EMBO reports

Licochalcone B specifically inhibits NLRP3 inflammasome by disrupting NEK7-NLRP3 interaction

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Dear Prof. Bai,

Thank you for the transfer of your research manuscript to EMBO reports. We have now received the reports from the three referees that were asked to evaluate your study, which can be found at the end of this email.

As you will see, the referees think that these findings are of interest. However, they have several comments, concerns, and suggestions, indicating that a major revision of the manuscript is necessary to allow publication of the study in EMBO reports. As the reports are below, and all the points need to be addressed, I will not detail them here. It will be of high importance, though, to address point 1 of referee #1 and to provide experimental insight how LicoB interferes with inflammasome activation in human cells. Please also have your revised manuscript carefully proofread by a native speaker.

Moreover, you state in the beginning of the results that 'To provide potential candidates for treatment of NLRP3-mediated diseases, we screened inhibitors of NLRP3 and found that LicoB could block NLRP3 inflammasome activation.' To provide a complete picture of the experiments done, we require that this screen is documented in the manuscript. We thus ask you to add these data.

Given the constructive referee comments, we would like to invite you to revise your manuscript with the understanding that all referee concerns must be addressed in the revised manuscript or in the detailed point-by-point response. Acceptance of your manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision. We are aware that many laboratories cannot function at full efficiency during the current COVID-19/SARS-CoV-2 pandemic, and we have therefore extended our 'scooping protection policy' to cover the period required for full revision. Please contact me to discuss the revision should you need additional time, and also if you see a paper with related content published elsewhere.

When submitting your revised manuscript, please also carefully review the instructions that follow below.

PLEASE NOTE THAT upon resubmission revised manuscripts are subjected to an initial quality control prior to exposition to re-review. Upon failure in the initial quality control, the manuscripts are sent back to the authors, which may lead to delays. Frequent reasons for such a failure are the lack of the data availability section (please see below) and the presence of statistics based on n=2 (the authors are then asked to present scatter plots or provide more data points).

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the final manuscript text (including legends for main figures, EV figures and tables), but without the figures included. Please make sure that changes are highlighted to be clearly visible. Figure legends should be compiled at the end of the manuscript text.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure), of main figures and EV figures. Please upload these as separate, individual files upon re-submission.

The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines

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5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq, structural and array data) are deposited in an appropriate public database. If no primary datasets have been deposited, please also state this a dedicated section (e.g. 'No primary datasets have been generated and deposited'), see below.

See also: http://embor.embopress.org/authorguide#datadeposition

Please remember to provide a reviewer password if the datasets are not yet public.

The accession numbers and database should be listed in a formal "Data Availability" section (placed after Materials & Methods) that follows the model below. This is now mandatory (like the COI statement). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
- [data type]: [name of the resource] [accession number/identifier/doi] ([URL or identifiers.org/DATABASE:ACCESSION])

*** Note - All links should resolve to a page where the data can be accessed. ***

Moreover, I have these editorial requests:

6) We strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure. If you would like to use this opportunity, please submit the source data (for example scans of entire gels or blots, data points of graphs in an excel sheet, additional images, etc.) of your key experiments together with the revised manuscript. If you want to provide source data, please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure.

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at: http://www.embopress.org/page/journal/14693178/authorguide#referencesformat

8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
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10) For microscopic images, please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Achim Breiling
Editor

EMBO Reports

Referee #1:

In this study, the authors discovered that Licochalcone B (LicoB) from the widely used Chinese traditional medicinal herb licorice specifically inhibits the NLRP3 inflammasome. Mechanistically, the authors demonstrated that LicoB directly bound to NEK7, disrupting the interaction between NEK7 and NLRP3. In addition, the authors also provided compelling evidences that LicoB showed strong protective effects in LPS induced-septic shock mouse model and MCD diet-induced NASH mouse model. Overall, their data are clean and convincing. Below are the specific comments to be addressed:

Major comments:

1. In human cells, the activation of NLRP3 inflammasome bypasses NEK7. Therefore, blockade of NLRP3 inflammasome by targeting NEK7 is not an applicable strategy for patients in clinic. The authors observed similar inhibitory effect in THP-1 cells and human PBMCs as in mouse BMDMs. This suggests that LicoB may act at additional step(s) inhibiting NLRP3 inflammasome activation.

2. In line 97, "To provide potential candidates for treatment of NLRP3-mediated diseases, we screened inhibitors of NLRP3 and found that LicoB could block NLRP3 inflammasome activation." The authors should provide the details of the screen. Including: How was it done? What was the readout for the screen? How many compounds were tested? The outcome? Etc.

3. NLRP3 inflammasome can be activated by potassium efflux-dependent or potassium efflux-independent activators. The authors showed that LicoB could efficiently inhibit NLRP3 inflammasome activation in response to ATP, Nigericin, MSU, cytosolic polyI:C, which are potassium efflux-dependent. However, it will be important to test whether LicoB could also inhibit the NLRP3 inflammasome activated by potassium efflux-dependent activators, such as Imiquimod.

4. Even though the kinase activity of NEK7 is dispensable for activation of the NLRP3 inflammasome, NEK7 is a mitotic kinase. For potential clinical application, it will be of great value to test whether LicoB affects the kinase activity of NEK7.

5. Another study (PMID: 30281174) showed that Licochalcone A inhibited the NLRP3 inflammasome by blocking the production of mtROS. Licochalcone A and LicoB show very high structural similarity. The author showed that LicoB didn't affect the production of mtROS. However, it will be very interesting to test whether Licochalcone A also bind to NEK7.

Minor comment:

The English writing of the manuscript need to be improved. There are many sentences that are difficult to understand.

For example:
Line 50, "frozen protein-related with cryopyrin-associated autoinflammatory syndrome"
Line 52 "In addition, the NLRP3 inflammasome has a sensitive stress response to some host-derived "risk signals", ..."

Referee #2:

The authors list compounds that inhibit the activation of NLRP3 but they do not write which of these are specific for NLRP3. They do not list Sulforaphran, which had been tested in humans with austism and published in Proceedings of the National Academy of Sciences. Unlike MCC950, there was no toxicity with Sulfphoraphran. The authors continue and state that "the development of safe and effective NLRP3 inhibitors is an urgent need for NLRP3-inflammatory-mediated diseases". They authors do cite OLT1177 published in a 2020 paper in Proceedings of the National Academy of Sciences using the mouse Alzheimer model (APP) and another paper on OLT1177 in mouse arthritis. However, they fail to cite the effectiveness and safety of OLT1177 in humans with acute gout flares. That paper was published in Lancet Rheumatology. Another paper published is OLT1177 in humans with heart failure, although this is a recent paper but was online 4 months ago. Why is these publication on OLT1177 important for the manuscript now being evaluated? Because these papers contradict the authors statement that "the development of safe and effective NLRP3 inhibitors is an urgent need for NLRP3-inflammatory-mediated diseases".

The present study does not have any data that the inhibitor (be it specific or non-specific) of NLRP3 is effective in the mouse model used and in vitro testing in mouse macrophages. These are standard assays used in most papers on NLRP3. The Authors provide data that LicoB is a specific inhibitor of NLRP3. The authors also show data that LicoB blocks ASC. Nothing unexpected. The ability of Lico B to bind to NEK is again not unexpected as others have shown that NEK is a component of the inflammasome complex (shown 5 years ago). The paper continues with the LPS model (not specific for IL-1beta but for TNFalpha an IFNgamma). In my opinion, the use of the LPS model for IL 1beta (or IL-18) is dose-dependent and varies so much that it is not used correctly. Although IL-1beta is decreased and the authors state there was no effect on TNFalpha, the model is not specific enough for IL-1beta medicated diseases. The use of the NASH model is also not specific enough for NLRP3 blockade. In the NASH model, the authors show that LicoB reduces gene expression for IL-1beta, IL-18 and TNFalpha. Well, this is not valid data for the mechanism of Lico B in the NASH model. IL-18 is constitutively expressed in healthy tissues and the efficacy of ANY NLRP3 inhibitor must be demonstrated to reduce the mature, processed form on IL-18.

The use of DMSO in Figure 2 connotes that LicoB must be not water soluble. They use DMSO as a control for for LicoB. I cannot find at any place in this manuscript about why they use DMSO. How they treat then with LicoB for IP and gavage. These details are missing for correct evaluation of this manuscript.

