EMBO reports

cGAS facilitates sensing of extracellular cyclic dinucleotides to activate innate immunity

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1st Editorial Decision 23 May 2018

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 the manuscript is of interest, but requires major revisions to allow publication in EMBO reports. All three referees 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.

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 or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

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When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision). Please insert page numbers in the checklist to indicate where the requested information can be found.
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
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Please also note that we now mandate that all corresponding authors list an ORCID digital identifier that is linked to their EMBO reports account!

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 this work, Liu et al. describe a novel function of cGAS, which they find is involved in the direct sensing of extracellular cyclic di-nucleotides (e.g. cGAMP) endocytosed by cells. The authors provide evidence that cGAS contributes to sensing of extracellular cGAMP/CDN by directly binding to it, and forming a perinuclear complex with STING. This discovery implies that the activity of cGAS is not restricted to nucleic acids sensing, and may also act as a scaffold for sensing of extracellular cGAMP/CDN by STING. It has important implications for the use of cGAMP as adjuvant or in cancer immunotherapies.
Although well-written, the line of thought of the manuscript is disjointed at times (see below), and

the story could be simplified to go more to the point. There are also a few issues which need addressing to strengthen the claims of the manuscript.

Major points:
1) The paper starts with a panel of cells lines which do not have cGAS or STING (e.g. HeLa and HEK293T do not have functional STING, and A549 also have been found not to have STING) - so the conclusion that this pathway only works in phagocytes is not supported. In fact, given that the ecGAMP can get internalised in HEK (Supp Figure 2D-F), it is most likely that it does not activate STING in this set up (unlike icGAMP), because cGAS is absent. Adding demonstration that cGAS expression in HEKSTING cells restores responsiveness to ecGAMP would strengthen the claims (and also open the door to an activity of ecGAMP in cancer cells which have retained cGAS function).

2: Line of thought. The paper is hard to follow at times, and a rearranging of the order of the figures could help make the point that this paper makes about cGAS being involved in ecGAMP sensing. It would make more sense to compare ecGAMP to icGAMP as early as in Figure 1A - which would have revealed the issue raised above. Assuming that HEK STING cells expressing cGAS are responsive to ecGAMP (suggested experience above), the early use of this point would immediately help orientate the story on the new role of cGAS.

As such, the following line would be more logical:
I) eCDNs can activate IFN production in human and mouse macrophages, although less than iCDNs
II) eCDNs activate IFN production through STING, but this response is not seen in HEK293T STING cells - although eCDN can enter these cells perfectly fine (as per S. Fig 2).
III) since HEK293T do not have cGAS, but macrophages do, investigation of the role of cGAS > cGAS expression in HEK STING restores sensitivity to eCDNs (assuming this works). Similarly cGAS deficiency in cGAS KO cells impacts eCDNs.
IV...) then look at endocytosis, binding of cGAS to CDNs, cGAS/STING complex/Dynein.

3) Some of the conclusions need to be toned down. cGAS and STING deficiency do not ablate sensing of eCDNs (hence the word "critical" is not appropriate). There is still a strong activation of IFN production in cGAS KO THP-1/BMMs (in Fig 3/7), and STING KO BMMs also still have a residual ~2 fold increase of IFN activity with cGAMP and c-di-AMP in fig 2D/E. In fact Fig 2C should be redrawn to show WT and STING KO BMMs separately (as the Y axis clearly mislead the potential low induction in KO BMMs). It would be more appropriate to change the claim to say that cGAS contributes to ecGAMP/CDN sensing.

4) The effect of DMA in Fig 2A is clearly significant in THP-1 cells and yet the text states that it did not affect ecGAMP uptake (this is incorrect). Fig 6E is really not convincing and should either be removed or a better blot shown. Fig 4B/F/G use poly(dA:dT) which can clearly signal independently of cGAS (through RIG-I for instance PMID: 19609254). This is a misleading control, which should be replaced by transfected ISD for instance.

5) Figure 5 indicates that c-di-GMP does not bind cGAS as well as c-di-AMP. This is in line with the observation that human cells were more sensitive to c-di-AMP in Fig 1. It would be interesting to repeat this experiment with mouse cGAS to see if it is more equally binding to c-di-GMP and c-di-AMP (which is suggest in Fig 1B/3).

6) The paper does not really provide evidence of the biological significance of their finding in response to naturally produced eCDN. The doses used in vitro are high, and it is hard to tell how this would be involved in sensing of cGAMP released by apoptotic cells/gut bacteria. This point should at least be discussed, but if the authors could substantiate activation through cGAS by extracellular CDN released by other cells it would strengthen their claims.

7) One of the issues of this work is that the study does not allow to know for sure whether the cGAMP made by cGAS upon CDN binding is required (although the need for catalytic activity suggest this). One way to address this could be to reconstitute mouse STING KO cells with STING mutants (e.g. R231) which are poorly responsive to c-di-GMP but can sense cGAMP. Again, this experiment would strengthen their argument.

Minor comments:
1- There are some sections of the Methods which introduce reagents not used anywhere... like the THP-1 cGAS shRNA cells... Where are they used? 2- Also, please clarify whether THP-1 were differentiated with PMA (how long) in all experiments?
3- There is mention of monocyte differentiation - where is this used?
4- the source of FITC-cGAMP is not provided.
5- The use of circles in the bar graphs should be consistent throughout. It cannot be used to show technical replicate in one graph, and mean of biological replicate in another (this is very misleading - e.g. Fig 1H). Also SD of technical replicate is useless - remove error bars when they refer to technical replicates.

Referee #2:

Innate responses to cyclic di-nucleotides (CDNs) are a timely topic, especially considering the growing interest in their use as adjuvants. In this manuscript, Liu and colleagues report an intriguing conclusion, saying that sensing of extracellular CDNs needs not only STING but also cGAS, and that cGAS and STING form a protein complex. This would constitute a new mechanism of how cGAS and STING operate and as such, would be of high interest to the community if appropriately supported by the data. However, this reviewer has concerns about a number of the experiments presented here and about the overall conclusion reached by the authors. Therefore, publication will require major revisions. For example, taking into consideration all the data presented in this manuscript, it is possible that CDNs, by binding to cGAS and inducing the formation of a dimer, facilitate DNA binding to cGAS, which in turn will lead to more cGAMP produced by cGAS that then activates STING via the canonical pathway.

