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Murine cytomegalovirus IE3-dependent transcription is required for DAI/ZBP1-mediated necroptosis

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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 06 February 2017

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 support the publication of your paper in EMBO reports. Nevertheless, they have a number of concerns and/or suggestions to improve the manuscript, which we ask you to address in a revised manuscript. As the reports are below, I will not detail them here, also as I think that all points should be addressed. In particular, I feel that the manuscript would benefit greatly if data regarding the molecular nature of the RNA PAMP that activates DAI could be added, as outlined by referee #2 (e.g. in his point 1). Further, data from human cells would further strengthen the relevance of the findings.

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 a 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 or rejection 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; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

Regarding data quantification and statistics, can you please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends? This information must be provided in the figure legends. Please provide statistical testing where applicable.

Supplementary/additional data: 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 labeled Appendix. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure Sx throughout the text and also label the figures according to this nomenclature. For more details please refer to our guide to authors.

We now 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. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

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.

REFEREE REPORTS

Referee #1:

In the presented manuscript, Sridharan et al. investigate the molecule DAI/ZBP1 in cytomegalovirus-induced necroptosis. DAI is a molecule of outstanding interest as currently high impact journals have been focusing on this molecule that might explain the in vivo relevance of necroptosis better than the TNFR-family members. In this paper, the authors identify the IE3-dependent viral transcription to be required for necroptosis induced by DAI, thereby adding important novel information to our understanding of the signaling pathway of necroptosis and the role of necroptosis inhibition by viruses to unleash their full virulence. The authors use the most current readout systems available to investigate necroptosis (e.g. pMLKL). The biochemistry of this paper is generally acceptable with some highlights (such as Fig. 4). The design of the experiments, e.g. the newly generated mutants in Figures 4 and 5, is generally excellent. The paper answers a clear question and follows a straight line in the sequence of experiments - a very concise but still well-controlled setup. Therefore, my criticism is generally minor and concerns the interpretation and the need for providing a more general perspective on the presented data. In addition, the clinical consequences of these experiments are potentially tremendous and should be discussed.

Major concerns:
• Fig. 1: it is highly appreciated that the authors provide a short and a long exposition of the western blots of pMLKL. However, why is there a clear signal. If these cell do not succumb to necroptosis, how is it possible that pMLKL becomes positive, even upon longer exposure. Currently, the accepted model (although subject to severe discussions) may favor a pMLKL-containing pore that is capable of killing the cells. Please provide a working model for why these cells survive pMLKL positivity.
• Why would there be less MLKL expression in the M45mutant in Fig. 2C?
• The mutant z alpha 2 in Fig. S4D is somehow strange compared to the other mutants. Is there any good interpretation of this "intermediate" effect?
• vIRA does not require the RIPK1-RHIM. Is there potentially a IE3-dependent factor that inhibits the RIPK1-RHIM?

Minor remarks:
• There are concepts currently discussed that interpret necroptosis as a form of regulated necrosis which is comparably less potent, e.g. in comparison with ferroptosis or pyroptosis. How do the results of these authors fit into such concepts of necroinflammation?
• It is interesting that PFA does not prevent necroptosis in all cell lines tested. This implies that DNA replication is dispensable for necroptosis induction. What might be the driver of necroptosis in this setting? Does a mechanism like that explain the findings of the IAV-DAI pathway?
• The oligomerized pMLKL may form a pore directly in the plasma membrane, according to some authors. Others challenged this view. How do the experimental data presented here support either concept?
• In Fig. S2B, the significance should be included for all three groups, not only for the TZ control.

Referee #2:

In this work Upton and coworkers have explored the viral molecular entity stimulating ZBP1-dependent necroptosis during MCMV infection. The work follows a previous paper demonstrating that ZBP1 induces necroptosis during infection with the MCMV M45mutRHIM mutant (Upton, 2012). The authors find that MCMV-induced necroptosis is dependent RNA transcription and IE3-dependent viral transcription, but independent of viral DNA replication. Mutagenesis experiments demonstrate the Zalpha2 domain and the RHIM-A motif to be essential. Based on these experiments, the authors conclude that viral RNA transcripts are the activating ligand for ZBP1 during MCMV infection. The work is well designed, the results are solid, and the conclusions are supported by the data. The main unresolved question is the molecular nature of the RNA PAMP that enables the cell to distinguish self from non-self.