Supplement Figure using THP-1 cells and PBMC is fundamental to the mechanism of action (MOA). But PBMC require fresh cells and I am not sure this is the case here. Also, fresh human PBMC already have active caspase-1 (see attachment). The manuscript is essentially a "me too" paper in that these investigators perform all the assays that others have published. They have a natural compound and as such adds to the list of natural compounds that inhibit NLRP3. The best example is sulforaphane.

The authors state that there is a need to develop drugs that reduce NLRP3, but they fail to cite studies published on OLT1177 in humans, although they cite basic science papers of OLT1177. There is a paper in Lancet Rheumatology that shows the safety and efficacy of

OLT1177 in the treatment of acute gout flares. Another paper on the efficacy of OLT1177 to treat patients with Heart Failure. These should have been cited and the something written about the safety of OLT1177. MCC950 is toxic and is irrelevant.

The sepsis model is poor, but the MSU model is useful.

I do not see why the manuscript is new news about NLRP3 inhibition. It's a "me too" paper and the authors fail to inform the Readers that it will take a lot of time for Loco B to enter clinical trials and reach the level of development of others (such as OLT1177) that are now in clinical trials.

Is the mechanism of action of NEK7 unique for Loco B? Each NLRP3 inhibitor may has a different molecular location to arrest the oligomerization of NLRP3 to activate caspase 1. This is because of the size and complexity of NLRP3. Look at how many mutations of NLRP3 result in activation and serious human diseases. That information is reveals that there is no one single area that can be blocked and results in inhibition of NLRP3 activation. Clearly NEK 7 seems to be the MOA for Loco.

Overall, the paper is a "me too" paper for another NLRP3 inhibitor and adds very little if anything to the biology and clinical use of NLRP3 inhibitors.

Referee #3:

The authors aimed to characterise the effects on LicoB on NLRP3 inflammasome function. Using a range of both in vitro and in vivo approaches, the authors show that LicoB selectively inhibits the NLRP3 inflammasome by interacting with NEK7, a protein required for NLRP3 inflammasome activation, and that LicoB has beneficial effects in an LPS-induced sepsis model, as well as a non-alcoholic steatohepatitis model. Overall, this is a nicely organised and convincing study.

1. In Fig 1, the authors demonstrate that caspase-1 activation is reduced by LicoB, and that LDH release is also reduced. Could the authors also assess whether Gasdermin D cleavage is reduced?
2. In Fig 2A, the authors show that LicoB inhibits non-canonical NLRP3 activation by using Pam3CSK4 priming followed by LPS transfection. Given that LicoB does not appear to be toxic over prolonged treatments, could the authors also show whether LicoB blocks alternative NLRP3 inflammasome activation in human monocytes in response to prolonged LPS treatment alone, as described by Gaidt et al 2016 (PMID: 27037191).
3. In Fig 4C, the authors nicely show that LicoB inhibits NLRP3 activation without blocking K+ efflux. Could the authors strengthen this data by also assessing whether LicoB blocks K+ efflux-independent NLRP3 activation e.g. via imiquimod stimulation?
4. Given that the authors suggest that LicoB binds to NEK7, can the authors address whether this interaction affects NEK7's role in mitotic spindle formation / cell division, or can they speculate about this?
5. The use of multiple unpaired t tests is often not the best way to compare datasets that contain multiple groups. One-way and two-way ANOVAs would be a more appropriate way to analyse differences between multiple groups. For example, in Figure 1D, a one-way ANOVA with Dunnett's post-hoc test (comparing all groups to Vehicle + Nig treatment) would be a better way to analyse the data.
6. A minor point, but it would be nice if the authors could include individual data points in the graphs in Figures 1-4 and Extended Data Figure 1 for greater transparency.
7. A very minor point, but in Figure 2B, C, E, F, the use of + and - in the labels on X axis is slightly confusing, as to me it suggests that the stimulus (e.g. nigericin) is either absent or present, when in fact the stimulus is present in both conditions. Labels more similar to those used in Figure 2A/D would be clearer.

Response to reviewers

Referee #1:

In this study, the authors discovered that Licochalcone B (LicoB) from the widely used Chinese traditional medicinal herb licorice specifically inhibits the NLRP3 inflammasome. Mechanistically, the authors demonstrated that LicoB directly bound to NEK7, disrupting the interaction between NEK7 and NLRP3. In addition, the authors also provided compelling evidences that LicoB showed strong protective effects in LPS induced-septic shock mouse model and MCD diet-induced NASH mouse model. Overall, their data are clean and convincing. Below are the specific comments to be addressed:

Response: We appreciate the reviewer's encouraging comments on our manuscript and the kind suggestions. We have performed several experiments to address the concerns and revised the manuscript. The point-to-point response is listed below.

Major comments:

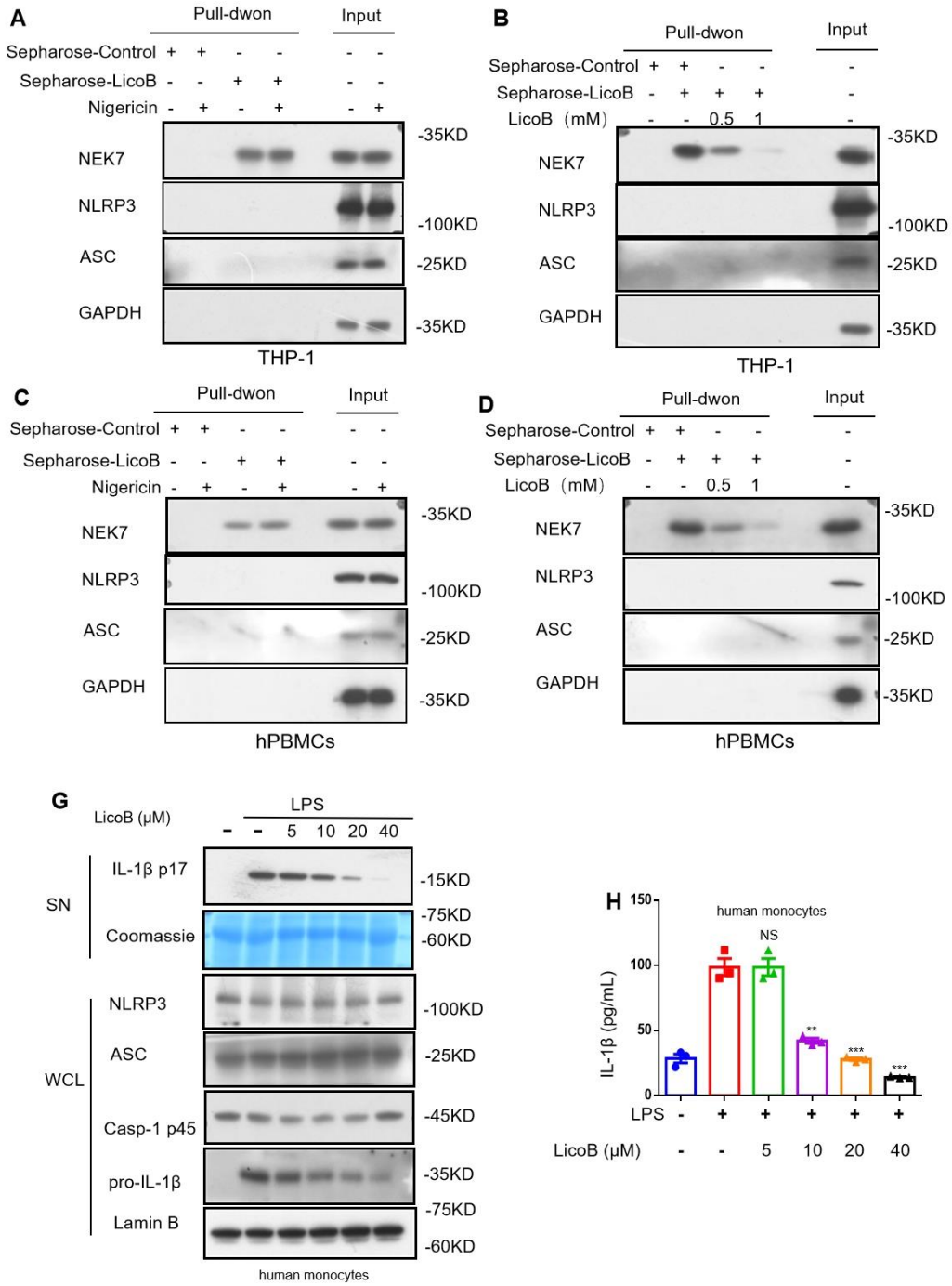
1. In human cells, the activation of NLRP3 inflammasome by passes NEK7. Therefore, blockade of NLRP3 inflammasome by targeting NEK7 is not an applicable strategy for patients in clinic. The authors observed similar inhibitory effect in THP-1 cells and human PBMCs as in mouse BMDMs. This suggests that LicoB may act at additional step(s) inhibiting NLRP3 inflammasome activation.