Major points:

1. The authors do not rule out the possibility that CDNs facilitate sensing of DNA by cGAS. The authors should test if cGAS mutated in its DNA binding site still binds cGAMP and induces an IFN response to extracellular CDNs.
2. When comparing extracellular cGAMP with intracellular cGAMP, the authors find that intracellular cGAMP is much more potent in inducing IFN. It is possible that this strong induction is saturating in the case of intracellular cGAMP, thereby masking any effect of drugs (Fig 2 and 7) or cGAS/STING expression (Fig 4 and S2). The authors should therefore titrate down the concentration of intracellular cGAMP to normalize it with extracellular cGAMP in terms of the IFN induction.
3. Do the CDNs used in these experiments contain any contaminant that could stimulate cGAS such as DNA or manganese (Wang et al, Immunity, 2018)? The authors should stimulate THP-1 cells or mouse BMDMs with DNase treated CDNs. The authors should also repeat their experiments in TLR4 KO BMDMs to show that this IFN induction is not due to endotoxins.
4. Are FITC-cGAMP and Biotin-cGAMP bioactive? Quite a few experiments in this manuscript use FITC-cGAMP (for flow cytometry and immunofluorescence) or Biotin-cGAMP (for pull-down) but in order to validate these experiments, the authors should show that FITC-cGAMP and Biotin-cGAMP are as active as untagged cGAMP in terms of IFN induction, in particular in THP-1 cells.
5. This manuscript needs proper statistics. The authors need to do non-parametric ANOVA instead of t-test.
6. The authors should reconstitute HA-STING HEK 293T cells with cGAS to see if this restores induction of IFN by extracellular cGAMP.
7. The model proposed here raises an interesting question that the authors should discuss in the manuscript: why and how would there be 2 different mechanisms for sensing CDNs, i.e. following rupture of endosomes containing extracellular cGAMP as opposed to bacterial CDNs (also released from ruptured vesicles) and cGAS-derived cGAMP?

Minor points:
1. Authors should cite and discuss their finding in the context of the following papers: McFarland et al, Immunity,2017; Morchikh et al, Molecular Cell, 2017.
2. The authors show a lot of data represented as fold induction. They should include the corresponding raw data, in particular in experiments where STING and cGAS are overexpressed, in

order to show how this overexpression is affecting the basal expression of IFN.
3. This reviewer has some concerns regarding the RAW264.7 ISRE luciferase reporter assay: If RAW264.7 can detect cGAMP in the supernatant (Fig 1A), then how can this assay distinguish between IFN produced by mBMDMs stimulated with cGAMP and IFN produced by RAW246.7 upon sensing of cGAMP carry-over from the supernatant of mBMDMs? Could the cGAMP carry-over be responsible for the background of IFN in Fig 3D-E?
4. Fig 1, there are discrepancies between qPCR results and the RAW264.7 bioassay as well as the IFN ELISA in human monocytes: in Fig 1B, c-di-AMP induces less IFN than 2'3'-cGAMP but it seems to induce similar levels in the RAW264.7 bioassay. The same discrepancy can be seen in Fig 1G vs 1H in human monocytes. Could the authors explain this?
5. Fig 2A, the authors should show controls that the various drugs are efficient at blocking internalisation. In this figure, the difference between the DMSO treated cells and the poly-I treated cells should be significant. Moreover, the authors should clarify whether this internalisation assay has been done by flow cytometry and, if yes, the gating strategy needs to be supplied at least in the supplementary figures.
6. Fig 2C, the EEA1 staining looks like an aggregate close to the nucleus whereas it should look more like punctae found in the entire cytoplasm. Could the authors show a better picture?
7. In other figures showing IF stainings (Fig 3H, 5C, 7A), the authors should show the individual channels as bigger images as these are quite hard to see.
8. Fig 3, the authors should show the knock-down efficiency in THP-1 cells.
9. Fig 4C, authors should include a positive control such as poly(I:C).
10. Fig 4D, the authors use a linear cGAMP that they need to describe in the material and methods. This figure is also missing the data points for cGAS KO cells.
11. Fig 4F-G, could the authors show ecGAMP and icGAMP on the same blot? This is linked to major point number 2: the authors should repeat the experiment shown in Fig 4E with a lower dose of icGAMP and do a western blot where they would be able to do a side-by-side comparison of both conditions.
12. Fig 6A, the tubulin signal is too saturated and hides what the authors are trying to show. In the quantification, how were the perinuclear aggregates defined? It might be clearer if the authors showed that colocalisation of cGAMP with STING is impaired in the absence of cGAS.
13. Fig 6E: Could the authors show a better image for His-STING staining in GST-pulldown?
14. Fig 6D-E: It is unclear whether these experiments are IP or GST pull-downs? The authors should also do the reverse HIS pull-down in the same conditions.
15. Fig 6C and 6F only show that both cGAS and STING are able to interact with cGAMP but do not demonstrate a direct interaction between cGAS and STING.
16. Fig 7A, the tubulin and dynein signals are both saturated and the colocalisation with FITC-cGAMP is not convincing.
17. In Fig S4, the authors claim that cGAMP induces autophagy in these cells but this is not evident looking at the LC3-II induction in the western blot. The authors need to include a positive control that induces autophagy and is blocked by 3-MA.
18. In Fig 2B, the legend should read "ecGAMP+Dynasore" and "icGAMP+Dynasore". Same in Fig 7 "ecGAMP+Ciliobrevin D" and "icGAMP+Ciliobrevin D".
19. This reviewer is unable to judge the technical quality of Fig 5 F to H.

Referee #3:

In this manuscript, Liu et al. show that extracellular cyclic di-nucleotides (CDNs) such as cGAMP can enter cells and and stimulate the production of IFNb and IL-6 in human and murine macrophages. The response to extracellular CDNs was dependent uptake via clathrin-coated pits and detection by the DNA sensing adaptor STING, which is also senses intracellular CDNs. Unexpectedly, the authors find that the cytosolic DNA sensor cGAS is required for the sensing of extracellular CDNs but not CDNs delivered into the cytosol via digitonin. The catalytic residues of cGAS (required for the synthesis of cGAMP by cGAS) are essential for cGAS function in this context, but cGAS also seems to bind STING in the presence of cGAMP, so the authors propose that the cGAS-STING interaction is required for STING activation in this context.

Many of the experiments are reasonably well controlled and convincing - but the concept of cGAS-dependent extracellular CDN recognition raises some important questions about how this can

happen, without also affecting the detection of intracellular CDNs and cytosolic DNA. How would, after escape from endolysosomes, extracellular cGAMP be different from cGAMP introduced by digitonin permeabilisation, or indeed the cGAMP produced by cGAS itself? Or is this a positive feedback mechanism that amplifies STING activation under circumstances where the intracellular levels of CDNs are low (such as when only little cGAMP leaks from endolysosomes)?

1.) This manuscript relies heavily on the quantification of IFNb production by real-time PCR or ELISA etc. It is not always clear how these experiments were carried out (in terms of biological or technical replicates, or independent experiments) and what the averages and error bars represent. For instance, in Fig 1, it seems that the data are averages of several experiments, each performed in triplicate - so are the error bars SD of the averages of each triplicate (without taking into account individual variation of triplicates), SD of all individual values, or SEM based on the SDs of the triplicates?

2.) Data should also include the mock treatment - while the average may be normalised to 1, this data point would still have an error/variation associated with it, which would be useful for comparison with the other data points. Mock stimulation would be particularly important to see where wt and ko/kd cells are used (e.g. Fig. 3 and 4) - in that case real-time PCR or luciferase data should be normalised to mock treatment of wt cells, to see whether basal responses are affected by the ko/kd.

