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Toll-like receptor 4 is activated by platinum and contributes to cisplatin-induced ototoxicity

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DOI: 10.15252/embr.202051280

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Review Timeline:

Submission Date:	9th Jul 20
Editorial Decision:	31st Jul 20
Revision Received:	28th Jan 21
Editorial Decision:	15th Feb 21
Revision Received:	18th Feb 21
Accepted:	23rd Feb 21

Editor: Achim Breiling

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 31st Jul 2020

Dear Dr. Bhavsar,

Thank you for the submission of your research manuscript to EMBO reports. We have now received 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, all referees think that the findings are of interest, but they also have several comments, concerns and suggestions, indicating that a major revision of the manuscript is necessary to allow publication in EMBO reports. As the reports are below, and I think all points need to be addressed, I will not detail them here. In particular, all the points by referee #1 need to be addressed, and the downstream signalling pathways indiced by platinum need to be explored further (also mentioned by referee #2).

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

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

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

When submitting your revised manuscript, we will require:

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

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The Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. You can submit up to 5 images as Expanded View. Please follow the nomenclature Figure EV1, Figure EV2 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure

Legends after the main Figure Legends section. Additional Supplementary material should be supplied as a single pdf file labeled Appendix. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

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

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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

5) that primary datasets produced in this study (e.g. RNA-seq, ChIP-seq and array data) are deposited in an appropriate public database. This is now mandatory (like the COI statement). If no primary datasets have been deposited in any database, please state this in this section (e.g. 'No primary datasets have been generated and deposited').

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. Please note that the Data Availability Section is restricted to new primary data that are part of this study.

Data availability

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

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

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

Moreover, I have these editorial requests:

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

7) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at:
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8) Regarding data quantification and statistics, can you please specify, where applicable, the number "n" for how many independent experiments (biological replicates) were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. Please provide statistical testing where applicable, and also add a paragraph detailing this to the methods section. See:
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10) Please add a conflict of interest statement to the manuscript, next to the author contributions, and move both next to the acknowledgements at the end of the manuscript text..

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

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

Referee #1:

In this manuscript, Babolmorad et al. investigate the role of TLR4 in cisplatin-induced ototoxicity, a major complication of cisplatin-induced chemotherapy in children resulting in bilateral hearing loss. While TLR4 has previously been implicated with platinium compound-induced ototoxicity and cytotoxicity this previously was proposed to result from sensitization for LPS sensitivity by inducing

TLR4 expression (Oh et al., (2011), J Immunol, 186(2):1140-50) or TLR4 activation by release of endogenous damage-associated molecular patterns (DAMPs) such as HMBG1 (Tesniere et al., (2010) Oncogene. 29(4):482-91). The major merit of this study is the finding that platinium compounds, similarly to other metals or LPS, may directly activate TLR4 signaling but unlike those apparently do not require presence of the TLR4 receptor MD2 to trigger TLR4-mediated signaling. While potentially of interest, the provided study unfortunately fails to provide sufficient mechanistic insight and evidence for the claimed direct activation of TLR4 independently of MD2 by cisplatin. In particular, flaws in the experimental setup, statistical evaluation, lack of important controls and inconsistencies with findings of other groups decrease the potential impact of the study in its present form.

Major points:
1) Fig. 1. Given that the proposed direct activation of TLR4 by cisplatin is the key novel finding distinguishing the study from previous ones it is unclear why the authors decided to study NFkappa B activation and IL-8 production at such late time points (36-48h according to the materials and methods part). The authors should provide proper kinetics using the respective stimuli (cisplatin, Nickel, LPS) especially including early time points (6-8h) to support their claim that cisplatin directly activates TLR4. Since TLR4 activation usually include an early NF kappa B response via Myd88 and a late activation via TRAM-mediated signaling such kinetics experiments would also provide insight whether both arms are equally stimulated and allow comparison to other metals and LPS. Those kinetics experiments should also be complemented by analysis of the release of potential DAMPs such as HMBG1, which may allow exclusion of DAMP-mediated TLR4 activation to account for their findings. It is also not clear why the authors chose to employ such a low dose of Nickel (200 µM) in 1B despite the finding that this concentration only induced a minor 1.5 fold induction of NF kappa B activation in 1A and other groups consistently found nickel to be effective at doses {greater than or equal to}0.5 mM in the employed cell type. Similarly, LPS is usually employed at higher doses (>100ng/ml). It thus would also be helpful if at least one higher dose (1.0 mM Ni, 100 µg/ml LPS) was included in Fig. 1A to judge the relative capacity of platinium compounds to trigger NF kappa B activation in relation to established direct TLR4 agonists.

2) Statistical evaluation: In several of the figures is seems that statistics were calculated from technical replicates rather than from biological replicates. E.g. in Fig. 1 the authors state that data are derived from 2 (1A) or 3 (1B, C) independent experiments, whereas in 1D shown data are representative of two independent experiments. Yet, in the legend for the respective subfigures they state that data are derived from n=4 (A), n=20 (B), n=9 (C) and n=4 (D) experiments, which probably refers to the number of technical replicates in the single experiments. Please explain. Generally, statistical evaluation should be done comparing the means of at least 3-5 independent biological experiments than from multiple technical replicates in one representative experiment. Likewise, the SD of the means of biological replicates not of technical replicates should be provided throughout the manuscript to judge the reproducibility of the data.