Response: We thank the reviewer for the suggestion. There are three kinds of NLRP3 inflammasome activation: canonical, non-canonical and alternative NLRP3 activation. The critical role of Nek7 in NLRP3 inflammasome activation has been shown in three independent studies (He, Zeng et al., 2016, Schmid-Burgk, Chauhan et al., 2016, Shi, Wang et al., 2016). Notably, in a cultured human monocyte cell line (THP-1) and in primary human monocytes, knockdown of endogenous NEK7 reduced secretion of IL-1B in response to priming with LPS plus stimulation with nigericin or ATP (Shi et al., 2016). Moreover, a study has reported a cryo-electron microscopy structure of human NLRP3 in complex with NEK7 and demonstrated that NEK7 bridges adjacent NLRP3 subunits with bipartite interactions to

mediate the activation of the NLRP3 inflammasome (Sharif, Wang et al., 2019). Overall, NEK7 is essential to NLRP3 inflammasome activation in human cells.

As shown in the original Figure 4I (Revised Figure 4I) , we found that LicoB interacted with NEK7 in BMDMs, we also performed the pulldown assay in human monocyte cell line (THP-1) and primary human monocytes, the result showed that LicoB also interacted with NEK7(Revised Figure EV3A-D). Moreover, our result showed that LicoB interfered the interaction of human-NLRP3(Flag-NLRP3) and endogenous NEK7 in 293T cells (original Figure 4K Revised Figure4K). LicoB also inhibited canonical NLRP3 inflammasome activation (induced by LPS treatment plus Nigericin stimulation) in THP-1 and PBMCs (Original Extended Fig 1A-D, Revised Figure EV1D-G).These results suggest that in human cells, LicoB interacts with NEK7 to affect NEK-NLRP3 interaction and subsequent NLRP3 activation.

Besides, a new type of NLRP3 inflammasome activation (Alternative NLRP3 inflammasome) has been reported. In human monocytes, lipopolysaccharide alone induced an ‘‘alternative inflammasome’’, the activation was propagated by TLR4- TRIF- RIPK1- FADD -CASP8 signaling upstream of NLRP3, and the mechanism did not extend to canocanical NLRP3 activation. As mentioned before, NEK7 is essential for canonical NLRP3 inflammasome activation in both human and murine cells (Shi et al., 2016), but the role of NEK7 in alternative NLRP3 inflammasome remains unclear. We also tested the effect of LicoB on alternative NLRP3 inflammasome activation in human monocytes. The result showed that pretreatment of LicoB inhibited the IL-1β secretion induced by 14 hours of LPS treatment in human monocytes, but the expression of pro-IL-1β was also inhibited (Revised Figure EV3G and H), suggesting that LicoB may affect alternative NLRP3 inflammasome in human monocytes at least partially via inhibition of production of pro-IL-1β (LPS-induced transcriptional priming).

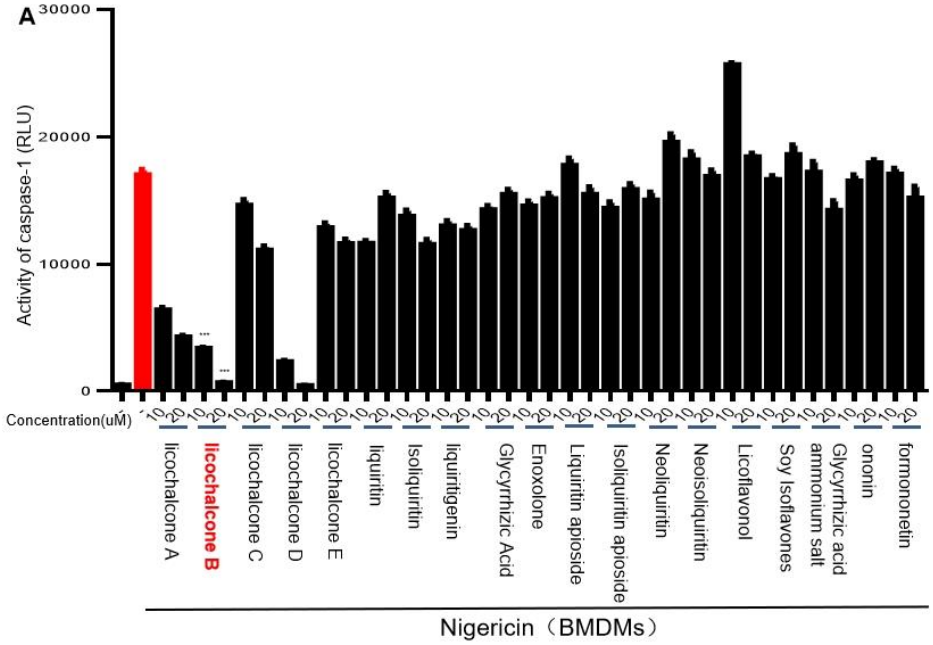


Responded Fig.1 (Revised Fig. EV3 A-D, G and H) (**A**) Cell lysates of PMA-primed THP-1 treated with nigericin or not were incubated with sepharose or LicoB-sepharose. The pull-down samples and input were analyzed by Western blot. (**B**) Cell lysates of PMA-primed THP-1 were incubated with sepharose or LicoB-sepharose in the presence of different concentrations of free LicoB（0.5mM and 1mM). The pull-down samples and input were analyzed by Western blot. (**C**) Cell lysates of LPS-primed hPBMCs treated with nigericin or

not were incubated with sepharose or LicoB-sepharose. The pull-down samples and input were analyzed by Western blot. (**D**) Cell lysates of LPS-primed hPBMCs were incubated with sepharose or LicoB-sepharose in the presence of different concentrations of free LicoB（0.5mM and 1mM). The pull-down samples and input were analyzed by Western blot. (**G and H**) Human monocytes were treated with LicoB for 1 h, prior to stimulation with LPS (200ng/mL) for 14 h. Western blot analyses of pro-caspase-1 (p45), pro-IL-1β, NLRP3, and ASC in the whole cell lysate (WCL); cleaved IL-1β (p17) in the culture SN of BMDMs were shown(**G**). Coomassie Blue staining was used as the SN loading control, while lamin B was used as the lysate loading control. IL-1β secretion (**H**) in the SN were measured by ELISA. Data are presented as mean ± SEM from biological replicates (n=3). NS: not significant (One-way ANOVA with Dunnett's post-hoc test).

2. In line 97, "To provide potential candidates for treatment of NLRP3-mediated diseases, we screened inhibitors of NLRP3 and found that LicoB could block NLRP3 inflammasome activation." The authors should provide the details of the screen. Including: How was it done? What was the readout for the screen? How many compounds were tested? The outcome? Etc.

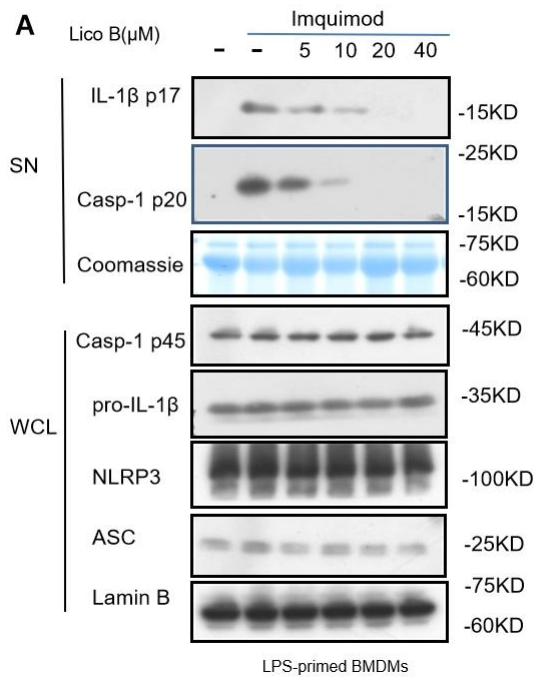
Response: We appreciate the suggestions and added the screening data accordingly. BMDMs were first primed with lipopolysaccharide (LPS) for 4 hours and then pretreated with 20 compounds from *Glycyrrhiza plants* (*licorice*) 1 hour prior to stimulation with nigericin at the doses of 10μM, 45 minutes later, the activity of caspase-1 in the culture supernatant was tested using the Caspase-Glo 1(A Caspase-Glo® 1 Inflammasome Assay, G9951, Promega) reagent. LicoB showed an obviously inhibitory effect on the caspase-1 activity.



