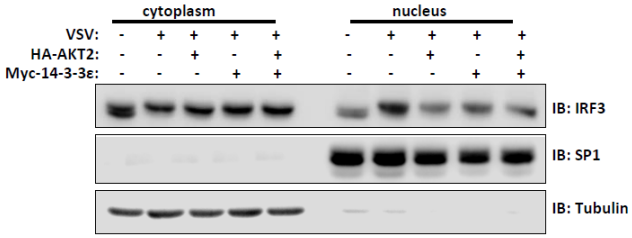
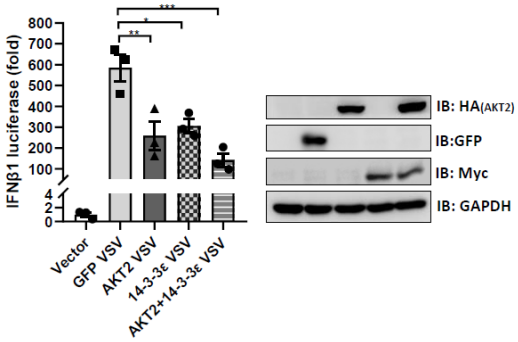
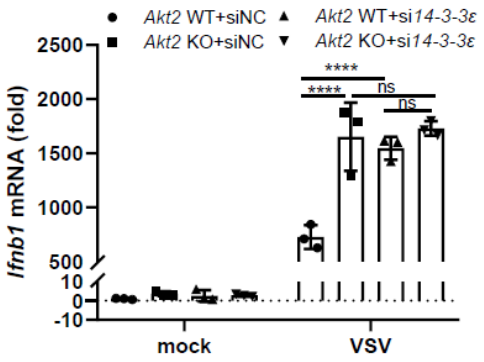


Figure 3J



In addition, we performed a new strategy (i.e. knockdown of 14-3-3ε alone, *Akt2* KO alone or si*14-3-3ε* + *Akt2* KO) and added the new data (Figure 3K). Knockdown of *14-3-3ε*, *Akt2* deficiency, or si*14-3-3ε* + *Akt2* KO all enhanced *Ifnb1* mRNA levels (i.e. around 1800 folds), when compared to WT PEMs (i.e. around 700 folds) (Figure 3K). These data together support that AKT2 and 14-3-3ε are in the same pathway to suppress IRF3-induced IFNβ1 production.

Figure 3K



-IRF3 nuclear translocation was not apparent.

We have followed the Reviewer's advice, and repeated this experiment under the optimal conditions. The new data were replaced in Figure 3H, S3G, 4A (also see Page 3 in this letter).

-The KO studies in mice were more about Akt2 than IRF3-IFN and one may question that modification of a growth regulatory pathway could affect a range of unrelated pathways by 2-fold.

This is an important question. As a serine/threonine kinase, AKT2 can phosphorylate different target proteins and controls a variety of cellular functions, including cell growth. In this study, we observed that *Akt2* full KO mice enhanced antiviral defense, and we agree with the Reviewer that this phenotype of *Akt2* full KO mice could not rule out the impact of *Akt2* deficiency on other types of cells or other cellular functions including the growth pathway. We therefore used different strategies to verify the function of Akt2/I-IFN in mice:

(1) We detected the normal percentages of $CD11b^+$ $F4/80^+$ macrophages in bone

marrow (BM) and spleen from *Akt2* KO mice, and macrophages were developed normally *ex vivo* from BM of *Akt2* KO mice (Figure 5A and S5A). Different from the conclusion that *Akt2* promotes tumor cells survival, WT and *Akt2* KO macrophages did not show significant difference in cell apoptosis under resting status or upon viral infection (Figure S5B). This suggests that *Akt2* has no impact on the survival of macrophages. *Akt2* KO mice also showed normal percentages of $CD4^+$ T cells, $CD8^+$ T cells or $B220^+$ B cells in thymus, spleen or BM (Figure S5C).

(2) WT or *Akt2* KO BMs were adoptively transferred into the irradiated WT CD45.1 recipient mice (Figure S5D, 5F-H), and the WT recipient mice carrying *Akt2* KO immune cells could increase I-IFN levels and anti-viral response.

To further focus on the function of *Akt2* deficient macrophages, WT or *Akt2* KO macrophages were adoptively transferred into clodronate liposome-treated mice, in which the endogenous macrophages were deleted (Figure S5E, S5F). Using these mice, we confirmed that *Akt2* KO macrophages (without effect by *Akt2* deficiency in any other cell types) indeed enhanced IFNβ1 production against viral infection *in vivo*.

(3) Moreover, to confirm the importance of the AKT2-IRF3-IFNβ1 module rather than other functions of AKT2 against infections in animal level, we used the zebrafish model (Figure S2F-G, 2G-H, 4F-H, S4G). After AKT2 and its mutation (Figure 2G-H & S2F-G), or IRF3 and its mutation (Figure S4F) were overexpressed alone or in combination in zebrafish embryos (Figure 4F-H), we observed that AKT2 was dependent on IRF3-T207/I-IFN to regulate VSV infection in zebrafish embryos by measuring tissue damage, virus copies and the survival rates.

All together, we confirmed that AKT2 regulates antiviral response through IRF3/IFNβ1 *in vivo*.

-huge amount of effort placed on this study - one wonders why the authors did not make a Ser207 phospho-specific Ab to determine more precisely the phosphorylation status of IRF3.

We agree with the Reviewer that it is critical to develop a Thr207 phospho-specific Ab. We have worked with one company to develop phospho-specific Abs of IRF3, including the critical site p-IRF3-Ser97 that negatively regulates IRF3 activation. But until now, we have not successfully obtained this antibody. And previous studies have identified many key phosphorylated residues in IRF3 to regulate I-IFN production, but only several commercial antibodies are available for detecting 2 or 3 phosphorylation sites in IRF3. This may due to the technique difficulty to generate these antibodies.

We appreciate the Reviewer' agreement about "huge amount of efforts placed on this study". We have performed multiple strategies to confirm the importance of IRF3-Thr207, including the luciferase assay (Figure 4C), immunofluorescence and

immunoblotting (Figure 4D), and the zebrafish model (Figure 4F-4H).

In the revised version, we performed two new experiments and added these new data in Figure 4E and S4A. Using Phos-tag gels, we confirmed higher IRF3 phosphorylation levels in *Akt2* KO macrophages after VSV challenge (Figure S4A). Next, we overexpressed AKT2 with WT IRF3 or the IRF3-T207A mutation to detect IRF3 phosphorylation levels (Figure 4E). As shown in the Phos-tag gels, AKT2 overexpression markedly promoted IRF3 phosphorylation, but failed to enhance IRF3-T207A phosphorylation. Together, these data have demonstrated that AKT2 affects IRF3 phosphorylation mainly on Thr207.

Figure S4A

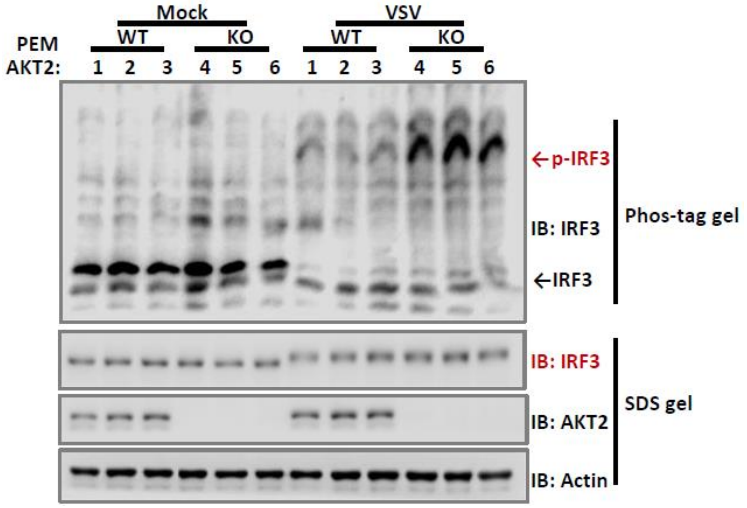
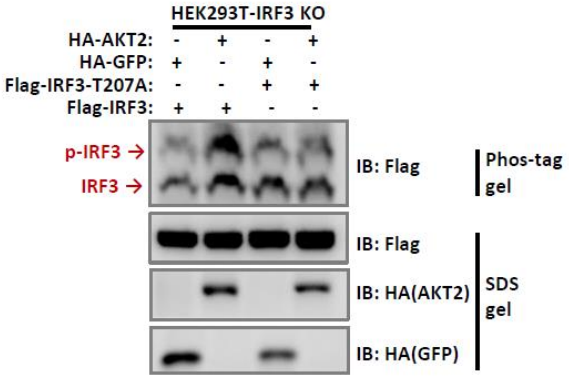


Figure 4E



Referee #2:

The paper by Zheng et al describes how Akt 2 can phosphorylate Ser207 at IRF3, which leads to a modest reduction in IFNb expression. They further show that the absence of Akt2 I a mouse model confer increased susceptibility to VSV infection and decreased severity of SLE. While **the amount of different data is impressive, and merit publication in EMBO,** the writing is often compressed to the degree where it is difficult to follow the logic of the experiments. The figure legends and methods are also lacking in several cases. Some key control experiments to ensure the specificity of the proposed model is needed (listed below). Finally I find that the author exaggerate their result in a number of ways, properly they think the use of wording like Akt2 is crucial for IFNb ..... is needed to get accepted in EMBO, however I find this quite disturbing and also unnecessary, and therefore quite irritating when reading the paper.

1. We appreciate the Reviewer's positive comments that "the amount of different data is impressive, and merit publication in EMBO". We apologize for the compressed writing that leads to lack of logical flow. As for the comment about "Exaggerate their result", we used "significantly" to describe the statistical significance and used "critical, crucial" in the previous version; we have now followed the advice to replace them in an appropriate way.

2. To response the Reviewer's comment about the modest changes in *Ifnb1* expression, we have carefully considered the Reviewer's criticism, and cited the data from others' studies that also show "about a 2-fold difference in Ifnb1 mRNA expression levels" (see below).

Figure 2G: HDAC6 regulates cellular viral RNA sensing by deacetylation of RIG-I. ***EMBO J.*** 2016 Feb 15;35(4):429-42.

