EMBO reports

# Tonic TNF conditioning of macrophages safeguards stimulus-specific inflammatory responses

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## Review Timeline:


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## 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.)

**1st Editorial Decision** **26th Sep 2022**

Dear Prof. Hoffmann,

Thank you for the submission of your research manuscript to EMBO reports. I 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 high 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 referee concerns need to be addressed, I will not detail them here.

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 and/or in a 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. Please contact me to discuss the revision (also by video chat) if you have questions or comments regarding the revision, or should you need additional time.

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. 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 (up to 8) 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.

For more details, please refer to our guide to authors:
http://www.embopress.org/page/journal/14693178/authorguide#manuscriptpreparation

Please consult our guide for figure preparation:
http://wol-prod-cdn.literatumonline.com/pb-assets/embo-site/EMBOPress_Figure_Guidelines_061115-1561436025777.pdf

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

3) a complete author checklist, which you can download from our author guidelines (https://www.embopress.org/page/journal/14693178/authorguide). Please insert page numbers in the checklist to indicate where the requested information can be found in the manuscript. The completed author checklist will also be part of the RPF.

Please also follow our guidelines for the use of living organisms, and the respective reporting guidelines:
http://www.embopress.org/page/journal/14693178/authorguide#livingorganisms

4) 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 in 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. This section is mandatory. As indicated above, if no primary datasets have been deposited, please state this in this section

# 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, 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 for potential EV figures and all those in the final Appendix). Please also check that all the p-values are explained in the legend, and that these fit to those shown in the figure. Please provide statistical testing where applicable. Please avoid the phrase 'independent experiment', but clearly state if these were biological or technical replicates. See also:
http://www.embopress.org/page/journal/14693178/authorguide#statisticalanalysis

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10) 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 themselves. Please do not write on or near the bars in the image but define the size in the respective figure legend.

11) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

12) We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. See also guide to authors:
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Please upload the blurb, the bullet points (these can be combined) and the synopsis image (in jpeg, png or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) as separate files.

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.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

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Referee #1:

This work connects the tonic TNF-α signalling with the ability of macrophages (MF) to distinguish between NF-κB activating stimuli of diverse origins.
The works is novel and of potential great interest for the field since neither TNFR recycling nor TNF-α tonic signalling have never been studied in living cells as modulator of NF-κB dynamics.

Furthermore, cracking the "stimulus code" for NF-κB activations would greatly improve our understanding of chronic and autoimmune inflammatory disease.

The concepts that are put forward are important, and have the potential to change our thinking on the role of NF-kB activation in chronic inflammatory pathologies. To increase the impact of the manuscript and provide more confidence in the conclusions several additional questions need to be addressed.

STRENGTHS: TNF-α KO primary MF, live imaging and mathematical modelling are exploited to reach several conclusions.

CONCLUSIONS in summary: Tonic TNF-α signalling a) maintains oscillatory NF-κB signalling in TNF proficient cells, b) if missing, the NFkB oscillatory pattern is deregulated and the overall response increased; c) controls the abundance of TNFR, for a correct feedback through IKK signalling and IkBα degradation, d) and prevents aberrant epigenetic modifications on NF-κB enhancers.

Although novel and interesting, some of the conclusions are not fully supported by results with statistical significance. Indeed, the authors state that "Several subtle changes, often near the limit of experimental detection, may combine to have a surprisingly pronounced effect on NFκB oscillations". This sentence implies that challenging the experimental systems to confirm data is needed.

Along the same line, some results are mere correlations which might benefit from some experiments of phenotype rescue to support conclusions.

In addition, if I'm not mistaken, there are several discrepancies between raw data in the figures and how they are described in the text.

In more detail:

Figure 1:
- Panel B: SD for the blue bar is high indicating high experimental variability leading to a non significant result. What about increasing the replicates?
- Panel D: image quality must be improved

Figure 2:
Overall, the comprehension of this figure is very difficult for the average reader, I would suggest to spend some more words in the Results section or in Supplementary giving definitions that can be connected to the NFkB biology.
- The plot in panel B is strange: it seems that normalization has been performed on maxTNF-/- point and not on the wt as stated in the text. Please check.
- In the plot in panel B I cant see a 3.6x, 1.4x increase, and so on..., as stated in the sentence "In Tnf-/- cells, IKK phosphorylation followed a similar temporal pattern, but was slightly increased compared to WT across all time points, to 3.6x, 1.4x, 1.4x, and 1.2x of WT levels at 2, 5, 10, and 15 min and to 1.7x - 8.9x of WT levels from 30 - 120 min, although many of these differences were not statistically significant (Figure 2D). The same holds true for p-JNK quantifications in panel E. Please check.
- Panel F shows A20 baseline quantification: these data are insufficient to draw conclusions because A20 transcription in NF-κB regulated and might change at later timepoints in the two genotypes.

Figure 3:
- It describes TNFR turnover and signalling. Analyses with mathematical modelling are very informative and provide interesting predictions. However, the authors correctly state that "Several subtle changes, often near the limit of experimental detection,

may combine to have a surprisingly pronounced effect on NFκB oscillations". This is a major flaw in the paper. Therefore, theoretical conclusions should be validated in living cells by perturbing the inflammatory environment or mildly overexpressing the TNFR to rescue the phenotype in wt cells, or in any other way.
- Panel E suffer from the same flaw as for A20 above.
- Panel E contains different data from those reported in this sentence "Even after much of the receptor was internalized upon TNF stimulation, the cell surface level appeared to be higher in Tnf-/- than in WT cells (130% of WT, p-value of 0.09). Baseline TNFR2 surface level was not altered in Tnf-/- cells (100% of WT) (Figure 3E, S3F, center)."

Figure 4:
- The experimental designing exploiting the IKBsome stability is poorly described making data interpretation difficult.
- Again, conclusions derive mainly from correlations and experimental validations of any kind are mandatory to support non-significant differences as in plot 4D and 4E.

Figure 5 contains two sets of data that are mutually exclusive and poorly connected, scRNA seq and epigenetic studies of enhancers. Please address this problem.

Single cells RNA seq should provide info on the response homogeneity to stimuli, but is poorly exploited. It would also be relevant to analyse changes in the transcription profiles. The comparison of two populations at a time, beside the UMAP with all the populations, should provide interesting information relevant for the molecular understanding of the phenotype.

In addition, the "TNF-/- + Mock" sample is missing precluding the evaluation of molecular differences with wt MF.
- Cherry-picked genes in panel B are almost non-sense unless volcano plots are shown.
- The following sentence is wrong: "TNF-stimulated cells seemed to hyper- respond to overlap with P3C4-stimulated cells" in that, UMAPs show only qualitative, but not quantitative, differences between cell populations due to the fact that calculation is based on the global transcriptome.
- Therefore, the conclusion "This suggests that the lack of tonic TNF conditioning changes TNF-induced gene expression to be more similar to gene expression responses to the PAMP P3C4" should be better commented.
- In panel G, if I understand correctly the legend, the symbols for significantly downregulated genes should be represented in red.

In the text, I do not agree with the sentence reported in the Results section: "thus, Tnf -/- cells showed increased de novo enhancer formation". In my opinion it is more a matter of " de novo opening in a larger fraction of cells"

In the Discussion, the sentence "In vivo, the levels of tonic TNF might be higher in a location-dependent manner due to the presence of microbiome PAMPs and DAMPs released during tissue turnover and repair" is in contrast with presented data showing that TNF exposure of TNF-/- MF can not rescue the wt/tonic phenotype. Please discuss

Some minor points below to be addressed, to facilitate comprehension from a broader audience

- Overall, axis descriptions are often inaccurate (Fig4D, e.g.)
- Ms pages should be numbered for the saneness of the reviewers
- Figure 2 should be described with a twist to biology

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Referee #2:

The paper by Luecke and colleagues considers how tonic TNF signaling contributes to the acute inflammatory response to different innate immune stimuli in macrophages. To explore tonic TNF signaling, which they define as constitutive signaling by background or baseline TNF prior to stimulation, they use BMDMs from Tnf-/- mice. Using live-cell imaging of NF-kB translocation dynamics, they observe that BMDMs from Tnf-/- have less oscillatory NF-kB signaling than WT BMDMs following TNF stimulation. Using computational modeling, they show that this is consistent with a role for tonic signaling in regulating TNFR turnover that promotes these oscillations. Functionally they show that BMDMs from Tnf-/- are less able to distinguish between acute stimulation between TNF and P3C4 as evidenced by gene programs that are more similar to each other.

The main conclusion, i.e. that tonic TNF signaling helps macrophages to distinguish between acute TNF vs PAMP stimuli, is significant. However, loss of tonic TNF signaling is not the only thing that would be changed in Tnf-/- mice and therefore it's unclear if the conclusion is fully supported. Tnf-/- mice also do not have autocrine TNF signaling, which has been shown by this group and others to be important in generating responses to PAMPs, and therefore this issue should be addressed.

1. In reference to Fig. 1, the authors cite their own work about autocrine signaling, stating that "the duration of signaling induced solely by the MyD88 adaptor was shorter in Tnf-/- cells (presumably due to the lack of autocrine signaling by newly synthesized TNF)". Why do the authors assume that this change can be attributed to loss of autocrine signaling while other changes are due to loss of tonic signaling? One experiment that could get at this more directly would be to block TNF signaling after the initial stimulation (e.g. with soluble TNFR) to attenuate autocrine signaling and try to separate these effects. Another possibility is to

use the computational model to look for evidence of the relative importance of baseline v. subsequent autocrine signaling.