Responded Fig.2 (Revised Fig. EV 1A) (**A)** BMDMs were primed with LPS for 4 hours and then treated with LicoB 1 hour prior to stimulation of nigericin for 45 minutes. Activity of caspase-1 in culture supernatants of BMDMs were shown. Data are presented as mean ± SEM from biological replicates (n=3). *P < 0.05, **P < 0.01, ***P < 0.001, NS: not significant (One-way ANOVA with Dunnett's post-hoc test).

3. NLRP3 inflammasome can be activated by potassium efflux-dependent or potassium efflux-independent activators. The authors showed that LicoB could efficiently inhibit NLRP3 inflammasome activation in response to ATP, Nigericin, MSU, cytosolic polyI:C, which are potassium efflux-dependent. However, it will be important to test whether LicoB could also inhibit the NLRP3 inflammasome activated by potassium efflux-dependent activators, such as Imiquimod.

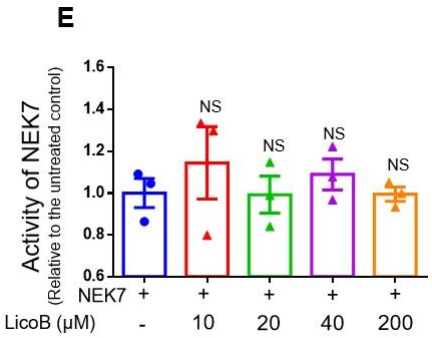
Response: We appreciate the constructive comments of the reviewer. To address the comments, we tested the effect of LicoB on Imiquimod-induced NLRP3 activation. BMDMs were primed with LPS and then treated with LicoB 1 hour prior to stimulation of Imiquimod(70μM) for 1 hour. The result suggested that LicoB can also effectively inhibit the activation of NLRP3 inflammasomes induced by the potassium efflux-independent activator imiquimod (Groß, Mishra et al., 2016).



Responded Fig.3 (Revised Fig. EV 2A) BMDMs were primed with LPS and then treated with LicoB 1 hour prior to stimulation of Imiquimod(70μM) for 1 hour. Western blot analysis of the pro-caspase-1 (p45), pro-IL -1β, NLRP3 and ASC in the whole cell lysate (WCL), activated caspase-1 (p20) and cleaved IL-1β (p17) in culture supernatants (SN) of BMDMs were shown.

4. Even though the kinase activity of NEK7 is dispensable for activation of the NLRP3 inflammasome, NEK7 is a mitotic kinase. For potential clinical application, it will be of great value to test whether LicoB affects the kinase activity of NEK7.

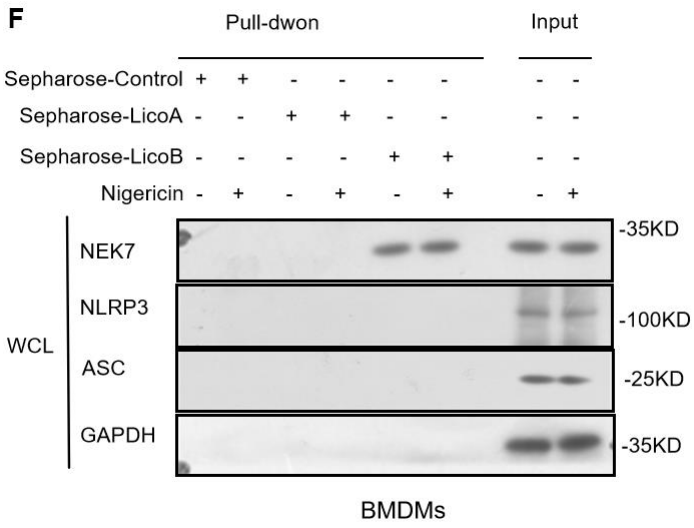
Response: We thank the reviewer for the comments. To address this concern, we investigated whether LicoB affected the kinase activity of NEK7. The in vitro kinase assay was performed with NEK7 and β-casein as a substrate, with a Universal Kinase Activity Kit according to the manufacturer's instructions (R&D Systems). Our experimental results showed that LicoB did not affect the kinase activity of NEK7.



Responded Fig.4 (Revised Fig. EV 3E) NEK7 was incubated with β-casein and ATP in the presence of different concerntrations of LicoB . NEK7 kinase activity was measured using an ADP-based phosphatase coupled kinase assay.Data are presented as mean ± SEM from biological replicates (n=3). NS: not significant (One-way ANOVA with Dunnett's post-hoc test).

5. Another study (PMID: 30281174) showed that Licochalcone A inhibited the NLRP3 inflammasome by blocking the production of mtROS. Licochalcone A and LicoB show very high structural similarity. The author showed that LicoB didn't affect the production of mtROS. However, it will be very interesting to test whether Licochalcone A also bind to NEK7.

Response: We thank the reviewer for the suggestion and perfomed the experiment accordingly. Licochalcone A (LicoA) was incubated with cyanogen bromide-activated -sepharose® 4B overnight to form a complex (sepharose-LicoA), then the coupled sepharose-LicoA was incubated with LPS-primed BMDMs cell lysate with or without nigericin stimulation. The proteins that interact with LicoA were analyzed and detected by immunoblotting. Our data showed that LicoA did not bind to NEK7.



Responded Fig.5 (Revised Fig. EV 3F). Cell lysates of LPS-primed BMDM treated with nigericin or not were incubated with sepharose , Sepharose-LicoA or Sepharose-LicoB . The pull-down samples and input were analyzed by Western blot.

Minor comment:

The English writing of the manuscript need to be improved. There are many sentences that are difficult to understand.

For example:

Line 50, "frozen protein-related with cryopyrin-associated autoinflammatory syndrome"

Line 52 "In addition, the NLRP3 inflammasome has a sensitive stress response to some host-derived "risk signals", ..."

Response: We thank the reviewers for their suggestions and corrected the corresponding language description accordingly. Moreover, we have the revised manuscript carefully proofread by a native speaker.The sentences mentioned in the comment have also been corrected to：

“Several mutations in the NLRP3 gene can result in spontaneous activation of NLRP3 inflammasome, which is central to the development of cryopyrin-associated autoinflammatory syndromes”.

“In addition, NLRP3 inflammasome also responds to some host-derived danger signals”.

Referee #2

The authors list compounds that inhibit the activation of NLRP3 but they do not write which of these are specific for NLRP3. They do not list Sulforaphran, which had been tested in humans with austism and published in Proceedings of the National Academy of Sciences. Unlike MCC950, there was no toxicity with Sulfphoraphran. The authors continue and state that "the development of safe and effective NLRP3 inhibitors is an urgent need for NLRP3-inflammatory-mediated diseases". They authors do cite OLT1177 published in a 2020 paper in Proceedings of the National Academy of Sciences using the mouse Alzheimer model (APP) and another paper on OLT1177 in mouse arthritis. However, they fail to cite the effectiveness and safety of OLT1177 in humans with acute gout flares. That paper was published in Lancet Rheumatology. Another paper published is OLT1177 in humans with heart failure, although this is a recent paper but was online 4 months ago. Why is these publication on OLT1177 important for the manuscript now being evaluated? Because these papers contradict the authors statement that "the development of safe and effective NLRP3 inhibitors is an urgent need for NLRP3-inflammatory-mediated diseases".

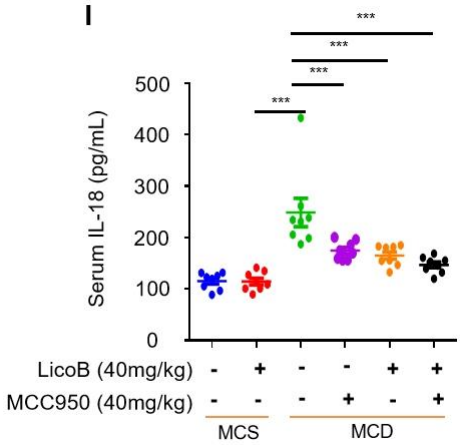
Response: We appreciate the constructive comments of the reviewer. Sulforaphane is an isothiocyanate found in broccoli sprout extracts(Fahey, Zhang et al., 1997), it has been reported to show cytoprotective effects in neurologic, cardiovascular, and other diseases(Dinkova-Kostova & Kostov, 2012) tested in humans with autism and exhibited negligible toxicity(Singh, Connors et al., 2014). Sulforaphane has also been demonstrated to inhibit the NLRP1b,NLRP3, NAIP/NLRC4, and AIM2 inflammasomes independent of Nrf2(Greaney, Maier et al., 2016), so it may not be a specific inhibitor of NLRP3 inflammasome. OLT1177(also known as dapansutrile) has been reported to specifically inhibit NLRP3 inflammasome(Marchetti, Swartzwelter et al., 2018), its safety and efficacy in the treatment of gout flares in an open-label, proof-of-concept, phase 2a trial has been demonstrated(Kluck, Jansen et al., 2020).Moreover, OLT1177 has been tested in a phase 1B trial and the result showed that treatment with OLT1177 for 14 days was safe and well

tolerated in patients with heart failure and reduced ejection fraction(Wohlford, Van Tassell et al., 2020). Further studies are needed to confirm the clinical potential of OLT1177. Overall, inhibitors of NLRP3 inflammasome show great potential in the treatment of NLRP3-mediated diseases. We thank the reviewer for the suggestion again and have revised our manuscript accordingly (line 79-93).