3) The authors repeatedly speculate that Pt compounds may act similarly to other group 10 transition group metals. However, cisplatin also triggers TLR4-dependent responses in mouse cells that are non-responsive to nickel. It thus is unlikely that the mechanism is similar to that of other group 10 compounds. Nickel- and cobalt-induced TLR4 activation were previously shown to require presence of human-specific histidines at the dimerization interface of TLR4, which are missing in mice (Raghavan et al.(2012), EMBO Rep 13(12):1109-15, Schmidt et al.,(2010) Nat Immunol. 11(9):814-9). The authors should also discuss why in their experiments nickel could trigger TLR4-mediated signaling in absence of MD2 whereas two different groups independently reported a requirement of MD2 for TLR4-mediated signaling (Oblak et al., (2015) 24;10(3):e0120583,

Raghavan et al.(2012), EMBO Rep 13(12):1109-15). Given that IL-8 inductions in Fig. 2 A are relatively low, I suggest clarifying this issue by using HEK293 cells stably expressing TLR4 versus TLR4 together with MD2/CD14. In these experiments at least one higher nickel dose (e.g. 1.0 mM) should be included to verify MD2-independent TLR4 activation by cisplatin. The authors should also consider to include stimulations with HMBG1 to study the role of MD2 in DAMP-mediated TLR4 activation. In case HMBG1-induced TLR4 activation expectedly requires MD2 this would further support their conclusion that cisplatin activates TLR4 directly and not via DAMP release.

4) Fig. 3: The authors suggest that cisplatin-induced ototoxicity is mediated by TLR4-dependent ROS production. However, in Fig. 3A a significant difference in the apoptotic response is already evident at 50 µM cisplatin whereas differences in ROS production are only found at higher doses. How do the authors account for this result? What is the impact of antioxidants such as NAC on cisplatin-induced apoptosis in TLR4-/- and WT HEI-OC cells? What is the impact of other TLR4 agonists such as LPS on the viability of TLR4-/- and WT HEI-OC1 cells?

5) Given that TLR4 deficiency does not fully abrogate IL6 production in Hei-OC1 cells (Fig. 3C) the authors should investigate the impact of TLR4 deficiency on time-dependent IL-6 production and include the data in Fig. 4A

Minor points:
-Is there a reason why the authors chose different readouts for ROS detection in Fig. 3 and Fig. 6B?
-Recently, Bregio et al. reported that release of exosomes carrying the TLR4-binding DAMP HSP70 could protect hair cells from ototoxicity by aminoglycolytic drugs (J Clin Invest. (2020) 130(5):2657-2672. The authors should discuss this finding with respect to their proposed mechanism of cisplatin-induced TLR4 activation and its suggested role in ototoxicity.

Referee #2:

Cisplatin ototoxicity is a significant health issue, and preventing such ototoxicity is important for reducing the side effects of treatments of certain cancers. The manuscript by Ghazal Babolmorad et al., reports a study aiming at defining molecular mechanisms for cisplatin-induced cochlear damage. The authors demonstrate that Tlr4 plays a vital role in mediating cisplatin ototoxicity and that preventing Tlr4 activation alleviates the damage. Overall the experiments are well-conceived and carried out. The paper will be of significant interest to readers who are working on solving the issues of cisplatin ototoxicity.

I have one major point, and a few minor suggestions.

Major:
The authors should analyse also direct downstream events of Tlr4 activation, such as those associated with TLR4-MyD88 and TLR4-TRIF pathways.

Minor suggestions
Page 2, line 18: "identify" should be "identities".

Page Lines 14-15; A concentration of 15µM was chosen for subsequent experiments. Please provide the rationale for this selecting this concentration.

Figure 1, plot A. it is recommended to increase the space between the bars separating conditions, that is, increase the space between the second bar (LPS) and the third bar (Ni). So, readers can easily discern the groups associated with each treatment condition.

Figure 4 presents data from an experiment using Hela cells. However, the condition for using this cell line was not presented in the Methods section.

Figure 6. The data presented in plot B were derived from 2 samples (n=2). I am not sure how can a statistical analysis be performed with only 2 samples?

Referee #3:

Authors show in the manuscript that TLR4 is activated by platinum and contributes to cisplatin-induced ototoxicity describes activation of TLR4 by platinum and platinum containing compounds in a MD2/CD14 independent manner. Furthermore, they demonstrate that cisplatin-triggered TLR4 activation correlates with increase in IL8/IL6 cytokines, ROS production and cell viability. Inhibition of TLR4 activity by small compound TAK reverses the effect of cisplatin, suggesting possible means of inhibiting unwanted TLR4 activation by cisplatin.

Minor corrections:

First sentence of abstract might be rewritten:
TLR4 recognizes bacterial LPS and can also be activated by some....
One might not refer to a compound cisplatin as derivative of platinum, but rather a platinum-based compound.

Introduction:
P3,ine 17: name individual DAMPs, viral proteins and transition metals for better overview
Results:

P7, last paragraph: The authors are mentioning that cisplatin via TLR4 induces ROS formation, which was corroborated with experiments on cells with TLR4 deletion. However, is ROS production cisplatin-dependent, or rather a consequence of TLR4 activation? As experiment was set, one can not distinguish between cisplatin-TLR4-ROS and TLR4-ROS. A control of LPS-TLR4-ROS is required to clarify the dilemma.

Some explanation is required why in HEK cells IL8 was used as indicator of TLR4 activation and what is the reason for selecting IL6 in HEI-OC1 cells (and a reference should be included).

P12, line 21: instead of 'In aggregate' 'In summary' should be used.

P16, line 1: list the type of flow cytometer

P17, line 3-9: number of cells used in experiment

Fig 1A. Describe how the fold activity was calculated and what exactly is null for each experiment.

Fig 3A. The figure presenting viability and apoptotic cells should be generated differently for better

understanding. If I understand correctly % of apoptotic cells was calculated from number of viable cells. I suggest that you present only % of viable cells since to determine apoptotic cells different reagents should be used.