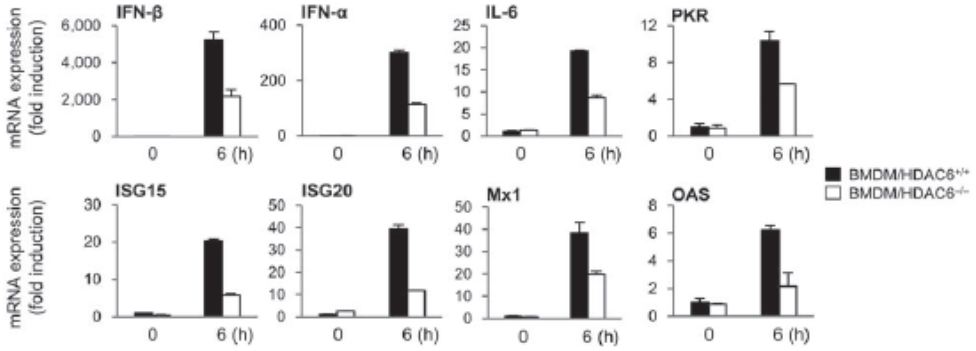


Figure 2A: The Zinc-Finger Protein ZCCHC3 Binds RNA and Facilitates Viral RNA Sensing and Activation of the RIG-I-like Receptors. ***Immunity.*** 2018 Sep 18;49(3):438-448.e5.

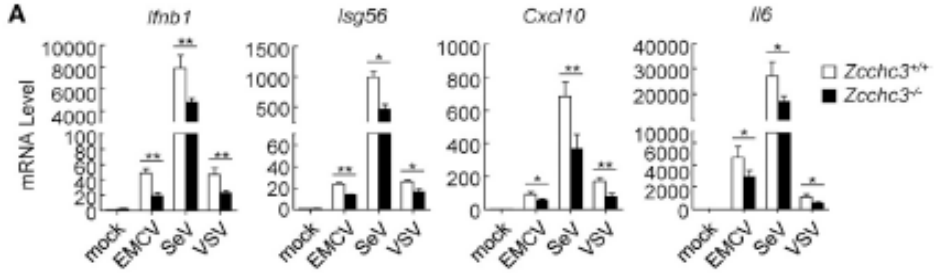
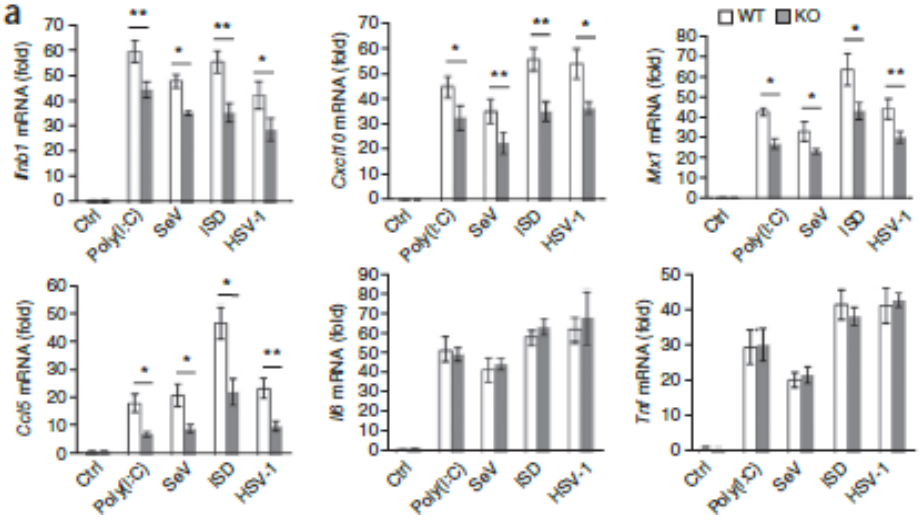


Figure 2a: E3 ubiquitin ligase RNF128 promotes innate antiviral immunity through K63-linked ubiquitination of TBK1. ***Nat Immunol.*** 2016 Dec;17(12):1342-1351.



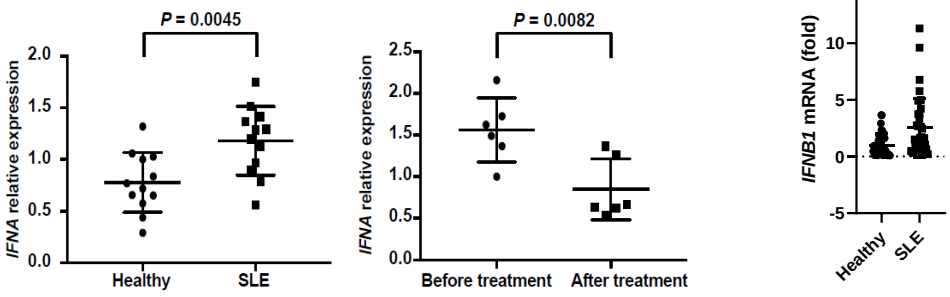
Then, our and others' findings noticed that a large number of macrophages were accumulated in the infected organs and viral-infected macrophages could enhance I-IFN levels over thousands folds compared to resting macrophages. *Akt2* deficiency further enhanced expression of I-IFN as well as a series of IFN-stimulated genes

(ISGs) about 2-folds; together with the accumulation of macrophages in the infected organs, this could indeed significantly affect the pathological process of infection.

Additionally, previous studies showed that IFNα expression levels were enhanced less than 2 folds in PBMCs from SLE patients when compared with the healthy controls, or reduced less than 2 folds after effective treatment (see below). Our data also confirmed the 2-fold enhancement of IFNβ1 levels in PBMCs from SLE patients (see below). These evidences suggest that the differences of I-IFN expression between WT and *Akt2* KO macrophages could play a critical role during viral infection and SLE.

Figure 7A, 7C: LncRNA Malat1 inhibition of TDP43 cleavage suppresses IRF3-initiated antiviral innate immunity. *PNAS*. 2020 Sep 22;117(38):23695-23706.

Our data:



Major comment:

End of page 7: I do not find the following conclusion merited by the data: "Together, we have demonstrated that AKT2 kinase activity is critical for Ifnb1 production and virus clearance in macrophages ex vivo and in zebrafish in vivo." At best their data show that Akt2 has a modest effect on IFNb expression. In connection with this I will argue that the data presentation is biased, normally IFNb expression (with and without inducer) is shown on a logarithmical scale, whereas the authors consequently use a linear scale. It is unclear to me how relative IFNb expression is defined? And using the linear scale it is impossible to judge the IFNb expression in the mock samples. The authors should show all IFNb expression data as IFNb mRNA expression relative to the chosen house keeping gene, on a logarithmical scale. The figure legend should clearly state how the data was obtained. Importantly, they should avoid for further normalization.

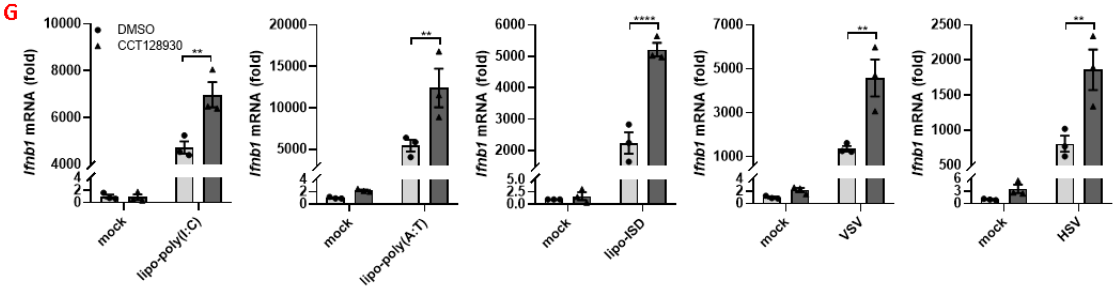
We have followed the Reviewer's advice to revise our conclusion: "Together, we have demonstrated that AKT2 inhibits *Ifnb1* expression and this is dependent on AKT2 kinase activity" (Page 8 in the revised manuscript).

We appreciate the Reviewer's key concern about the scale of *Ifnb1* expression. Many references indeed show the linear scale and fold changes of the *Ifnb1* mRNA expression, and please see the cited data from other references (see Page 9 in this letter).

We also followed this advice, and defined "relative" and "fold" in Materials and

Methods (Page 22 in the revised manuscript): "β-Actin in human and mouse samples, or GAPDH in zebrafish samples were used to normalize gene expression. The "relative" mRNA levels were calculated using the $2^{-\Delta Ct}$ method with the normalized gene. The "fold" changes were calculated using the $2^{-\Delta\Delta Ct}$ method with the normalized gene, and then compared to the control groups including PBS, mock, WT mock, DMSO mock, siNC, Vector, PBS mock, WT siNC mock *et al*". In order to show the levels of *Ifnb1* in the mock samples, the Y axis were prepared as two segments using GraphPad Prism software, and all of the related figures have been corrected. The revised Figure 2G is shown below as one example.

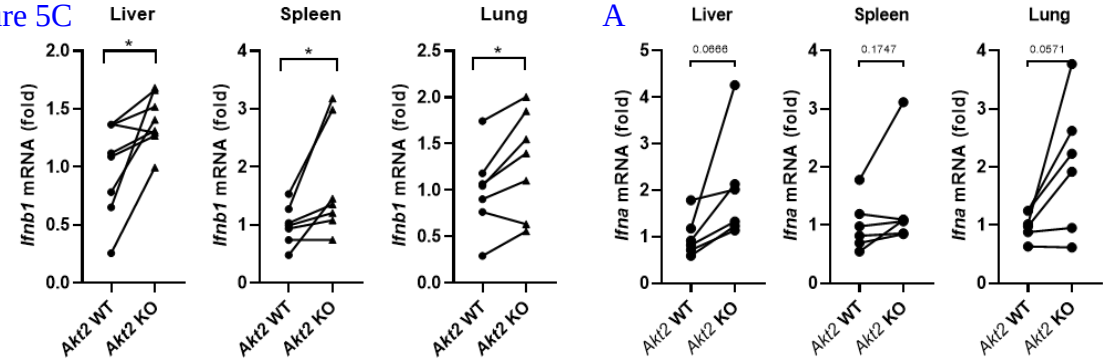
Figure 2G

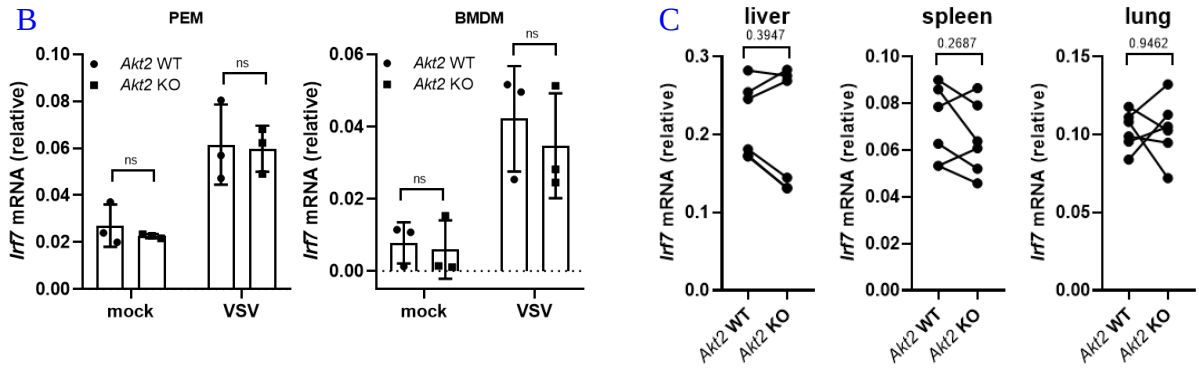


To verify the model the authors put forward in vivo please conduct the following exp: In figure 5 where they characterize a Akt2 KO mouse, would they please measure both IFNb and IFNa expression post infection with VSV and Influenza virus (nasal infection for influenza). According to their model IFNb expression should be altered by the absence of Akt2 but not IFNa (which is driven by IRF7)