2. The discussion around Fig. 2 is confusing. The authors first hypothesize that decreased IkBa feedback might lead to the sustained signaling observed in Tnf-/- cells. When they observe a modest but significant increase in IkBa mRNA (Fig. 2B) they assume this is a result of the sustained signaling. This perhaps has to do with the timing of protein versus mRNA readouts; if so, it would be helpful to explain. Although their conclusion that there is no impairment in the IkBa feedback loop in Tnf-/- mice seems like a reasonable one, placed together the arguments appear circular.

3. Related to this, the conclusion in Fig. 3-4 that Tnf-/- cells have a modest but significant increase in IKK activity and strengthening IkBa negative feedback can restore oscillations also seems at odds with the finding in Fig. 2B. The pairing of the prediction in Fig. 4A that strengthening will increase IkBa mRNA, and the experiment in Fig. 4D-F with the p100 KO validating this is striking. However, Fig. 2B showed that Tnf-/- cells already have increased IkBa relative to WT and yet oscillations are dampened. Does the model predict both these results? Can context be added to help to interpret these results together?

4. Fig. 4: These are very nice results regarding the functional significance of the small changes in NF-kB signaling. However, as in Fig. 1, it seems possible that loss of TNF autocrine signaling might also have a role to play and some additional experiments to control for this would be more convincing.

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Referee #3:

This study seeks to build upon and extend this group's prior interesting and important work to understand why acute TNF stimulation results in oscillatory NF-kB dynamics, which results in limited epigenomic remodeling and a different pattern of gene expression than that induced by PAMPs, which induce more sustained NF-kB activation, broad chromatin remodeling and potent gene induction. The authors' hypothesis that tonic TNF signaling affects the NF-kB response to subsequent TNF challenge is strongly supported by single cell analysis of NF-kB dynamics using Tnf KO mouse cells (Figure 1). The remainder of the paper uses analysis of bulk macrophage cultures and computational modeling to probe mechanisms underlying changes in NF-kB dynamics, and suggests that lower TNFR1 expression that results from tonic TNF signaling leads to decreased magnitude of signaling (IKK and JNK phosphorylation) and chromatin remodeling. There are several issues with these experiments leading to substantial concerns about whether the conclusions of the study are adequately supported by the experimental data.

Major points:

1. As the authors acknowledge, the differences in signaling between WT and Tnf KO (Fig. 2) and TNF KO and TNF p100 DKO (Fig. 4) are very modest and not statistically significant. Although I take the authors' point that effects that occur at single cell level can be difficult to detect with bulk cultures, it is still in my view not reasonable to use results that are minimal and not significant to draw mechanistic conclusions. Ideally, the authors should develop additional single cell assays, e.g. for IKK activation.

2. The epigenomic analysis of H3K4me1 appears very incomplete as only 1515 inducible peaks were detected even when LPS and Pam3Cys were used, which is much less than expected for a histone mark. There were only a small number of differentially induced peaks (37 peaks, Fig 5G) and the p value was not corrected for multiple comparisons, weakening the ability to draw rigorous conclusions. Additionally, only 2 replicates were analyzed. Overall, it is difficult to draw conclusions based on incomplete data.

3. The soluble basal TNF levels are extremely low and add-back of TNF to TNF KO cultures did not effectively reverse the effect of deletion. This suggests an important role for membrane TNF, which can be measured using flow cytometry. The role of membrane TNF should be investigated.

4. The causal relationship between low TNFR1 and NF-kB dynamics is solely supported by computational modeling (Fig. 3). The differences in TNFR1 expression are minimal both before and after TNF stimulation, although TNF stimulation induces a large fractional (appr. 70%) decrease in cell surface TNFR1. Given these results, the causal relationship between TNFR1 expression levels and NF-kB dynamics should be directly experimentally tested by varying TNFR1 expression using knockdown or Tnfr1-/+ heterozygous null mice, and by the complementary approach of increasing TNFR1 levels in WT cells to those observed in TNF KO cells using a forced expression system.

Minor points:

5. In the absence of tonic TNF signaling, the authors state that macrophages showed less oscillatory NF-kB responses that are similar to PAMP exposure. This point would be strengthened if a positive control of stimulation with a PAMP was included in the figures (e.g. 1D, 2).

6. It would be helpful to show expression of several canonical NF-kB targets such as IL6, Il12b, TNF etc. throughout the figures

and in the single cell data in Fig. 5A.

## Referee #1:

This work connects the tonic TNF-α signalling with the ability of macrophages (MF) to distinguish between NF-κB activating stimuli of diverse origins.
The works is novel and of potential great interest for the field since neither TNFR recycling nor TNF-α tonic signalling have never been studied in living cells as modulator of NF-κB dynamics.
Furthermore, cracking the "stimulus code" for NF-κB activations would greatly improve our understanding of chronic and autoimmune inflammatory disease.
The concepts that are put forward are important, and have the potential to change our thinking on the role of NF-kB activation in chronic inflammatory pathologies. To increase the impact of the manuscript and provide more confidence in the conclusions several additional questions need to be addressed.

STRENGTHS: TNF-α KO primary MF, live imaging and mathematical modelling are exploited to reach several conclusions.

CONCLUSIONS in summary: Tonic TNF-α signalling a) maintains oscillatory NF-κB signalling in TNF proficient cells, b) if missing, the NFkB oscillatory pattern is deregulated and the overall response increased; c) controls the abundance of TNFR, for a correct feedback through IKK signalling and IkBα degradation, d) and prevents aberrant epigenetic modifications on NF-κB enhancers.

We thank the reviewer for their concise summary and appreciative assessment of the work.

Although novel and interesting, some of the conclusions are not fully supported by results with statistical significance. Indeed, the authors state that "Several subtle changes, often near the limit of experimental detection, may combine to have a surprisingly pronounced effect on NFκB oscillations". This sentence implies that challenging the experimental systems to confirm data is needed.
Along the same line, some results are mere correlations which might benefit from some experiments of phenotype rescue to support conclusions.

We address these concerns in detail below. In brief: We agree with the reviewer that the effect sizes of several of the molecular changes are small. To interpret the correlated experimental data, we leverage a mathematical model of the molecular network that represents the TNF to NFκB signaling pathway. This model is not new but was first developed in 2008 (Werner *et al*, 2008) and has been refined in a number of publications by our lab (e.g. (Caldwell *et al*, 2014)) and others, and has most recently been refined and adapted to primary macrophages in 2021 (Adelaja *et al*, 2021). Thus, the model was not merely created to advance a specific interpretation in the current work.

Using this mathematical model, we found that within the molecular network, very subtle changes in biochemical reactions may combine to have more pronounced effects on NFκB dynamics. The computational simulations alone do not provide “proof” that the mechanistic interpretation of the “correlated” experimental data is correct, but indicates that such a mechanistic interpretation is very plausible.

Importantly, we now provide siRNA-mediated reduction of TNFR1 expression in *Tnf*$^{-/-}$ cells, showing that this is able to partially rescue the oscillatory character of NFκB dynamics in *Tnf*$^{-/-}$ cells. This experimentally confirms the computationally predicted causal relationship between TNFR1 expression levels and oscillatory NFκB dynamics.

In addition, if I'm not mistaken, there are several discrepancies between raw data in the figures and how they are described in the text.

Figure legends and descriptions of the figures in the manuscript text were written with great care. We have double checked them and not been able to find inaccuracies. We apologize for any unclear descriptions. We have made some adjustments to figures, legends, and descriptions to clarify any confusions (please see below for details).

In more detail:
Figure 1:
- Panel B: SD for the blue bar is high indicating high experimental variability leading to a non significant result. What about increasing the replicates?

Since the SD on the blue bar in panel 1B (nuclear NFκB fluorescence at baseline) is comparably small, we assume the reviewer is referring to the right side of panel 1A, which shows the levels of tonic TNF (close-up of 0 h data point from panel 1A, left), with a p-value of 0.069 for the difference between WT and *Tnf*$^{-/-}$ BMDM supernatants. We have now added an additional replicate for the 0 h time point, and the p-value of the two-tailed paired-sample t-test for the comparison of tonic TNF levels in WT vs *Tnf*$^{-/-}$ is now 0.031.

- Panel D: image quality must be improved

We have now incorporated images with improved and hopefully satisfactory quality (tiff instead of png, enhanced contrast, increase size of scale bar). To image the number of cells sufficient for reliably representing the cell-to-cell heterogeneity of signaling, we acquire images using a 20x objective. Due to the automated microscopy stage having to move quickly to image up to 40 positions within 5 min, we use an air objective. Therefore, the images have sufficient resolution to segment and quantify nuclear fluorescence (with a nuclear marker), but do not have a resolution comparable to images acquired with e.g. higher magnification oil objectives. Only a small section of one field of view is shown here.

To improve understanding of this data type, from which the heatmaps throughout the manuscript are derived, we've now also added an example DIC and nuclear DNA stain image to the figure.

Figure 2:
Overall, the comprehension of this figure is very difficult for the average reader, I would suggest to spend some more words in the Results section or in Supplementary giving definitions that can be connected to the NFkB biology.

