Cell Systems

Transparent Peer Review Record

Stimulus-Response signaling dynamics characterize macrophage polarization states

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Summary

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2 original, 0 new
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Editorial decision letter with reviewers' comments, first round of review

Dear Alex,

I am enclosing the comments that reviewers provided on your paper. Unfortunately, the recommendation is against publication in Cell Systems. While the Reviewers appreciate the dataset presented in the paper, they raise a number of comments and criticisms, including with regards to the conceptual advance that the findings represent It is a matter of general policy that we do not propose resubmission of manuscripts when the reviewers present extensive criticisms and there is no clear path forward to publication without extensive further work, and we therefore cannot offer to consider this paper further.

Although we cannot proceed further with this manuscript at Cell Systems, I'd like to offer you the opportunity to transfer it to our sister journal, iScience, a broad-scope, open-access journal dedicated to championing interdisciplinary research. Their team is spectacular, and they specialize in finding constructive routes to publication in circumstances like these. I have taken the liberty to briefly discuss your manuscript and the reviewers' comments with my colleagues at iScience, who are satisfied with the conceptual advance made in the manuscript and who would be interested in taking forward a revised version of the manuscript that addresses the points the reviewers' raise largely through textual changes and acknowledgement of limitations. However, the editors are concerned about Reviewer 3's point about the whether HoxB4-immortalised macrophages represent "normal" macrophages and the concern about the L929 media, and would be looking for these concerns to be addressed in full.

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All the best,

Bernadett

Bernadett Gaal, DPhil
Editor-in-Chief, Cell Systems

Reviewers' comments:

Reviewer #1: Singh et al. aim to address an exciting and important question: how does cytokine pre-treatment (representing cellular context or "polarization") inform macrophage integration of microbial stimuli (immune threats). In doing so, they built upon the group's recent Immunity paper (Adelaja et al 2021) and generate an unprecedented dataset, including 6 polarization conditions and 8 stimuli indicating an immune threat (7 microbial PAMPs and TNF). While the significance of the problem, innovative approach, and dataset used are strengths of the paper, the authors do not take the study beyond descriptive analysis. Thus, the conceptual advances are modest. The major finding is that polarization impacts dynamic features of NFkB dynamics (codons) in a cytokine and PAMP specific manner; M1 conditions drive PAMPs to appear more similar by encoding increased threat, while M2 conditions drive PAMPs to appear more similar by encoding decreased threat. There are extensive visualizations of various machine learning readouts, drilling down on which codons vary, contrasting specific PAMPs or cytokines. However, the authors do not attempt to demonstrate the biological significance of any of the cytokine-dependent changes in codons or determine the mechanisms that underlie any of the cytokine-dependent NFkB codon changes.

The authors conclude by saying that "Future studies may further describe this functional landscape of macrophage polarization states … by combining our signaling data with other single-cell measurements or using mathematical models of the signaling network that account for the observed signaling dynamics." It is this reviewer's opinion that progress in one of these two directions is needed to reach the level of novelty warranting publication in Cell Systems. The former could include further analysis of macrophage function: do specific cytokine-regulated codons predict altered production of inflammatory mediators (eg. TNF, IL-6, iNOS, etc; mRNA or protein measured in the same single cells as the NFkB trajectories)? The latter could include using the authors' previously published mechanistic models (Adelaja et al 2021) to predict mechanisms likely shaping cytokine-dependent changes in NFkB codons. Mechanistic modeling predictions related to protein expression could be validated using available antibodies (eg. cytokine-dependent changes in TLRs, a likely source of the PAMP specific results). These studies could be pursued for a small subset of polarization and stimulation conditions.

Some additional comments and questions are listed below.