MAJOR POINTS
1. The authors should challenge their cells with different synthetic RNA PAMPs and characterize the ZBP1 agonist in more details (including Z-RNA if possible).
2. MCMV is a beta herpesvirus. It would be interested to also test an alpha-hepesvirus (e.g HSV-1) and a gamma-herpesvirus (e.g MHV68) to see whether they also induce necroptosis in a manner driven by viral transcripts.
3. All data are from mouse cells. There is little data linking human ZBP1 to Z-RNA-induced necroptosis in human cells. This is important, since the nucleic acid sensing field has revealed significant species differences.
4. In the discussion, the authors note that ZBP1 is involved in sensing nuclear replicating viruses but not viruses replicating in the cytoplasm. To at least start to address this point, Ssbcellular fractionation and confocal microscopy should be used to identify the subcellular location of ZBP1 in resting and infected cells.

Referee #3:

In this manuscript Sridharan et al examined the mechanism of MCMV-induced activation of DAI-mediated necroptosis. While previous work by the authors identified DAI as the inducer of necroptosis in response to infection with mutant MCMV lacking the M45/vIRA inhibitor, it remained unclear how MCMV triggers DAI activation. Here they show that neither the presence of virus in the cytoplasm nor new synthesis of viral DNA were required for M45mutRHIM MCMV-induced necroptosis. In contrast, inhibition of virus transport to the nucleus or inhibition of gene transcription protected cells from M45mutRHIM-induced necroptosis, suggested that transcription of the viral genome in the nucleus was required for DAI activation. Furthermore, using a virus expressing unstable IE3, a key activator of early viral gene transcription, they provide evidence that IE3-dependent transcription is important for activation of DAI-induced necroptosis. Finally, using cells reconstituted with different mutants of DAI they show that the RHIM1 of DAI and the two Za domains are critical for M45mutRHIM-induced necroptosis. The authors conclude that viral RNA transcripts activate DAI through its Za domains to induce necroptosis. Overall, this is a nice paper presenting convincing results that answer a central question on the mechanisms of virus induced DAI activation and necroptosis.

Specific comments

The authors conclude that the Za2 domain of DAI is critical for M45mutRHIM-induced necroptosis, but this is based on the finding that deletion of both Za domains prevented necroptosis while deletion of Za1 did not. Another interpretation of these results could be that the two Za domains are functionally redundant, therefore either the role of Za2 needs to be specifically addressed in deletion mutants lacking this domain, or the conclusions need to be more carefully drawn to include alternative interpretations of the data. In this context, it is interesting that mutations previously shown to abolish the RNA binding properties of the Za domains could not recapitulate the results obtained in Za deletion mutants. Data in Fig 5E-G and S4D indicate partial protection by Za2 mutation, but it would be helpful to include the Za1Za2 double mutant result in Fig S4D as it seems that mutation of both Za domains might have a stronger effect. Although it would be unfair to ask the authors to resolve this discrepancy in this manuscript, it would be important to discuss these findings more carefully, particularly when comparing this study with earlier work in IAV infection where the same Za2 mutations were sufficient to prevent necroptosis.

There is no information on statistics and reproducibility throughout the figures of the paper. This includes lack of information on the replicates used for the calculation of mean and SD in the different graphs (number of replicates, biological or technical), but also on how many times the experiments were independently repeated. This is essential information that needs to be included before considering the paper for publication.

The authors refer to unpublished data showing that IE3 overexpression could not induce necroptosis in DAI-expressing cells. It would be important to include these results in the manuscript to support the conclusion that IE3 or IE3-dependent transcription of host genes cannot activate DAI-induced necroptosis.

It would be helpful to include data showing the protein levels of IE3-DD in the presence or absence of shield in figure 4. Also, assessment of MLKL phosphorylation in IE3-DD mutant virus infected cells would further strengthen the data presented in this figure.