We appreciate this feedback and have now added a full paragraph to the beginning of the Figure 2 Results section thoroughly explaining NFκB activation via IKK, the NFκB-IκBα feedback loop, and the

control of oscillatory vs. non-oscillatory responses by IKK. We have also clarified some of the time scales of interest in the investigation in the IκBα feedback loop (also in response to Reviewer 2's comment). Apart from that, every Panel/experiment description is already preceded with a brief explanation of the molecular mechanisms under investigation, motivation for the experiment, and expected results. We hope that, together, this will allow the reader to follow our reasoning.

- The plot in panel B is strange: it seems that normalization has been performed on maxTNF-/- point and not on the wt as stated in the text. Please check.

The normalization is indeed performed on the maximum WT value for each biological replicate individually as stated in figure legend and axis, not on max *Tnf*$^{-/-}$ value. The timepoint at which the WT cells reach max IκBα expression varies between replicates (40, 50, 50, 60, and 80 min in the different replicates). Thus, when displaying the average of normalized WT time courses from 5 replicates, none of the average values reach 1.

We are not sure exactly what about the plot the reviewer considers strange looking. We believe this way of normalizing is an accurate representation of variability in the time course data. In our opinion, the figure legend describes this accurately, but for increased clarity we have now rephrased 'each experiment' to 'each time course replicate': "IκBα expression was normalized to GAPDH expression and then to maximum WT value in each ~~experiment~~ *time course replicate*. Data are represented as mean ± SD of 5 ~~independent experiments~~ *biological replicates*."

- In the plot in panel B I cant see a 3.6x, 1.4x increase, and so on..., as stated in the sentence "In Tnf-/- cells, IKK phosphorylation followed a similar temporal pattern, but was slightly increased compared to WT across all time points, to 3.6x, 1.4x, 1.4x, and 1.2x of WT levels at 2, 5, 10, and 15 min and to 1.7x - 8.9x of WT levels from 30 - 120 min, although many of these differences were not statistically significant (Figure 2D). The same holds true for p-JNK quantifications in panel E. Please check.

Since the cited text refers to Figure 2D, we're assuming the reviewer is referring to Panel 2D, not 2B. After double-checking, we can confirm that the fold difference between *Tnf*$^{-/-}$ data compared to WT data are indeed as stated in the text. For example, pIKK at the 10 min timepoint is 0.85x (of the max WT value across the time course) for *Tnf*$^{-/-}$ cells and 0.59x (of the max WT value across the time course) for WT cells – thus, *Tnf*$^{-/-}$ cells have 1.4x of the pIKK levels in WT cells at this timepoint.

To make the fold changes easier to see from the graphs, especially for the close-to-baseline values at later timepoints, we've added a y-line at 0 in Panel 2D (p-IKK), 2E (p-JNK), as well as EV2D (p-ERK) and EV2E (p-p38).

- Panel F shows A20 baseline quantification: these data are insufficient to draw conclusions because A20 transcription in NF-κB regulated and might change at later timepoints in the two genotypes.

We chose to show comparisons of the baseline levels of A20, because, within the time frame of the observed differences in NFκB oscillations, it is the baseline levels of A20 that are important for

determining the strength of the initial NFκB response to stimulation (Werner *et al*, 2008). A20's inducibility, while an important mediator of e.g. determining the level of tolerance to sequential stimuli, is likely not essential to its control of early timepoints. NFκB dynamics in WT and *Tnf*$^{-/-}$ showed appreciable differences as early as 30 - 45 min and pathway activation showed possible differences even earlier.

A Western Blot analysis of A20 levels up to 8 h after TNF stimulation, provided below (Figure RL1), failed to reveal any appreciable decrease in A20 levels in *Tnf*$^{-/-}$ cells.

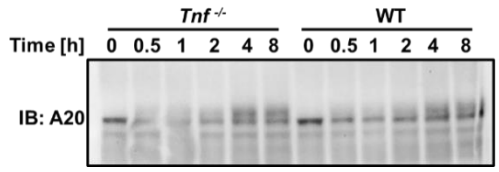


**Figure RL1: A20 levels determined by Western Blotting in *Tnf*$^{-/-}$ and WT BMDM upon TNF stimulation at the indicated time points.**

Figure 3:

- It describes TNFR turnover and signalling. Analyses with mathematical modelling are very informative and provide interesting predictions. However, the authors correctly state that "Several subtle changes, often near the limit of experimental detection, may combine to have a surprisingly pronounced effect on NFκB oscillations". This is a major flaw in the paper. Therefore, theoretical conclusions should be validated in living cells by perturbing the inflammatory environment or mildly overexpressing the TNFR to rescue the phenotype in wt cells, or in any other way.

We thank the reviewer for the suggestion to further validate the conclusions made in Figure 3 experimentally. The mathematical model predicted and experiments confirmed a small increase in TNFR1 receptor levels in *Tnf*$^{-/-}$ cells. While indeed the difference in TNFR1 expression between WT and *Tnf*$^{-/-}$ cells is not very large (126% of WT cells), this difference is very reliably reproducible and statistically significant (p-value of 0.0027 with 3 biological replicates).

To experimentally test the predicted causal relationship between TNFR1 expression levels and oscillatory character of NFκB dynamics, we have now performed siRNA-mediated reduction of TNFR1 expression in *Tnf*$^{-/-}$ cells. TNFR1 levels similar to levels in WT were achieved in *Tnf*$^{-/-}$ cells (confirmed by flow cytometry, Figure 3F left). We then performed microscopy to compare NFκB dynamics. Compared to control siRNA transfected *Tnf*$^{-/-}$ cells (60 % oscillatory NFκB trajectories), *Tnf*$^{-/-}$ cells with reduced TNFR1 levels had a higher percentage of oscillatory NFκB trajectories (69%, p = 0.023), with control siRNA transfected WT cells having 73% oscillatory NFκB trajectories (Figure 3F, right). Thus, a reduction of TNFR1 levels in *Tnf*$^{-/-}$ cells partially rescues the WT phenotype. This increases our confidence in the conclusion that increased TNFR1 surface expression is a mechanism contributing to the decrease in oscillatory NFκB trajectories in *Tnf*$^{-/-}$ cells.

- Panel E suffer from the same flaw as for A20 above.

In Panel 3E, we show that TNFR1 expression is increased at baseline and that that difference is largely maintained at 30 min, despite internalization of a proportion of the receptors. Thus, in contrast to the panel describing A20 expression, we a) show a difference at baseline and b) already provide a second,

later timepoint (30 min), which shows a similar difference. The differences in the NFκB dynamics between WT and *Tnf*$^{-/-}$, such as the downregulation of NFκB activity after the first peak, which is much weaker in *Tnf*$^{-/-}$ cells (one of the defining features of the less oscillatory NFκB phenotype in the *Tnf*$^{-/-}$), already start around that timepoint. Therefore, we do not believe adding TNFR1 surface expression at additional, later time points to the manuscript would provide a benefit to this study and may confuse the reader with regards to the mechanisms mediating the differences in NFκB dynamics.

Below, we provide a time course of TNFR1, TNFR2, and CD11b expression with more detail at earlier timepoints and extending beyond 30 min up to 240 min (Figure RL2). This experiment indicates that the increase in TNFR1 expression seen at baseline is maintained throughout the time course and the similar expression of TNFR2 and CD11b in WT and *Tnf*$^{-/-}$ is similarly consistent across time points.

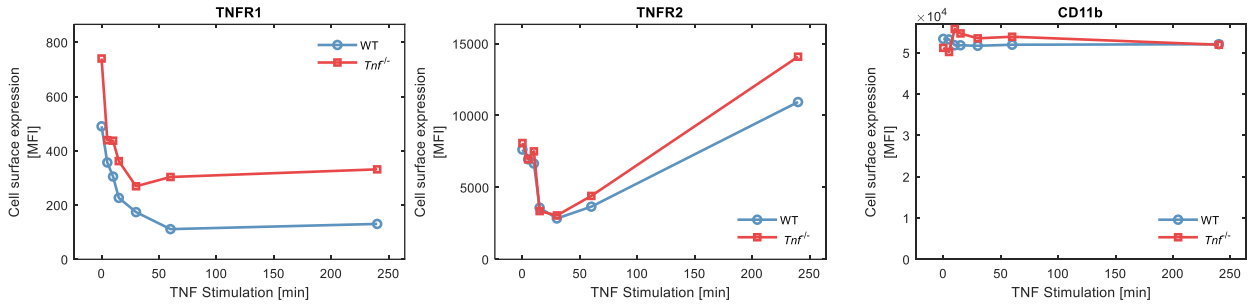


**Figure RL2: TNFR1, TNFR2, CD11b cell surface expression in WT and *Tnf*$^{-/-}$ cells stimulated with TNF for up to 240 min.**

- Panel E contains different data from those reported in this sentence "Even after much of the receptor was internalized upon TNF stimulation, the cell surface level appeared to be higher in Tnf-/- than in WT cells (130% of WT, p-value of 0.09). Baseline TNFR2 surface level was not altered in Tnf-/- cells (100% of WT) (Figure 3E, S3F, center)."