We have performed the experiments as suggested. *Ifnb1* expression levels in liver, spleen, lung of *Akt2* KO mice were enhanced and have been included in Figure 5C (*p < 0.05). As suggested that *Ifna* is driven by IRF7, we next used the same samples to measure the expression levels of *Ifna* of *Akt2* KO mice (A), which showed no significant change. Furthermore, the mRNA levels of *Irf7* were similar in WT and *Akt2* KO macrophages (B) or tissues (C). These data indicate that AKT2 might not affect IRF7-induced *Ifna* production, which have been added into Discussion.

Figure 5C





General for all luciferase assay: Please show a western blow which verify expression of all relevant constructs. Also, please specify how data are reproduced. Normally I would do 3 side by side replicate using the same DNA prep for transfection and label them as technical replicate, than repeating the experiment 3 times using different DNA preparations, which would give biological replicate. Would the authors please specify how the experiment is carried out!

We had repeated the luciferase reporter assay more than 3 times independently. In each experiment, we collected 3 wells that were transfected with the same plasmids in a 24-well plate to represent one sample. The representative data from 3 independent experiments were shown in the figures.

We thank the reviewer's advice, and have included all Western blot data to verify the expression levels of the relevant constructs (Figure S2D, S2E, 3A, 3B, 3J, S3A, S3I, 4C, S4C, S4E).

Figure S2D

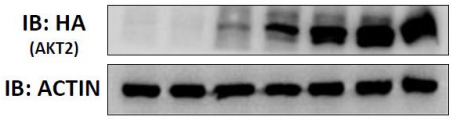


Figure S2E

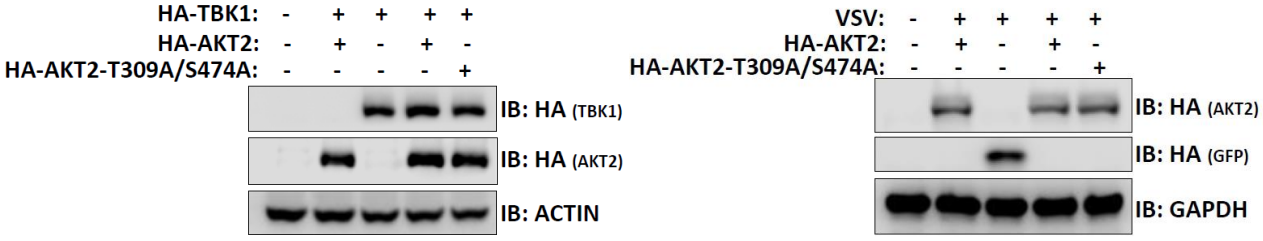


Figure 3A

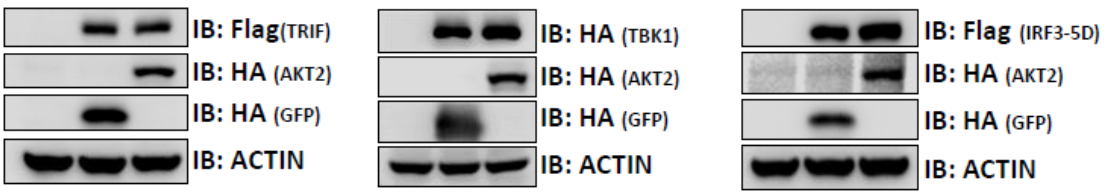


Figure 3B

Figure 3J

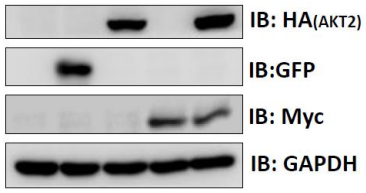


Figure S3A

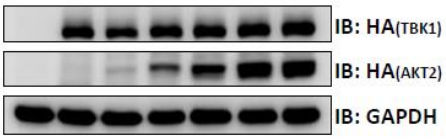


Figure S3I

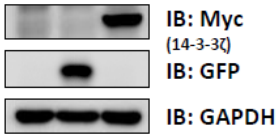


Figure 4C

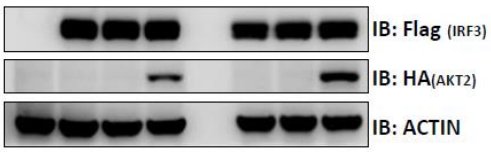


Figure S4C

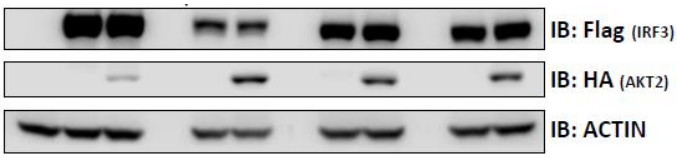


Figure S4E

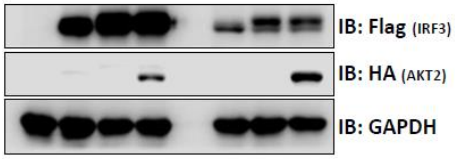


Figure 3A, while interesting the author should expand the data collected in the HEK293 system, TRIF is notorious difficult in the HEK system due to cell toxicity and TBK1 overexpression is a very general way of activating the IFNb reporter. Could the authors please use MAVS overexpression in an additional exp just to confirm the observation in figure 3A.

Our protocol: The pcDNA3.0-Flag-TRIF plasmid (0.25μg), IFNβ1 luciferase reporter (0.25μg) and the internal control TK-renilla (0.01μg) were transfected using 1.53μl PEI into HEK293T cells in a 24-well plate when HEK293T cells were 80–90% confluent in the plate. After 6-8 hours, the culture medium was changed followed by at least another 18 hours culture. Before cells were harvested, cells were examined under the microscope and the LDH release levels were measured (as below), which did not show cell toxicity after TRIF overexpression.

We thank the reviewer's good suggestion and have included the new data in Figure S3A, left panel. Consistently, AKT2 also suppressed MAVS-induced IFNβ1 production in the luciferase reporter assay.

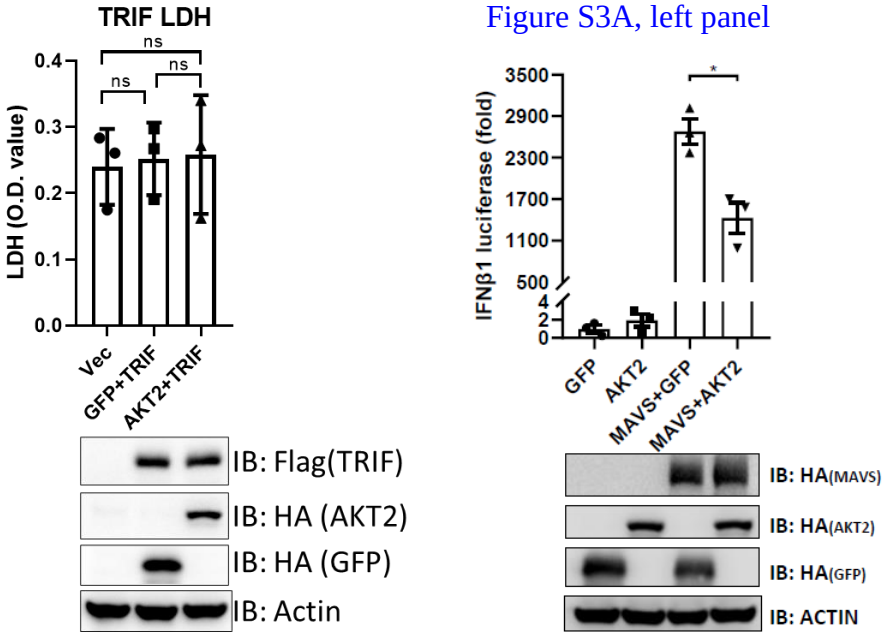


Figure S3A, left panel

This is the entire figure legend to figure 3D: "Immunoblot analysis of the interaction between AKT2 and IRF3 in PEMs infected with VSV for 6 hours." it difficult to decipher the data based upon this. Please be much clearer about how experiments are conducted throughout the paper!

We thank the reviewer's criticism and have enriched the figure legend to Figure 3D (Page 34 in the revised manuscript): "WT and *Akt2* KO PEMs were infected with or without VSV for 6 hours. Then, cell lysates of WT or *Akt2* KO PEMs were prepared for immunoprecipitation using anti-AKT2 antibody, followed by immunoblotting using anti-IRF3 antibody (left panel). Alternatively, cell lysates were prepared for immunoprecipitation using anti-IRF3 antibody, followed by immunoblotting using anti-AKT2 antibody (right panel) to detect the endogenous interaction between AKT2 and IRF3". We have followed the Reviewer's advice and modified the methods and figure legends to make them much clearer throughout the paper.

Figure 3D. I am not sure why they are looking at Ser396-P as several recent papers show that phosphorylation at this site has little impact on IRF3 function. Same for figure 4D

We appreciate this key question. We agree with the Reviewer that the papers PMID:32826280, 3320582 conclude IRF3-Ser386 as a critical site rather than IRF3-Ser396. The process of IRF3 activation is conducted as phosphorylation→dimerization→nuclear localization→Ifnb1 introduction, the user-friendly phosphorylation antibody is needed to indicate IRF3 activation status. p-IRF3-Ser396 has been used as a marker to show the activation status of IRF3 by many articles. Similarly, our manuscript used p-IRF3-Ser396 to indicate the activation

status of IRF3. Furthermore, in order to confirm IRF3 activation, we examined the levels of IRF3 dimerization in most of our figures. We apologize for this confusion and have now modified this description properly in the Introduction and cited the references PMID:32826280, 3320582.