3.) In Fig 1A, the authors use 4h stimulation in macrophage-like cells (which response to ecGAMP), but 24h in other cell types (which don't respond) - all shown in the same chart. This is misleading - all cells should be tested at the same time point (4h). This is important, as after conventional stimulation with DNA or icGAMP, IFNb mRNA levels often return to basal levels by 24h of stimulation.

4.) One concern could be that the ec CDNs could be contaminated with something else that stimulates the response (e.g. DNA). This could be excluded by stimulating cells with cGAMP solution treated with snake venom phosphodiesterase, which will cleave cGAMP, but leave potential trace amounts of contaminants intact.

5.) The authors observe very different extents of stimulation with c-di-AMP, c-di-GMP and cGAMP in murine and human cells - does this correlate with the affinity for human CDNs of human and murine cGAS (e.g. does murine cGAS bind c-di-GMP better than human cGAS - as in Fig. 5D?).

6.) The authors show very little IFNb mRNA induction in human monocytes after 4h stimulation with ec c-di-AMP or c-di-GMP (Fig. 1G), but robust IFNb secretion by ELISA for c-di-AMP but not c-di-GMP at the same time point. How can that be? Is 4h really the best time point for both those read outs? Peak times for mRNA production and protein secretion should be established using timecourse analyses for the different CDNs, in case responses are missed.

7.) In Fig. 2, the requirements of stimulation with ecGAMP should be compared to lower doses of icGAMP to give a comparable stimulation of IFN production. icGAMP induces responses that are 50-fold more potent at the same dose, so a lack of inhibition could be due to saturation of the signalling pathway. Same for Fig 7c.

8.) poly(dA-dT) is not suitable as a control for DNA sensing, as it also activates the RNA sensing pathway via RNA pol III (Ablasser et al., 2009; Chiu et al., 2009). Any other source of DNA (ISD, herring testis DNA, plasmid etc). would be far preferable as DNA sensing control, as that would be entirely dependent on STING and cGAS. This is particularly important in Fig. 4, where the absence of cGAS only causes a reduction in ecGAMP-induced responses, and poly(dA-dT) also still induces IFNb production (Fig. 4B) and IRF3 phosphorylation (Fig. 4F). Inclusion of a more appropriate DNA sensing control would show whether the cGAS ko is not complete, or whether cGAS acts to boost the ecGAMP response, rather than being completely essential. This should be clarified in the text.

9.) In Fig. 4F and 4G, total IRF3 levels should be shown as well. Similarly, total protein levels for ULK1 in Fig. 4J, and total TBK1 in S2E. Phospho-STING (Ser366) can also be detected more directly using a commercially available antibody, which may be more convincing than the faint

upper band in Fig. 1J. This would also be useful in Fig. 4G.

10.) More cGAS ko THP1 cell clones should be tested, as elevated STING protein levels have not previously been reported. The higher STING levels in the ko could confound any additional effect of cGAS deletion on the sensing of intracellular CDNs.

11.) Co-localisation of ecGAMP with cGAS (Fig. 5c) is not very convincing. Can this be quantified, and compared with intracellular cGAMP?

12.) The pull-down in Fig. 6c needs a control that doesn't interact with the resin, e.g. GST and a different His-tagged protein.

13.) It would be interesting to see whether the cGAS and STING interaction also occurs after treatment with intracellular cGAMP.

14.) The authors show that cGAS with mutations in its catalytic domain cannot rescue the response to ecGAMP in cGAS ko THP1 cells, implying that the production of cGAMP may be important in the amplification of the response by cGAS. This could be tested, e.g. using in vitro cGAS activity assays in the presence of low doses of other CDNs (similar to those in Fig S8), or by stimulating cells with an extracellular CDN such as c-di-AMP, and then measuring the endogenous cGAMP produced in response by LC-MS.

Minor points:

1.) In Fig. 1A, is the NT data the same for all three CDNs? If so, only show once.

2.) Data points are not shown in Fig 4D cGAS KO.

3.) In Fig. 6D, should the GST-cGAS and His-STING labels on the right be swapped?

1st Revision - authors' response 23 November 2018

Point to point responses

Referee #1:

In this work, Liu et al. describe a novel function of cGAS, which they find is involved in the direct sensing of extracellular cyclic di-nucleotides (e.g. cGAMP) endocytosed by cells. The authors provide evidence that cGAS contributes to sensing of extracellular cGAMP/CDN by directly binding to it, and forming a perinuclear complex with STING. This discovery implies that the activity of cGAS is not restricted to nucleic acids sensing, and may also act as a scaffold for sensing of extracellular cGAMP/CDN by STING. It has important implications for the use of cGAMP as adjuvant or in cancer immunotherapies.
Although well-written, the line of thought of the manuscript is disjointed at times (see below), and the story could be simplified to go more to the point. There are also a few issues which need addressing to strengthen the claims of the manuscript.

Major points:
1) The paper starts with a panel of cells lines which do not have cGAS or STING (e.g. HeLa and HEK293T do not have functional STING, and A549 also have been found not to have STING) - so the conclusion that this pathway only works in phagocytes is not supported. In fact, given that the ecGAMP can get internalised in HEK (Supp Figure 2D-F), it is most likely that it does not activate STING in this set up (unlike icGAMP), because cGAS is absent. Adding demonstration that cGAS expression in HEKSTING cells restores responsiveness to ecGAMP would strengthen the claims (and also open the door to an activity of ecGAMP in cancer cells which have retained cGAS function).

We highly appreciate the reviewer's insightful comments. In the revised manuscript, we include that data supporting the requirement of cGAS for STING activation by eCDNs as measured by the production of interferon β transcripts in HEK293T cells stably expressing both cGAS and STING after ecGAMP stimulation **(Appendix Fig S5A and B)**. It has been reported that DNA tumor virus oncogenes namely E1A of human adenovirus 5 (hAd5) and Large T antigen of simian virus 40 (SV40) impaired cGAS-STING pathway **(Lau, Gray et al. 2015)**. However, a weak but apparent induction of type I IFN induction in HEK293T cells stably expressing both cGAS and STING in response to ecGMAP was observed. These data support the point raised by the reviewer that cGAS contributes to eCDNs sensing not only in phagocytes. We have therefore corrected the claims and discussed the correlation of cGAS function and potential ecGAMP application for cancer therapy **(p. 17, line 1-2)**.

2: Line of thought. The paper is hard to follow at times, and a rearranging of the order of the figures could help make the point that this paper makes about cGAS being involved in ecGAMP sensing. It would make more sense to compare ecGAMP to icGAMP as early as in Figure 1A - which would have revealed the issue raised above. Assuming that HEK STING cells expressing cGAS are responsive to ecGAMP (suggested experience above), the early use of this point would immediately help orientate the story on the new role of cGAS.