Please, explain what null (nil) represents (buffer, solvent-for cisplatin,TAK, metals) also for other figures.

Authors' Comments:

We thank the reviewers for their insightful comments and key issues they raised, which we have addressed in full point-by-point in this letter and in the revised manuscript. We believe that their comments and suggestions have strengthened the manuscript ensuring that it will be an important contribution to this field.

Referee #1:

Major points:
1) Fig. 1. Given that the proposed direct activation of TLR4 by cisplatin is the key novel finding distinguishing the study from previous ones it is unclear why the authors decided to study NFkappa B activation and IL-8 production at such late time points (36-48h according to the materials and methods part). The authors should provide proper kinetics using the respective stimuli (cisplatin, Nickel, LPS) especially including early time points (6-8h) to support their claim that cisplatin directly activates TLR4. Since TLR4 activation usually include an early NF kappa B response via Myd88 and a late activation via TRAM-mediated signaling such kinetics experiments would also provide insight whether both arms are equally stimulated and allow comparison to other metals and LPS. Those kinetics experiments should also be complemented by analysis of the release of potential DAMPs such as HMBG1, which may allow exclusion of DAMP-mediated TLR4 activation to account for their findings. It is also not clear why the authors chose to employ such a low dose of Nickel (200 µM) in 1B despite the finding that this concentration only induced a minor 1.5 fold induction of NF kappa B activation in 1A and other groups consistently found nickel to be effective at doses {greater than or equal to}0.5 mM in the employed cell type. Similarly, LPS is usually employed at higher doses (>100ng/ml). It thus would also be helpful if at least one higher dose (1.0 mM Ni, 100 µg/ml LPS) was included in Fig. 1A to judge the relative capacity of platinum compounds to trigger NF kappa B activation in relation to established direct TLR4 agonists.

We thank the reviewer for their comments and suggestions. We have conducted the experiments suggested by the reviewer and our data is consistent with a model where platinum and cisplatin induce TLR4 activity. We provide the details of these results below:

*We agree with the reviewer that previous studies using nickel and LPS have used higher concentrations in their experiments. As suggested by the reviewer, we investigated higher agonist concentrations in the HEK-hTLR4 IL-8 secretion assay, as this assay was more sensitive than the NF-κB SEAP reporter assay. We did observe enhanced activation of TLR4 at high LPS and nickel concentrations consistent with reports in the literature (*Schmidt et al. (2010) Nat Immunol. 11(9):814-9). *Notably, platinum and cisplatin compounds exhibit profound toxicity at concentrations >100µM in the HEK-hTLR4 cells that precluded our ability to test higher concentrations of these agonists and perform*

*direct concentration comparisons with LPS and nickel. Our investigations used cisplatin concentrations that are in line with therapeutic levels of this drug. Serum levels of cisplatin are reported to be 7-30 μM following infusion and 3-10 μM after 24 hours (Himmelstein et al. Clin Pharmacol Therapeut. 1981;29:658; Lanvers-Kaminsky et al. Pediatr Blood Cancer. 2006;47:183; Rajkumar et al. J Clin Diagnost Res. 2016; 10:7860). Accordingly, in the main manuscript, we used sub-millimolar concentrations of metal agonists to better facilitate comparisons with therapeutically-relevant concentrations of cisplatin. Nevertheless, extrapolating the activation of TLR4, particularly by Pt(IV), would be in line with that observed with high concentrations of LPS and nickel (see response to reviewers **Fig. RR1**).*

*We appreciate the insightful comments offered by the reviewer regarding the merits of kinetic experiments. We performed the suggested kinetic experiments, and here, to facilitate comparisons with platinum and cisplatin, we used lower concentrations of LPS and nickel. As shown **in Fig. RR2A**; Pt(IV) showed significant induction of IL-8 at 6 and 8 hr time points, which was comparable to LPS and nickel. Pt(II) also showed > 10 fold induction of IL-8 secretion at 6 and 8 hrs post-stimulation, compared to 3-fold or less for nil treated cells. We similarly observed significant induction of IL-8 secretion elicited by cisplatin at 6 and 8 hour time points (**Fig. RR2B**).*

Consistent with the kinetic data from HEK-hTLR4 cells, we observed significant increases in IL-6 secretion in HEI-OC1 cells within 4 hours post-cisplatin treatment that was dependent on Tlr4, an informative experiment suggested by the reviewer (see revised Fig. 4A,B).These data were supported by the results of cisplatin-induced signaling at early timepoints. As suggested by the reviewer we examined MyD88 and TRAM-mediated signaling events. Notably, our NF-kB reporter assay was not sensitive enough to allow kinetic analysis prior to 36 hours post-agonist treatment, therefore we assessed TLR4-MyD88 phospho-signaling events and observed p42/44 and NF-κB phosphorylation within 30 min. of cisplatin treatment (see Figure EV1A). Although we could not detect TRIF-dependent phosphorylation events within 30 min., we did observe activation of an IRF3-luciferase reporter within 24 hours of cisplatin treatment (see Figure EV1B). Thus, our results are consistent with the signaling kinetics noted by the reviewer and we thank them for this suggestion. These data demonstrate that cisplatin can activate TLR4 signaling at early time points and are consistent with reports in the literature of cisplatin activating NF-κB and ERK signaling (So et al. JARO, 2007,8:338; So et al. JARO, 2008,9:290; Chung et al. Acta Oto-Laryngologica, 2008,128:1063).