1st Revision - authors' response 10 April 2017

Referee #1:

In the presented manuscript, Sridharan et al. investigate the molecule DAI/ZBP1 in cytomegalovirus-induced necroptosis. DAI is a molecule of outstanding interest as currently high impact journals have been focusing on this molecule that might explain the in vivo relevance of necroptosis better than the TNFR-family members. In this paper, the authors identify the IE3-dependent viral transcription to be required for necroptosis induced by DAI, thereby adding important novel information to our understanding of the signaling pathway of necroptosis and the role of necroptosis inhibition by viruses to unleash their full virulence. The authors use the most current readout systems available to investigate necroptosis (e.g. pMLKL). The biochemistry of this paper is generally acceptable with some highlights (such as Fig. 4). The design of the experiments, e.g. the newly generated mutants in Figures 4 and 5, is generally excellent. The paper answers a clear question and follows a straight line in the sequence of experiments - a very concise but still well-controlled setup. Therefore, my criticism is generally minor and concerns the interpretation and the need for providing a more general perspective on the presented data. In addition, the clinical consequences of these experiments are potentially tremendous and should be discussed.

Major concerns:
• Fig. 1: it is highly appreciated that the authors provide a short and a long exposition of the western blots of pMLKL. However, why is there a clear signal. If these cell do not succumb to necroptosis, how is it possible that pMLKL becomes positive, even upon longer exposure. Currently, the accepted model (although subject to severe discussions) may favor a pMLKL-containing pore that is capable of killing the cells. Please provide a working model for why these cells survive pMLKL positivity.

We acknowledge that the inclusion of the long exposure in Fig 1 suggests that low levels of pMLKL may be present in cells that do not undergo necroptosis. It is likely that a critical threshold of

pMLKL may be necessary to undergo necroptosis. Importantly work by Doug Green and colleagues has revealed that the ESCRT-III machinery buffers cells from membrane damage (Gong et al., 2017, Cell, In press), and it is likely that this is the case in our necroptoissis sensitive cell lines. Also, efforts by the Silke and Murphy groups (Tanzer et al., 2015 Biochem J) group have demonstrated that MLKL phospho-mutants that efficiently induce pore formation in in vitro settings are not as effective in cells, suggesting that a minimum of activation is necessary to 'switch on' MLKL. It is also worth noting that previous investigations of Human CMV suppression of necroptosis revealed a block to the signal transduction pathway downstream of MLKL phosphorylation (Omoto et al., 2015 JBC). These studies also showed a 'basal' level of pMLKL in untreated and HCMV infected cells, which increases with TNF-zVAD-Smac mimetic treatment. While this doesn't resolve either issue mentioned above, it is similar to the very conspicuous increase of pMLKL over time in cells infected with M45mutRHIM virus (evident in both Fig 1 short and long exposures), and consistent with this MCMV inducing DAI-RIPK3-MLKL dependent necroptosis. We have added text (Discussion p15) to address the these issues.

• Why would there be less MLKL expression in the M45mutant in Fig. 2C?

We have now repeated this experiment for a fourth time, and continue to see slightly lower levels of total MLKL in Noc treated cells (new Figure 2C). We hypothesize that the lower MLKL expression is due to MLKL's known upregulation by Type I interferons. Blocking capsid transport to the nucleus with Noc treatment prevents viral infection and the early ISG inducing effects of CMV infection (Le et al., 2008). Figure 1a supports that levels of total MLKL do increase with infection with WT and M45mutRHIM. Although there seem to be slightly reduced levels of total MLKL, the significant reduction in levels of p-MLKL in Noc treated cells suggests that it is blocking MLKL activation.

• The mutant z alpha 2 in Fig. S4D is somehow strange compared to the other mutants. Is there any good interpretation of this "intermediate" effect?

While endpoint measurement indicate that DAI mutZa2 does induce necroptosis in response to MCMV-M45mutRHIM virus at levels similar to WT DAI (Figure 5D, F), Fig. S4D (now EV4D) suggests that the mutZa2 DAI shows a kinetic delay in the induction of death. These date support a role for these known nucleic acid binding residues in initiating necroptosis, but also indicate that a residual capacity to sense viral infection remains in the mutant, as indicated by the delayed kinetics of killing. We have modified the text (p12) to highlight this point.

• vIRA does not require the RIPK1-RHIM. Is there potentially a IE3-dependent factor that inhibits the RIPK1-RHIM?