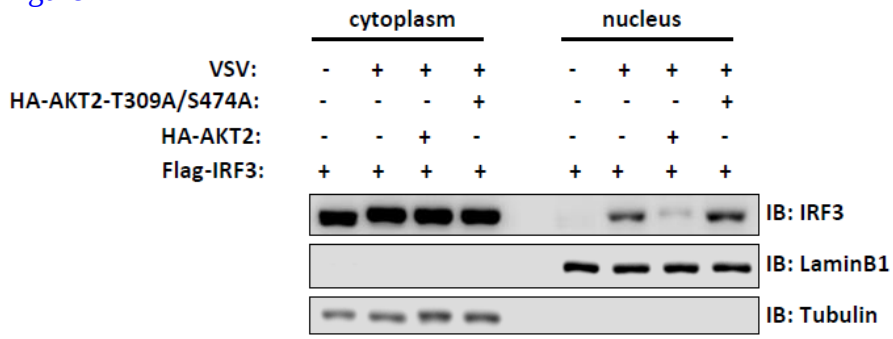
Figure 3I, I am not sure there is a significant difference between AKT2 alone and the combination of AKT2 and 14-3-3e, was this tested statistically?

We have suggested that 14-3-3ε interacts with AKT2 to restrain the AKT2-IRF3 module in the cytoplasm (Figure 3I), leading to the reduced I-IFN production (Figure 3J). The combination of AKT2 and 14-3-3 slightly further inhibits the amount of IRF3 in the nuclei and the induction of IFNβ1, but not statistically significant when compared to AKT2 alone. This indicates that AKT2 depends on 14-3-3ε (i.e. in the same pathway) to regulate IRF-3-induced I-IFN production.

Figure 4A, the difference in nuclear IRF3 are small land hard to make out from a single WB, the authors should show the duplicated experiments as supplementary data.

We thank the reviewer's suggestion and have revised Figure 4A with the improved quality, which are representative data from 3 independent experiments.

Figure 4A



Based upon the description, I cannot understand (yet reproduce) figure 4B, what is a phosphate-gel? How does this work, no information is given.

We apologize for this and have now added the detailed information about phosphate-gel in Materials and Methods (Page 22 in the revised manuscript): "To measure AKT2 kinase activity, HA-AKT2 was immunoprecipitated from HEK293T cells by anti-HA antibody, and anti-IgG antibody was used as a control. Samples were then incubated with 6*His-hIRF3 at 30℃ for 30min with gentle shaking in the kinase reaction buffer (Cell Signaling Technology). This kinase reaction was stopped by adding SDS loading buffer, and the supernatants were subjected to a phosphate-gel for immunoblotting to measure the *in vitro* phosphorylation reaction. Phosphate-gel was 8% acrylamide gel with the addition of 5 mmol/L Phos-tag™

(#034-93521(AAL-107)) and 10 mmol/L Mn^{2+} in the resolving gel. Before western transfer, the gel was washed 3 times every 10 min in transferring buffer containing EDTA (10mM), which is required to increase the transferring efficiency. Phos-tag™ and Mn^{2+} cooperates to bind a phosphorylated protein; when the phosphorylation levels are increased, the migration velocity of this protein is slower. Therefore, non-phosphorylated and phosphorylated proteins are separated in the gel." We have added the detailed information in figure legend to Figure 4B and the Results section.

Minor comment

This statement in the introduction is incorrect and do not take the newest literature into consideration "Phosphorylation of Ser396 and the five serine or threonine residues in the C-terminal of IRF3 (Ser396, Ser398, Ser402, Thr404, and Ser405) has been demonstrated as the major sites that represent the activated IRF3 via enhancing IRF3 dimerization(Fitzgerald et al, 2003; Lin et al, 1998; Lin et al, 1999)." Phosphorylation of Ser386 is clearly the defining event. Please site the following papers PMID: 32826280; 33205822

Thanks for the reviewer's correction and we have modified the description in the Introduction (Page 3 in the revised manuscript): "Phosphorylation sites of the cluster 1 (Ser385/Ser386) and the cluster 2 (Ser396, Ser398, Ser402, Thr404, and Ser405) in the C-terminal of IRF3 have been demonstrated to indicate IRF3 activation status in antiviral immunity (Lin et al, 1998; Lin et al, 1999; Panne et al, 2007). Further studies suggest that Ser386 is critical, while Ser396 plays a moderate role in IRF3 dimerization and activation (Dalskov et al, 2020; Jing et al, 2020)".

There is a data not shown in the discussion, do EMBO allow this?

Thanks for this question and the EMBO Journal requires to avoid "data not shown". We have removed this sentence from the revised manuscript.

The authors should distinguish between zebrafish larvae and adult zebrafish.

We thank the reviewer's advice. We have clarified this and used "zebrafish embryos" (0-48 hpf) and "zebrafish larvae" (48-120 hpf) in the manuscript.

Referee #3:

The authors describe the role of the AKT serine/threonine kinase 2 (AKT2) in the regulation of interferon (IFN) and IFN induced transcription upon pathogen infection and IFN-driven pathogenic autoimmune disorders driven by IRF3 activation. While the role of PI(3)K-AKT pathway in the regulation of innate immunity is controversial, this is a very interesting work that demonstrated a novel negative regulation of IRF3 activation and type I IFN production by the specific isoform AKT2 isoform. AKTs are a critical kinase family that can phosphorylate different target proteins and control a variety of cellular functions. Here, they showed that upon infection

with various pathogens, induction of IFN and IFN signaling decreases AKT2 expression (knockdown of negative regulator of IFN signaling pathway SOCS1/SOCS3 further reduce AKT2 expression). Mechanistically, they reported that AKT2, through the assist of 14-3-3ε, binds IRF3 and phosphorylates IRF3 at the position Thr207, a newly identified phosphorylation site, to attenuate IRF3 nuclear translocation and type I IFN production without affecting IRF3 dimerization. Overexpression of the AKT2 kinase-dead mutant or IRF3-T207A in zebrafish improved protection against infection. Akt2 deficient mice increased type I IFN induction and reduced mortality in response to virus infection, but aggravated severity of systemic lupus erythematosus (SLE). The treatment of mice with AKT2 selective inhibitor CCT128930 enhanced the survival rates in response to VSV infection. Adoptive transfer experiments of Akt2 KO BMs into irradiated CD45.1 recipient mice followed by VSV challenge showed higher survival rates, increased concentrations of serum Ifnb1, reduced VSV copies and titers from livers, spleens or lungs confirming that deletion or blocking AKT2 activity could be an effective strategy to enhance I-IFN production and facilitate the host against viral infection. Heightened type I IFN is a prominently hallmark in SLE patients and correlate with disease severity. TMPD-treated Akt2 KO mice (and adoptive transfer of Akt2 KO BM) developed a more serious diffuse pulmonary hemorrhage (DPH) that occur in some SLE patients with the increased mortality. They further confirmed the significantly reduced expression of AKT2 in PBMCs isolated from SLE patients, when compared that from healthy donors, strongly suggesting that AKT2 plays a protective role in the pathology of SLE. The study identifies AKT2 kinase as a novel target to either promote innate immune responses upon pathogen infection (by inhibition) and to reduce pathogenic type I IFN production upon autoimmune disorders as SLE (by promoting AKT2 kinase activity and phosphorylation of T207-IRF3).

Specific comments

IRF3 is a master transcription factor for I-IFN production, and IRF3 transcriptional activity and biological function are precisely regulated via its phosphorylation. This is an interesting perspective into IRF3 activation as it is generally thought that phosphorylation is a positive post-transcriptional modification that mediates the progression of antiviral signaling. Such inactivation via phosphorylation is relevant to the reported study describing phosphorylation of IRF3 at Ser97 (Thr75/Thr253) that is release by PTEN to activate IRF3 and the subsequent nuclear import [Li S, et al. PMID: 26692175]. Here, the authors provide strong evidence for the requirement of the kinase activity of AKT2 and phosphorylation of residue T207 of IRF3 to attenuate IRF3 nuclear translocation and to negatively regulate type I IFN production upon various PRR-mediated activation. This novel regulatory mechanism is strongly supported by the experimental evidence in this manuscript, and it adds relevant and highly significant information to our understanding of IRF3 activation and antiviral signaling pathways. Overall, the data clearly support the author's conclusions and ultimately the model provided.

We do appreciate the Reviewer #3's positive comments about our study.

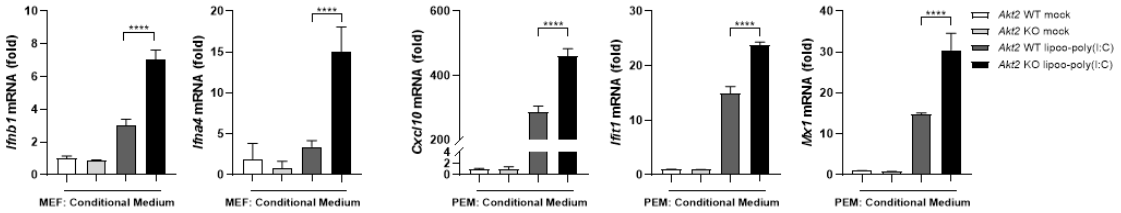
Nonetheless, several points remain to be addressed to fully appreciate the significance and generality of the findings. Antiviral signalling pathways is differentiated from one another based on their recognition of specific pathogen associated molecular patterns (PAMP) or their spatial distribution inside the cell by various pathogen recognition receptors (PRR) leading to IFN production. Despite that upstream divergence, it can be observed that all the antiviral pathways converge on a limited set of downstream transcription factors, namely IRF3/7 and NF-κB (p65/p50). Once activated, theses transcription factors will mediate the first wave of antiviral defences by initiating the transcription of type I IFN (α/β) and pro-inflammatory cytokines (early phase). Then, in an autocrine and paracrine fashion, these IFNs will pledge to stimulate the second wave of antiviral defences through the cytoplasmic receptors and downstream effectors of the JAK/STAT mediated IFN amplification loop (IFNAR1/2-JAK-STAT1/2-IRF9 pathway) (late phase). For the clarity of the manuscript, the authors may wish to add a few sentences to make this explicit.