As such, the following line would be more logical:
I) eCDNs can activate IFN production in human and mouse macrophages, although less than iCDNs.
II) eCDNs activate IFN production through STING, but this response is not seen in HEK293T STING cells - although eCDN can enter these cells perfectly fine (as per S. Fig 2).
III) since HEK293T do not have cGAS, but macrophages do, investigation of the role of cGAS > cGAS expression in HEK STING restores sensitivity to eCDNs (assuming this works). Similarly cGAS deficiency in cGAS KO cells impacts eCDNs.
IV...) then look at endocytosis, binding of cGAS to CDNs, cGAS/STING complex/Dynein.
We highly appreciate the reviewer's advice on the reorganization of manuscript and have changed accordingly as follows:

I) eCDNs trigger innate immune responses.
II) eCDNs are less potent than iCDNs in inducing innate immune responses.
III) eCDNs require clathrin-dependent endocytosis to activate type I IFN
IV) STING is important, but not sufficient for eCDNs-induced type I IFN response
V) cGAS facilitates eCDNs detection in macrophages
VI) CDNs bind cGAS directly leading to its dimerization
VII) eCDNs promote formation of the cGAS/STING complex
VIII) eCDNs promote cGAS-mediated DNA sensing.

3) Some of the conclusions need to be toned down. cGAS and STING deficiency do not ablate sensing of eCDNs (hence the word "critical" is not appropriate). There is still a strong activation of IFN production in cGAS KO THP-1/BMMs (in Fig 3/7), and STING KO BMMs also still have a residual ~2 fold increase of IFN activity with cGAMP and c-di-AMP in fig 2D/E. In fact Fig 2C should be redrawn to show WT and STING KO BMMs separately (as the Y axis clearly mislead the potential low induction in KO BMMs). It would be more appropriate to change the claim to say that cGAS contributes to ecGAMP/CDN sensing.
We thank the reviewer for these suggestions. Indeed, the deficiency of cGAS only led to partial reduction of type I IFN response **(Fig 5)**. Interestingly, as pointed out, marginal type I IFN response were observed in STING KO BMDMs in response to stimulation with c-di-AMP and 2'2'-cGAMP **(Fig 4C and D, Appendix Fig S4A)**, indicating that other CDN receptors such as ER adaptor protein (ERAdP) and mouse oxidoreductase RECON as well as the cytosolic DNA receptor DDX41 **(Burdette, Monroe et al. 2011, Parvatiyar, Zhang et al. 2012, McFarland, Luo et al. 2017, Xia, Wang et al. 2018)** may contribute to the residual type I IFN responses which warrants further investigation. We have redrawn the corresponding figures to show the marginal induction of type I IFN in STING KO BMDM cells **(Fig 4C and Appendix Fig S4A)**. Following the reviewer's suggestion, we have changed the claim to say that cGAS contributes to eCDNs sensing **(p. 9, line 8 and p. 10, line 6-7)**.

4) The effect of DMA in Fig 2A is clearly significant in THP-1 cells and yet the text states that it did not affect ecGAMP uptake (this is incorrect). Fig 6E is really not convincing and should

either be removed or a better blot shown. Fig 4B/F/G use poly(dA:dT) which can clearly signal independently of cGAS (through RIG-I for instance PMID: 19609254). This is a misleading control, which should be replaced by transfected ISD for instance.
We appreciate the reviewer's comments. DMA significantly inhibited ecGAMP uptake in THP-1 cells but not in HEK293T cells **(Fig 3A and Appendix Fig S2C)**. Therefore, the effect of DMA on ecGAMP uptake is cell type specific. We have changed the text accordingly **(p. 7, line 5-7)**. The experiments have been repeated and the data in previous **Fig. 6E** have been updated in the revised manuscript **(Fig 7F)**. Meanwhile, interferon stimulatory DNA (ISD) has been included as the appropriated control throughout the revised manuscript **(Fig 4 and 5)**.

5) Figure 5 indicates that c-di-GMP does not bind cGAS as well as c-di-AMP. This is in line with the observation that human cells were more sensitive to c-di-AMP in Fig 1. It would be interesting to repeat this experiment with mouse cGAS to see if it is more equally binding to c-di-GMP and c-di-AMP (which is suggest in Fig 1B/3).
We thank the reviewer for this question and have performed the requested experiment to test the binding affinity of c-di-GMP and c-di-AMP to mouse cGAS. By overexpressing HA-tagged mouse cGAS in HEK293T cells, the immunoprecipitation experiment demonstrates that the binding affinity of both c-di-GMP and c-di-AMP to mouse cGAS is equivalent but less than that of cGAMP **(Appendix Figure S6G)**. Moreover, the binding affinity of eCDNs to mouse or human cGAS is closely correlated with cell type specific potency to stimulate a type I IFN response **(Appendix Figure S6H)**. These results indicate that the binding of eCDNs to cGAS is important for promoting its effect on STING activation.

6) The paper does not really provide evidence of the biological significance of their finding in response to naturally produced eCDN. The doses used in vitro are high, and it is hard to tell how this would be involved in sensing of cGAMP released by apoptotic cells/gut bacteria. This point should at least be discussed, but if the authors could substantiate activation through cGAS by extracellular CDN released by other cells it would strengthen their claims.
We appreciate the reviewer's comments. Indeed, the response of macrophages to eCDNs is dose-dependent **(Fig 2)** and the abundances of eCDNs released by apoptotic cells/gut bacteria alone may fail to induce an apparent type I IFN response. Interestingly, we found eCDNs dramatically enhanced the macrophage response to dsDNA **(Fig 8A-C)** or DNA virus HSV-1 infection **(Fig 8D-F)**. Of note, eCDNs at a concentration as low as 0.1 μg/ml synergistically enhanced macrophage responses to dsDNA HSV-1 infection **(Fig 8D-F)**, indicating that eCDNs sensing is of biological relevance.

7) One of the issues of this work is that the study does not allow to know for sure whether the cGAMP made by cGAS upon CDN binding is required (although the need for catalytic activity suggest this). One way to address this could be to reconstitute mouse STING KO cells with STING mutants (e.g. R231) which are poorly responsive to c-di-GMP but can sense cGAMP. Again, this experiment would strengthen their argument.
We appreciate the reviewer's concern and suggestion. To determine whether cGAMP synthesized by cGAS upon CDN binding is involved in eCDNs sensing, we reconstituted STING KO RAW264.7 cells with mouse STING R231A mutant (mSTING^{R231A}) **(Appendix Fig S7A)**. The cells still can initiate responses to dsDNA by sensing noncanonical 2'3'-cGAMP generated by cGAS while lacking responsiveness to canonical CDNs **(Burdette, Monroe et al. 2011)**. As expected, stimulation of extracellular c-di-GMP failed to induce a type I IFN response in STING KO cells **(Appendix Fig S7B and C)**. Of note, reconstitution with mSTING^{R231A} did not rescue the c-di-GMP-induced type I IFN response, but restored the responses to 2'3'-cGAMP and ISD in STING KO cells **(Appendix Fig S7B and C)**. Therefore, the binding of eCDNs to cGAS and its subsequent dimerization apparently does not lead to production of cGAMP. These data indicate that the failure in restoration of the impaired type I IFN response in cGAS KO THP-1 cells by complementation with enzyme inactive cGAS$^{E225A\ D227A}$ **(Appendix Fig S7E and F)** are because the amino acids E225A D227A themselves rather than the enzyme activity of cGAS are critical for the sensing of eCDNs.