* With limited biological advances, it was unclear if there was significant conceptual advances in terms of approach. According to the authors: "We addressed these challenges using two innovations. The first is the utilization of 'signaling codons'… Our second approach to address the challenges of time-series data analysis was to utilize a novel machine learning approach that allowed for trajectory distinguishability to be explored in a feature-free manner." The signaling codon approach was previously published in Adelaja et al 2021. Not clear what about the LSTM is novel? This is a standard neural network approach commonly used for time series data. Was there an alteration to the standard model or was the novelty the application?

* Given that LDA is critical to the narrative, it should be explained in more detail. What is this analysis telling you? Why do the authors equate the LDA projection to immune threat?

Minor points:

* Is it possible the 6 signaling codons identified in Adelaja et al 2021 are not optimal for this classification task? This possibility should be discussed at a minimum.

* Figure 2B: Upon first read, it seemed as though only M0:TNF and IFNg:LPS were used to do this ranking. It is clarified a bit by Figure 2E, but the authors should revise to make it clear when discussing 2B that all conditions were considered. In Figures 2B-D, it may help to show the average for each condition, perhaps with a dotted line?

* Figures 4G-H: It seems like the Pam3CSK (H) is driving the bacterial response (G). If you take Pam3CSK4 out of the F1 average, is the bacterial score more comparable to the viral F1 score?

* What was the software/package used for cell segmentation?

* How was the Jensen-Shannon distance threshold (0.3) determined? Also, "we found that the maximum JSD between replicates were in general much smaller than between cells stimulated in different conditions" should be shown.

* Figure 1D is too small and minimally labeled to be easily interpreted. I would suggest moving it to the supplement and labeling the rows and columns in more detail. Figure 1B could also be moved to the supplement.

Reviewer #2: "Stimulus-response signaling dynamics characterize macrophage polarization states" by Singh et al. presents findings from systematic quantitative analyses of polarization-dependent macrophage NF-kappaB signaling responses to a host cytokine and pathogen-associated ligands (PAMPs). This is based on an extensive dataset generated with cells from the Hoffmann lab's mice endogenously expressing fluorescent fusion of the RelA subunit. The data covers a panel of commonly used PAMPs for probing macrophage NF-kappaB signaling after 24 hours of polarization with another panel of cytokines, allowing for multiple polarizing factors within each of M1 or M2 category. The authors used TNF-alpha as a host (non-PAMP) cytokine against which PAMPs were assessed for "immune (or pathogenic) threat" in a linear discriminant analysis and a set of "confusion" analyses.

Overall, the results provide an unprecedented insight about the multidimensionality of macrophage

polarization states that exert distinct signaling responses, which challenges the prevailing notion that M1 and M2 represent the two extremes of macrophage states along a linear spectrum. Leveraging the live cell imaging data, specific dynamic features of signaling (termed "signaling codons"), such as duration and speed, were linked to the polarization-induced effects of macrophage responses to PAMPs and TNF. Among the important behaviors identified was an increased confusion between viral and bacterial ligands by polarized macrophages, suggesting a greater role of naïve macrophages in specific threat recognition.

In addition, the authors present a suite of statistical and machine learning tools (LDA, LSTM, FPCA) that they customized and developed to extract the relevant features of signaling and functional states from a large set of single cell time series data. The Hoffmann group has been well known for this strength, and again applied advanced computational methods for the questions in this study. These analysis frameworks are likely to be useful for other studies of single cell signaling dynamics. I believe that this will be an exciting contribution to Cell Systems. Below I have specific comments for the authors to address as much as possible. If some issues cannot be resolved by additional experiments, I recommend acknowledging the caveats in the main text regarding the limitations of the study (related to comments # 2 - 5).