Our collective data strongly support that TLR4 is activated by cisplatin e.g. TLR4 activation in isogenic cell lines with and without TLR4; chemical inhibition of TLR4; genetic deletion of TLR4 in an outer hair cell line; gene silencing in zebrafish; and activation of signaling events downstream of TLR4. Our data describe MD-2 independent activation of TLR4 by cisplatin (see revised Fig. 2A),

marking an important distinction from the literature, where LPS was proposed to be a major contributor to cisplatin ototoxicity through TLR4. Other mechanistic details of TLR4 activation by cisplatin remain to be elucidated. We agree with the reviewer that we should avoid claiming that cisplatin "directly" activates TLR4, and we have carefully revised the manuscript to remove this inference. We have specifically discussed this point in the revised manuscript (page 12, lines 20-28). We also agree with the reviewer that further characterizing the role of potential DAMPs in cisplatin activation of TLR4 is of great interest to determine. Indeed, in a separate project we are systematically defining DAMPs elicited by cisplatin over long exposures through fractionation studies. While HMGB1 may play a role in this process, it is only one example of a potential DAMP, as pointed out by the reviewer, and more study will be required to exclude DAMP-mediated TLR4 activation.

2) Statistical evaluation: In several of the figures is seems that statistics were calculated from technical replicates rather than from biological replicates. E.g. in Fig. 1 the authors state that data are derived from 2 (1A) or 3 (1B, C) independent experiments, whereas in 1D shown data are representative of two independent experiments. Yet, in the legend for the respective subfigures they state that data are derived from n=4 (A), n=20 (B), n=9 (C) and n=4 (D) experiments, which probably refers to the number of technical replicates in the single experiments. Please explain.

The reviewer is correct that we used n to refer to all replicates in the experiment (technical replicates x biological replicates) in the previous version of this manuscript. We have revised the manuscript as suggested by the reviewer to clearly indicate the number of biological replicates as "n". All experimental data now include a minimum of 3 biological replicates. As indicated in the Author Guidelines, we have plotted actual individual data from each experiment where possible to better depict experimental variability (shown as box and whiskers for clarity). We thank the reviewer for pointing this out.

3. The authors repeatedly speculate that Pt compounds may act similarly to other group 10 transition group metals. However, cisplatin also triggers TLR4-dependent responses in mouse cells that are non-responsive to nickel. It thus is unlikely that the mechanism is similar to that of other group 10 compounds. Nickel- and cobalt-induced TLR4 activation were previously shown to require presence of human-specific histidines at the dimerization interface of TLR4, which are missing in mice (Raghavan et al.(2012), EMBO Rep 13(12):1109-15, Schmidt et al.,(2010) Nat Immunol. 11(9):814-9). The authors should also discuss why in their experiments nickel could trigger TLR4-mediated signaling in absence of MD2 whereas two different groups independently reported a requirement of MD2 for TLR4-mediated signaling (Oblak et al., (2015) 24;10(3):e0120583, Raghavan et al.(2012), EMBO Rep 13(12):1109-15). Given that IL-8 inductions in Fig. 2 A are relatively low, I suggest clarifying this issue by using HEK293 cells stably expressing TLR4 versus TLR4 together with MD2/CD14. In these

experiments at least one higher nickel dose (e.g. 1.0 mM) should be included to verify MD2-independent TLR4 activation by cisplatin. The authors should also consider to include stimulations with HMBG1 to study the role of MD2 in DAMP-mediated TLR4 activation. In case HMBG1-induced TLR4 activation expectedly requires MD2 this would further support their conclusion that cisplatin activates TLR4 directly and not via DAMP release.

We thank the reviewer for the excellent suggestion of using a stably expressing TLR4 cell line. We obtained a cell line that stably expresses TLR4 in the absence of MD-2 (we refer to this cell line as HEK-isoTLR4). We treated these cells with LPS, nickel chloride, cisplatin, platinum(II) chloride and platinum(IV) chloride after transfection with an empty vector or MD-2. MD-2 transfection acted as a control to confirm that TLR4 is active in the HEK-isoTLR4 cell line. As expected, LPS did not elicit significant IL-8 secretion unless transfected with MD-2. By contrast, cisplatin, platinum(II) chloride and platinum(IV) chloride all significantly induced IL-8 secretion in the absence of MD-2 transfection. We also observed no significant secretion of IL-8 in response to nickel chloride treatment in the absence of MD-2 transfection. We thank the reviewer for highlighting the literature on MD-2 dependency of TLR4 activation by nickel and we note that our results in a cell line that stably expresses TLR4 are consistent with these reports. Moreover, we also observed that HMGB1 treatment did not elicit significant IL-8 secretion in the absence of MD-2 transfection. As indicated by the reviewer, these data further support a model where cisplatin can activate TLR4 in a primary manner. These data have been included as Fig. 2A in the revised manuscript.

*We agree with the reviewer that cisplatin may activate TLR4 through a mechanism that differs from the reported histidine-mediated metal activation of human TLR4. Our findings also raise the possibility that platinum activation of TLR4 differs from nickel because our data suggest that platinum is not dependent on MD-2 to activate TLR4 (Fig. 2A). Furthermore, platinum(IV) chloride and cisplatin activate mouse TLR4 suggesting they do not strictly require His 456/458 (see **Fig. RR3**). Nevertheless, this needs to be further investigated and we are targeting a follow-up manuscript to describe the cisplatin mechanism of TLR4 activation re: role of histidines, species-specificity and role of DAMPs. As suggested by the reviewer we have included a section to the discussion that contrasts nickel and platinum's requirement for MD-2 (page 12, lines 13-19):*

"Our in vitro analyses showed that LPS and HMGB1 required MD-2 for significant TLR4 activation, which is consistent with the literature (Kawai & Akira, 2006; Yang et al, 2015). By contrast, cisplatin, platinum(II) and platinum(IV) were able to activate TLR4 signaling in the absence of the TLR4 co-receptor, MD-2. This is notable because it has been reported that this co-receptor is required for effective TLR4 activation by nickel, suggesting possible functional differences within the group 10 metals in their capacity to activate TLR4 (Oblak et al., 2015; Raghavan et al., 2012)."