Minor comments:
1- There are some sections of the Methods which introduce reagents not used anywhere... like the THP-1 cGAS shRNA cells... Where are they used?

We thank the reviewer for the correction. We have checked the manuscript thoroughly and revised it accordingly.

2- Also, please clarify whether THP-1 were differentiated with PMA (how long) in all experiments?
As per the reviewer's suggestion, we have further described the procedure for THP-1 cell preparation in detail in the Method section of the revised manuscript **(p. 23, line 5-8)**. Briefly, the THP-1 cells were treated with PMA (100 ng/ml) for 24 hours and then left rested for another 48 hours for differentiation followed by subsequent experiments.

3- There is mention of monocyte differentiation - where is this used?
We thank the reviewer for the correction. We have checked the manuscript thoroughly and the specific information has been removed from the revised manuscript accordingly.

4- the source of FITC-cGAMP is not provided.
FITC-cGAMP was purchased from BIOLOG Life Science Institute and we have included this information in the revised manuscript **(p. 21, line 3).**

5- The use of circles in the bar graphs should be consistent throughout. It cannot be used to show technical replicate in one graph, and mean of biological replicate in another (this is very misleading - e.g. Fig 1H). Also SD of technical replicate is useless - remove error bars when they refer to technical replicates.
We thank the reviewer for the correction. We have checked the data thoroughly and revised corresponding graphs as per the reviewer's suggestions.

Referee #2:

Innate responses to cyclic di-nucleotides (CDNs) are a timely topic, especially considering the growing interest in their use as adjuvants. In this manuscript, Liu and colleagues report an intriguing conclusion, saying that sensing of extracellular CDNs needs not only STING but also cGAS, and that cGAS and STING form a protein complex. This would constitute a new mechanism of how cGAS and STING operate and as such, would be of high interest to the community if appropriately supported by the data. However, this reviewer has concerns about a number of the experiments presented here and about the overall conclusion reached by the authors. Therefore, publication will require major revisions. For example, taking into consideration all the data presented in this manuscript, it is possible that CDNs, by binding to cGAS and inducing the formation of a dimer, facilitate DNA binding to cGAS, which in turn will lead to more cGAMP produced by cGAS that then activates STING via the canonical pathway.
We thank the reviewer for the encouraging comments and highly appreciate the concern. The reviewer raised an interesting alternative interpretation of the data and argued that eCDNs may facilitate cGAS-mediated DNA sensing by inducing cGAS dimerization. This is consistent with our observation that eCDNs synergistically amplified the type I IFN response in macrophages to DNA stimulation **(Fig 8A-C)** or DNA virus HSV-1 infection **(Fig 8D-F)**. However, of note, the deficiency of cGAS only resulted in a moderate reduction but not a complete loss of type I IFN response to eCDNs **(Fig. 5)**. This observation argues for a promoting effect of cGAS on STING-mediated sensing of eCDNs rather than for an augmented cGAS-mediated sensing of endogenous DNA since cGAS is essential for DNA-induced type I IFN responses. Overexpression of cGAS restores the responsiveness of HA-STING HEK293T cells to eCDNs stimulation **(Appendix Fig S5A and B)**, further strengthening an important role of cGAS in eCDNs sensing. Taken together, our data indicate that eCDNs promote the formation of a cGAS/STING complex which may be important for sensing of eCDNs. eCDNs facilitate cGAS-mediated sensing of DNA as well **(Fig 8A-C)**.

Major points:
1. The authors do not rule out the possibility that CDNs facilitate sensing of DNA by cGAS. The authors should test if cGAS mutated in its DNA binding site still binds cGAMP and induces an IFN response to extracellular CDNs.

We appreciate the reviewer's concern and thank for the suggestion. We demonstrated that eCDNs priming synergistically amplified the type I IFN response in macrophages to DNA stimulation **(Fig 8A-C)** or DNA virus HSV-1 infection **(Fig 8D-F)**, supporting that eCDNs may facilitate cGAS-mediated DNA sensing by inducing cGAS dimerization. We therefore fully agree that the identification of cGAS mutated in its DNA binding site still binds cGAMP would be ideally to preclude the possibility that eCDNs activate the canonical pathway by facilitating DNA sensing by cGAS. However, the deficiency of cGAS only resulted in a moderate reduction but not loss of type I IFN response to eCDNs **(Fig. 5)**, indicating that cGAS promotes STING-mediated sensing of eCDNs. Moreover, overexpression of cGAS restored the responsiveness of HA-STING HEK293T cells to eCDNs stimulation **(Appendix Fig S5A and B)**, further strengthening an important role of cGAS in eCDNs sensing. Our data indicate that the contributing effect of cGAS on eCDNs sensing and the promoting effect eCDNs sensing on DNA-induced cGAS activation are not mutually exclusive **(Appendix Fig S10)**. We therefore did not perform the requested experiments.

2. When comparing extracellular cGAMP with intracellular cGAMP, the authors find that intracellular cGAMP is much more potent in inducing IFN. It is possible that this strong induction is saturating in the case of intracellular cGAMP, thereby masking any effect of drugs (Fig 2 and 7) or cGAS/STING expression (Fig 4 and S2). The authors should therefore titrate down the concentration of intracellular cGAMP to normalize it with extracellular cGAMP in terms of the IFN induction.
We appreciate the reviewer's concern. We compared macrophage responses to different concentrations of eCDNs and iCDNs and observed that iCDNs are 1~2 log10 fold more potent than icGAMP **(Fig 2)**. Therefore, we have titrated down the concentration of icGAMP (0.1 μg/ml) to normalize it with extracellular cGAMP (5 μg/ml) in terms of IFN induction and repeated corresponding experiments **(Fig 3B-F and H-K, Fig 5E-G, Fig 6C, Fig. 7G, Appendix Fig S9)**. The data have been updated in the revised manuscript.

3. Do the CDNs used in these experiments contain any contaminant that could stimulate cGAS such as DNA or manganese (Wang et al, Immunity, 2018)? The authors should stimulate THP-1 cells or mouse BMDMs with DNase treated CDNs. The authors should also repeat their experiments in TLR4 KO BMDMs to show that this IFN induction is not due to endotoxins.
We appreciate the reviewer's concern and thank for the helpful suggestions. As reviewer #3 suggested, we stimulated mouse BMDM cells with ecGAMP solution treated with snake venom phosphodiesterase which can cleave cGAMP, but leavse potential trace amounts of contaminants intact **(Ablasser, Goldeck et al. 2013, Bridgeman, Maelfait et al. 2015)**. The results demonstrate that snake venom phosphodiesterase treatment completely blocked the effect of ecGAMP on the induction of the type I IFN response in mBMDMs **(Appendix Fig S1B)**. Our data demonstrate that only the cyclic cGAMP but not the linear cGAMP induces a type I IFN response in macrophages **(Fig R1)**. These data indicate that CDNs themselves but not the contamination of DNA, manganese or endotoxin impact on the responsiveness of macrophages.