1. The study provides strong evidence of the transcriptional regulation of AKT2 upon the late phase of IFN signaling. It is expected to take place following IFN production in infected cells (autocrine) and in bystander cells of infection site (paracrine) when exposed to IFN-α/β. The authors should provide evidence or at least discuss the contribution of AKT2 in both phases, and specifically upon the antiviral priming of bystander cells and the impact on propagation of viral infection. Second, as IRF3 is responsible for the early phase of the induction of IFN-α/β, whereas IRF7 is involved in the late phase, whether AKT2 expression regulates IRF7-mediated activation IFN-α/β (PTEN was reported to regulate only IRF3-mediated activation of Ifnb1).

We appreciate the key question about I-IFN function via autocrine and paracrine manners. Our data showed that *Akt2* KO cells increased the expression of *Ifnb1* and some ISGs upon infection at the early stage (6 hours, autocrine) (Figure 2A and 2B).

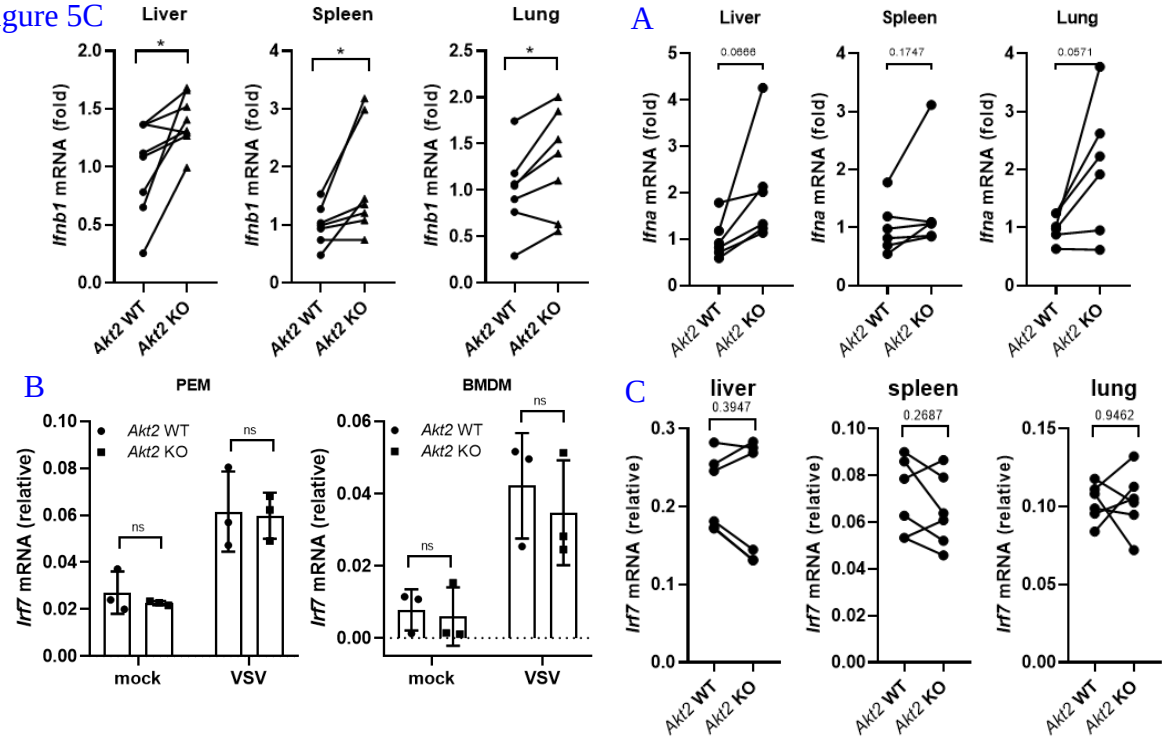
We conducted a new experiment as shown in Figure S2B to answer the effect to bystander cells at the late stage (paracrine). “Following I-IFN production in *Akt2* KO macrophages, we further asked whether this affected bystander cells. WT or *Akt2* KO PEMs were stimulated with lipo-poly(I:C) for 3 hours, and washed several times to remove any residual lipo-poly(I:C). Fresh medium was added to WT or *Akt2* KO PEMs for another 9 hours to collect cell medium, which was used to stimulate MEFs or PEMs. The culture medium from *Akt2* KO PEMs indeed induced higher *Ifnb1*, *Ifna4* production in MEFs or *Cxcl10*, *Ifit1*, *Mx1* production in PEMs (Figure S2B). This suggests that *Akt2* KO macrophages not only enhance I-IFN production, but also enhance the ISG expression in bystander cells such as PEMs and MEFs, therefore better preventing the viral infection and propagation” (Page 7 in the revised manuscript).

Figure S2B



Both Reviewer 2 and Reviewer 3 have asked whether IRF7 was affected by AKT2. We have performed the experiments as suggested. *Ifnb1* expression levels in liver, spleen, lung of *Akt2* KO mice were enhanced and have been included in Figure 5C (*p < 0.05). As suggested that *Ifna* is driven by IRF7, we next used the same samples and measured the expression levels of *Ifna* in different organs of *Akt2* KO mice (see below: A), which showed no significant change. This indicates that AKT2 might not affect IRF7-induced *Ifna* production. In addition, we detected that IRF7 mRNA levels were not affected by *Akt2* in macrophages or in different organs after VSV infection (see below: B, C). We have added this into the Discussion about whether AKT2 affects IRF7-induced IFN production.

Figure 5C



2. Li S et al., 2016 (PMID: 26692175) described a mechanism of negative regulation by phosphorylation of IRF3 at Ser97 in prevention of IRF3 dimerization and subsequent nuclear import and regulation by PTEN. This is briefly indicated in the discussion. PTEN was shown to promote antiviral response and IFN production by acting on IRF3 (dephosphorylation Ser97) but not on IRF7 induced in the late IFN amplification phase. PTEN is also known to antagonize the PI(3)P/AKT pathway which could contribute to a release of IRF3 activation via inactivation of

AKT2 and reduced phosphorylation of T207-IRF3. The author should address this aspect. Author should mention this study in the introduction of the manuscript.

Thanks for the reviewer's suggestion. Considering the structure of this manuscript, we have cited this article in the Discussion and described in details (Page 14 in the revised manuscript): "Previous work has suggested that PTEN antagonizes the PI(3)K/AKT pathway (Maehama & Dixon, 1998; Stambolic et al, 1998), and PTEN can positively facilitate IRF3 nuclear localization through de-phosphorylating IRF3 at Ser97(Li et al, 2016). Those clues imply a possibility about whether PTEN could inactivate AKT2, which results in the reduced phosphorylation levels of IRF3 at Thr207 and allows IRF3 activation and I-IFN production".

3. AKT2 mRNA is almost undetectable in PBMCs (Fig.S1A). Is this consistent with the main findings of the study?

The relative expression of *mAkt2* (Cp 22-24) is normalized to *mActin* (Cp 14-17), therefore the value is only about 0.005 in PBMCs (Fig.S1A). But it does not mean that PBMCs express *Akt2* at undetected levels. For example, resting macrophages express *Il6* at undetectable levels (*mIl6*-Cp 32-35; *mActin*-Cp 14-16), however LPS-treated macrophages enhanced *Il6* levels about thousands folds (*mIl-6*-Cp 20-22; *mActin*-Cp 14-16). The relative *Il6* values in LPS-treated macrophages are comparable to *mAkt2*-Cp. Additionally, the relative expression of *Akt2* in murine PBMCs is consistent with that in human PBMCs (hAKT2-Cp 24-26; hACTIN-Cp 17-19) and $CD14^+$ cells (hAKT2-Cp 23-25; hACTIN- Cp 15-18) from SLE patients. Nevertheless, the relative mRNA level of *Akt2* in PBMCs is lower when compared to other tissues.

4. The authors may wish to discuss a role of the cytoplasmic phosphorylated T207-IRF3 in the process of RIG-I-like receptor-induced IRF3 mediated pathway of apoptosis (RIPA) (Chattopadhyay S, PMID: 27815826; PMID: 27178468).

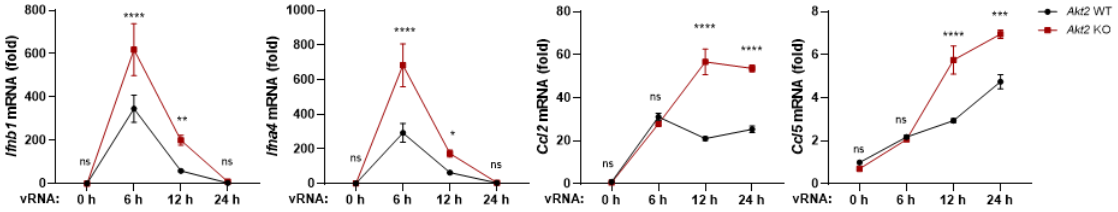
We have followed this good advice and added it into the Results section (Page 11 in the revised manuscript): "We have shown that *Akt2* KO PEMs enhanced IRF3 activity, and previous work suggests that the host could induce RIPA (RLR-induced IRF3 mediated Pathway of Apoptosis) to eliminate viral infection and pathogenesis(Chattopadhyay et al, 2016; Chattopadhyay & Sen, 2017). Therefore, we examined and found that in the absence or presence of viral infection, *Akt2* deficiency in PEMs did not obviously affect cell apoptosis (Figure S5B). This excludes the possibility that AKT2 might influence macrophage apoptosis to contribute to its anti-viral function".

5. The main functions of IFN-Stimulated Genes (ISGs) are to restrict viral replication, balance

viral recognition by PRRs, balance downstream activation of transcription factors, such as IRF3, and balance the induction of the IFN amplification loop. A subset of ISGs are themselves components of the IFN pathway or promote it signaling. However, IFN also induces several negative regulators which can target PRR, IRFs, or JAK/STAT to dampen the response. The authors may discuss a time frame and existence of a diverse set of genes for the novel regulatory mechanism by AKT2 (in early and late phase of antiviral responses) using a similar approach than Goulet et al. (PMID: 23633948) for analysis of the genes induced early (6 hrs.) and late (24 hrs.) following the activation of the RLR pathway by 5'pppRNA as a viral surrogate.

We extracted vRNA from VSV to mimic 5'pppRNA and treated macrophages as suggested by PMID: 23633948. We assessed expression of several genes at different time points, which were differentially regulated by *Akt2* deficiency; furthermore, difference of the individual gene caused by *Akt2* deficiency varies at the early or late stages (Figure S7A).