Although is possible that MCMV encodes another IE3-dependent RIPK1 inhibitor, we have previously shown by multiple biochemical, chemical and genetic methods that RIPK1 plays no role in MCMV-induced, DAI/RIPK3/MLKL-dependent necroptosis (Upton et al 2010, Upton et al 2012, Kaiser et al 2014). However, additional functions for RIPK1 during MCMV remain relatively uncharacterized. vIRA does modulate pro-inflammatory signaling via RIPK1 and NEMO in a RHIM-independent manner (Krause et al., 2014), although the biological function for these other interactions in vivo have not been evaluated. We have addressed this possibility in the text (Discussion P19).

Minor remarks:
• There are concepts currently discussed that interpret necroptosis as a form of regulated necrosis which is comparably less potent, e.g. in comparison with ferroptosis or pyroptosis. How do the results of these authors fit into such concepts of necroinflammation?

The results presented here, nor our previous work, have addressed the potency of MCMV-induced necroptosis when compared to other immunogenic forms of death. Currently, we are not aware of any data regarding cytokine release, vascular permeability or inflammatory infiltrates during M45mutRHIM infection. We would, however, speculate it is possible that infection with M45*mut*RHIM could drive the characteristic auto-amplification of inflammation. Infection with WT

MCMV of HCMV would not be expected to elicit necroinflammation, due to the active inhibitor of necroptosis by these viruses.

• It is interesting that PFA does not prevent necroptosis in all cell lines tested. This implies that DNA replication is dispensable for necroptosis induction. What might be the driver of necroptosis in this setting? Does a mechanism like that explain the findings of the IAV-DAI pathway?

Actually, the reviewer's observations and assertions are at the heart of the manuscript. We apologize for failing to clearly illustrate that point, and have modified the text to stress this detail. The data from both PFA experiments and the IE3.DD virus demonstrate that DNA replication is dispensable, while the amanitin and IE3.DD data imply viral transcription generates the necroptotic signal. While the specific identity of the MCMV-derived DAI ligand remains to be determined, the results do align with our previous findings with IAV and the DAI pathway. We have made text changes throughout the manuscript in attempts to clarify this confusion.

• The oligomerized pMLKL may form a pore directly in the plasma membrane, according to some authors. Others challenged this view. How do the experimental data presented here support either concept?

We focused our current study on DAI activation by MCMV and have not investigated the distal execution phase of necroptosis, in part as this step is common among all pro-necroptotic stimuli and not unique to virus-induced necroptosis.

• In Fig. S2B, the significance should be included for all three groups, not only for the TZ control.

We have now performed statistical analyses on the requested groups and report them as not significant (ns).

Referee #2:

In this work Upton and coworkers have explored the viral molecular entity stimulating ZBP1-dependent necroptosis during MCMV infection. The work follows a previous paper demonstrating that ZBP1 induces necroptosis during infection with the MCMV M45mutRHIM mutant (Upton, 2012). The authors find that MCMV-induced necroptosis is dependent RNA transcription and IE3-dependent viral transcription, but independent of viral DNA replication. Mutagenesis experiments demonstrate the Zalpha2 domain and the RHIM-A motif to be essential. Based on these experiments, the authors conclude that viral RNA transcripts are the activating ligand for ZBP1 during MCMV infection. The work is well designed, the results are solid, and the conclusions are supported by the data. The main unresolved question is the molecular nature of the RNA PAMP that enables the cell to distinguish self from non-self.

MAJOR POINTS
1. The authors should challenge their cells with different synthetic RNA PAMPs and characterize the ZBP1 agonist in more details (including Z-RNA if possible).

Poly(dAdT) has been demonstrated to activate DAI/ZBP1 (Takaoka et al., 2007); however, the response was shown to interferon induction and not death and these finding remain controversial given the redundancy on nucleic acid signaling pathways. Synthetic nucleic acid PAMPs have so far proven too non-specific as they can lead to AIM2, cGAS, RIG-I, MDA-5, as well as DAI activation Thus, one would need to eliminate all other known nucleic acid sensors to isolate the contribution from DAI. It is also worth noting that the only stimuli capable of eliciting DAI-dependent death thus far has been viral infection (Upton et al., 2012: Thapa et al., 2017; Kuriakose et al., 2017), and may suggest that while a specific ligand is necessary, it may alone not be sufficient for triggering death. Thus, to date, viral infection is the only means to specifically activate DAI.

2. MCMV is a beta herpesvirus. It would be interested to also test an alpha-hepesvirus (e.g HSV-1) and a gamma-herpesvirus (e.g MHV68) to see whether they also induce necroptosis in a manner driven by viral transcripts.