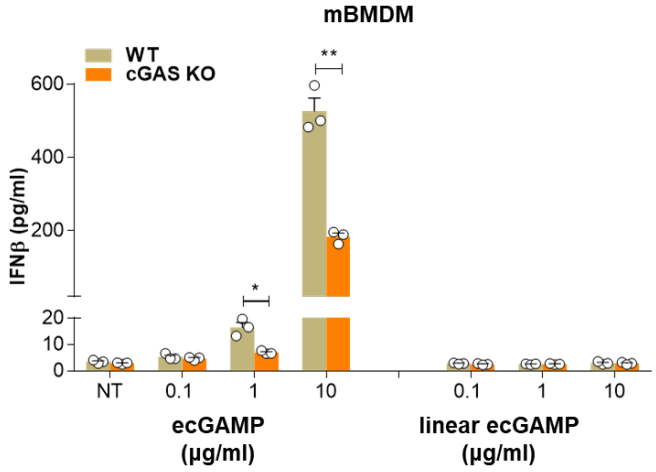

Figure R1. ELISA detection of IFNβ in the supernatants of mBMDMs from WT and cGAS KO mice stimulated with ecGAMP or control linear ecGAMP at indicated concentrations for 4 h.

4. Are FITC-cGAMP and Biotin-cGAMP bioactive? Quite a few experiments in this

manuscript use FITC-cGAMP (for flow cytometry and immunofluorescence) or Biotin-cGAMP (for pull-down) but in order to validate these experiments, the authors should show that FITC-cGAMP and Biotin-cGAMP are as active as untagged cGAMP in terms of IFN induction, in particular in THP-1 cells.
We appreciate the reviewer's concern. We detected bioactivity of FITC-cGAMP and biotin-cGAMP. The data demonstrate that both FITC-cGAMP and Biotin-cGAMP are bioactive with less potency but in the same order in inducing type I IFN in THP-1 cells and mouse macrophages. These data have been included in the revised manuscript **(Fig S6H and Fig. R2)**.

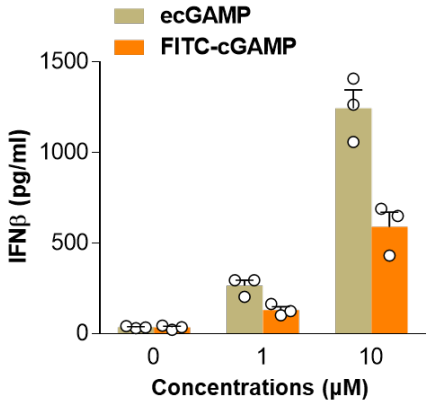

Figure R2. ELISA detection of IFNβ release in the supernatants of THP-1 cells stimulated with ecGAMP and FITC-ecGMAP at indicated concentrations for 4 h.

5. This manuscript needs proper statistics. The authors need to do non-parametric ANOVA instead of t-test.
We thank the reviewer for the correction and have performed the appropriate statistical analysis where necessary. Two-tailed Student's t-test or One-way ANOVA followed by Dunnett's post hoc test or Two-way ANOVA followed by Tukey's post hoc test or Mann-Whitney U test were employed for statistical analysis. The updated information has been included in the Materials and Methods section **(p. 33, line 5-7)**.

6. The authors should reconstitute HA-STING HEK293T cells with cGAS to see if this restores induction of IFN by extracellular cGAMP.
We appreciate the reviewer's advice. By performing the suggested experiment, the data demonstrate that reconstitution of HA-STING HEK293T cells with cGAS restored the responsiveness of HA-STING HEK293T cells to eCDNs stimulation, further strengthening an important role of cGAS in eCDNs sensing **(Appendix Fig S5A-B)**. Of note, ecGAMP induced less type I IFN response in HA-STING HEK293T cells reconstituted with cGAS than icGAMP did **(Appendix Fig S5B)**, consistent with the observation in macrophages, indicating that additional factor(s) other than cGAS are involved in the differential responses to eCDNs and iCDNs.

7. The model proposed here raises an interesting question that the authors should discuss in the manuscript: why and how would there be 2 different mechanisms for sensing CDNs, i.e. following rupture of endosomes containing extracellular cGAMP as opposed to bacterial CDNs (also released from ruptured vesicles) and cGAS-derived cGAMP?
We appreciate the reviewer's inspiring question. Although production of type I IFNs is essential for control of most viruses, type I IFNs have often been found to be detrimental in the response to bacterial infections **(Monroe, McWhirter et al. 2010)**. Therefore, a tight regulation of the magnitude of response to the same type of stimulus, but of different origin is very important. This is supported by the finding that R232H variant of human STING and R231A variant of mouse STING may provide a selective advantage by impairing responses to canonical bacterial CDNs, while still retaining responsiveness to endogenous non-canonical 2'3'-cGAMP produced by cGAS in response to dsDNA **(Diner, Burdette et al. 2013) (Burdette, Monroe et al. 2011)**. Therefore, the contribution of the newly formed cGAS/STING complex in sensing of eCDNs may add a novel layer of modulation of the host response to CDNs, which warrants further investigations.

Minor points:

1. Authors should cite and discuss their finding in the context of the following papers: McFarland et al, Immunity, 2017; Morchikh et al, Molecular Cell, 2017.
As per the reviewer's suggestion, we have cited those papers and discussed those findings accordingly in the revised manuscript. Mouse oxidoreductase RECON has been classified as a sensor for some bacterial CDNs which modulate NF-κB activation independently of STING through which they shape a proinflammatory antibacterial response **(McFarland, Luo et al. 2017) (p. 4, line 12; p. 16, line18-21)**. HEXIM1-DNA-PK-paraspeckle components-ribonucleoprotein complex (HDP-RNP) was established as a key nuclear regulator of DNA sensing through the cGAS-STING pathway. The remodeling of HDP-RNP in response to DNA stimulation leads to the release of paraspeckle proteins and recruitment of STING, which thereby serve as a signal platform for subsequent signaling activation **(Morchikh, Cribier et al. 2017) (p. 19, line 10-13)**.

2. The authors show a lot of data represented as fold induction. They should include the corresponding raw data, in particular in experiments where STING and cGAS are overexpressed, in order to show how this overexpression is affecting the basal expression of IFN.
As per the reviewer's suggestion, we have included the corresponding raw data to show the basal expression in the updated data of the revised manuscript unless indicated otherwise. HEK293T cell stably expressing both STING and cGAS show a higher basal expression of IFN **(data not shown)**. To render the data comparable, we show the fold induction of IFNβ normalized to corresponding unstimulated cells in **Appendix Fig S5B**. This is comparable to the scenario of comparing interferon transcripts in different cells **(Fig. 1A)** and in the monocytes from different donors **(Fig. 1G and Appendix Fig S1D)** where the basal IFN expression varied. Moreover, we compared macrophage responses to different concentration of eCDNs and iCDNs and observed that iCDNs are 1-2 log10 fold more potent than icGAMP in induction of IFNβ transcripts **(Fig 2A, B, E, F)**. Therefore, the data shown are represented as log10 of the fold induction of IFNβ **(Fig 2A, B, E, F)**.