4) Fig. 3: The authors suggest that cisplatin-induced ototoxicity is mediated by TLR4-dependent ROS production. However, in Fig. 3A a significant difference in the apoptotic response is already evident at 50 μM cisplatin whereas differences in ROS production are only found at higher doses. How do the authors account for this result? What is the impact of antioxidants such as NAC on cisplatin-induced apoptosis in TLR4-/- and WT HEI-OC cells?

In response to the reviewer's comments we used a more sensitive ROS reagent as a standardized method in this revised manuscript (Total ROS-ID instead of DCFD-HA). This has allowed us to detect ROS formation at lower cisplatin concentrations (20μM) which is in line with the significant change in Annexin V^{+} cells observed at 33μM cisplatin (revised Fig. 3B). These data have been included in Fig. 3C in the revised manuscript.

It has been reported that the antioxidants α-tocopherol and erdosteine reduce cisplatin induced apoptosis in wild-type HEI-OC1 cells (Kim et al; Toxicol Appl Pharmacol 2015; 288:192 and Kim et al; Int J Pediatr Otorhinolarngol 2016; 86: 9). As we did not detect substantial apoptosis in TLR4$^{-/-}$ cells until very high concentrations of cisplatin (100μM, see Fig. 3B), the effects of antioxidants in this cell line might be equivocal.

5) Given that TLR4 deficiency does not fully abrogate IL6 production in Hei-OC1 cells (Fig. 3C) the authors should investigate the impact of TLR4 deficiency on time-dependent IL-6 production and include the data in Fig. 4A

We thank the reviewer for this suggestion. We transfected our TLR4$^{-/-}$ cells with an empty vector plasmid or a plasmid expressing mouse Tlr4 and treated these cells with 20μM cisplatin for 0, 6 and 8 hours. We quantified secreted IL-6 and observed significant secretion when cells were complemented with mouse TLR4 but no significant secretion with the empty vector. These data are included as Fig. 4B in the revised manuscript.

Minor points:
-Is there a reason why the authors chose different readouts for ROS detection in Fig. 3 and Fig. 6B?

We thank the reviewer for raising this point. In the revised manuscript we have standardized our ROS detection reagent throughout the manuscript. Due to COVID-19 restricting access to our flow core facility, we have used both flow cytometry and fluorescence quantification (platereader), to quantify the same reagent.

-Recently, Bregio et al. reported that release of exosomes carrying the TLR4-binding DAMP HSP70 could protect hair cells from ototoxicity by aminoglycolytic drugs (J Clin Invest. (2020) 130(5):2657-2672. The authors should discuss this finding with respect to their proposed mechanism of cisplatin-induced TLR4 activation and its suggested role in ototoxicity.

We thank the reviewer for highlighting this recent publication. As suggested by the reviewer we have commented on this manuscript in the discussion (page 13, lines 19-32):

*"*Recently Breglio *et al* reported that sensory hair cells could be protected from aminoglycoside-induced ototoxicity through exosome-mediated activation of TLR4 by the DAMP, HSP70 (Breglio *et al*, 2020). This work, and our data, position TLR4 as a critical mediator of ototoxicity. Here we show that TLR4 contributes to cisplatin-induced ototoxicity, while Breglio *et al* studied aminoglycoside-induced ototoxicity. It is notable that in contrast to cisplatin, as we report here, aminoglycosides are not known to activate TLR4 as a primary event. Moreover, Breglio *et al* reported that the TLR4-activating DAMP, HSP70 required an exosomal context to mediate otoprotection through TLR4, which raises the possibility that other factors in the exosome may modulate TLR4 responses. In their report, Bregio et al. did not characterize the signaling event downstream of TLR4 that correspond to HSP70-mediated protection from aminoglycoside-induced ototoxicity. However, it is known that TLR4 can activate both pro-inflammatory signaling (NF-κb, MAPK) and anti-inflammatory signaling (PI3K, AKT)(Siegemund & Sauer, 2012).*"*

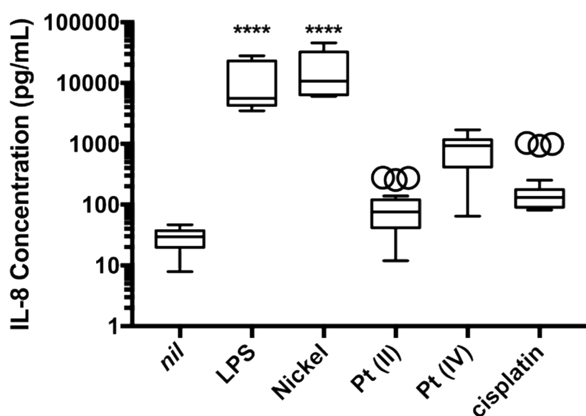

Fig. RR1. Selective high concentration agonist treatment of HEK-hTLR4 cells. Human embryonic kidney cells that express TLR4 (hTLR4) were unstimulated (*nil*) or treated with LPS (100 ng/mL), nickel chloride (1 mM), platinum (II) chloride (100 ∝M), platinum (IV) chloride (100 ∝M) or cisplatin (100 ∝M). Secreted IL-8 was monitored as a metric of TLR4 activation. Data are shown as box and Tukey whiskers from n= 3-7 independent experiments. **** denotes *P*<.0001 by one-way ANOVA with multiple comparisons to *nil* treatment performed using Dunnett's test.