1. Concentrations of the TNF and PAMP ligands couldn't be found in the Methods or figure legends. Please specify the ligand concentrations used and how they were selected (e.g. half maximal in a dose response or something like that?).
2. Immortalized myeloid precursor cells from bone marrow may behave differently from primary BMDMs. Any data on the shared features between immortalized (and differentiated) cells and BMDMs would help readers assess the immunological implications of the results.
3. While M0 condition was used as a no-polarization control for all the analyses, it is not clear if the M0 cells also had the same 24 culture period (that were applied to polarized macrophages) before ligand stimulation. If the naïve macrophages were subject to one-day shorter in vitro culture, their ligand responsiveness might be affected solely from that effect. Was the pre-stimulation in vitro culture period fixed for all the conditions? If not, was control experiment done to show the different culture periods (with/without 24 hr) did not affect the M0 responses?
4. While Hoechst 33342 is a common dye for labeling nuclei, it has two properties that make it a poor choice for live cell imaging. First, it intercalates DNA and affects cell physiology (Ku H et al. https://doi.org/10.3389/fcell.2022.822026). Second, highly phototoxic UV laser must be used for acquiring the images (Purschke M et al. https://doi.org/10.1039/C0PP00234H). Alternative choices for nuclear labeling in live cell imaging are available, such as SPY650, which uses a low-energy laser line and does not intercalate DNA. The authors used a low concentration of Hoechst to minimize undesired effects, but detrimental effects may have caused hard-to-notice changes in signaling dynamics.
5. How was the photobleaching of mVenus accounted for in the quantification of live cell imaging time series?
6. Figure S1 should include scale bars and the ligand stimulation time points of the images shown.

Reviewer #3: Singh et al. use a combinatorial macrophage stimulation approach to try and understand if NF-kB signalling is associated with "threat" level in the different stimulation conditions. The primary approach was quantification of RelA-mVenus reporter immortalised "macrophages". Overall, this is a weak manuscript that treads down a familiar path (see Xue et al) and suffers from two fundamental flaws: (i) the theory and outcomes/results, and (ii) the methodological approach.

Let's conducer these independently and then later, the many small errors and misconceptions that minimise the manuscript's value.

Theory. A key question here concerns the overall relevance of the approach and outcomes. Previously, Xue et al. defined (in human Mo-derived macrophages) a wide range of different gene expression states. This paper, along with discontent in the field over the Mills classification (and the Mantovani classification) led to a push to acknowledge a continuum of states (see Ginhoux et al., Murray et al, Immunity, and others). A key recommendation was to abandon terms like M2a (used by Singh). The concept of continuous states has been taken further by scRNAseq (a couple of papers by Miller-Jensen, Nature Comms, and Dichtl et al. Life Science Alliance), which shown even with highly "homogeneous" BMDMs, many different sub-populations exist in macrophages from the same mouse grown under highly controlled conditions. None of this prior work is properly considered by Singh because in doing so would expose the fact that we already know that different stimuli result in different magnitudes of stimuli, and that there is intrinsic noise in the system. Thus, the conceptual advance of Singh is negligible. For example, on pg. 6 at the top, Singh states "that macrophage polarisation states do not merely fall into two discrete classes of M1 and M2, but may be represented on a continuum" (see, supra). A fact known to every serious person working in this field for over a decade.

Additional comments: (1) in the introduction and results (in toto) the actual purpose and results are not articulated. (2) The choice of the initial stimulation and second stimulation is arbitrary, as is the time chosen to analysis. This is a limitation never stated. (3) There is no gene of protein expression data to confirm or extend the findings.

Methodology. Singh uses HoxB4 immortalised macrophages, then grown in L929 medium. There are two problems here. First, extensive experiments are needed to confirm that the HoxB4 system represents a "normal" macrophage. Second, use of L929 media will distort all the data because it contains thousands of factors that can influence macrophage function (Heap et a. 2021). There are also batch-to-batch variations in the preparation of the supernatant. Without an explicit experiment against recombinant CSF-1, one will always question the influence of the L929 media on the system.