3. This reviewer has some concerns regarding the RAW264.7 ISRE luciferase reporter assay: If RAW264.7 can detect cGAMP in the supernatant (Fig 1A), then how can this assay distinguish between IFN produced by mBMDMs stimulated with cGAMP and IFN produced by RAW246.7 upon sensing of cGAMP carry-over from the supernatant of mBMDMs? Could the cGAMP carry-over be responsible for the background of IFN in Fig 3D-E?
We appreciate the reviewer's concern. Indeed, cGAMP carry-over does affect the readout of the luciferase activity in RAW264.7 ISRE luciferase reporter assay and is likely responsible for the background of IFN in **Fig 3D-E** of previous manuscript. Though the data shown still reflect differential type I IFN production in WT and STING KO mBMDM, we validated those experiments by ELISA detection of IFNβ in the supernatants. The validation experiments have been included in the revised manuscript **(Fig 1C and D, Fig 4D)**.

4. Fig 1, there are discrepancies between qPCR results and the RAW264.7 bioassay as well as the IFN ELISA in human monocytes: in Fig 1B, c-di-AMP induces less IFN than 2'3'-cGAMP but it seems to induce similar levels in the RAW264.7 bioassay. The same discrepancy can be seen in Fig 1G vs 1H in human monocytes. Could the authors explain this?
We appreciate the reviewer's concern. The discrepancies between qPCR results and the RAW264.7 bioassay may result from the inappropriate application of RAW264.7 ISRE luciferase reporter assay in detecting macrophage response to eCDNs as mentioned above. We have therefore repeated related experiments by ELISA detection of IFNβ in the supernatants **(Fig 1C and D, Fig 4D)**. The data demonstrate that 2'3'-cGAMP induced significantly more IFNβ at both mRNA and protein level than c-di-AMP did. The discrepancies between qPCR results and the IFN ELISA in human monocytes may be due to variation of the response among different donors. When we enrolled more healthy donors, 2'3'-cGAMP appeared to be more potent than c-di-AMP in inducing IFNβ at both mRNA and protein level **(Fig 1G and H)**. The data has been updated and included in the revised manuscript.

5. Fig 2A, the authors should show controls that the various drugs are efficient at blocking internalisation. In this figure, the difference between the DMSO treated cells and the poly-I treated cells should be significant. Moreover, the authors should clarify whether this internalisation assay has been done by flow cytometry and, if yes, the gating strategy needs to be supplied at least in the supplementary figures.

We appreciate the reviewer's concern. Here we did not include a positive control because we used compounds that have been widely used elsewhere **(Marina-Garcia, Franchi et al. 2009)**. Poly-I treatment led to a reduction of internalization, however the difference is not statistically significant (P=0.052). The internalization assay was done by flow cytometry and the gating strategy has been supplied in the revised manuscript **(Appendix Fig S2A)**.

6. Fig 2C, the EEA1 staining looks like an aggregate close to the nucleus whereas it should look more like punctae found in the entire cytoplasm. Could the authors show a better picture?
We appreciate the reviewer's concern. The experiment has been repeated and representative data have been included in the revised manuscript **(Figure 3F)**.

7. In other figures showing IF stainings (Fig 3H, 5C, 7A), the authors should show the individual channels as bigger images as these are quite hard to see.
Following the reviewer's suggestion, larger images in individual channels have been included in the revised manuscript **(Fig 2F and G, Fig 6C, Fig 7A and B, Appendix Fig S9A and B)**.

8. Fig 3, the authors should show the knock-down efficiency in THP-1 cells.
As mentioned in the Materials and Methods section, the cGAS knockout THP-1 cells were a gift of Veit Hornung (Ludwig-Maximilians-Universität München, Germany). The immunoblot of cGAS showed that cGAS is totally absent in the knockout cells **(Wassermann, Gulen et al. 2015)** .

9. Fig 4C, authors should include a positive control such as poly(I:C).
As per the reviewer's suggestion, both ISD and poly(I:C) have been included as controls in the revised manuscript **(Fig 4 and 5)**.

10. Fig 4D, the authors use a linear cGAMP that they need to describe in the material and methods. This figure is also missing the data points for cGAS KO cells.
We thank the reviewer for the correction. The linear cGAMP has been purchased from Invivogen and the data for cGAS KO cells have been included **(Fig R1)**.

11. Fig 4F-G, could the authors show ecGAMP and icGAMP on the same blot? This is linked to major point number 2: the authors should repeat the experiment shown in Fig 4E with a lower dose of icGAMP and do a western blot where they would be able to do a side-by-side comparison of both conditions.
As per the reviewer's suggestion, we have scaled down the dose of icGAMP (0.1 µg/ml) and performed a side-by-side comparison of ecGAMP and icGAMP in inducing signaling activation on the same blot. ISD have been used as a control. The data demonstrate that cGAS contributes to ecGAMP - but not icGAMP - or ISD-induced phosphorylation of IRF3 **(Fig 5G)**, which has been included in the revised manuscript.

12. Fig 6A, the tubulin signal is too saturated and hides what the authors are trying to show. In the quantification, how were the perinuclear aggregates defined? It might be clearer if the authors showed that colocalisation of cGAMP with STING is impaired in the absence of cGAS.
We thank the reviewer for the suggestion. We have repeated the experiment and did not include tubulin staining in the updated data **(Fig. 7B)**. Representative perinuclear aggregates showing the accumulation of FITC-cGAMP close to the nucleus are presented in the revised manuscript **(Fig. 7B)**. cGAS is critical for the formation of perinuclear cGAMP aggregates **(Fig. 7C)**, with which STING partially colocalizes **(Fig 7A).**

13. Fig 6E: Could the authors show a better image for His-STING staining in GST-pulldown?
As per the reviewer's suggesting, we have repeated the experiment and updated data have been included in the revised manuscript **(Fig 7F)**.

14. Fig 6D-E: It is unclear whether these experiments are IP or GST pull-downs? The authors should also do the reverse HIS pull-down in the same conditions.
GST pull-downs were performed in these experiments. We consider it sufficient to support the direct interaction. We therefore did not perform the reverse HIS pull-down under the same condition.

15. Fig 6C and 6F only show that both cGAS and STING are able to interact with cGAMP but do not demonstrate a direct interaction between cGAS and STING.
As per the reviewer's suggestion, we have rephrased the text accordingly and conclude that cGAMP promotes the formation of a cGAMP/cGAS/STING complex **(p. 13, line 20-21)**.

16. Fig 7A, the tubulin and dynein signals are both saturated and the colocalisation with FITC-cGAMP is not convincing.
We have repeated the experiment and updated the data to show the colocalization of FITC-cGAMP with dynein in the revised manuscript **(Appendix Fig S9A)**.

17. In Fig S4, the authors claim that cGAMP induces autophagy in these cells but this is not evident looking at the LC3-II induction in the western blot. The authors need to include a positive control that induces autophagy and is blocked by 3-MA.
We have repeated the experiment by introducing ISD stimulation as a positive control for autophagy induction. The data demonstrate that ecGAMP induces robust autophagy as measured by LC3 lipidation, and 3-MA significantly inhibits this process. The data has been updated in the revised manuscript **(Fig. S3A)**.