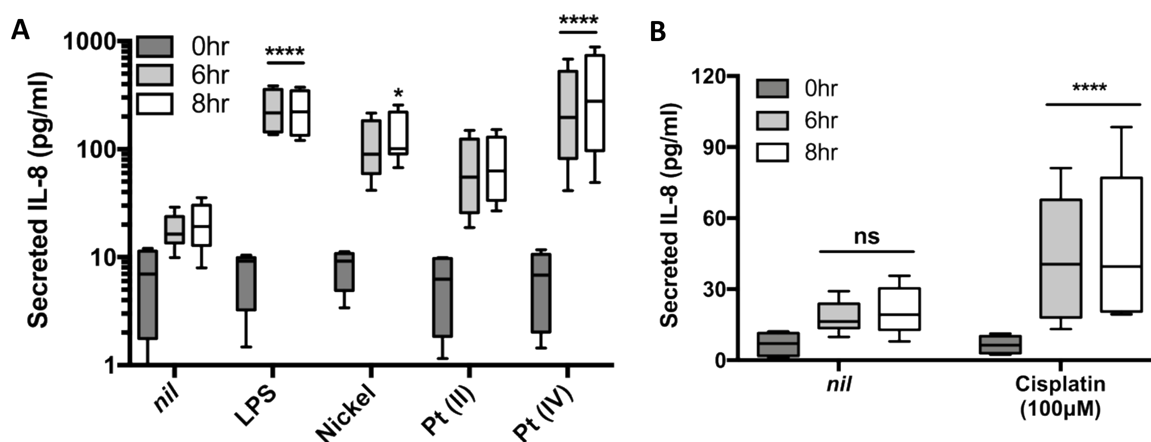

Fig. RR2. Kinetic analysis of agonist treatment of HEK-hTLR4 cells. A) Human embryonic kidney cells that express TLR4 (hTLR4) were unstimulated (*nil*) or treated with LPS (50 pg/mL), nickel chloride (200 ∝M), platinum (II) chloride (100 ∝M) or platinum (IV) chloride (100 ∝M). Secreted IL-8 was monitored as a metric of TLR4 activation after the indicated time point. B) The experiment was performed as in A) except cells were left untreated (*nil*) or treated with 100∝M cisplatin. Data are shown as box and Tukey whiskers from n= 3 or 4 independent experiments. ns, not significant; *, *P*<.05; ***, *P*<.001; ****, *P*<.0001 by 2-way ANOVA with multiple comparisons to 0hr time points performed using Dunnett's test.

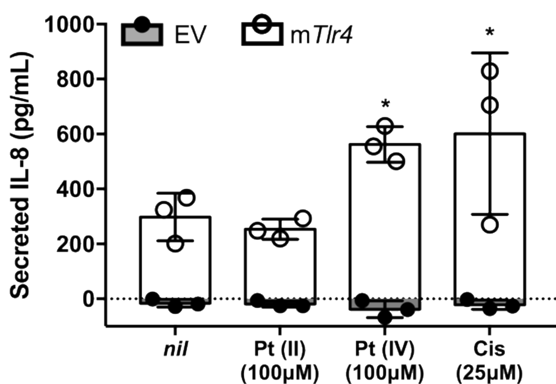

Fig. RR3. Activation of murine TLR4 by platinum and cisplatin. Human embryonic kidney cells that do not express TLR4 (HEK-null2) cells were transfected with empty vector (EV; shaded) or mouse *Tlr4* (m*Tlr4;* open). Transfected cells were unstimulated (*nil*) or treated with platinum (II) chloride, platinum (IV) chloride or cisplatin at the indicated concentrations. Secreted IL-8 was monitored as a metric of Tlr4 activation. Data are presented with mean and standard deviation indicated from n= 3 independent experiments. *, *P*<.05 by 2-way ANOVA with multiple comparisons to *nil* treatment performed using Dunnett's test.

Referee #2:

Major:
The authors should analyse also direct downstream events of Tlr4 activation, such as those associated with TLR4-MyD88 and TLR4-TRIF pathways.

We thank the reviewer for this suggestion, we agree that this is an interesting area for further study. We have conducted experiments to examine cisplatin activation of TLR4-MyD88 and TLR4-TRIF pathways. We used phospho-specific antibodies to detect phosphorylation events at 0, 10, 20 or 30 minutes post cisplatin treatment. We observed increases in NF-kB and p42/44 phosphorylation after cisplatin treatment. These data are included in the revised manuscript as Figure EV1A and demonstrate that cisplatin can activate TLR4 signaling at early time points and are consistent with reports in the literature of cisplatin activating NF-kB and ERK signaling (So et al. JARO, 2007,8:338; So et al. JARO, 2008,9:290; Chung et al. Acta Oto-Laryngologica, 2008,128:1063). We did not observe appreciable phosphorylation of IRF3 within 30 min but did observe activation of an IRF3-reporter 24hr post-treatment (see Figure EV1B). These data suggest that cisplatin can activate TLR4-MyD88 signaling at early time points and TLR4-TRIF signaling at later time points.

Minor suggestions
Page 2, line 18: "identify" should be "identities".

As suggested by the reviewer we have replaced “identify” with “identifies”.

Page 9 Lines 14-15; A concentration of 15μM was chosen for subsequent experiments. Please provide the rationale for this selecting this concentration.

As requested by the reviewer we have now included the rationale for using 15μM cisplatin in zebrafish experiments. The following sentences are included on Page 10, lines 1-5:

“A concentration of 15 μM was chosen for subsequent experiments because the dose response curve indicated that this concentration yielded significant, but not total, loss of neuromast cell viability as determined through DASPEI staining. This concentration also yielded consistent results in morpholino experiments.”