We anticipate that MHV68, like other herpesviruses, encodes an as of yet unidentified inhibitor of necroptosis that prevents detection and/or death. Recent work from the lab of Astar Winoto demonstrated that MHV68 can induce necroptosis in L929 cells in a STING/TNF-dependent manner (Schock et al., 2017), suggesting that a DNA-trigger is sufficient to drive cytokine mediated death in infected cells. This would also suggest that an MHV68 encoded inhibitor would be upstream of RIPK3 activation, possibly at the level of DAI/ZBP1 itself. Unfortunately, without the identity of the inhibitor and recombinant viruses to study it, it will be difficult to interpret experiments of that nature with MHV68.

As for alpha-herpesviruses, we have previously shown that HSV-1 inhibits necroptosis via similar mechanism as MCMV, utilizing the RHIM in ICP6 to inhibit RIPK3-dependent necroptosis in human cells (Guo et al., 2015). HSV-1 is much more complicated in some regards than MCMV in that the RR1 triggers necroptosis in mouse cells, but blocks necroptosis in human cells. Given the species-specific complexity, we are submitting a separate manuscripts on HSV-1. We have added text to the manuscript to address the reviews comment (p16).

3. All data are from mouse cells. There is little data linking human ZBP1 to Z-RNA-induced necroptosis in human cells. This is important, since the nucleic acid sensing field has revealed significant species differences.

We appreciate the reviewers comment. The significant difference in virus- and species-specific cell death is quite intriguing, and purported lack of function for DAI/ZBP1 in human cells lends itself to questions of conservation between organisms. Moreover, our previous work has also shown that M45*mut*RHIM virus infection of human foreskin fibroblasts expressing RIPK3 results in necroptosis (Omoto et al., 2015), indicating that this pathway is intact and active in human cells. MCMV is able to enter and initiate gene expression in human fibroblasts, but fails to progress to DNA replication, which is consistent with the results presented here. In addition, we have now included new Extended View data showing that human HT-29 cells undergo M45*mut*RHIM induced necroptosis in a DAI dependent manner (Figure EV4C,D). We also currently have a manuscript under consideration detailing the role of DAI/ZBP1 in HSV1 induced necroptosis in human cells, further supporting a role for DAI in sensing viruses in human cells. We have included text in the manuscript to clarify this point (Results p12-13)

4. In the discussion, the authors note that ZBP1 is involved in sensing nuclear replicating viruses but not viruses replicating in the cytoplasm. To at least start to address this point, Ssbcellular fractionation and confocal microscopy should be used to identify the subcellular location of ZBP1 in resting and infected cells.

We have included an additional figure panel (Fig. 2E) showing the accumulation of DAI in the nucleus in response to viral infection utilizing subcellular fractionation as suggested by the reviewer. We have included additional text in the manuscript to this effect (Results p7-8)

Referee #3:

In this manuscript Sridharan et al examined the mechanism of MCMV-induced activation of DAI-mediated necroptosis. While previous work by the authors identified DAI as the inducer of necroptosis in response to infection with mutant MCMV lacking the M45/vIRA inhibitor, it remained unclear how MCMV triggers DAI activation. Here they show that neither the presence of virus in the cytoplasm nor new synthesis of viral DNA were required for M45mutRHIM MCMV-induced necroptosis. In contrast, inhibition of virus transport to the nucleus or inhibition of gene transcription protected cells from M45mutRHIM-induced necroptosis, suggested that transcription of the viral genome in the nucleus was required for DAI activation. Furthermore, using a virus expressing unstable IE3, a key activator of early viral gene transcription, they provide evidence that IE3-dependent transcription is important for activation of DAI-induced necroptosis. Finally, using cells reconstituted with different mutants of DAI they show that the RHIM1 of DAI and the two Za domains are critical for M45mutRHIM-induced necroptosis. The authors conclude that viral RNA transcripts activate DAI through its Za domains to induce necroptosis. Overall, this is a nice paper presenting convincing results that answer a central question on the mechanisms of virus induced DAI activation and necroptosis.