Other comments:

1. Introduction. The introduction is very poorly written and ignores key literature. Reference #3, for example, is hardly a definitive source of up-to-date knowledge. M2a… (see supra).
2. "threat level" is an arbitrary and imprecise term the authors use to "sell" their story. This should be omitted.
3. "Codons" Creates confusion relative to the genetic code. (The prior Immunity paper may have got through using this term but that does not mean is also escapes subsequent confusion).
4. Gratuitous adjective use: "rich" (pg. 4). Why is it "rich"?
5. Polarisation should be avoided and instead "stimulation" used.
6. The figures are below the standard normally shown from this group.

Authors' response to the reviewers' first round comments

Attached.

Cell Systems Transparent Peer Review Record

Editorial decision letter with reviewers' comments, second round of review

Dear Alex,

I'm very pleased to let you know that your manuscript is now "accepted in principle," that is, provisionally accepted pending our receipt of final files that meet the journal's formatting requirements. Congratulations!

I have included some editorial requests in the Editorial Notes section below my signature. Please review the information below along with the detailed formatting requirements listed in the Final Files Checklist. We've also put together this FAQ (click the Final Formatting Checks tab) for your convenience. Please ask any questions you may have, make any necessary changes to your manuscript files, and then upload your final files into Editorial Manager. Once we receive your formatted files, we will go through our formatting checks and let you know if further changes are needed.

Introducing new referencing style

To standardize the referencing style across Cell Press journals, starting from October 2022, we ask that all in-text citations be formatted as superscripted numbers (e.g. "Multiple reports support this observation.[1,2]"). Moving away from the Harvard referencing style (e.g. Smith *et al.*, 2020) will improve author and reader experiences. All manuscripts accepted from now on must use **the superscript numbered Cell Press referencing style**. Make sure to use this numbered referencing style for all new and revised submissions as well. Switching is easy. Just use the updated CSL and EndNote referencing styles for Cell Press articles.

Below my signature, you'll find specific information about what to expect next regarding formatting checks and working with our Production Department after acceptance. It's been a pleasure working with you, please feel free to contact our journal team with questions.

All the best,

Bernadett

Bernadett Gaál, DPhil
Editor-in-Chief, Cell Systems

Editorial Notes

Transparent Peer Review: Thank you for electing to make your manuscript's peer review process transparent. As part of our approach to Transparent Peer Review, we ask that you add the following

sentence to the end of your abstract: “A record of this paper’s Transparent Peer Review process is included in the Supplemental Information.” Note that this ***doesn't*** count towards your 150 word total!

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Manuscript Text:

- House style disallows editorializing within the text (e.g. strikingly, surprisingly, importantly, etc.), especially the Results section. These terms are a distraction and they aren't needed—your excellent observations are certainly impactful enough to stand on their own. Please remove these words and others like them. “Notably” is suitably neutral to use once or twice if absolutely necessary.
- Please double-check that you use the word "significantly" in the statistical sense only.

Figures and Legends:

Also, please look over your figures keeping the following in mind:

- When data visualization tools are used (e.g. UMAP, tSNE), please ensure that the dataset being visualized is named in the figure legend and, when applicable, its accession number is included.
- When color scales are used, please define them, noting units or indicating "arbitrary units," and specify whether the scale is linear or log.
- Please ensure that every time you have used a graph, you have defined "n's" specifically and listed statistical tests within your figure legend.
- When figures include micrographs, please ensure that scale bars are included and defined within the legend, montages are made obvious, and any digital adjustments (e.g. brightness) have been applied equally across the entire image in a manner that does not obscure characteristics of the original image (e.g. no "blown out" contrast). ***Note that all accepted papers are screened for image irregularities, and if this advice is not followed, your paper will be flagged.***
- Please ensure that if you include representative images within your figures, a "representative of XXX individual cells"-type statement is made in the legend.
- Please ensure that all figures included in your point-by-point response to the reviewers' comments are present within the final version of the paper, either within the main text or within the Supplemental Information.

STAR Methods:

Data and Code Availability:

- Please ensure that ***all datatypes*** reported in your paper are represented the data availability statement, not only standardised data such as sequencing data. For more information, please consult this list of standardized datatypes and repositories recommended by Cell

Press. Non-standardized datatypes can either be deposited to a general repository or made available by the lead contact upon request.