The present study does not have any data that the inhibitor (be it specific or non-specific) of NLRP3 is effective in the mouse model used and in vitro testing in mouse macrophages. These are standard assays used in most papers on NLRP3. The Authors provide data that LicoB is a specific inhibitor of NLRP3. The authors also show data that LicoB blocks ASC. Nothing unexpected. The ability of Lico B to bind to NEK is again not unexpected as others have shown that NEK is a component of the inflammasome complex (shown 5 years ago). The paper continues with the LPS model (not specific for IL-1beta but for TNFalpha an IFNgamma). In my opinion, the use of the LPS model for IL 1beta (or IL-18) is dose-dependent and varies so much that it is not used correctly. Although IL-1beta is decreased and the authors state there was no effect on TNFalpha, the model is not specific enough for IL-1beta medicated diseases. The use of the NASH model is also not specific enough for NLRP3 blockade. In the NASH model, the authors show that LicoB reduces gene expression for IL-1beta, IL-18 and TNFalpha. Well, this is not valid data for the mechanism of Lico B in the NASH model. IL-18 is constitutively expressed in healthy tissues and the efficacy of ANY NLRP3 inhibitor must be demonstrated to reduce the mature, processed form on IL-18.

Response: We thank the reviewer for the suggestion. The septic shock and production of IL-1β induced by i.p. injection of LPS has been reported to be NLRP3 dependent, NLRP3 deficiency inhibits inflammatory responses and enhances the survival of septic mice(He, Franchi et al., 2013, Kanneganti, Body-Malapel et al., 2006).Our result also showed that LicoB protected against LPS-induced septic shock and reduced the amout of IL-1β (Orignial Figure5A-C, Revised Figure5A-C).NLRP3 activation is mechanistically important for

progression of NASH evidenced by genetic knockout mice and NLRP3 pharmacological inhibitor (Mridha, Wree et al., 2017, Szabo & Petrasek, 2015, Wree, McGeough et al., 2014). Our results showed the protective effects of LicoB on the MCD-induced NASH (Orignial Figure6, Revised Figure7), the protein level of active caspase-1 in liver tissue in NASH mice was reduced by LicoB treatment (Orignial Figure6D, Revised Figure7D). Moreover, we also detected the amount of IL-18 in serum of mice by ELISA and the data showed that LicoB reduced the serum level of IL-18 (Revised Figure 7I).



Responded Fig.10 (Revised Fig. 7I) Eight-week-old male C57BL/6 mice were continuously fed with methionine- and choline-supplemented (MCS) or methionine- and choline-deficient (MCD) diets for 6 weeks, and at the same time, gavaged with LicoB, MCC950, or a combination of LicoB and MCC950 (n=8). The levels of IL-18 in the serum was measured using ELISA (n=8). Data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, and NS: not significant vs. control group (One-way ANOVA with Dunnett's post-hoc test).

The use of DMSO in Figure 2 connotes that LicoB must be not water soluble. They use DMSO as a control for for LicoB. I cannot find at any place in this manuscript about why they use DMSO. How they treat then with LicoB for IP and gavage. These details are missing for correct evaluation of this manuscript.

Response: We thank the reviewer for the comment. LicoB was purchased from MCE (MedChemExpress, Catalog Number: HY-N0373), for the in vitro experiments, we dissolved

LicoB in DMSO as the instruction (product data sheet). The maxium solubility of LicoB in DMSO is 83.33 mg/mL (291.08 mM; Need ultrasonic). In the IP experiment (Original Fig. 4K, Revised Fig.4K), HEK-293T cells were transfected with Flag-NLRP3 or Flag-vector and then treated with LicoB (40μM). Immunoprecipitation was performed with anti-DYKDDDDK (Flag) affinity gel agarose beads. We prepared stock solution of 40mM, the working concerntration of LicoB was 40μM, so for 1ml of cell culture media, 1μl of the stock solution was added into, the LicoB could be easily dissolved and no precipitation was observed during the experiment.

In the pulldown assay (Original Fig. 4I and J, Revised Fig. 4I and J), to prepare the sepharose-LicoB, we first dissolve 10mg of LicoB in 700μL DMSO (concerntration is 14.3mg/ml, which is much lower than the maxium solubility, 83.33 mg/mL), then the solution was added into 500uL Coupling Buffer (Changzhou Tiandi Renhe Biotechnology Co., Ltd.) and incubated with Sepharose 4B magnetic beads overnight to obtain the Sepharose-LicoB. The Sepharose-LicoB was then incubated with cell lyasates of LPS-primed BMDMs treated with/without nigericin. Furthermore, in the original Fig.4J (Revised Fig. 4J), free LicoB(dissolved in DMSO) was added into the mixture to test whether it interfered with the binding of Sepharose-LicoB to NEK7 , the final concerntration was 0.5mM and 1mM (the concerntration of stock solution was 100mM, much lower than the maxium solubility, 291.08 mM), no precipitation was observed during the experiment.

As for the in vivo experiment (gavage), we prepared the solution as a protocol modified from the instruction (product data sheet). In the instruction, LicoB is dissvolved in DMSO and then added with 10% DMSO and then 90% (20% SBE-β-CD in saline), solubility: ≥ 2.08 mg/mL (7.27 mM); C lear solution. In our experiment (NASH mice model, original figure 6, revised figure7), the maximum dose of LicoB we use is 40mg/kg , LicoB was dissolved in DMSO and then added with 5% DMSO and then 95% (5.26% Tween 80 in saline) to prepare a 2mg/mL solution,no precipitation was observed during the preparation. For a 20-gram mouse, it was gavaged with 0.4ml. Thanks for the suggestion again and we have revised our manuscript accordingly(line 630-632).

Supplement Figure using THP-1 cells and PBMC is fundamental to the mechanism of action (MOA). But PBMC require fresh cells and I am not sure this is the case here. Also, fresh human PBMC already have active caspase-1 (see attachment). The manuscript is essentially a "me too" paper in that these investigators perform all the assays that others have published. They have a natural compound and as such adds to the list of natural compounds that inhibit NLRP3. The best example is sulforaphane.