Specific comments

The authors conclude that the Za2 domain of DAI is critical for M45mutRHIM-induced necroptosis, but this is based on the finding that deletion of both Za domains prevented necroptosis while deletion of Za1 did not. Another interpretation of these results could be that the two Za domains are functionally redundant, therefore either the role of Za2 needs to be specifically addressed in deletion mutants lacking this domain, or the conclusions need to be more carefully drawn to include alternative interpretations of the data. In this context, it is interesting that mutations previously shown to abolish the RNA binding properties of the Za domains could not recapitulate the results obtained in Za deletion mutants. Data in Fig 5E-G and S4D indicate partial protection by Za2 mutation, but it would be helpful to include the Za1Za2 double mutant result in Fig S4D as it seems that mutation of both Za domains might have a stronger effect. Although it would be unfair to ask the authors to resolve this discrepancy in this manuscript, it would be important to discuss these findings more carefully, particularly when comparing this study with earlier work in IAV infection where the same Za2 mutations were sufficient to prevent necroptosis.

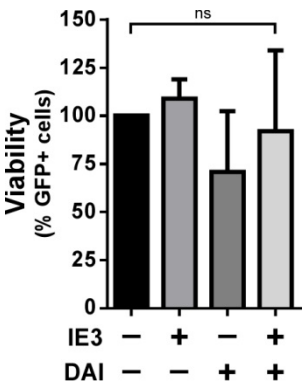
We thank the reviewer for pointing out this oversight. We have now included the mZa1a2 data in Figure EV4B as requested. We have now also characterized a deltaZa1/mutZa2 mutant DAI and its role in virus induced necroptosis. Our findings demonstrate that the Za1 and Za2 are not functionally redundant (Figure EV4E). We have also modified the manuscript to more carefully discuss the roles of Zα1 and Zα2 (Discussion, p16).

There is no information on statistics and reproducibility throughout the figures of the paper. This includes lack of information on the replicates used for the calculation of mean and SD in the different graphs (number of replicates, biological or technical), but also on how many times the experiments were independently repeated. This is essential information that needs to be included before considering the paper for publication.

Information on Statistical analyses, replicates and reproducibility has been added to the figure legends and Materials and Methods.

The authors refer to unpublished data showing that IE3 overexpression could not induce necroptosis in DAI-expressing cells. It would be important to include these results in the manuscript to support the conclusion that IE3 or IE3-dependent transcription of host genes cannot activate DAI-induced necroptosis.

We have re-evaluated, and repeated, our unpublished data concerning IE3 expression and necroptosis and concluded . While our conclusion has not changed, and we maintain that IE3 overexpression does not induce DAI-dependent necroptosis (Fig. Rev1), these negative data are not sufficiently strong enough to support the strongly worded statement we included in the original draft of the manuscript. Additional experiments will be necessary to positively exclude a role of IE3 protein in necroptosis, and are not feasible within the allotted time of revision. We have altered the text to address this (Discussion p19).



Reviewer Figure 1. DAI knockout 29-11 cells were transfected with the expression plasmids indicated and GFP. 24hours post transfection, cells were fixed and observed by fluorescence microscopy. GFP positive cells were counted (10-15 fields/condition), and percent viability normalized to vector alone transfections. n.s., not significant ($p>0.05$) by one way Anova analysis with Dunnett's multiple comparisons test, compared to EV. Error bars indicate standard deviation from the mean (SD; n=4 biological replicates).

It would be helpful to include data showing the protein levels of IE3-DD in the presence or absence of shield in figure 4. Also, assessment of MLKL phosphorylation in IE3-DD mutant virus infected cells would further strengthen the data presented in this figure.

We have included an additional Expanded view figure panel (EV 4D) showing expression of IE3-DD and E1 in infected cells in the presence or absence of Shield-1. We have included additional text to this effect (Results p9-10)

2nd Editorial Decision 02 May 2017

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 that you will find enclosed below. As you will see, all three referees support the publication of your manuscript in EMBO reports. However, referee #3 has some final minor concerns, we ask you to address in a final revised version.

Further, I have these editorial requests:

The title is currently too long. Please provide a new title with not more than 100 characters (including spaces).

Please upload table EV1 as single file and remove the table from the main manuscript file. But, add a legend for the table to the figure legends in the main manuscript. Also change the callout for the table in the manuscript text from Table S1 to Table EV1. Please remove the file "Expanded View Info" from the manuscript files, as the EV legends are already contained in the main manuscript text.