- If you are using GitHub, please follow the instructions here to archive a “version of record” of your GitHub repo at Zenodo, then report the resulting DOI in the Key Resources Table.
- Please add the following sentence to the end of your Cod and Data Availability statement: "Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request."

Thank you!

Response to Reviewers

Reviewer #1: I appreciate the clarifications and additions to the manuscript. The new results shown in Figure 7 represent a novel approach and exciting research direction. However, the results presented are not robust enough or validated sufficiently to address my major concern. Without (a) demonstrating the biological significance of the cytokine-dependent changes to NFkB codons or (b) thorough exploration of the signaling mechanisms that underly the cytokine-dependent NFkB codon changes, the advances are modest.

We're glad that the reviewer appreciates the new work in Figure 7 as an important research direction.
We understand that the reviewer is interested in a) the biological significance of the cytokine dependent changes in NFκB signaling codons, and b) the molecular mechanisms that underly the cytokine-induced changes in NFκB signaling codon deployment. However, the goals of this particular study were different, and so we do not claim to provide insights to these questions.

The primary goal of the study is to determine how much information the stimulus-responsive dynamics of one analyte provides about the cell state. This is quite innovative as the current state-of the art to characterize cell states is NGS: i.e. a single timepoint of many thousands of analytes (mRNAs or ATAC locations). While measuring a single analyte at one timepoint does not give much information, it is unclear whether examining the timecourse trajectory (many timepoints) of a single analyte provides sufficient information to separate cell states. This is what we have done here.

Dimensionality reducing a complex timecourse trajectory in a meaningful manner is a challenge for which no standardized approach has been established. We show that functional PCA, signaling codons, or model-inferred kinetic parameters can all be applied, but that the latter two are of particular use. Dimensionality reduction via signaling codons provides interpretable distinctions of cell clusters, and kinetic parameters provide potential mechanistic descriptions of the molecular network of the cells in the population.
The above analysis is conceptually novel and unprecedented.

The reviewer is asking about questions that are important, and that are related to the present study but are not within the scope of the present paper. They are also not tractable within the available experimental system.
(a) Are the cytokine-induced changes of NFκB codons of biological significance? The most proximal biological assay for NFκB activation is gene expression. Polarizing cytokines affect the activation of many transcription factors and also chromatin modifying enzymes, altering the epigenomic landscape. It will not be possible to determine whether changes in signaling codons will affect downstream gene expression. However, in a prior series of publications several signaling codons were shown to control different kinds of genes: Peak fold change (Lee et al 2014), speed (Ando et al 2022), duration (Sen et al 2020), oscillation (Cheng et al 2021). This prior work establishes the biological significance of signaling codons. Based on that, we posit that measuring their deployment is of interest, even if in specific polarizing conditions it is not experimentally tractable to determine whether the specific changes are biologically significant.
(b) What are the signaling mechanisms that generate the changes in signaling codons? While there is an extensive literature about molecular mechanisms that affect NFκB signaling (reviewed in introduction and discussion), it is not currently possible to determine at the single cell level which mechanisms are responsible. That is because the NFκB dynamics are insufficient to uniquely identify all kinetic parameters that are responsible; additional measurements such as kinase activities, complex formations etc. are required, but those can only be done in bulk assays. In the manuscript, we describe the strategy that allowed us to side-step the parameter non-identifiability problem by limiting the number of free parameters and assigning several parameter fits to each cell. This side-step still allows us to address the question we posed: whether inferred parameters can be used as means of dimensionality reducing the trajectories to map cell states. But it does not allow us to determine with confidence all of the biochemical rate constants that are responsible for altering the signaling codons.