We appreciate the feedback that this might be confusing, since the numbers cited in the parenthesis refer to the supplemental figure (S3F), not to the main figure. As stated in the figure legends, the supplemental figure showed surface expression values in *Tnf*$^{-/-}$ as % of WT over 3 biological replicates, while the main figure showed MFI from 3 culture replicates from 1 of the 3 biological replicates. Based on this feedback, we have now switched main and supplemental figures and refer to those panels more specifically in the different parts of that sentence:

> "Even after much of the receptor was internalized upon TNF stimulation (Figure S3F, left), the cell surface level appeared to be higher in Tnf-/- than in WT cells (130% of WT, p-value of 0.093) (Figure 3E, left). Baseline TNFR2 surface level was not altered in Tnf-/- cells (100% of WT) (Figure 3E, S3F, center)."

Figure 4:
- The experimental designing exploiting the IKBsome stability is poorly described making data interpretation difficult.

We apologize if the description of the experimental design was unclear. We have now expanded the explanation as follows and hope that this improves understanding:

> Before: "To experimentally test the model predictions, we utilized a genetic perturbation that renders more cellular NFκB responsive to TNF. The IκBsome traps NFκB in a complex that is not responsive to the canonical IKK pathway (Basak et al, 2007; Shih et al, 2009; Savinova et al, 2009). By genetic removal of the nfkb2/p100 subunit, the IκBsome complex collapses (Yılmaz et al, 2014) and more NFκB can associate with canonical IκB proteins. Upon their stimulus-induced degradation, more nuclear NFκB drives a stronger IκBα negative feedback loop."
>
> Now: "To experimentally test the model predictions, we utilized a genetic perturbation that renders more cellular NFκB responsive to TNF. The IκBsome is a protein complex consisting of p105 and p100 subunits, which traps NFκB. It is not responsive to the canonical IKK pathway (Basak et al, 2007; Shih et al, 2009; Savinova et al, 2009). By genetic removal of the nfkb2/p100 subunit, the IκBsome complex collapses (Yılmaz et al, 2014), freeing more NFκB to associate with canonical IκB proteins. Upon stimulus-induced degradation of canonical IκB proteins, the additional NFκB can translocate to the nucleus to drive a stronger IκBα negative feedback loop. Therefore, to mimic the 'Tnf -/- with 1.25x NFκB' model conditions, we bred Tnf -/- p100-/- mice to increase the amount of stimulus-responsive NFκB and the strength of the IκBα negative feedback in Tnf -/- background."

- Again, conclusions derive mainly from correlations and experimental validations of any kind are mandatory to support non-significant differences as in plot 4D and 4E.

We apologize for not presenting this work clearly. Figure 4 actually describes work that is intended to challenge and experimentally test the proposed molecular mechanism. We use the mathematical model to generate a specific hypothesis: that greater availability of NFκB RelA will reverse the abrogation of oscillatory characteristics. We then test this hypothesis experimentally by increasing the pool of available NFκB RelA using the p100 knockout. This is a targeted perturbation experiment to test the proposed molecular mechanism, not merely correlative studies exploiting natural variation. We hope the revised description does a better job in clarifying the work:

> "As the computational modeling analysis suggested that the reduced oscillatory content of NFκB responses in the absence of tonic TNF conditioning may be mediated by increased IKK activity, we aimed to provide further experimental evidence to support this conclusion. If the excess IKK activity observed in the absence of tonic TNF neutralizes the IκBα negative feedback loop and thus diminishes post-activation repression of NFκB and NFκB oscillations, we reasoned that strengthening IκBα feedback control may compensate and restore NFκB oscillations."

The increase in available NFκB by modeling or experiment represents the perturbation of the system. Therefore, Figure 4 in its entirety represents a rescue experiment, providing supporting evidence for previous conclusions.

The analysis of the difference in IκBα induction (Figure 4D) and nuclear NFκB (Figure 4E) in *Tnf*$^{-/-}$ vs *Tnf*$^{-/-}$ p100$^{-/-}$ serve only as control experiments to confirm that the increase in available stimulus-responsive NFκB through removal of p100 closely matches the mathematical model as intended and that *Tnf*$^{-/-}$

p100$^{-/-}$ cells are thus an appropriate experimental system to experimentally confirm the predictions of the mathematical model. Panels 4C and 4F-H, showing the increase in oscillatory dynamics in *Tnf*$^{-/-}$ cells in presence of more stimulus-responsive NFκB depict the relevant readouts.

Therefore, while we agree that Figure 3 greatly benefited from the suggestion to further experimentally test the predicted causal relationship between TNFR1 expression levels and oscillatory character of NFκB dynamics (which we did using the siRNA experiment), we do not see what kind of experimental validation would be required or useful in Figure 4. We hope that the expanded explanation of the experimental setup (described above) may clarify the purpose of Figure 4.

Figure 5 contains two sets of data that are mutually exclusive and poorly connected, scRNA seq and epigenetic studies of enhancers. Please address this problem.

We attempt to address this as follows: In Figure 5, we investigate two functional consequences of the absence of tonic TNF conditioning in *Tnf*$^{-/-}$ cells with regards to processes known to be dependent on NFκB dynamics: 1) altered TNF-induced gene expression at 4 h and 2) altered TNF-induced epigenomic reprogramming, specifically *de novo* enhancers measured by H3K4me1 signal, at 8 h. Nuclear NFκB induces both expression of target genes and formation of *de novo* enhancers (which affect responses to subsequent signals).

These data are not meant to be directly connected, except that they both address the functional importance of tonic TNF conditioning. We do not draw any direct connections between the data, e.g. stating that any of the altered enhancers are directly responsible for expression changes in any of the specific genes, etc. To make that clearer, we have now separated the data into two separate figures (Figure 5 and Figure 6). In the text, the data are discussed in two distinct paragraphs (already in the first submission). We have expanded the introductory sentence for the epigenetic data slightly to emphasize that we're discussing a separate functional consequence of tonic TNF:

> "Next, we investigated the effect of tonic TNF conditioning on another important function of stimulus-induced NFκB: the ability to alter the epigenome through formation of *de novo* enhancers which affects transcriptional responses to subsequent stimulations. Our previous work demonstrated that non-oscillatory NFκB has much greater capacity to form *de novo* enhancers than oscillatory NFκB (Cheng *et al*, 2021). We therefore hypothesized that tonic TNF may restrict the formation of *de novo* enhancers."

Single cells RNA seq should provide info on the response homogeneity to stimuli, but is poorly exploited. It would also be relevant to analyse changes in the transcription profiles. The comparison of two populations at a time, beside the UMAP with all the populations, should provide interesting information relevant for the molecular understanding of the phenotype.

Based on this suggestion, we've now expanded the analysis of the single cell RNA seq data. In addition to the UMAP plot (Figure 5A), we present comparisons TNF vs P3C4 stimulated transcriptomes using the first two principal components for *Tnf*$^{-/-}$ and for WT cells separately (Figure 5B). These better demonstrate the larger overlap of the P3C4-and TNF-stimulated transcriptomes in *Tnf*$^{-/-}$ cells and the

shift of the TNF-stimulated transcriptome in *Tnf*$^{-/-}$ compared to WT cells. We additionally compare the average pairwise distances between the TNF vs P3C4 stimulated transcription profiles in WT and *Tnf*$^{-/-}$ cells and find that they are smaller in *Tnf*$^{-/-}$ cells (Figure 5C). The fold change between P3C4 vs TNF stimulated expression of genes differentially expressed in WT upon P3C4 stimulation is also smaller in *Tnf*$^{-/-}$ compared to WT cells (Figure 5D). These analyses demonstrate the decreased distinguishability of TNF and P3C4 transcription profiles in the absence of tonic TNF.

In addition, the "TNF-/- + Mock" sample is missing precluding the evaluation of molecular differences with wt MF.

Since we were limited in the number of samples to include in these resource-intensive and technically challenging Fluidigm experiments, and since in this experiment we were most interested in how gene expression after stimulation is altered in *Tnf*$^{-/-}$ vs. WT, we did not obtain a *Tnf*$^{-/-}$ mock stimulation sample. We do agree that it would have been helpful to have this control for extending the conclusions drawn from the data. However, the main conclusions from this experiment, namely that transcriptomes induced upon TNF and P3C4 stimulation are less distinguishable in *Tnf*$^{-/-}$ than WT cells and that *Tnf*$^{-/-}$ cells show increased gene expression upon TNF stimulation can be drawn without a *Tnf*$^{-/-}$ + Mock sample. Although not conclusive, several experimental results suggest that baseline differences might be small: a) there were no detectable differences in NFκB activity in WT vs *Tnf*$^{-/-}$ at baseline (by EMSA or microscopy, Figure 1B, 1C), b) *Nfkbia* (IκBα) expression was very similar at baseline (Figure 2B) but different after TNF stimulation (Figure 2B, 5F), and c) H3K4me1 signal was similar at baseline (Figure RL3).

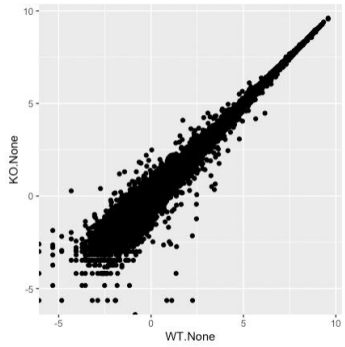


**Figure RL3: Scatterplot of H3K4me1 counts (log2) in WT vs *Tnf*$^{-/-}$ BMDMs at baseline; average of 2 biological replicates.**

- Cherry-picked genes in panel B are almost non-sense unless volcano plots are shown.