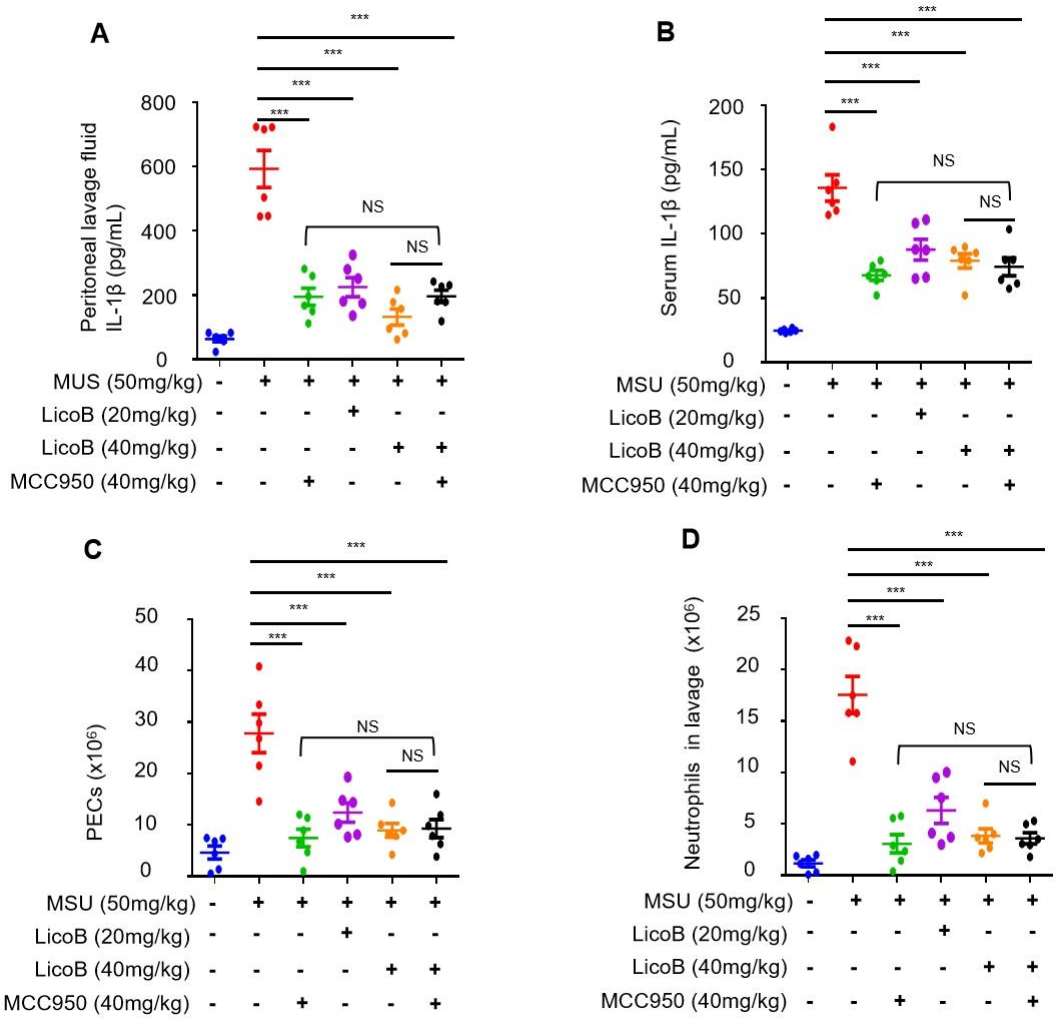
Response: We thank the reviewer for the question. The PBMCs used in the original supplementary figure (Original Extended Data Fig.1 D and E, Revised Figure EV 1G and H) were fresh cells, after isolation from the peripheral blood of healthy volunteers, the cells were seeded into the cell culture plates and cultured overnight, then the cells were primed with LPS for 4 hours and then treated with LicoB 1hour prior to nigericin or ATP stimulation.The activity of caspase-1 in the supernantant was measured. The data showed that LicoB inhibited caspase-1 activity induced by nigercin or ATP, suggesting it blocks canonical NLRP3 inflammasome activation in PBMCs. Several natural compounds including sulforaphane have been reported to inhibit NLRP3 inflammasome, our data demonstrated LicoB (a component of *licorice*)as a specific inhibitor of NLRP3 inflammasome, and it prevented against NLRP3-mediated diseases in mice models, suggesting it may be a candiate for the treatment of NLRP3-mediated diseases, but further studies are needed to evaluate its therapeutic potential. Thanks again for the reviewer's comment and we have revised our manuscript accordingly(line 389-392, 447-454).

The authors state that there is a need to develop drugs that reduce NLRP3, but they fail to cite studies published on OLT1177 in humans, although they cite basic science papers of OLT1177. There is a paper in Lancet Rheumatology that shows the safety and efficacy of OLT1177 in the treatment of acute gout flares. Another paper on the efficacy of OLT1177 to treat patients with Heart Failure. These should have been cited and the something written about the safety of OLT1177. MCC950 is toxic and is irrelevant.

Response: We appreciated the suggestion. OLT1177(also known as dapansutrile) has been reported to specifically inhibit NLRP3 inflammasome(Marchetti et al., 2018), its safety and efficacy in the treatment of gout flares in a open-label, proof-of-concept, phase 2a trial has been demonstrated(Kluck et al., 2020).Moreover, OLT1177 has been tested in a phase 1B trial and the result showed that treatment with OLT1177 for 14 days was safe and well tolerated in patients with heart failure and reduced ejection fraction(Wohlford et al., 2020). We thank the reviewer for the suggestion again and have revised our manuscript accordingly (line 84-93).

The sepsis model is poor, but the MSU model is useful.

Response: We thank the reviewer for the comment and have tested the effect of LicoB in the MSU model. Intraperitoneal injection of MSU induced peritonitis which is NLRP3 -dependent, charaterzied by Il-1β production and neutrophil influx (Martinon et al., 2006) . Our data showed that LicoB obviously reduced MSU injection–induced IL-1β production and neutrophil influx (Revised Fig.), suggesting that LicoB could inhibit NLRP3 inflammasome activation induced by MSU in vivo.



Responded Fig.10 (Revised Fig.6) (**A-D**) Mice were pre-treated with LicoB or MCC950 for 1 h, then i.p. injected with MSU (50 mg/kg) and treated for 6 h (n=6). The levels of IL-1β in the peritoneal lavage fluid (**A**) and serum (**B**) were measured using ELISA. Quantification of peritoneal exudate cells (PECs) (**C**) and neutrophils(Ly6G and CD11b) (**D**) using flow cytometry. Data are presented as mean ± SEM. *P<0.05, **P<0.01, ***P<0.001, and NS: not significant vs. control group (One-way ANOVA with Sidak's post-hoc test).

I do not see why the manuscript is new news about NLRP3 inhibition. It's a "me too" paper and the authors fail to inform the Readers that it will take a lot of time for Loco B to enter clinical trials and reach the level of development of others (such as OLT1177) that are now in clinical trials.

Response: We thank the reviewer for the suggestion. In our study, we demonstrated that LicoB (a component of *licorice*)as a specific inhibitor of NLRP3 inflammasome, and it prevented against NLRP3-mediated diseases in mice models, suggesting it may be a candiate for the treatment of NLRP3-mediated diseases. However, it may not be soon to enter clinical trials and reach the level of development of other NLRP3 inhibitors (such as OLT1177) that are now in clinical trials, further studies are needed to evaluate its therapeutic potential. Thanks for the reviewer's comment and we have included above discussion in the discussion part of the revised manuscript (line 389-392).

Is the mechanism of action of NEK7 unique for Loco B? Each NLRP3 inhibitor may has a different molecular location to arrest the oligomerization of NLRP3 to activate caspase 1. This is because of the size and complexity of NLRP3. Look at how many mutations of NLRP3 result in activation and serious human diseases. That information is reveals that there is no one single area that can be blocked and results in inhibition of NLRP3 activation. Clearly NEK 7 seems to be the MOA for Loco.

Response: We appreciate the comment.The critical role of NEK7 in NLRP3 inflammasome activation has been shown in three independent studies(He et al., 2016, Schmid-Burgk et al., 2016, Shi et al., 2016). NEK7 binds to the leucine-rich repeat domain of NLRP3 in a kinase-independent manner, its kinase activity is dispensable for NLRP3 activation(He et al., 2016, Shi et al., 2016). Our data showed that LicoB directly binds to NEK7 and interrupts NEK7-NLRP3 interaction to block NLRP3 activation. It has been demonstrated that oridonin covalently binds to Cys279 of NLRP3 and block NEK7-NLRP3 interaction(He, Jiang et al., 2018). A recent study demonstrates that berberine directly targets NEK7 to inhibit the interaction between NLRP3 and NEK7(Zeng, Deng et al., 2021). These studies demonstrate that inhibition of NEK7-NLRP3 interaction may be a good strategy to inhibit NLRP3 inflammasome activation. Thanks for the reviewer's comment and we have included above discussion in the discussion part of the revised manuscript (line 370-375).

Overall, the paper is a "me too" paper for another NLRP3 inhibitor and adds very little if anything to the biology and clinical use of NLRP3 inhibitors.

Response: We thank the reviewer for the comment. In our study, we demonstrated that LicoB (a component of *licorice*) as a specific inhibitor of NLRP3 inflammasome, it directly binds to NEK7 and interrupt NEK7-NLRP3 interaction. Moreover, LicoB prevents against NLRP3-mediated diseases in mice models, suggesting it may be a candiate for the treatment of NLRP3-mediated diseases. However, further studies are needed to evaluate its therapeutic potential.

Referee #3:

The authors aimed to characterise the effects on LicoB on NLRP3 inflammasome function. Using a range of both in vitro and in vivo approaches, the authors show that LicoB selectively inhibits the NLRP3 inflammasome by interacting with NEK7, a protein required for NLRP3 inflammasome activation, and that LicoB has beneficial effects in an LPS-induced sepsis model, as well as a non-alcoholic steatohepatitis model. Overall, this is a nicely organised and convincing study.