Specific comments related to Figure 7:

For Fig. 7F they the authors state, "We found with both the parameter and feature KNN, IL10 polarized cells have on average the greatest proportion of their own polarization state in their neighborhoods, followed by IFNγ cells which have a greater proportion of their neighborhoods occupied by IFNβ cells." Is the last part about IFNβ cells being in the IFNγ neighborhood a typo or are the authors making a point that the two IFNs are quite similar?

We apologize for this confusion. The reviewer's interpretation is correct in that several IFNγ cells are neighbors to IFNβ cells, hence similar in their signaling codons or inferred biochemical parameters. There is a smaller average proportion of IFNγ cells in IFNγ neighborhoods compared to IL10 cells in IL10 neighborhoods. This smaller proportion is due to some IFNβ cells in IFNγ neighborhoods.

The differences shown in the parameter distributions and the UMAPs in Fig. S7C and Fig. 7G are subtle. At the very least, the distributions should be tested to see if they are significantly different across stimuli.

Yes, we agree they are subtle, and in our minds this is indeed one of the key points of interest: very small parameter changes can affect the stimulus-responsive NFκB dynamics. In fact, the cell-to-cell heterogeneity of a parameter may be larger than the change of the median induced by a polarizing cytokine. We have also added statistical testing of the parameter distributions we highlight in the results text which do demonstrate the differences mentioned are statistically significant.

Given that the first parameter in Fig. 7G (IkBa mRNA synthesis) is higher in IL-10, but the next parameter (IkBa protein degradation) is lower in IL-10, could the different directionality of these closely related parameters by be due to non-identifiability in the model?

This is an interesting point. From steady-state observations, the two parameters (the Km of NFκB induced synthesis of IκBα, and the IκBα protein degradation rate) would not be identifiable, however we are using the stimulus-response timecourse to fit parameters. The precise balance of the two parameters is actually an important control point for stimulus-responsiveness (O'Dea, et al Mol Cell 2009). Furthermore, for all our analyses we did not consider only a single parameter fit, but rather a collection of parameter fits that reflect the uncertainty in identified parameters

The authors have made some predictions about biochemical differences resulting from cytokine treatment ("polarization") but do not pursue these predictions. For example, can they use these parameter differences (altered cell state) to predict how the cytokine pre-treatment will impact response to stimuli other than Pam3CSK4? Can staining for relevant proteins (IkBa, TLR2, etc) be used to validate the single cell predictions?

We thank the reviewer for this question and interest. We do wish to highlight that there are model predictions mentioned in the text that align with prior studies (increased IκBα degradation rate (Mitchell et al. 2019) and TLR2 synthesis (Matsuguchi et al. 2000, Sheu et al. 2023) with IFNγ), as predicted. However, as described above, a complete biochemical mechanistic inference is not possible with the current technologies. NFκB signaling dynamics are not sufficient to identify all parameters within the network. Hence, our results and discussion section has not focused on biochemical mechanism, but have focused on the kinetic parameters as a means of dimensionality reduction in order to map the cellular state space. This in itself is novel in the literature.

Reviewer #2: The authors have addressed my previous comments #1-3 and #6, but have not provided adequate responses to comments #4-5. Referring to unpublished data from a previous publication is insufficient to address the technical concerns that might have potentially affected the entire dataset.

We thank the reviewer for their continued interest. We have now included in Supplemental Figure 1 prior unpublished data that reviewers of previous manuscripts had reviewed.

For comment #4, I was hoping to see time course data or at least end point data (e.g. cell viability or death monitored with or without Hoechst dye under same imaging conditions). There are multiple ways to test the effects of Hoechst. Different time lapse intervals (delta T = 5 min, 15 min, or 1 hour) could be used for live imaging (each producing a varying radiation load of the short-wavelength laser), which would produce useful information about the photo-stress of UV laser imaging frequency on imaged cells. Alternatively, a direct comparison of imaging with Hoechst and SPY DNA dyes (not intercalating DNA; available in the red to far red spectral range) would also be useful in determining whether the signaling dynamics depend on the dye or whether there are differences in cell viability/death.