We thank the reviewer for this important suggestion. We now show a volcano plot of log2 fold change of TNF-induced gene expression in *Tnf*$^{-/-}$ over WT cells, which is asymmetric in shape (towards increased expression in *Tnf*$^{-/-}$ cells), and identified 10 genes that have higher expression in *Tnf*$^{-/-}$ cells with p-value threshold of 0.01 and absolute log2(FC) threshold of 0.5 (Figure 5E). We include only genes shown to be TNF- or P3C4-inducible in WT cells with lenient thresholds (p-value threshold of 0.05 and log2(FC) threshold of 0.25). The genes upregulated in *Tnf*$^{-/-}$ are shown individually in Figure 5F.

- The following sentence is wrong: " TNF-stimulated cells seemed to hyper- respond to overlap with P3C4-stimulated cells" in that, UMAPs show only qualitative, but not quantitative, differences between cell populations due to the fact that calculation is based on the global transcriptome.

We appreciate this important note and have rephrased the description of the UMAP plot as follows: “On the UMAP plot, the two *Tnf*$^{-/-}$ populations overlapped more than the WT populations (Figure 5A) and on the PCA plot the population of TNF-stimulated *Tnf*$^{-/-}$ cells appeared shifted compared to WT to overlap with P3C4-stimulated *Tnf*$^{-/-}$ cells (Figure 5B).”

Only after also discussing the newly added P3C4 vs TNF transcriptome comparisons (Figures 5B – 5D) and, importantly, the volcano plot (Figure 5E), which shows a number of upregulated and very few downregulated genes in *Tnf*$^{-/-}$ cells, do we now conclude that “[I]n the absence of tonic TNF, TNF-stimulated cells seem to hyper-respond to overlap with P3C4-stimulated cells.”

- Therefore, the conclusion "This suggests that the lack of tonic TNF conditioning changes TNF-induced gene expression to be more similar to gene expression responses to the PAMP P3C4" should be better commented.

We believe that this conclusion is now better supported the expanded analysis of the scRNAseq described above (TNF vs P3C4 stimulated transcriptomes using the first two principal components (Figure 5B); Average pairwise distances between the TNF vs P3C4 stimulated transcription profiles (Figure 5C); Fold change between P3C4 vs TNF stimulated expression of genes differentially expressed in WT upon P3C4 stimulation (Figure 5D)).

- In panel G, if I understand correctly the legend, the symbols for significantly downregulated genes should be represented in red.

The reviewer is right that, while the figure legend described the color code correctly, in the plot’s legend, the red symbols should have been labeled ‘Diff up’, rather than ‘Diff’. This plot has now been removed from the figure in response to a comment from Reviewer 3 (see below).

In the text, I do not agree with the sentence reported in the Results section: "thus, Tnf -/- cells showed increased de novo enhancer formation". In my opinion it is more a matter of " de novo opening in a larger fraction of cells"

We thank the reviewer for raising this nuanced point of discussion. We appreciate that in a bulk ChIP-seq assay the peak height is indeed a measure of the fraction of cells in which a given genomic region was immunoprecipitated, and that the probability of immunoprecipitating a given region is a function of the abundance of H3K4me1 in the region. Thus, we have reworded the sentence in question to, “Tnf-/- cells were more likely to form *de novo* enhancers.”

In the Discussion, the sentence "In vivo, the levels of tonic TNF might be higher in a location-dependent manner due to the presence of microbiome PAMPs and DAMPs released during tissue turnover and

repair" is in contrast with presented data showing that TNF exposure of TNF-/- MF cannot rescue the wt/tonic phenotype. Please discuss

We agree that extrinsic TNF exposure of *Tnf*$^{-/-}$ cells for four days did not fully rescue the WT phenotype, leading only to a partial rescue with a small increase in oscillatory trajectories. Co-culture with WT cells (newly added to the manuscript in this revised version) also did not rescue the phenotype. However, in addition to autocrine or cell intrinsic mechanisms, mechanisms requiring a longer exposure to tonic TNF and/or mechanisms acting earlier in macrophage differentiation or precursor development may still play a role. We have added this note to an earlier paragraph in the discussion. For these types of mechanism, the possibly higher physiological levels of tonic TNF are of relevance. Therefore, we think this statement in this general form is still helpful in the context of this discussion.

Some minor points below to be addressed, to facilitate comprehension from a broader audience

- Overall, axis descriptions are often inaccurate (Fig4D, e.g.)

Axis description and figure legends were written with great care. We have double checked them and have been not able to find inaccuracies.

As stated in the figure legend, the data in Fig 4D and Fig 4E are indeed normalized to max WT values obtained together with each *Tnf*$^{-/-}$ vs *Tnf*$^{-/-}$ p100KO replicate. We have rephrased the figure legends slightly to make this clearer. Generation of 3 of 5 replicates shown in Figure 2B and 3 of 3 replicates shown in Figure EV2C comparing WT to *Tnf*$^{-/-}$ also included *Tnf*$^{-/-}$ p100KO comparisons. These are shown in Fig 4D and Fig 4E. This is noted in the Figure legends of the initial submission:

> Figure 2B: “Some replicates for Tnf-/- BMDMs overlap with data in Figure 4D.”
>
> Figure EV2C: “Data for Tnf-/- BMDMs is also shown in Figure 4E.”
>
> Figure 4D: “Data for Tnf-/- BMDMs is also included in Figure 2B.”
>
> Figure 4E: “Data for Tnf-/- BMDMs is also shown in Figure S2C.”

- Ms pages should be numbered for the saneness of the reviewers

We sincerely apologize for this oversight. The revised manuscript includes page numbers.

- Figure 2 should be described with a twist to biology

Unless we’re misunderstanding this comment, this point is already made and addressed above by adding a more thorough description of the IKK-NFκB-IκBα signaling system.

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## Referee #2:

The paper by Luecke and colleagues considers how tonic TNF signaling contributes to the acute inflammatory response to different innate immune stimuli in macrophages. To explore tonic TNF signaling, which they define as constitutive signaling by background or baseline TNF prior to stimulation, they use BMDMs from Tnf-/- mice. Using live-cell imaging of NF-kB translocation dynamics, they observe that BMDMs from Tnf-/- have less oscillatory NF-kB signaling than WT BMDMs following TNF stimulation. Using computational modeling, they show that this is consistent with a role for tonic signaling in regulating TNFR turnover that promotes these oscillations. Functionally they show that BMDMs from Tnf-/- are less able to distinguish between acute stimulation between TNF and P3C4 as evidenced by gene programs that are more similar to each other.

The main conclusion, i.e. that tonic TNF signaling helps macrophages to distinguish between acute TNF vs PAMP stimuli, is significant. However, loss of tonic TNF signaling is not the only thing that would be changed in Tnf-/- mice and therefore it's unclear if the conclusion is fully supported. Tnf-/- mice also do not have autocrine TNF signaling, which has been shown by this group and others to be important in generating responses to PAMPs, and therefore this issue should be addressed.

We thank the reviewer for their appreciation of the significance of our work. We address their main concern regarding the importance of autocrine signaling in detail below and have expanded the explanations of Figure 2 and Figure 4 results in the manuscript to address their other two points.

1. In reference to Fig. 1, the authors cite their own work about autocrine signaling, stating that "the duration of signaling induced solely by the MyD88 adaptor was shorter in Tnf-/- cells (presumably due to the lack of autocrine signaling by newly synthesized TNF)". Why do the authors assume that this change can be attributed to loss of autocrine signaling while other changes are due to loss of tonic signaling? One experiment that could get at this more directly would be to block TNF signaling after the initial stimulation (e.g. with soluble TNFR) to attenuate autocrine signaling and try to separate these effects. Another possibility is to use the computational model to look for evidence of the relative importance of baseline v. subsequent autocrine signaling.

We assume that the changes in signaling and NFκB dynamics seen with TNF stimulation are due to the absence of tonic TNF rather than the absence of autocrine/paracrine signaling, because:

1) The differences in NFκB dynamics between WT and *Tnf*$^{-/-}$, importantly the loss of TNF-responsive NFκB oscillations, start at very early timepoints (e.g. the much less prominent trough at 30 – 60 min after the first NFκB activity peak) (Figure 1D-G). Signaling differences are also observed (and modeled) at early timepoints already (e.g. IKK activity, Figure 2D, Figure 3C). Importantly, the experimentally observed differences in TNFR1 levels, which we identify via modeling (and now in the revised version confirm experimentally using siRNA experiments to partially rescue oscillations in *Tnf*$^{-/-}$ cells) as one of the main mechanisms mediating the altered TNF-responsive NFκB dynamics, are already present at baseline.

On the other hand, differences in P3C4-induced NFκB responses affect the later timepoints of NFκB signaling, when we might expect autocrine TNF signaling to be acting. As can be seen from the NFκB trajectory heatmaps (Figure 1H), early P3C4-induced NFκB activity up to approximately 3 h is very similar between WT and *Tnf*$^{-/-}$ , with the activity in *Tnf*$^{-/-}$ cells then declining faster. Correspondingly, dynamic features dependent on duration (e.g. duration itself, integral, time to half max activity) are most significantly altered, while features such as oscillation power, and amplitude difference between peak 1 to trough 1, etc. are much less different. Thus, it is unlikely that absence of tonic TNF is the main mediator of these later differences.