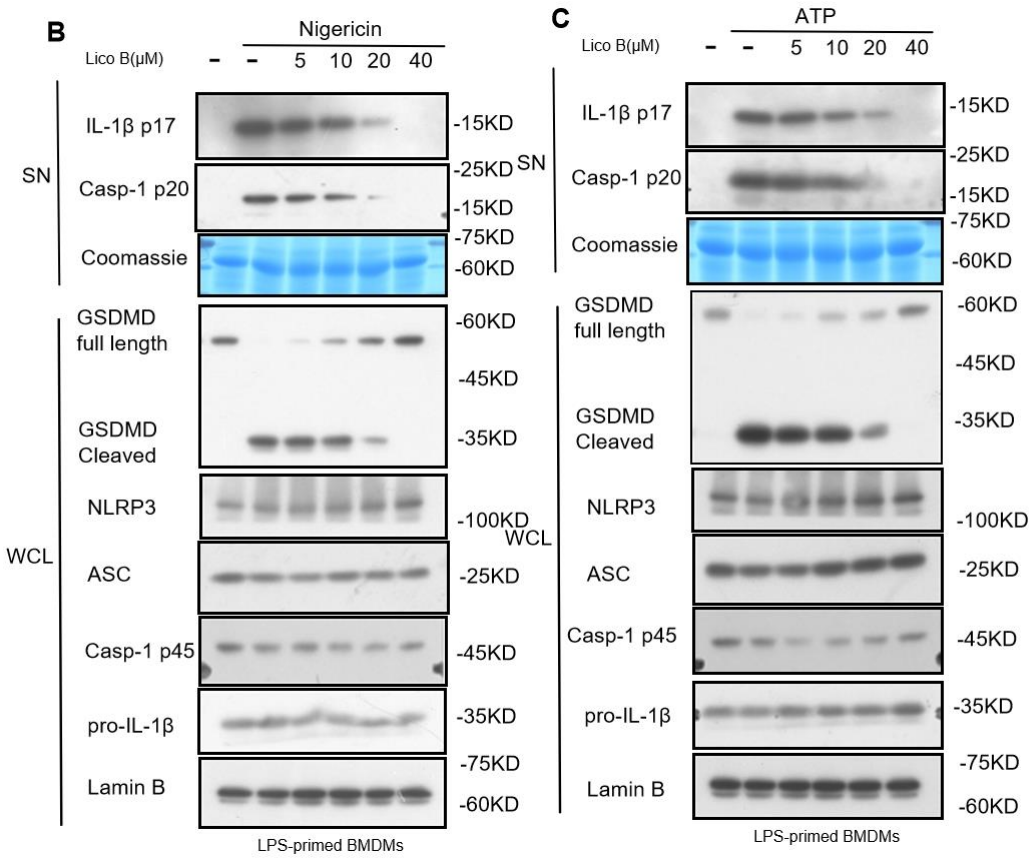
Response: We appreciate the careful and constructive comments of the reviewer and we are keen to address these concerns noted. We have responded to each comment in a point-by-point response below and hope that the new data and revised text address the concerns.

Major comments:

1. In Fig 1, the authors demonstrate that caspase-1 activation is reduced by LicoB, and that LDH release is also reduced. Could the authors also assess whether Gasdermin D cleavage is reduced?

Response: We appreciate the reviewer's suggestion. We have perfomed the related experiments, BMDMs were first primed with lipopolysaccharide (LPS) and then pretreated with LicoB before stimulation with nigericin at the dose of 10μM or ATP at the dose of 5mM

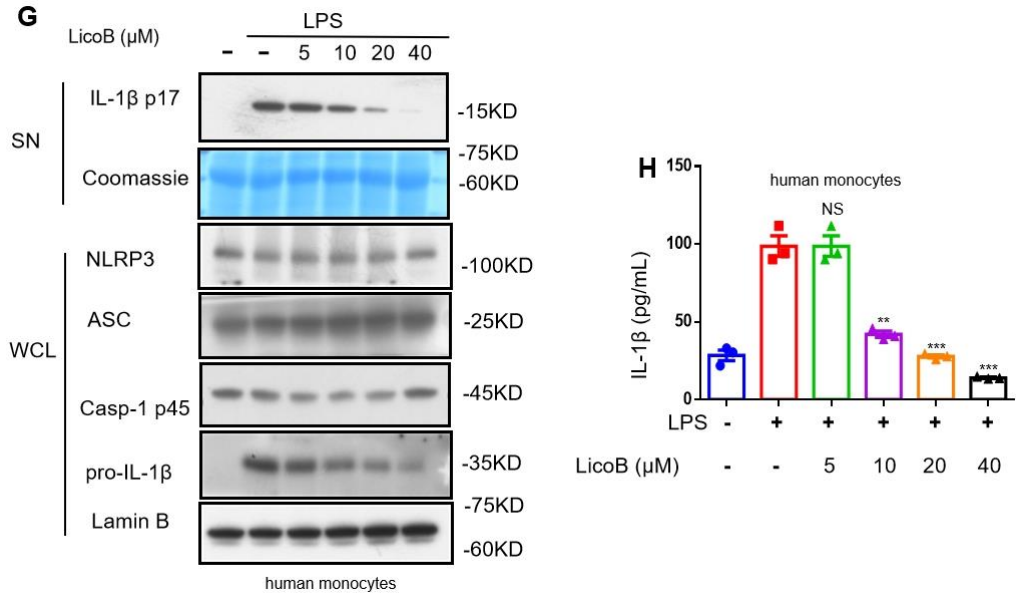
to induce NLRP3 inflammasome activation, the Gasdermin D (GSDMD) cleavage was detected by Immunoblotting. The data showed that LicoB could also reduce Gasdermin D cleavage.



Responded Fig.6 (Revised Fig. EV 1B and C) **(B and C)** BMDMs were primed with LPS and then treated with LicoB 1 hour prior to stimulation of Nigericin (10μM) for 45min (B) or ATP(5mM) for 1hour (C). Western blot analysis of the GSDMD in the whole cell lysate (WCL) of BMDMs were shown.

2. In Fig 2A, the authors show that LicoB inhibits non-canonical NLRP3 activation by using Pam3CSK4 priming followed by LPS transfection. Given that LicoB does not appear to be toxic over prolonged treatments, could the authors also show whether LicoB blocks alternative NLRP3 inflammasome activation in human monocytes in response to prolonged LPS treatment alone, as described by Gaidt et al 2016 (PMID: 27037191).

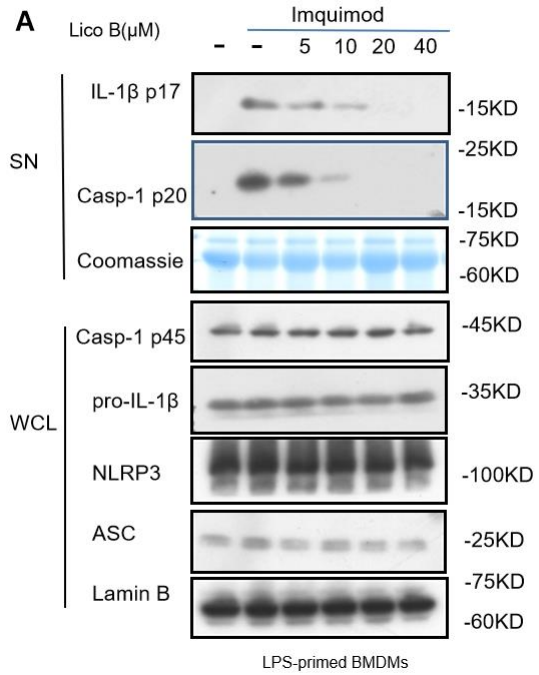
Response: Thank the reviewer's kindly suggestion, and we have done the experiments accordingly. Moritz M. Gaidt et al. demonstrated the alternative inflammasome activation in human monocytes, lipopolysaccharide alone induced an alternative inflammasome, the activation was propagated by TLR4-TRIF-RIPK1-FADD-CASP8 signaling upstream of NLRP3, and the mechanism did not extend to canocanical NLRP3 activation(Gaidt, Ebert et al., 2016). We tested the effect of LicoB on alternative NLRP3 inflammasome activation in human monocytes. The result showed that pretreatment of LicoB inhibited the IL-1β secretion induced by 14 hours of LPS treatment in human monocytes, but the expression of pro-IL-1β was also inhibited (Revised Figure EV3G and H), suggesting that LicoB may affect alternative NLRP3 inflammasome in human monocytes at least partially via inhibition of production of pro-IL-1β (LPS-induced transcriptional priming).