In the conditions we have used there is no change in cell death or other overt toxicity feature with various nuclear marker options (after optimization). This was previously reviewed in prior manuscripts from our group. To address the reviewer's concern, we have included a sample comparison of mock, LPS, or TNF signaling dynamics when either one of three different nuclear maker strategies are used (Supplemental Figure 1D): Hoechst dye, SiR-DNA dye (far red), and endogenously expressed H2B-mCherry. All three show comparable NFκB signaling dynamics. No artefactual signaling is observed using Hoechst dye. Thus, all are workable, but while H2B-mCherry may be preferred, Hoechst is a close second, while our experience with SiR-DNA dye with regards to shelf life / stability and need for Verapamil is less good.

For comment #5, I was hoping to see photobleaching kinetics data from a control imaging of mVenus-RelA without any stimulations. This can be done by a simple test with repeated imaging, in a fast time lapse (delta T can be small, e.g. 1 min, but the number of repeated imaging should be high enough to see signal decay). If significant decay is observed by, say, the 100th repetition of image captures, then the actual time lapse imaging should be spread and completed before reaching this threshold. Otherwise, the mVenus intensity would decay into non-linear ranges where it no longer is a faithful reporter of RelA concentration. For low endogenous protein signals from knock-in reporters such as the current one in this study, photobleaching and detection sensitivity become particularly important issues.

We have not seen concerning levels of photobleaching after optimizing our workflow. This was reviewed in prior manuscripts from our group. To address the reviewer's concern, we have included a dataset that shows that photobleaching has little impact on mVenus-RelA nuclear fluorescence and on the NFκB dynamics (Supplemental Figure 1E-F). There is a dip in the baseline upon stimulus addition, which we compensate for with appropriate baseline deduction, but there is very little decline in mVenus-RelA nuclear fluorescence in mock stimulation over 12 h. Furthermore, the mVenus-RelA fluorescence behaves similarly over 2 h when imaged with a 1 min frame rate (120 exposures) and 5 min framerate (24 exposures). Thus, photobleaching does not appear to affect the quantitation of NFκB dynamics in our workflow.

I do not recall seeing data addressing the above issues in previous publications from the Hoffmann group. If relevant data had already been shown in a previous publication that I missed, the authors can provide references to look up. If prior papers have only mentioned unpublished data, then the data need to be shown in a supplemental figure or at least shared with the reviewers. This reviewer believes that these are important issues for validity of the imaging data used in the current study.

We hope that the above will satisfy the reviewer's concerns. We apologize that we referred to prior papers. Previous reviewers were content in reviewing these controls within the review process and they did not enter supplemental materials due to space constraints.

In response to my previous comment #2, the new supplementary figure S1 shows a side-by-side comparison of the BMDM, and hMPDM. This is a very useful additional dataset, although the comparison was limited to 3 stimuli. A question: heatmaps suggest that all the cells are responding after TNF or LPS stimulation. Only for Poly(I:C) stimulation, a small fraction of cells seem non-responsive. However, in Figure 1D shows that for every condition, there is a significant fraction of cells which are non-responsive. Where does the discrepancy come from? Also, in Figure S1C, it will be better to show log2CPM of all the genes (not just LPS-induced ones) so that differences in basal gene expression can be assessed.

We thank the reviewer for their comments. In Figure S1 we focused on the signaling dynamics of NFκB and therefore focused on responding cells. In Figure 1D we included non-responding cells as they affect the downstream quantitation of stimulus-response-specificity.

For the analysis in Figure S1C we focused on stimulus-response genes. Given that the present analysis is exclusively about stimulus-responses, focusing on stimulus-response genes is appropriate. The steady state of cells grown in replicate conditions at different times is substantially more distinct. However, our work here is exclusively on stimulus-responses, and so stimulus-response gene expression is what is relevant.