2) TNF stimulation, in contrast to PAMPs, induces little TNF production. TNF-induced mRNA production is much lower than P3C4-induced mRNA production, as we can see e.g. from our scRNAseq data (Figure RL4). TNF protein synthesis and secretion additionally requires p38/ERK MAPK signaling at multiple steps (e.g. for mRNA splicing, mRNA stabilization, pro-protein processing) (Luecke *et al*, 2021; Caldwell *et al*, 2014). MAPK activity is low in response to TNF, but high in response to PAMP stimulation (Figure RL5)(Cheng *et al*, 2017). Therefore, it is expected that, in response to TNF stimulation, the secreted TNF protein levels are even lower than the TNF mRNA levels may suggest.

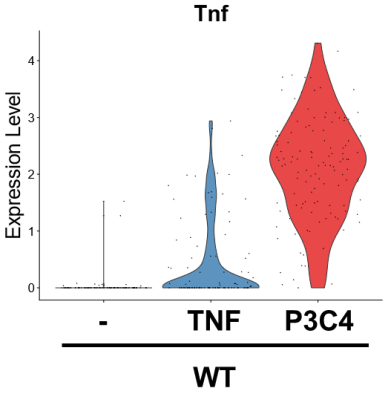


**Figure RL4: TNF mRNA in WT BMDM stimulated with TNF or P3C4 for 4 h, measured by single-cell RNA-seq.**

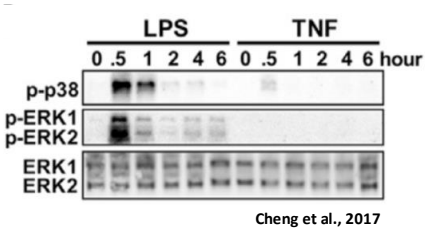


**Figure RL5: p38 and ERK1/2 phosphorylation (indicative of activity) in BMDMs upon LPS or TNF stimulation. From (Cheng *et al*, 2017).**

3) Moreover, any potential post-stimulation autocrine TNF signaling would be expected to increase IKK activity, diminishing the oscillatory troughs in NFκB activity, rather than making them more prominent. Thus, based on knowledge of the dynamical system mediating NFκB oscillations, the increase in non-oscillatory NFκB trajectories observed in TNF-stimulated *Tnf*$^{-/-}$ cells is not compatible with stimulus-induced *de novo* produced TNF.

In sum, while the loss of autocrine/paracrine TNF signaling likely plays some role in the differences in PAMP-induced NFκB responses between WT and *Tnf*$^{-/-}$, it is very unlikely that this plays a role in differences seen with TNF stimulation.

2. The discussion around Fig. 2 is confusing. The authors first hypothesize that decreased IkBa feedback might lead to the sustained signaling observed in Tnf-/- cells. When they observe a modest but significant increase in IkBa mRNA (Fig. 2B) they assume this is a result of the sustained signaling. This perhaps has to do with the timing of protein versus mRNA readouts; if so, it would be helpful to explain. Although their conclusion that there is no impairment in the IkBa feedback loop in Tnf-/- mice seems like a reasonable one, placed together the arguments appear circular.

We appreciate this feedback. This point is indeed about timing (but not so much of protein vs mRNA synthesis, rather the timing of IκBα level differences in relation to the timing of the oscillatory troughs (or absence thereof) in NFκB activity): A delay or early reduction (within the first hour after signaling) of IκBα induction in *Tnf*$^{-/-}$ cells could lead to lack of oscillations/ more sustained NFκB response/ less prominent activity trough at 30 – 60 min due to less IκBα being available for the post-induction inhibition of the activated NFκB. However, as Figure 2B shows, this is not the case – IκBα induction up to 60 min is very similar between WT and *Tnf*$^{-/-}$.

The more sustained NFκB presence in the nucleus at 30 – 60 min in *Tnf*$^{-/-}$ cells (Figure 1) likely mediates the increased IκBα levels at the later timepoints (70 min and later).

We now attempt to explain the timing of these two effects more thoroughly in the Results section and have added a full paragraph more thoroughly describing the NFκB-IκBα feedback loop (also in response to Reviewer 1's comment).

3. Related to this, the conclusion in Fig. 3-4 that Tnf-/- cells have a modest but significant increase in IKK activity and strengthening IkBa negative feedback can restore oscillations also seems at odds with the finding in Fig. 2B. The pairing of the prediction in Fig. 4A that strengthening will increase IkBa mRNA, and the experiment in Fig. 4D-F with the p100 KO validating this is striking. However, Fig. 2B showed that Tnf-/- cells already have increased IkBa relative to WT and yet oscillations are dampened. Does the model predict both these results? Can context be added to help to interpret these results together?

As with Figure 2B, this is explained by the time at which the differences in signaling between genotypes occur and also by the narrow parameter range (both in the mathematical model and in cells) that allows for oscillations. At the given IKK activity in the *Tnf*$^{-/-}$ cells and the '*Tnf*$^{-/-}$' model (which is slightly higher than in WT), increasing the IκBα negative feedback strength by increasing available NFκB for IκBα expression levels *higher than those in Tnf*$^{-/-}$ is both predicted by modeling and shown by experiments to increase the number of oscillatory trajectories, thus overcoming the effect of the increased IKK activity in *Tnf*$^{-/-}$. The additional NFκB is available from the start of signaling and the additional increase in IκBα expression is observed already in the first hour of signaling (Figure 4D), which is important for the restoration of the first post-induction trough of NFκB activity (Figure 4F).

On the other hand, in the *Tnf*$^{-/-}$ vs WT comparison, the slightly increased IKK activity in *Tnf*$^{-/-}$ (Figure 2D, 3C) is likely responsible for the reduced post-activation repression in the first hour (with similar IκBα

levels in WT and *Tnf*$^{-/-}$ in the first hour) and is able to overcome the increased IκBα expression seen after the 1 h time point in *Tnf*$^{-/-}$ cells (Figure 2B) for less oscillatory signaling (Figure Figure 1E, Figure 3B).

Below, we provide simulations of IκBα expression in WT vs *Tnf*$^{-/-}$ and *Tnf*$^{-/-}$ vs *Tnf*$^{-/-}$ with 1.25x NFκB to show that the model indeed predicts both the increase in expression in *Tnf*$^{-/-}$ compared to WT (with a larger increase after 1 h) (Figure RL6, right) and a further increase, already at early time points, in the *Tnf*$^{-/-}$ with 1.25x NFκB model (Figure RL6, left).

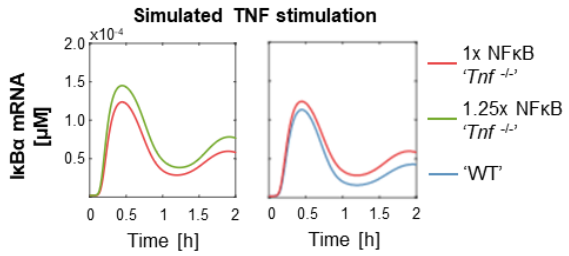


**Figure RL6: Simulated IκBα mRNA expression upon TNF stimulation in '*Tnf*$^{-/-}$ ','*Tnf*$^{-/-}$ ' with 1.25x NFκB, and 'WT' models.**

The '*Tnf*$^{-/-}$ ' vs '*Tnf*$^{-/-}$ ' with 1.25x NFκB is shown in Figure 4A (also in original submission). Since the IκBα simulations for the WT vs *Tnf*$^{-/-}$ model are not directly relevant to the mechanisms of altered dynamics investigated in Figure 3, but rather a functional consequence of the increase in NFκB activity, we chose not to include these in Figure 3.

We have added a few additional explanations to the first paragraph of the Figure 4 Results section and hope that this, together with the more thorough explanation of this dynamical system added to the Figure 2 Results section, will allow for a better understanding of the figure.

4. Fig. 4: These are very nice results regarding the functional significance of the small changes in NF-kB signaling. However, as in Fig. 1, it seems possible that loss of TNF autocrine signaling might also have a role to play and some additional experiments to control for this would be more convincing.

Since the reviewer mentions the functional significance of the tonic TNF deficiency, we assume they're referring to Figure 5, not Figure 4. We thank the reviewer for appreciating the quality of these results.

For the reasons explained above (Point 1 of Reviewer 3), we find it unlikely that loss of autocrine TNF signaling contributes to the differences in gene expression at 4 h of TNF stimulation. The newly added Figure 5B shows how TNF-stimulated cells have a greater shift from WT to *Tnf*$^{-/-}$ compared to P3C4-stimulated cells. We agree that the loss of TNF autocrine signaling may contribute to the more subtle changes in P3CSK4 -induced responses in the *Tnf*$^{-/-}$ vs WT BMDMs. We have added the following statement regarding this to the description of Figure 5 results: "The more subtle changes in P3C4-induced gene expression at 4 h in *Tnf*$^{-/-}$ compared to WT BMDMs (Figure 5B, 5F) may result from the decreased duration of signaling (Figure 1H, 1I), presumably due to lack of autocrine TNF signaling (Caldwell *et al*, 2014)."