Figure 1, plot A. it is recommended to increase the space between the bars separating conditions, that is, increase the space between the second bar (LPS) and the third bar (Ni). So, readers can easily discern the groups associated with each treatment condition.

As suggested by the reviewer we have modified Fig. 1A to allow the reader to more easily discern the groups associated with each treatment condition.

Figure 4 presents data from an experiment using Hela cells. However, the condition for using this cell line was not presented in the Methods section.

We apologize for this omission and thank the reviewer for pointing this out. We have now included growth and treatment conditions for HeLa cell experiments (Page 14, lines 31-33; page 17, lines 11-16.)

Figure 6. The data presented in plot B were derived from 2 samples (n=2). I am not sure how can a statistical analysis be performed with only 2 samples?

We thank the reviewer for pointing out this inadvertent error. We have conducted additional experiments and performed a new statistical analysis on three biological replicates. These data have been included in a revised Fig. 6B.

Referee #3:

First sentence of abstract might be rewritten:
TLR4 recognizes bacterial LPS and can also be activated by some....
One might not refer to a compound cisplatin as derivative of platinum, but rather a platinum-based compound.

We thank the reviewer for these suggestions and have modified the revised manuscript accordingly (page 2, lines 2-3).

Introduction:
P3,ine 17: name individual DAMPs, viral proteins and transition metals for better overview

We thank the reviewer for these suggestions and have modified the revised manuscript as follows (page 3, lines 17-23):

"It is also widely accepted that TLR4 is activated by other agonists including damage-associated molecular patterns (DAMPs *e.g.* HMGB1 and HSP70) and the fusion protein from respiratory syncytial virus (Gaikwad *et al*, 2017; Kurt-Jones *et al*, 2000; Lee & Seong, 2009; Rallabhandi *et al*, 2012; Yuan *et al*, 2018). TLR4 was also found to mediate immune hypersensitivity reactions to the Group 9/10 transition metals nickel, cobalt and palladium (Rachmawati *et al*, 2013; Raghavan *et al*, 2012; Schmidt *et al*, 2010)."

Results:

P7, last paragraph: The authors are mentioning that cisplatin via TLR4 induces ROS formation, which was corroborated with experiments on cells with TLR4 deletion. However, is ROS production cisplatin-dependent, or rather a consequence of TLR4 activation? As experiment was set, one can not distinguish between cisplatin-TLR4-ROS and TLR4-ROS. A control of LPS-TLR4-ROS is required to clarify the dilemma.

We thank the reviewer for this comment. It is unclear if LPS-induces ROS production in HEI-OC1. We have examined the literature and found no reports that directly assess LPS-induced ROS production in HEI-OC1 cells. LPS has been shown to induce ROS in many cell types, via NADPH oxidase activation (Lee et al. Cell Communication and Signaling, 2012). It has also been reported that LPS induces nitric oxide synthesis, via iNOS, in HEI-OC1. By contrast, it is widely reported that cisplatin induces ROS in HEI-OC1 cells (Langer et al Trends Pharmacol Sci 2013;34:458; Brock et al. J Clin Oncol 2012;30:2408; Kim et al J Neurosci, 2010;30:3933). Unpublished data from our laboratory indicate that LPS does increase cellular ROS levels, but this was not significant compared to untreated cells.

Some explanation is required why in HEK cells IL8 was used as indicator of TLR4 activation and what is the reason for selecting IL6 in HEI-OC1 cells (and a reference should be included).

As suggested by the reviewer, we have now included a rationale for cytokine selection in HEK and HEI-OC1 cells, along with supporting references to the methods section (page 16, line 29 – page 17, line 5):

"As an alternate method of assessing TLR4 activation, IL-6 secretion was quantified in HEI-OC1 because this cytokine is a key mediator of cisplatin toxicity in HEI-OC1 cells (So *et al.*, 2007). IL-8 secretion was previously reported as marker of TLR4 activation in HEK-hTLR4 cells and was chosen for our experiments using related cell lines (Schmidt *et al.*, 2010). In addition, both IL-6 and IL-8 have been reported to be upregulated by cisplatin in human cells (Kiss *et al*, 2020), while mice do not contain a true gene ortholog for IL8 precluding its direct characterization."

P12, line 21: instead of 'In aggregate' 'In summary' should be used.

We have modified the manuscript according to the reviewer's suggestion (page 14, line 1).

P16, line 1: list the type of flow cytometer

We have included this information in the Methods section, as requested by the reviewer (page 19, lines 9-11):

"Samples were diluted with 400 μL Annexin V binding buffer and acquired on an Attune NxT flow cytometer (ThermoFisher Scientific)."

P17, line 3-9: number of cells used in experiment

We have revised this section to better describe the methods used to quantitate ROS and have included the cell numbers used in these experiments as suggested by the reviewer (page 19, lines 15 – 31).

Fig 1A. Describe how the fold activity was calculated and what exactly is null for each experiment.

As suggested by the reviewer we have modified the revised manuscript to indicate that NF-κB activity In Fig. 1A was calculated relative to cells treated with the vehicle. The isogenic control cell line that does not express TLR4 is designated HEK-null2 (commercial name). Here we use it as a control to show that NF-κB responses are TLR4-dependent. We have included this information on page 28, lines 5-8.

Fig 3A. The figure presenting viability and apoptotic cells should be generated differently for better understanding. If I understand correctly % of apoptotic cells was calculated from number of viable cells. I suggest that you present only % of viable cells since to determine apoptotic cells different reagents should be used.