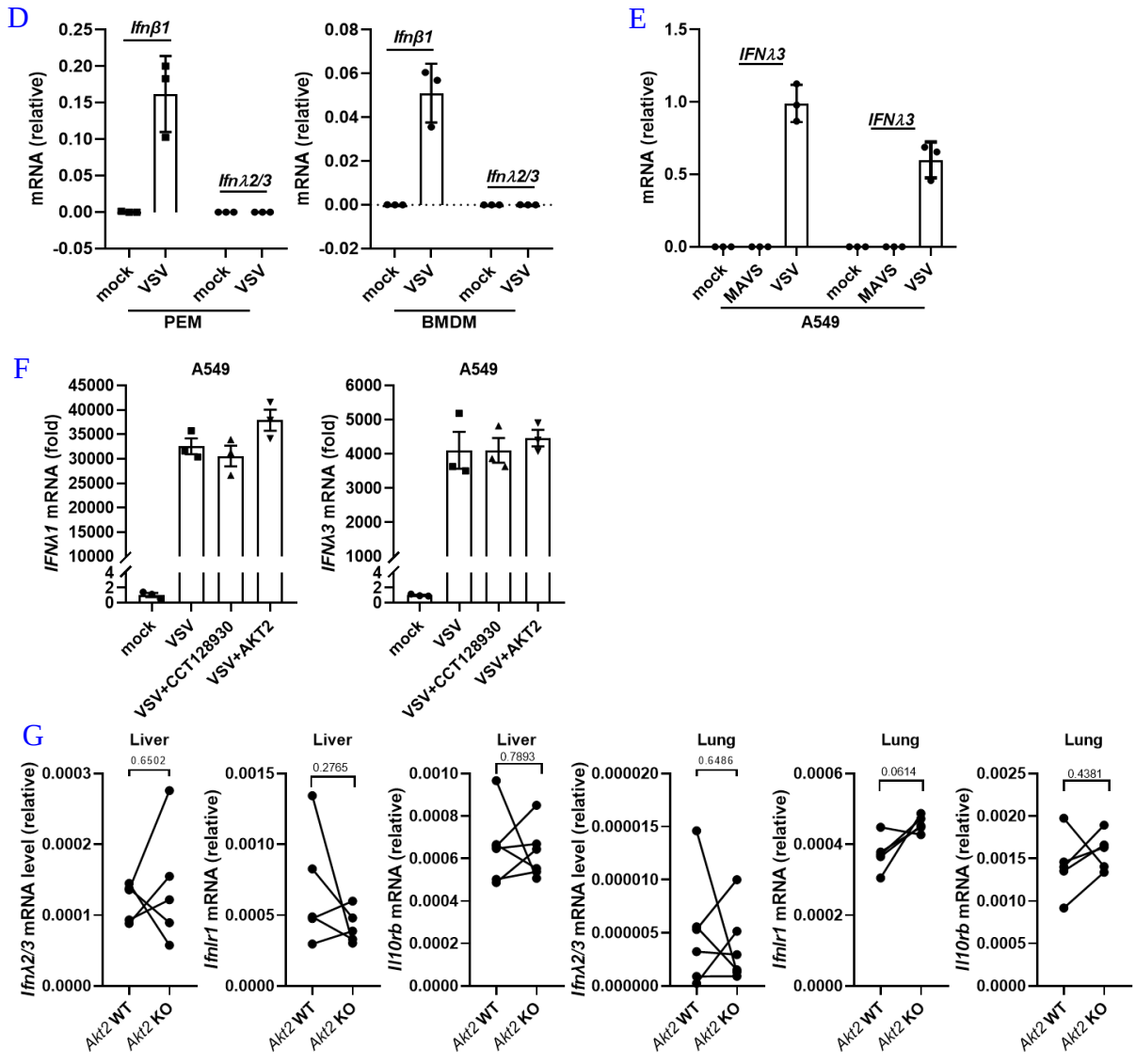
Figure S7A



We have discussed this in the manuscript (Page 15 in the revised manuscript): "Antiviral responses are complicated processes including the early and late stages. We confirmed that *Akt2* deficiency could enhance *Ifnb1*and ISGs production at the early stage (Figure 2A, 2B), which could also affect bystander cells for the induction of ISGs (Figure S2B). In addition, vRNA exacted from VSV was used to treat *Akt2* KO macrophages to mimic 5'pppRNA stimulation(Goulet et al, 2013), which differentially regulated the expression of several genes at the early or late stages. For example, *Akt2* KO PEMs enhanced the mRNA levels of *Ifnb1* and *Ifna4* at the early stage, but this enhancement was diminished at the late stage; in contrast, the mRNA levels of *Ccl2* and *Ccl5* were enhanced in *Akt2* KO PEMs at the late stage (Figure S7A). These results suggest that AKT2 might affect expression of multiple genes directly or indirectly during different phases of antiviral responses".

6. Does AKT2 also regulates PRR-mediated type III IFN production and requires IFN-λ receptor.

We have examined III-IFN levels in VSV-infected PEMs and BMDMs, which could produce *Ifnb1* at high levels, but failed to induce III-IFN (see below: D). We next tested the VSV-infected A549 cells, which could generate high levels of III-IFN (see below: E). When A549 cells were pretreated with the AKT2 inhibitor-CCT128930 or overexpressed with AKT2, we found that AKT2 did not affect VSV-mediated III-IFN production in A549 cells (see below: F). Besides, we measured IFNλ2/3 and III-IFN receptor levels in liver and spleen of WT and *Akt2* KO mice after VSV infection, which were not affected by *Akt2* deficiency (see below: G).



7. While this paper focused mainly on phosphorylation, it cannot be ignored that many other PTM, such as ubiquitination, methylation, acetylation, SUMOylation, ADP-ribosylation and glutamylation, oversee the activation of IRF3, the authors should discuss any potential relationship.

We have added this in the Discussion (Page 15 in the revised manuscript): “Besides phosphorylation, other types of posttranslational modifications (PTMs) are also critical for IRF3 activation, including ubiquitination, methylation, acetylation, SUMOylation, ADP-ribosylation. Further studies suggest that one type of PTMs might be related to other types of PTMs to affect IRF3 activation. For example, the prolyl isomerase Pin1 phosphorylates IRF3 at Ser339/Pro340, leading to IRF3 polyubiquitination and then degradation (Saitoh et al, 2006); lysine methyltransferase nuclear receptor-binding SET domain 3 (NSD3) is associated with IRF3 directly and methylates IRF3 at K366, which can maintain IRF3 phosphorylation to promote I-IFN production(Wang et al, 2017). These studies suggest that different signal pathways are participated and cooperated to precisely regulate IRF3 activation. It is interestingly to further reveal whether AKT2-mediated IRF3-Thr207 phosphorylation could affect or to be affected by other types of PTMs”.

8. TBK1 kinase phosphorylate the adaptor proteins (MAVS, TRIF STING) that in turn recruit IRF3, thereby licensing IRF3 for phosphorylation by TBK1. Phosphorylated IRF3 dissociates from the adaptor proteins, dimerizes, and then enters the nucleus to induce IFNs. As the phosphorylated Thr207-IRF3 is still able to dimerize it must not affect the licensing of IRF3. This should be discussed.

Thanks for this reviewer' suggestion. We have added this into Discussion in our revised manuscript (Page 14): “Upon viral infection, TBK1 phosphorylates MAVS, TRIF and STING to recruit IRF3, which then allows TBK1 to phosphorylate IRF3. Once phosphorylated, IRF3 is licensed to dissociate from them, dimerization and entry into the nuclei. Since IRF3-Thr207 has no impact on IRF3 dimerization, we could speculate that like WT IRF3, IRF3-Thr207 is recruited to and then dissociated from the other adaptor proteins (STING/TRIF/TBK1)”.

9. Mutation of the 5ST sites constitutively activates IRF3 following phosphomimic (aspartic acid) substitution. Phosphorylation of 5ST residues enable transition from an inactive to an active conformation. The authors demonstrated that IRF3-5D driven IFNB1 production is not regulated by AKT2. The authors may discuss if active conformation of IRF3 cannot be phosphorylated by AKT2.

We agree with the Reviewer and have added this speculation in the revised version (Page 14 in the manuscript): “In addition, the luciferase reporter assays in Figure 3A and 3B have shown that IRF3-5D-induced IFNβ1 production was not affected by AKT2 overexpression. This suggests that IRF3-5D might form a dimer and is not accessible to be phosphorylated by AKT2 at Thr207.”.

10. How zebrafish (Danio rerio) IRF3 (DrIRF3) amino acid sequence related to IRF3 in relation to

the Thr207.

This is a very good question. Thr207 is a conserved site in many species including Mus musculus, Macaca mulatta, Sus scrofa, Bos taurus and Rattus norvegicus, but zebrafish (Danio rerio) does not have Thr207. Instead, zebrafish has the IAD motif (β-helix) and further studies should address whether it might play similar function as hIRF3.

Minor comments

Intro Page 3 - Phosphorylation of Ser396 and the five serine or threonine residues in the C-terminal of IRF3 (Ser396, Ser398, Ser402, Thr404, and Ser405) has been demonstrated as the major sites that represent the activated IRF3 via enhancing IRF3 dimerization (Fitzgerald et al, 2003; Lin et al, 1998; Lin et al, 1999). The authors probably mean: Phosphorylation of Ser385/Ser386 and the five .....

We appreciate this correction and have revised it in the manuscript.

Fig. 1D PEM - At 12h post VSV infection, both AKT2 and IFNB1 mRNA are reduced. What does it mean?

At 12h post VSV infection, *Ifnb1* mRNA levels start to reduce; it indicates that some negative effectors are involved in reducing *Ifnb1* production. For example, lnc-Lsm3b is induced by I-IFN and then acts as a decoy to restrict RIG-I-induced downstream signaling (PMID: 29706547). Nevertheless, *Ifnb1* mRNA levels are still kept at high levels, and *Akt2* mRNA expression is reduced substantially; it suggests that the IFNβ1/JAK/STAT pathway could maintain its suppression on *Akt2* expression. We speculate that once the production of *Ifnb1* is diminished, *Akt2* mRNA expression is no longer reduced.

Fig. S1B and C (lung) - Relative AKT2 mRNA level of PBS control is different than 1.

We apologize for this mistake and have corrected it.

Fig. 2E and F- Legends should include more details of the transfection. In Fig. 2H What is the significance of the arrows (in other panels as well).

We have revised the figures legends throughout of the manuscript and enriched the details of the transfection (Page 20 and 32 in the revised manuscript). In Fig. 2H, the arrows indicate VSV-infected eye and skeletal muscle in zebrafish larvae, where tissue damages were observed.

Fig. 3 2F and 2G (right panel) - Explain why the data of VSV and ISD (WT and KO) are similar in panel 2G while quite different with analysis of IB:pIRF3 and dimer detection (2F).

We apologize for this confusion. This variation was caused by different stocks of

ISD that we used. We have repeated ISD treatment using the same new reagent (Figure S3F, S3G) and ensured the consistence of IB:pIRF3 and dimer detection as well as IRF3 nuclear localization (IF).