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## Referee #3:

This study seeks to build upon and extend this group's prior interesting and important work to understand why acute TNF stimulation results in oscillatory NF-kB dynamics, which results in limited epigenomic remodeling and a different pattern of gene expression than that induced by PAMPs, which induce more sustained NF-kB activation, broad chromatin remodeling and potent gene induction. The authors' hypothesis that tonic TNF signaling affects the NF-kB response to subsequent TNF challenge is strongly supported by single cell analysis of NF-kB dynamics using Tnf KO mouse cells (Figure 1). The remainder of the paper uses analysis of bulk macrophage cultures and computational modeling to probe mechanisms underlying changes in NF-kB dynamics, and suggests that lower TNFR1 expression that results from tonic TNF signaling leads to decreased magnitude of signaling (IKK and JNK phosphorylation) and chromatin remodeling. There are several issues with these experiments leading to substantial concerns about whether the conclusions of the study are adequately supported by the experimental data.

We thank the reviewer for their interest in our current and previous studies and their appreciation of our core conclusions. We address their concerns below, e.g. by providing experiments using siRNA-mediated partial reduction of TNFR1 to WT levels in *Tnf*$^{-/-}$ cells, a co-culture experiments with *Tnf*$^{-/-}$ and WT cells to attempt to address tmTNF exposure, and adjustments to the figure describing the H3K4me1 data.

Major points:

1. As the authors acknowledge, the differences in signaling between WT and Tnf KO (Fig. 2) and TNF KO and TNF p100 DKO (Fig. 4) are very modest and not statistically significant. Although I take the authors' point that effects that occur at single cell level can be difficult to detect with bulk cultures, it is still in my view not reasonable to use results that are minimal and not significant to draw mechanistic conclusions. Ideally, the authors should develop additional single cell assays, e.g. for IKK activation.

We agree with the reviewer that additional lines of evidence would strengthen the manuscript. While IKK activation is often scored by p-IKK immunoblot, anti p-IKK staining for flow-cytometry or immunofluorescence has not been established and does not work in our hands. Further, IKK activity in response to TNF stimulation is highly dynamic which means that a live cell assay would be preferable. FRET has been used to detect when specific kinases are active but their dynamic range is notoriously limited meaning that only large differences can be distinguished. Also, our prior attempts to generate a FRET reporter of IKK were unfortunately unsuccessful. The invention of the kinase translocation reporter (KTR) by the Covert lab, implemented for MAPKs, was inspired by the NFκB system: after all, NFκB is essentially a KTR for IKK. No additional KTR for IKK has been generated. Design of such a reporter for IKK that is faithful and can be implemented in primary macrophages would be an extensive project with several technical hurdles. We feel that this is beyond the scope of the current project.

The effect sizes of the signaling differences under investigation here are often small and given the technical and biological variability in the bulk assays used, make it difficult to establish statistical significance at a significance level of 5% using a number of replicates typical and feasible for these assays.

While the bulk assays do make it more difficult to detect differences at single cell levels, the effect size here is expected to be small even on a single cell level: In dynamical systems, such as that controlling NFκB dynamics, small changes to relevant parameters have a large effect on the system, especially on oscillations, as demonstrated in the parameter scans in Figure 3A, where altering certain parameters by a factor of only 10^0.1 or 10^-0.1 already mediates significant changes to the oscillatory character of NFκB dynamics. Thus, these small differences in signaling can have very significant downstream consequences (like misregulated NFκB dynamics with altered gene expression) and, in our opinion, are important to investigate and report, even if currently available assays are not optimal.

Thus, the small effect sizes seen experimentally match those predicted using this well-established mathematical model of NFκB signaling in macrophages (Table RL1), providing confidence in their biological relevance. Modeling also allows us to connect the multiple strands of evidence, which all point in a similar direction, into a coherent theory (Figure 2, 3: In *Tnf*$^{-/-}$: Increased TNFR1 levels → increased IKK (and JNK) signaling → less pronounced troughs in NFκB signaling → increased NFκB in nucleus → increased gene expression (IκBα)) and challenge that theory by predicting and then experimentally confirming the ability of the dynamic system to overcome increased IKK activity in *Tnf*$^{-/-}$ cells by increasing NFκB levels and thus increased IκBα-mediated negative feedback in the *Tnf*$^{-/-}$ system (Figure 4: In *Tnf*$^{-/-}$ with additional NFκB available for signaling ('1.25x NFκB' model, *Tnf*$^{-/-}$ p100$^{-/-}$ cells): more NFκB in nucleus → further increase of IκBα expression → partial restoration of oscillations). Moreover, while the signaling differences often do not reach the conventional, but arbitrary significance level of 0.05, they do often reach low p-values for multiple timepoints (Table RL2).

**Table RL1: Effect sizes predicted by mathematical modeling *vs*. experimental results.**

| | Comparison | Fold difference predicted by mathematical model | Fold difference *Tnf*$^{-/-}$ vs WT measured in experiment |
|---|---|---|---|
| IKK activity | *Tnf*$^{-/-}$ *vs*. WT | 1.2x - 1.6x in 1$^{st}$ two hours (Figure 3C) | 1.4x at peak activity and 1.7x at 2 h (Figure 2D) |
| IκBα expression | *Tnf*$^{-/-}$ p100KO *vs*. *Tnf*$^{-/-}$ | 1.2x at 30 min (first expression peak), 1.34x at 70 min (first expression trough) (Figure 4A) | 1.3x – 1.5x (20 - 50 min; 100 - 110 min) (Figure 4D) |
| TNFR1 levels | *Tnf*$^{-/-}$ *vs*. WT | 123% - 127% (Figure 3D) | 126% (Figure 3E) |

**Table RL2: p-values of differences in signaling between genotypes.**

| | Comparison | Time points | p-value | Figure |
|---|---|---|---|---|
| IKK activity | *Tnf*$^{-/-}$ *vs*. WT | 2, 5, 10, 15, 30 min | 0.0079, 0.14, 0.19, 0.22, 0.23 | Figure 2D |
| JNK activity | *Tnf*$^{-/-}$ *vs*. WT | 5, 10, 15, 30 min | 0.2, 0.29, 0.26, 0.25 | Figure 2EF |
| *Nfkbia*/IκBα expression | *Tnf*$^{-/-}$ *vs*. WT | 70, 80, 90, 100, 110, 120 min | 0.013, 0.044, 0.0031, 0.013, 0.024, 0.024 | Figure 2B |
| Nuclear NFκB (EMSA) | *Tnf*$^{-/-}$ *vs*. WT | 30, 60, 90, 120 min | 0.29, 0.28, 0.22, 0.14 | Figure EV2 |
| TNFR1 levels | *Tnf*$^{-/-}$ *vs*. WT | 0 min, 30 min | 0.0027, 0.093 | Figure 3E |
| *Nfkbia*/IκBα expression | *Tnf*$^{-/-}$ p100KO *vs*. *Tnf*$^{-/-}$ | 20, 30, 40, 50, 100, 110 min | 0.27, 0.063, 0.14, 0.086, 0.097, 0.2 | Figure 4 |
| Nuclear NFκB (EMSA) | *Tnf*$^{-/-}$ p100KO *vs*. *Tnf*$^{-/-}$ | 30, 120 min | 0.1, 0.18 | Figure 4 |

Importantly, one of main parameters determined experimentally to be altered, the increase in TNFR1 levels at baseline (Figure 3), is very reproducible and statistically significant, despite a modest effect size (126% of WT cells, p-value of 0.0027).

2. The epigenomic analysis of H3K4me1 appears very incomplete as only 1515 inducible peaks were detected even when LPS and Pam3Cys were used, which is much less than expected for a histone mark. There were only a small number of differentially induced peaks (37 peaks, Fig 5G) and the p value was not corrected for multiple comparisons, weakening the ability to draw rigorous conclusions. Additionally, only 2 replicates were analyzed. Overall, it is difficult to draw conclusions based on incomplete data.

There were indeed 1515 H3K4me1 peaks identified as being *de novo* enhancers, i.e. stimulus-inducible in any stimulation condition (LPS, P3C4, TNF) compared to unstimulated. These were identified using edgeR with FDR < 0.05 and log2FC > 1 (Robinson et al, 2010). To clarify, these 1515 *de novo* enhancers were found among the >89000 H3K4me1 ChIP-seq peaks identified overall. We have added this information to the Materials & Methods section to avoid misunderstandings.

We believe this is a reasonable number of *de novo* enhancers for innate immune stimulation in murine macrophages at 8 h. It is similar to the number identified in our earlier publication on NFκB-dependent *de novo* enhancers in macrophages, where we identified app. 4000 enhancers (using five innate immune stimuli and with a slightly better experimental signal-to-noise ratio) (Cheng *et al*, 2021).

For very resource-intense experiments, such as ChIP-seq, it is common to produce and analyze two, rather than more, biological replicates. Because the conclusions are not about specific genomic

locations, but about groups of genomic locations, the members within the group provide the basis for a statistical evaluation also. Thus, we do not think that our data or analysis is incomplete based on the number of identified *de novo* enhancers or the two biological replicates generated.