Responded Fig.7 (Revised Fig. EV 3G and H) (G and H) Human monocytes were pretreated with LicoB 1hour prior to LPS treatment for 14 hours. Western blot analysis of cleaved IL-1β (p17) in culture supernatants (SN) and the NLRP3,ASC, pro-caspase-1 (p45) and pro-IL -1β in the whole cell lysate (WCL) were shown(G). IL-1β secretion in SN was measured by ELISA (H).

3. In Fig 4C, the authors nicely show that LicoB inhibits NLRP3 activation without blocking K+ efflux. Could the authors strengthen this data by also assessing whether LicoB blocks K+ efflux-independent NLRP3 activation e.g. via imiquimod stimulation?

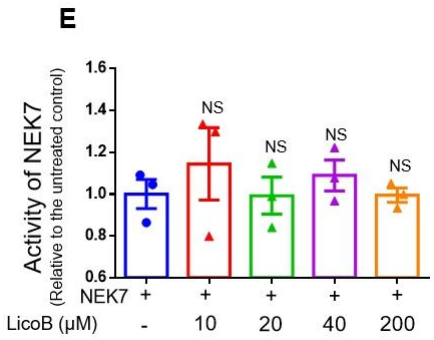
Response: We appreciate the constructive comments of the reviewer. To address the comments, we tested the effect of LicoB on Imiquimod-induced NLRP3 activation. BMDMs were primed with LPS and then treated with LicoB 1 hour prior to stimulation of Imiquimod(70μM) for 1 hour. The result suggested that LicoB can also effectively inhibit the activation of NLRP3 inflammasomes induced by the potassium efflux-independent activator imiquimod (Groß et al., 2016, He et al., 2016).



Responded Fig.8 (Revised Fig. EV 2A) (**A)** BMDMs were primed with LPS and then treated with LicoB 1 hour prior to stimulation of Imiquimod(70μM) for 1 hour. Western blot analysis of the pro-caspase-1 (p45), pro-IL -1β, NLRP3 and ASC in the whole cell lysate (WCL), activated caspase-1 (p20) and cleaved IL-1β (p17) in culture supernatants (SN) of BMDMs were shown.

4. Given that the authors suggest that LicoB binds to NEK7, can the authors address whether this interaction affects NEK7's role in mitotic spindle formation / cell division, or can they speculate about this?

Response: We thank the reviewer for the comments. It has been reported that NEK7 playa a role in the mitotic spindles formation and centrosomes separation (with NEK6 and NEK9), it kinase activity is essential for its function in in mitotic spindle formation / cell division(Belham, Roig et al., 2003, Bertran, Sdelci et al., 2011, O'Regan & Fry, 2009, Roig, Mikhailov et al., 2002, Yissachar, Salem et al., 2006) . To address whter the binding of LicoB would affect Nek7's role in mitosis, we tested the effect of LicoB on the kinase activity of NEK7. The in vitro kinase assay was performed with NEK7 and β-casein as a subtrate, with a Universal Kinase Activity Kit according to the manufacturer's instructions (R&D Systems). Our experimental results showed that LicoB did not affect the kinase activity of NEK7.These data suggested that LicoB may not affect the role of NKE7 in mitotic spindle formation / cell division.



Responded Fig.9 (Revised Fig. EV 3E) NEK7 was incubated with β-casein and ATP in the presence of different concerntrations of LicoB . NEK7 kinase activity was measured using an ADP-based phosphatase coupled kinase assay.Data are presented as mean ± SEM from biological replicates (n=3). NS: not significant (One-way ANOVA with Dunnett's post-hoc test).

5. The use of multiple unpaired t tests is often not the best way to compare datasets that contain multiple groups. One-way and two-way ANOVAs would be a more appropriate way

to analyse differences between multiple groups. For example, in Figure 1D, a one-way ANOVA with Dunnett's post-hoc test (comparing all groups to Vehicle + Nig treatment) would be a better way to analyse the data.

Response: Sincerely thank you for your suggestions. We have corrected the statistic method accordingly. For comparison of datasets that contain multiple groups, One-way ANOVA with Dunnett's or Sidak's post-hoc test was used to analyse the data. Thanks again for the reviewer's thoughtful suggestion and we have made the changes in our revised manuscript (line 639-642) and revised figure.

6. A minor point, but it would be nice if the authors could include individual data points in the graphs in Figures 1-4 and Extended Data Figure 1 for greater transparency.

Response: We appreciate the suggestion. We have included individual data points in the graphs in all related figures (including the original Figures 1-4 and Extended Data Figure 1).

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Dear Prof. Bai,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the two referees that were asked to re-evaluate your study, you will find below. As you will see, the referees support the publication of your study. Original referee #2 has declined to look into the revision but going through your point-by-point response I consider his/her points as adequately addressed.

Before we can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised manuscript:

- Please simplify and shorten the title to not more than 100 characters (including spaces).

- It seems in the author contributions Jiabo Wang is missing. Please check.

- Please remove the sentence 'Expanded View for this article is available online' from the manuscript main text.

- It seems there are no callouts for Figs. 4D and 4H. Please check.

- Please change the callouts for the panels of Fig. EV1 to 'Fig EV1 x' (delete the space between EV and 1).

- As there is only one panel, please change the callouts of Fig. EV2A to 'Fig EV2'.

- Reading the legends, Figs. 1H and 3B and Figs. 1I and 3C show the same experiment (Figure 1: BMDMs were primed with LPS for 4 h and then treated with LicoB for 1 h, prior to stimulation with ... ATP for 1 h. Caspase-1 activity (H), IL-1β secretion (I), ... in the SN were measured. - Figure 3: LPS-primed BMDMs were pre-treated with the indicated dose of LicoB for 1 h and then stimulated with ATP for 1 h. Caspase-1 activity (B) and IL-1β secretion (C) were measured in the SN). Indeed the diagrams look identical. Is it necessary to show these data twice? Or is some information missing? Could you lease clarify? If it is necessary to show this twice, please clearly indicate that it is the same experiment in the legends. Or are these two very similar replicates?

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also of the diagrams in the Appendix), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment' or 'independent ereplicate', but clearly state if these were biological or technical replicates. If statistical testing was done but there is no significant difference, please also mark this in the diagrams (n.s.).

- Please add scale bars of similar style and thickness to all the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend. Presently some of the scale bars are too thin and will not display well online, also because of their red colour.

- Please also note our reference format (there should be et al for references with more than 10 authors):
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- Finally, please find attached a word file of the manuscript text (provided by our publisher) with a few changes and queries we ask you to include in your final manuscript text. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short bullet points highlighting the key findings of your study.
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Best,

Achim Breiling
Editor
EMBO Reports

Referee #1:

We are happy with the revision as it is and don't have further comments.

Referee #3:

I am satisfied that the authors have sufficiently addressed my comments by adding extra data to the manuscript, and as such I believe this paper is now suitable for publication in EMBO reports.

The authors performed the requested editorial changes.

Prof. Zhaofang Bai
Department of Hepatology，Fifth Medical Center of Chinese PLA General Hospital, Beijing 100039, China. China Military Institute of Chinese Materia, The Fifth Medical Centre, Chinese PLA General Hospital, Beijing 100039, China.
China

Dear Prof. Bai,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ 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.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	To ensure a sufficient sample size, the LPS-induced septic shock animal model, toxicological evaluation and the NASH model have 8 mice in each group, and MSU model have 6 mice in each group.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	Yes. There was no subjective bias used.
For animal studies, include a statement about randomization even if no randomization was used.	Yes. The samples are randomly selected and grouped.
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. The investigator is blinded to the group information during assessing results in animal experiment.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Yes
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes. For comparison of two groups, unparied student's t test was used, for comparison of multiple goups, One-way ANOVA with Dunnett's post-hoc test or Sidak's post-hoc test was used.
Is there an estimate of variation within each group of data?	NA

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http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumc

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http://www.selectagents.gov/

Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Yes. We have made a detailed description in the reagent section of the manuscript.
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. BMDMs were isolated from the bone marrow of 8-week-old mice. hPBMCs were isolated from the peripheral blood of healthy volunteers. CD14+ were isolated from the fresh hPBMCs. THP-1 and HEK-293T cells in our laboratory were tested to be free of mycoplasma.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Yes
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Yes
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Yes

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Yes
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Yes
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	NA
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
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22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	NA