Further, I think the "A" in the legend to Fig. EV1 can be deleted. Finally, in the legend of Figure EV4 please change "G" to "E" (and check if this is called out correctly in the 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.

REFEREEE REPORTS
Referee #1:

The authors are congratulated to moving the field of necroptosis research a significant step forward! The paper is suitable for publication in this journal.

Referee #2:

The authors address the points raised by me mainly by arguing, rather than adding new data. However, I find the explenations (and additions to the text) acceptable, and have no further comments.

Referee #3:

The authors addressed the main points raised.

Some information is still missing on the replicates used for the calculation of mean and SD in the graphs shown in Figs 1B,C, 2B, 3B,D, 4C,D and EV2A.

On page 17 the reference to Figure 4D, F should be Figure 5D, E.

2nd Revision - authors' response 02 May 2017

Enclosed, please find a second revised version of our manuscript under consideration for publication at *EMBO Reports* [EMBOR-2017-43947V3]. We are very excited that the referees viewed our initial revisions favorably, and we appreciate the opportunity to address the minor points raised by Reviewer #3 and fix the editorial issues you described. As requested, we have altered the title of the manuscript to be "Murine cytomegalovirus IE3-dependent transcription is required for DAI/ZBP1-mediated necroptosis", which is 96 characters (including spaces). Below, we detail the rest of our responses to those concerns:

Editor concerns-

- The title is currently too long. Please provide a new title with not more than 100 characters (including spaces).
    - **Response:** We have altered the title to "Murine cytomegalovirus IE3-dependent transcription is required for DAI/ZBP1-mediated necroptosis"
- Please upload table EV1 as single file and remove the table from the main manuscript file. But, add a legend for the table to the figure legends in the main manuscript. Also change the callout for the table in the manuscript text from Table S1 to Table EV1. Please remove the file "Expanded View Info" from the manuscript files, as the EV legends are already contained in the main manuscript text.
    - **Response:** Table EV1 has been removed from main text to a separate uploaded document, and a legend added in the main text. Table description in text changed from S1 to EV1. Expanded View content file was removed from submission.
- the "A" in the legend to Fig. EV1 can be deleted. Finally, in the legend of Figure EV4 please change "G" to "E" (and check if this is called out correctly in the text).
    - **Response:** (A) in legend to Fig EV1 has been removed. In Fig. EV4, G has been changed to E, and has been confirmed within the main text.

Referee #3 -

- Some information is still missing on the replicates used for the calculation of mean and SD in the graphs shown in Figs 1B,C, 2B, 3B,D, 4C,D and EV2A.
    - **Response:** We have added information regarding biological replicates for Figs 1B,C, 2B, 3B,D, 4C,D and EV2A

- On page 17 the reference to Figure 4D, F should be Figure 5D, E.
    - **Response:** Text on p 17 fixed to read "(Fig. 5D, E)"

3rd Editorial Decision 05 May 2017

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.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

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Journal Submitted to:	EMBO reports
Manuscript Number:	EMBOR-2017-43947V1

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

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

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

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

2. Captions

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

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

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	SGenerally, sample size for these studies was n=3-4, which is used routinely in our groups and others in the field. Details are provided in figure legends. No power calculation was performed.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Not applicable
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Not applicable
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.	None.
For animal studies, include a statement about randomization even if no randomization was used.	Not applicable
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.	All quantitative data were collected by automated methods. Critical experiments were performed by multiple individuals to ensure reproducibility and avoid bias. Randomization was not perfomed.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Not applicable
5. For every figure, are statistical tests justified as appropriate?	Yes. T-tests were used to compare the means of two unmatched groups. One-way Anova was used to compare means fo three or more groups.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	Standard Deviation (SD) were used to estimate variation within groups where no less than three replicates were performed.
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

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

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	details provided in Methods Section p16 -17
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	details provided in Methods Sectionp16

* 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.	Not applicable.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Not applicable.
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.	Not applicable.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	Not applicable.
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.	Not applicable.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	Not applicable.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	Not applicable.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	Not applicable.
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.	Not applicable.
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.	Not applicable.

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under '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	Not applicable.
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).	Not applicable.
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).	Not applicable.
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	Not applicable.
22. 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.	Not applicable.

G- Dual use research of concern

23. 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.	Not Applicable.