We believe that we have adequately addressed all of the criticisms raised by the reviewers and that our manuscript has been substantially improved. We thus hope that our revised version is to be reconsidered for publication. Many thanks for your great efforts to evaluate our manuscript.

Best regards,

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hongyanwang@sibcb.ac.cn
http://www2.sibcb.ac.cn/ePI.asp?id=132
Shanghai Institute of Biochemistry and Cell Biology,
Chinese Academy of Sciences,
Shanghai, China

Dear Hongyan,

Thank you for submitting your manuscript to The EMBO Journal. I am sorry for the delay in getting back to you with a decision, but I have now received the needed input on the study.

This submission is a resubmission of MS 108016 that rejected post review earlier this year. In response to the revisions that you had carried out to address the originally raised concerns I sent it back to the referees. While referee #2 was able to look at this version, neither referees #1 nor 3 were able to do so. Given this, I involved a new referee (referee# 4) and asked the referee to look at your response to the originally raised concerns.

I have now heard back from both referees and they both find the revised version improved. Referee #2 has some remaining concerns that I would like you to address in a revised version. Referee #4 appreciates the added experiments and supports publication here.

When you submit your revised version will you also take care of the following issues:

- Please upload the MS text as a word document

- We need a Data Availability section. This is the place to enter accession numbers etc. Please place it after the Materials and Methods and before Acknowledgements. If the generated data does not need to be deposited in a database then please state: This study includes no data deposited in external repositories.

- "Competing Interests" should be re-labelled as Conflict of Interest.

- We need an author check list - please see guide to authors

- For the reference list => For references with more than 10 authors please cut after 10 and follow by et al.

- Is the same mock used in Figure 1G and 2A? If so, please state in the figure legend

- We are missing your ORCID

- please upload individual figure files

- You have 6 supplemental figures. Either they should be turned into 5 Expanded View figures or alternative turn 5 of them into EV figures and add the remaining one to an appendix. Please see guide to authors
https://www.embopress.org/page/journal/14602075/authorguide#expandedview

- The supplementary tables should be removed from the manuscript file and uploaded individually as Table EV# OR renamed as Tables 1 & 2 and left where they are.

- We 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 labelled 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 (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

- We also need a summary figure for the synopsis. The size should be 550 wide by [200-400] high (pixels). You can also use something from the figures if that is easier.

I have also attached a document with helpful tips on how to prepare the revised version.

Let me know if you have any further questions

With best wishes

Karin

Karin Dumstrei, PhD

Senior Editor
The EMBO Journal

Information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Revision to The EMBO Journal should be submitted online within 90 days, unless an extension has been requested and approved by the editor; please click on the link below to submit the revision online before 12th Jan 2022:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #2:

While the paper by Zheng et al has been substantially improved, with addition of the appropriate figure legend, methodical information etc. Furthermore, I acknowledge that the authors present a large amount of experimental work in this manuscript. However, I still have some doubt about data presentation, interpretation and the general readability of the manuscript. Below are a few examples. The sheer length prevent a systematic response (response letter is 25 pages).

1) I think AKT2 modulate IFNb production is a more accurate title.
2) I am still not happy with the data presentation using linear but broken axis, I would prefer logarithmic axis as is normal for IFN induction. It gives the impression of a selective data presentation.
3) Reviewer 2 explicit ask to see all three replicates of figure 4A, instead one gel is presented.
4) On page 12 R2 specifically ask how luciferase assays are performed and duplicated, I find the answer to this question somewhat cloudy, and therefore reproducing the data would be more difficult.
5) In figure 5C authors measure IFNb induction in wt and AKT2 KO mice. In response to request from both R2 and R3 the authors also measure IFNa induction in these mice, but those data are only shown in the rebuttal letter. They claim no effect on IFNa, but to me there appears to be an effect on IFNa, and it seems very close to reach statistical significance P 0.067 for liver and P 0.057 for lungs. Again to me that indicate som degree of selectivity in the way data are interpreted and presented. If AKT2 KO influences IFNa production this questions their mechanistically argument

Referee #4:

The authors have extensively and carefully addressed all the concerns raised in the previous reviews. One additional issue that could be discussed is the difference in effect between AKT2 KO and T207A mutation of IRF3. Is it likely that the difference represents additional functions of AKT2? It is unfortunate but understandable that the authors were not able to obtain an antibody against phosphorylated T207, but their data showing much less phosphorylation of the entire protein for the T207A mutant is convincing. Finally, the authors have extensively addressed the issue of the significance of the relatively minor changes in IFN levels, citing several other examples. Final validation will need to come from knockin mouse studies, which are beyond the scope of the current work and are not being suggested by this reviewer for the present manuscript. In summary this is a very nice contribution with solid data and it is quite suitable for publication in EMBO J.

Point-to-Point Response (#EMBOJ-2021-108016R)

(AKT2 modulates IFNβ1 to regulate viral infections and SLE)

TO: The EMBO Journal

Dec. 2nd, 2021

Dear Editors,

We appreciate your efforts to re-evaluate our manuscript. We are pleased to get the supportive comments from the reviewers and editors. We have addressed the remaining questions and highlighted the revised figures & text in red. A point-to-point response letter is included in this letter.

Editor's advice:

- Please upload the MS text as a word document.

We have attached the MS text as a word document.

- We need a Data Availability section…

"Data availability: This study includes no data deposited in external repositories. Expanded View for this article is available online."

- "Competing Interests" should be re-labelled as Conflict of Interest.

"Conflict of interest: The authors declare that they have no conflict of interest."

- We need an author check list - please see guide to authors…

We have downloaded the author check list and filled in the relevant information.

- For the reference list => For references with more than 10 authors please cut after 10 and follow by et al.

The reference list has been reformatted as suggested.

- Is the same mock used in Figure 1G and 2A? If so, please state in the figure legend.

The mock is different in Figure 1G and 2A, and the source data have been added in the attached files for per figure.

- We are missing your ORCID.

My ORCID is 0000-0002-0731-9685.

- please upload individual figure files.

We have uploaded individual figure files. The descriptions of P, the test and error bars (SD, SEM) were provided at the end of Figure Legend as “Data information”.

- You have 6 supplemental figures…

We have turned 5 supplemental figures into EV figures and the remaining one into an appendix.

- The supplementary tables should be removed from the manuscript file and uploaded individually as Table EV# OR renamed as Tables 1 & 2 and left where they are.

The supplementary tables have been uploaded individually as Table EV.

- We encourage the publication of source data, particularly for electrophoretic gels and blots… The PDF files will be published online with the article as supplementary "Source Data" files.

We have provided the "Source Data" files.

- We include a synopsis of the paper (see http://emboj.embopress.org/). Please provide me with a general summary statement and 3-5 bullet points that capture the key findings of the paper.

Interferon regulatory factor 3 (IRF3)-induced type I interferon (I-IFN) production is vital in anti-viral infection and systemic lupus erythematosus (SLE). This study has elucidated that AKT2 bound and phosphorylated IRF3 to attenuate IRF3 nuclear translocation, leading to the reduced I-IFN production. AKT2 expression was downregulated in macrophages after infection or in PBMCs from SLE patients. *Akt2* deficiency increased I-IFN induction to aggravate the severity of SLE, but protect the host from viral infection.

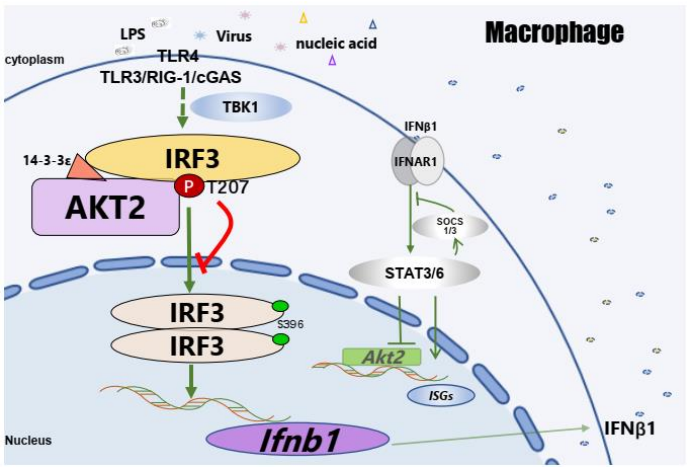
1. I-IFN stimulation reduces *Akt2* transcription, and AKT2 inhibits I-IFN production, which exerts a negative feedback loop.

2. AKT2 binds and phosphorylates IRF3 at Thr207 to reduce IRF3 nuclear localization.

3. *Akt2* deficiency promotes IFNβ1 production to favor antiviral response but aggravates SLE.

- We also need a summary figure for the synopsis...

The summary figure is from the ‘Appendix Figure S3’.



Referee #2's comments:

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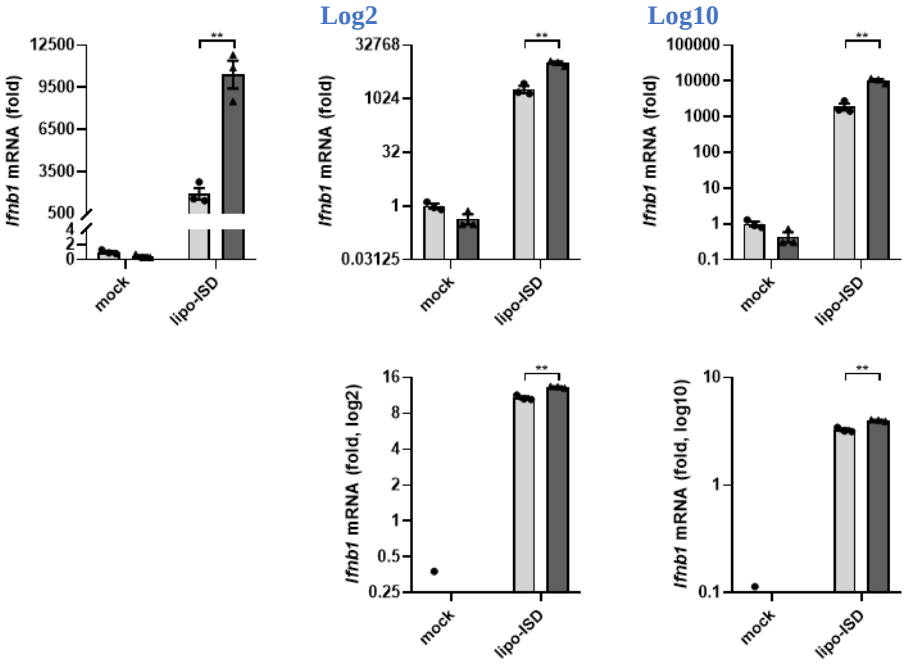
1) I think AKT2 modulate IFNb production is a more accurate title.