However, we do agree with the reviewer that the identification of specific differentially induced peaks between *Tnf*$^{-/-}$ and WT cells based on two replicates (and depiction as a volcano plot) as was done in the previous version of the manuscript does not have sufficient power to be very meaningful and generally does not add much information. We have thus removed the volcano plot from that figure (now Figure 6). The main conclusion of the figure, that there is increased H3K4me1 signal induction in *Tnf*$^{-/-}$ cells compared to WT cells in NFκB-motif containing regions, is well supported by Figure 6C and 6D (scatter plot and violin plot of Cluster 1), with the fold change distribution of TNF-inducible peaks in *Tnf*$^{-/-}$ cells having an increased median and being clearly distinct from the distribution in WT cells. For additional visualization of this point, we have added a heatmap depiction of fold change of peak counts in *Tnf*$^{-/-}$ vs WT cells to the heatmap in Figure 6A, which demonstrates the increase of counts in Cluster 1 in *Tnf*$^{-/-}$ vs WT cells with TNF stimulation. We also provide additional examples of *de novo* enhancers from Cluster 1 (Figure 6E).

3. The soluble basal TNF levels are extremely low and add-back of TNF to TNF KO cultures did not effectively reverse the effect of deletion. This suggests an important role for membrane TNF, which can be measured using flow cytometry. The role of membrane TNF should be investigated.

We agree that it would be very interesting to fully understand the mechanisms resulting in the altered TNFR1 levels at baseline and agree that tmTNF could play a role. To investigate whether supplying exposure to tmTNF on neighboring cells would rescue TNF-induced NFκB oscillations in *Tnf*$^{-/-}$ cells, we performed co-culture experiments: *Tnf*$^{-/-}$ cells were incubated with 85% non-fluorescent WT cells, which might provide exposure to both soluble TNF (sTNF) and transmembrane TNF (tmTNF) via intercellular contact. However, this did not detectably increase the percentage of cells with oscillatory trajectories compared to *Tnf*$^{-/-}$ cells cultures alone (23% in absence of WT cells vs. 23% in co-culture with WT cells; p = 0.43) (Figure EV1F). We have now included these results in the manuscript.

While these negative results to do not allow for a definitive conclusion, they, together with the only partial rescue of oscillatory trajectories achieved by addition of extrinsic TNF to the culture, point towards contributions of cell-intrinsic or autocrine mechanisms (rather than mechanisms involving neighboring cells) and/or mechanisms acting earlier in macrophage differentiation or precursor development.

4. The causal relationship between low TNFR1 and NF-kB dynamics is solely supported by computational modeling (Fig. 3). The differences in TNFR1 expression are minimal both before and after TNF stimulation, although TNF stimulation induces a large fractional (appr. 70%) decrease in cell surface TNFR1. Given these results, the causal relationship between TNFR1 expression levels and NF-kB dynamics should be directly experimentally tested by varying TNFR1 expression using knockdown or Tnfr1-/+ heterozygous null mice, and by the complementary approach of increasing TNFR1 levels in WT cells to those observed in TNF KO cells using a forced expression system.

With regards to the criticism that the TNFR1 expression difference is minimal, we'd like to mention that, while indeed the difference in TNFR1 expression between WT and *Tnf*$^{-/-}$ cells is not very large (126% of WT cells), a) this difference is very reliably reproducible and statistically significant (p-value of 0.0027 with 3 biological replicates) and b) in the control of oscillatory dynamics, small changes to relevant parameters have a large effect on the system as demonstrated in Figure 3A (discussed above in more detail) .

We'd also like to emphasize that the mathematical model of the molecular network of TNF to NFκB signaling used here to predict and interpret the correlated experimental results is a well-established model that has been refined over many years (Werner *et al*, 2008; Caldwell *et al*, 2014) and was most recently adapted to primary macrophages in 2021 (Adelaja *et al*, 2021). Thus, the model was not created merely to advance a specific interpretation in the current work.

That being said, we fully agree with the reviewer that an experimental test of the relationship between TNFR1 expression level and NFκB dynamics is very beneficial to increase the confidence in our conclusions and thank them for their suggestion. We have now performed siRNA-mediated reduction of TNFR1 expression in *Tnf*$^{-/-}$ cells. TNFR1 levels similar to levels in WT were achieved (confirmed by flow cytometry, Figure 3F left). We then performed microscopy to compare NFκB dynamics. Compared to control siRNA transfected *Tnf*$^{-/-}$ cells (60 % oscillatory NFκB trajectories), *Tnf*$^{-/-}$ cells with reduced TNFR1 levels had a higher percentage of oscillatory NFκB trajectories (69%, p = 0.023), with control siRNA transfected WT cells having 73% oscillatory NFκB trajectories (Figure 3F, right). This provides further support for a causal relationship between TNFR1 surface expression and percentage of oscillatory NFκB trajectories.

Minor points:

5. In the absence of tonic TNF signaling, the authors state that macrophages showed less oscillatory NF-kB responses that are similar to PAMP exposure. This point would be strengthened if a positive control of stimulation with a PAMP was included in the figures (e.g. 1D, 2).

As suggested, we have added microscopy images of P3CSK4 stimulation to Figure 1D, in addition to the heatmaps and quantitative features derived from P3CSK4 microscopy already shown in Figure H-J. We added a corresponding statement to the description of Figure 1D describing how the TNF-induced NFκB activity in *Tnf*$^{-/-}$ resembles the less oscillatory character of early P3CSK4-induced NFκB dynamics.

Figures 5 and 6, which describe the functional consequences of the altered NFκB dynamics for single cell gene expression and *de novo* enhancer formation, already include P3CSK4 data as a comparison/positive control to show how TNF-stimulated *Tnf*$^{-/-}$ cells functionally react more similarly to PAMP-stimulated cells. In the now extended analysis of the Fluidigm gene expression data (Figure 5), the differences between P3CSK4 and TNF stimulated cells in WT vs *Tnf*$^{-/-}$ cells are now explicitly investigated.

While the decreased oscillatory character of TNF-induced NFκB dynamics in *Tnf*$^{-/-}$ resembles the non-oscillatory character of P3CSK4-induced dynamics, and gene expression between the two stimulations is more similar in the *Tnf*$^{-/-}$ (Figure 5), the NFκB dynamics remain distinct in other aspects, e.g. with regards to amplitude and duration (compare Figure 1E and Figure 1H), and are expected to differ in the molecular mechanisms by which they are achieved. Therefore, we do not think adding P3CSK4 data to

Figure 2 would improve understanding of the molecular mechanisms governing TNF-stimulation in *Tnf*$^{-/-}$ cells.

6. It would be helpful to show expression of several canonical NF-kB targets such as IL6, Il12b, TNF etc. throughout the figures and in the single cell data in Fig. 5A.

Figure 5E/F now shows 10 genes significantly upregulated in *Tnf*$^{-/-}$ vs WT cells upon TNF stimulation, all which have been reported as NFκB-dependent genes (Cheng *et al*, 2017; Rouillard *et al*, 2016; Wu *et al*, 2022), including *Nfkbia*. *Nkfbia* (encoding IκBα) expression, a typical NFκB target gene, is already shown in Figures 2 and 4, as its expression levels are among the factors that control NFκB dynamics. We believe depicting expression data of other genes not hypothesized to be involved in the determination of NFκB dynamics at these timepoints before Figure 5 might distract the reader from the main focus of the earlier figures, which is the mechanism of differences in NFκB dynamics.

IL12b appears to be expressed only at very low levels in these cells upon TNF or P3C4 stimulation (Figure RL7) and IL6 did not appear in the list of expressed genes in the Fluidigm analysis.

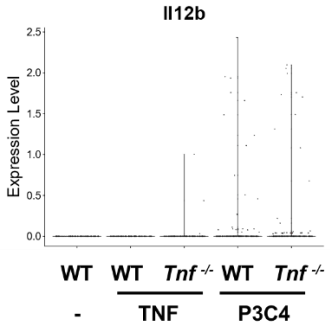


**Figure RL7: IL12b is not or very weakly expressed in *Tnf*$^{-/-}$ and WT BMDM at 4 h of TNF or P3C4 stimulation as determined by Fluidigm single cell gene expression analysis.**

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| If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI. | Not Applicable | |
| Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable. | Not Applicable | |

| Laboratory protocol | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| Provide DOI OR other citation details if **external detailed step-by-step protocols** are available. | Not Applicable | |

| Experimental study design and statistics | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| Include a statement about **sample size** estimate even if no statistical methods were used. | Yes | Materials and Methods section |
| Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described? | Not Applicable | |
| Include a statement about **blinding** even if no blinding was done. | Yes | Materials and Methods section |
| Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established?<br><br>If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification. | Yes | Materials and Methods section, Figure legends |
| For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared? | Yes | Materials and Methods section |

| Sample definition and in-laboratory replication | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| In the figure legends: state number of times the experiment was **replicated** in laboratory. | Yes | Figure legends |
| In the figure legends: define whether data describe **technical or biological replicates**. | Yes | Figure legends |

## Ethics

| Ethics | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. | Not Applicable | |
| Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | Not Applicable | |
| Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained. | Not Applicable | |
| Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations. | Yes | Materials and Methods section |
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| Dual Use Research of Concern (DURC) | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm | Not Applicable | |
| If you used a select agent, is the security level of the lab appropriate and reported in the manuscript? | Not Applicable | |
| If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript? | Not Applicable | |

## Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

| Adherence to community standards | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided. | Not Applicable | |
| 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. | Not Applicable | |
| 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. | Not Applicable | |

## Data Availability

| Data availability | Information included in the manuscript? | In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section) |
|---|---|---|
| Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section? | Yes | Data availability section |
| Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement? | Not Applicable | |
| Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided? | Yes | Data availability section |
| If publicly available data were reused, provide the respective **data citations in the reference list**. | Not Applicable | |