> *As suggested by the reviewer we have modified Fig. 3A of the revised manuscript to depict viable cells as a separate graph. We now refer to the other cell population as Annex V^+/propidium iodide$^-$ cells, rather than apoptotic cells. These new graphs are shown in Figs. 3A and 3B.*

Please, explain what null (nil) represents (buffer, solvent-for cisplatin,TAK, metals) also for other figures.

> *As suggested by the reviewer we have clarified that null2 refers to an isogenic cell line in the HEK background that lacks TLR4 (commercial name). We use the term nil to describe conditions where cells are left untreated e.g. no agonist or small molecule addition.*

1st Revision - Editorial Decision 15th Feb 2021

Dear Dr. Bhavsar,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, the referees now support the publication of your study in EMBO reports.

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

- Please provide the abstract written in present tense.

- Please add all the funding information to the acknowledgements and make sure that equivalent and complete information is provided in our submission system.

- Please remove the sentence 'This PDF file includes: Main Text; Figures 1 to 6; Expanded View Figures 1 to 4' from the main manuscript text.

- Please also remove the ORCID information from the manuscript title page. This is stored in our system, and the names in the online version of the paper will be automatically linked with the ORCID.

- Please change the name of the section 'Methods' to 'Materials and Methods'.

- Please move the data availability section up in front of the acknowledgements.

- The images in EV3B look rather fuzzy and out of focus. Could these be replaced with higher resolution images. Moreover, please please scale bars of similar style and thickness to the microscopic images, using clearly visible black or white bars (depending on the background). Please place these in the lower right corner of the images. Please do not write on or near the bars in the image but define the size in the respective figure legend.

- Throughout the legends, please indicate if 'independent experiments ' refers to technical or biological replicates.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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

Kind regards,

Achim Breiling
Editor
EMBO Reports

Referee #1:

The authors have adequately adressed my concerns with the previous version of their manuscript and now provide convincing and statistically solid data that cisplatin can activate TLR4 in an MD2-independent manner.

They now also provide improved data showing that TLR4 activation contributes to cisplatin-induced ototoxicity. From the translational point of view especially their new finding that cisplatin can activate TLR4 independently of MD2 is interesting as this may allow the development of inhbitors to prevent cisplatin-induced ototoxicity without interfering with the function of TLR4 in bacterial responsiveness.
Overall I feel that the manuscript significantly improved during the review process, so that I have no longer reservations to recommend publication of the manuscript in EMBO Reports.

Referee #2:

The authors have adequately addressed my previous comments and suggestions. I have no more comments.

Referee #3:
The manuscript is suitable for publication in EMBO reports without revision.

The authors have addressed all minor editorial requests.

2nd Revision - Editorial Decision 23rd Feb 2021

Dr. Amit Bhavsar
University of Alberta
Canada

Dear Dr. Bhavsar,

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

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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Yours sincerely,

Achim Breiling
Editor
EMBO Reports

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Corresponding Author Name: Amit P. Bhavsar
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2020-51280V2

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

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

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B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	We performed at least 3 independent experiments that consisted of typically 3 technical replicates
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	N/A - larval experiments are not considered animal studies
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Samples were not excluded.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	N/A - larval experiments are not considered animal studies
For animal studies, include a statement about randomization even if no randomization was used.	N/A - larval experiments are not considered animal studies
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	Researchers are blinded to treatment prior to quantifying neuromasts
4.b. For animal studies, include a statement about blinding even if no blinding was done	Researchers are blinded to treatment prior to quantifying neuromasts
5. For every figure, are statistical tests justified as appropriate?	Comparison groups are indicated and the most appropriate statistical test was used. For comparisons to a control sample, Dunnett's test's were used. For comparisons between samples Bonferroni or Tukey tests were used.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We used D'Agostino & Pearson normality tests and all samples passed or were too small to analyze
Is there an estimate of variation within each group of data?	Variation is shown by Tukey whiskers or standard deviation error bars

Is the variance similar between the groups that are being statistically compared?	This is dependent on the experiment type, e.g. presence of small molecule inhibitor, nature of control samples. All groups have variance depicted graphically.

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Page 17; lines 18-32: Cell Signaling Technologies: anti-IRF3 (4302S), anti-P-IRF3 (4947S), anti-NF-κB P65 (8242S), anti-P-NF-κB P65 (3033L), anti-p42/44 (9102S), anti-P-p42/44 (9101S) or anti-beta-Actin (3700S); LiCOR Biosciences: IRDye 680RD Goat anti-Rabbit IgG secondary antibody (92568071), IRDye 680RD Goat anti-mouse IgG secondary antibody (92668020); Invitrogen: mouse anti-TLR4 (13-9041-80); Jackson Immunoresearch: Alexa-488 Fluor Goat anti-mouse IgG secondary antibody (115-545-146). All primary antibodies are listed on Antibodypedia except biotinylated TLR4 antibody.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Page 14, lines 18-33: HEK-Blue null2, HEK-Blue htlr4 and 293-htlr4A were obtained from Invivogen. HeLa cells were obtained from the ATCC. HEI-OC1 cells were obtained from Dr. Federico Kalinec (UCLA) under MTA agreement. Cells were tested for mycoplasma contamination by PCR and authenticated by the commercial providers, but not subsequently.

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

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Page 21, lines 2-7: Wildtype (AB strain) zebrafish were kept at the University of Alberta following a 14:10 light/dark cycle at 28°C cycle in standard conditions as previously described.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Page 21, lines 1-5: Zebrafish were raised, bred and maintained following an institutional Animal Care and Use Committee approved protocol AUP00000077, operating under guidelines set by the Canadian Council of Animal Care.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Compliance confirmed.

E- Human Subjects

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

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	Page 31, lines 10-11: A Data availability section has been included in the manuscript
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	N/A
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	N/A
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	N/A

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No.