We have followed the advice and changed the title as "AKT2 modulates IFNβ1 to regulate viral infections and SLE".

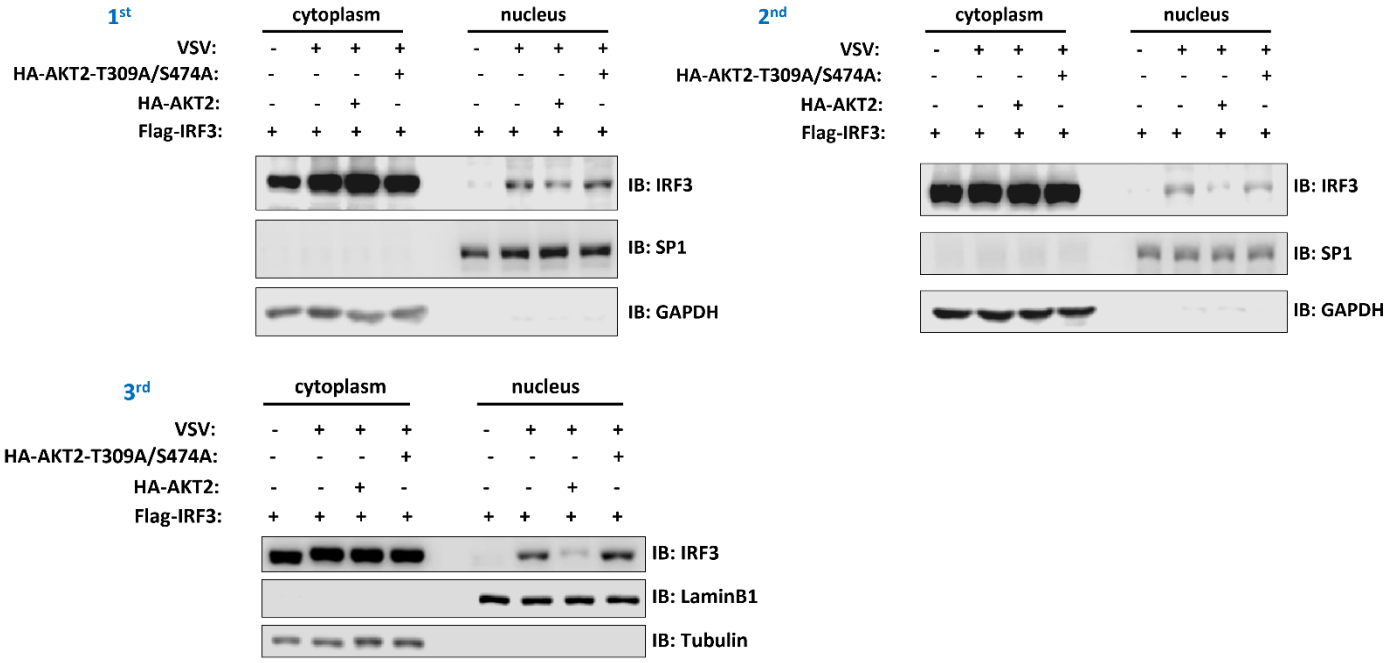
2) I am still not happy with the data presentation using linear but broken axis, I would prefer logarithmic axis as is normal for IFN induction. It gives the impression of a selective data presentation.

We tried to follow this advice to reformat these figures, but this seems not suitable for our data showing IFNβ1 induction. Firstly, we consulted a great deal of relevant literature on this issue, and very few papers have presented IFNβ1 induction using logarithmic axis. Second, our previous rebuttal letter has addressed that the 2-3-fold enhancement of IFNβ1 could significantly affect the pathological process of infection. Taking Figure 1H as an example, once we changed to the log2 or log 10 axis, the difference was so little that might cause confusion or misleading to get different conclusions.



3) ask to see all three replicates of figure 4A, instead one gel is presented.

We have attached three replicates in the below. The 3rd results were showed in Figure 4A.



4) On page 12 R2 specifically ask how luciferase assays are performed and duplicated, I find the answer to this question somewhat cloudy, and therefore reproducing the data would be more difficult.

Sorry for this. We have modified the description about the 'IFNβ1 Luciferase reporter assay' in the 'Materials and methods' and highlighted it in red.

"The plasmids expressing the indicated proteins (0.25μg), the IFNβ1 luciferase reporter (0.25μg) and the internal control TK-renilla (0.01μg) were transfected with 1.53μl PEI into HEK293T cells, which were 80–90% confluent in a 24-well plate. After 6-8 hours, fresh culture medium was changed to culture the cells for at least another 18 hours. If necessary, HEK293T cells were infected with or without VSV for 6 hours. Then cells were harvested to measure the luciferase readings with the guidance of a dual-luciferase assay kit (Promega, #0000456500). Briefly, culture supernatant was discarded, and cells were washed with PBS. Passive Lysis Buffer (diluted with water to 1×, 100 μL/well) was added to lyse cells, and supernatants were collected. Next, 20μL lytic supernatants of each well were mixed with 20μL Luciferase Assay Buffer II and measured as Results 1. Then 20μL Stop & Glo® Buffer was added for Results 2. Finally, we divided Result 1 by Result 2 to get the relative IFNβ1 Luciferase activity, and calculated the fold changes of IFNβ1 Luciferase activity by compared to the control groups.

Each experiment was performed in triple wells using the same plasmids synchronously for technical replicate; and three independent experiments were repeated. The data were shown from three independent experiments or from a representative experiment as the indicated."

5) In figure 5C authors measure IFNb induction in wt and AKT2 KO mice. In response to request from both R2 and R3 the authors also measure IFNa induction in these mice, but those data are only shown in the rebuttal letter. They claim no effect on IFNa, but to me there appears to be an effect on IFNa, and it seems very close to reach statistical significance P 0.067 for liver and P 0.057 for lungs. Again to me that indicate some degree of selectivity in the way data are interpreted and presented. If AKT2 KO influences IFNa production this questions their mechanistically argument.

We thank Reviewer 2's valuable question. We agree that the difference of *Ifnα* levels is close to reach statistical significance; but IRF7-induced *Ifnα* expression could be the downstream effect of the IFNβ/IFNAR signaling, which means that the increased *Ifnα* production might be driven by *Akt2* KO-induced enhancement of IFNβ1

production. Nevertheless, we agree with the possibilities that AKT2 might affect other targets or signaling, leading to a combined effect on *Ifnb1* production. Considering together with Reviewer #4's question, we added the *Ifnα* data into Appendix Figure S2 and discussed this in the revised manuscript (page 15, highlighted in red).

"These results suggest that AKT2 might affect expression of multiple genes directly or indirectly during different phases of antiviral responses. For instance, IRF7 is another IRF family member which could also induce *Ifnb1* transcription (Levy et al, 2002; Ning et al, 2011). We found that *Ifnα* was enhanced in livers and lungs of *Akt2* KO mice (Appendix Fig S2B), and *Ifnα* transcription is driven mainly by IRF7. IRF7 is also downstream of the IFNβ1/IFNAR pathway, therefore *Ifnα* enhancement might be indirectly induced by the increased IFNβ1 in *Akt2* KO macrophages. Future studies are needed to explore additional functions of AKT2, including its role in regulating IRF7 activation. In addition, AKT2 activation could induce glucose transporter 1 expression, glucose uptake, and lactate production in neoplastic cells (Moro et al, 2009). Interestingly, lactate was reported to inhibit RLR-mediated IFN production (Zhang et al, 2019). It is therefore worthy to test whether AKT2 might also affect lactate production to inhibit IFN production."

Referee #4's comments:

The authors have extensively and carefully addressed all the concerns raised in the previous reviews. One additional issue that could be discussed is the difference in effect between AKT2 KO and T207A mutation of IRF3. Is it likely that the difference represents additional functions of AKT2? It is unfortunate but understandable that the authors were not able to obtain an antibody against phosphorylated T207, but their data showing much less phosphorylation of the entire protein for the T207A mutant is convincing. Finally, the authors have extensively addressed the issue of the significance of the relatively minor changes in IFN levels, citing several other examples. Final validation will need to come from knockin mouse studies, which are beyond the scope of the current work and are not being suggested by this reviewer for the present

manuscript. In summary this is a very nice contribution with solid data and it is quite suitable for publication in EMBO J.

We do appreciate Reviewer #4's supportive comments about our revised manuscript. Many thanks for his/her valuable question, too.

We agree with the difference in effect between AKT2 KO and T207A mutation of IRF3, and we propose that other underline mechanism might exist to regulate IFN production in *Akt2* KO macrophages (i.e., except for IRF3-T207). According to others' studies of AKT2, AKT2 activation could induce glucose transporter 1 expression, glucose uptake, and lactate production in neoplastic cells (PMID: 19079138). Interestingly, lactate was demonstrated to inhibit RLR-mediated IFN production (PMID: 31155231). We therefore speculate the possibility that AKT2 might also affect lactate production to inhibit IFN production. We have added this discussion into the revised version of manuscript (see page 15).

After we have adequately addressed the remaining concerns, we hope that the revised manuscript is now suitable for publication in The EMBO Journal. Thanks again for your great efforts to evaluate our revision.

Yours sincerely,

Best regards,

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Shanghai, China

2nd Revision - Editorial Decision **23rd Dec 2021**

Dear Hongyan,

Thank you for submitting your revised manuscript to The EMBO Journal. I have now had a chance to take a careful look at everything and all looks good!

I am therefore very pleased to accept the manuscript for publication here.

With best wishes

Karin

Karin Dumstrei, PhD
Senior Editor
The EMBO Journal

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Corresponding Author Name: Hongyan Wang
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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.

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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.
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- 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 $\chi 2$ 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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Mainly refer to related similar literature reports.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Yes. For animal studies, include a statement about sample size estimate even if no statistical methods were used.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	The criteria were pre-established.
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.	Steps were taken to minimize the effects of subjective bias when allocating animals/samples to treatment. For example, when different batches of mice were counted together, pairing analysis was performed to ensure comparability between the same batches of mice.
For animal studies, include a statement about randomization even if no randomization was used.	NA.
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.	Yes, steps were taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator). For example, in order to avoid subjective bias, genotypes of the cell or mouse were unknown during experiment and collecting data , until the data analysis that the genotypes were checked.
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Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA.

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7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes.

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13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA.
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15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA.
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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.	NA.

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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	NA.
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