18. In Fig 2B, the legend should read "ecGAMP+Dynasore" and "icGAMP+Dynasore". Same in Fig 7 "ecGAMP+Ciliobrevin D" and "icGAMP+Ciliobrevin D".
We thank the reviewer for the correction and include corrections in the revised manuscript.

19. This reviewer is unable to judge the technical quality of Fig 5 F to H.

Referee #3:

In this manuscript, Liu et al. show that extracellular cyclic di-nucleotides (CDNs) such as cGAMP can enter cells and stimulate the production of IFNb and IL-6 in human and murine macrophages. The response to extracellular CDNs was dependent uptake via clathrin-coated pits and detection by the DNA sensing adaptor STING, which is also senses intracellular CDNs. Unexpectedly, the authors find that the cytosolic DNA sensor cGAS is required for the sensing of extracellular CDNs but not CDNs delivered into the cytosol via digitonin. The catalytic residues of cGAS (required for the synthesis of cGAMP by cGAS) are essential for cGAS function in this context, but cGAS also seems to bind STING in the presence of cGAMP, so the authors propose that the cGAS-STING interaction is required for STING activation in this context.

Many of the experiments are reasonably well controlled and convincing - but the concept of cGAS-dependent extracellular CDN recognition raises some important questions about how this can happen, without also affecting the detection of intracellular CDNs and cytosolic DNA. How would, after escape from endolysosomes, extracellular cGAMP be different from cGAMP introduced by digitonin permeabilisation, or indeed the cGAMP produced by cGAS itself? Or is this a positive feedback mechanism that amplifies STING activation under circumstances where the intracellular levels of CDNs are low (such as when only little cGAMP leaks from endolysosomes)?

We appreciate the reviewer's insightful questions. Our data demonstrate that extracellular cGAMP differs from cGAMP introduced by digitonin permeabilization in enabling STING activation in terms of the requirement of cGAS **(Fig. 5E-G)**. This may partially account for the difference in magnitude of type I IFN production in response to eCDNs and iCDNs. Although production of type I IFNs is essential for control of most viruses, type I IFNs have often been found to be detrimental in the response to bacterial infections **(Monroe, McWhirter et al. 2010)**. Therefore, the elaborate regulation of the magnitude of response to the same type of stimulus, but of different origin is very important. This is supported by the finding that R232H variant of human STING and R231A variant of mouse STING may provide a selective advantage by impairing responses to canonical bacterial CDNs, while still retaining responsiveness to endogenous non-canonical 2'3'-cGAMP produced by cGAS in response to viral dsDNA **(Burdette, Monroe et al. 2011, Diner, Burdette et al.**

2013).Therefore, it is highly possible that the formation of a cGAS/STING complex is specifically involved in sensing of eCDNs. However, cGAS did not affect the type I IFN response to cGAMP introduced by digitonin permeabilization in macrophages regardless of the abundance of icGAMP **(Fig. 5E-G)**. Therefore, cGAS is specifically involved in host sensing of eCDNs, but not of iCDNs. Moreover, eCDNs priming synergistically amplified the type I IFN response in macrophages to DNA stimulation **(Fig 8A)**, indicating that eCDNs facilitate DNA sensing by inducing cGAS dimerization.

1.) This manuscript relies heavily on the quantification of IFNb production by real-time PCR or ELISA etc. It is not always clear how these experiments were carried out (in terms of biological or technical replicates, or independent experiments) and what the averages and error bars represent. For instance, in Fig 1, it seems that the data are averages of several experiments, each performed in triplicate - so are the error bars SD of the averages of each triplicate (without taking into account individual variation of triplicates), SD of all individual values, or SEM based on the SDs of the triplicates?
We appreciate the reviewer's concern. The data shown are mean+SD of the averages of biological replicates from indicated number of independent experiments. The detailed information has been included in the revised manuscript **(p. 33, line 7-9)**.

2.) Data should also include the mock treatment - while the average may be normalised to 1, this data point would still have an error/variation associated with it, which would be useful for comparison with the other data points. Mock stimulation would be particularly important to see where wt and ko/kd cells are used (e.g. Fig. 3 and 4) - in that case real-time PCR or luciferase data should be normalised to mock treatment of wt cells, to see whether basal responses are affected by the ko/kd.
We appreciate the reviewer's concerns and suggestions. We have reanalyzed the data and normalised real-time PCR data to mock treatment of wild-type cells where necessary. The updated data have been included in the revised manuscript **(Fig 4 and 5)**.

3.) In Fig 1A, the authors use 4h stimulation in macrophage-like cells (which response to ecGAMP), but 24h in other cell types (which don't respond) - all shown in the same chart. This is misleading - all cells should be tested at the same time point (4h). This is important, as after conventional stimulation with DNA or icGAMP, IFNb mRNA levels often return to basal levels by 24h of stimulation.
We appreciate the reviewer's concern. In fact, type I IFN was not detectable in other indicated cell types including HEK293T cells, A549 cell and HeLa cells at either 4h or 24 h post stimulation with ecGAMP. We show the data at 24 h because the type I IFN response reaches the peak in HEK293T-STING cells at 24 hours post stimulation with icGAMP **(Fig 4G)**. Since our data indicate that the contribution of cGAS to eCDNs sensing is not limited to macrophages, the corresponding data have been removed in the revised manuscript.

4.) One concern could be that the eCDNs could be contaminated with something else that stimulates the response (e.g. DNA). This could be excluded by stimulating cells with cGAMP solution treated with snake venom phosphodiesterase, which will cleave cGAMP, but leave potential trace amounts of contaminants intact.
We appreciate the reviewer's concern and thank for the helpful suggestion. We have performed the suggested experiments and found that snake venom phosphodiesterase treatment completely abolished eCDNs-induced type I IFN response **(Appendix Fig S1B)**. The data has been included in the revised manuscript.

5.) The authors observe very different extents of stimulation with c-di-AMP, c-di-GMP and cGAMP in murine and human cells - does this correlate with the affinity for human CDNs of human and murine cGAS (e.g. does murine cGAS bind c-di-GMP better than human cGAS - as in Fig. 5D?).
We thank the reviewer for the question. We have performed an immunoprecipitation experiment to compare the binding affinity of different CDNs to mouse cGAS. The results demonstrate that c-di-AMP and c-di-GMP has equivalent binding affinity to mouse cGAS **(Appendix Fig S6G)**, while c-di-GMP is much less potent than c-di-AMP in binding human cGAS **(Appendix Fig S6G)**. The results indicate that the binding affinity of CDNs to cGAS correlates with their cell type specific induction of type I IFN in macrophages **(Appendix Fig S6H)**.

6.) The authors show very little IFNb mRNA induction in human monocytes after 4h stimulation with ec c-di-AMP or c-di-GMP (Fig. 1G), but robust IFNb secretion by ELISA for c-di-AMP but not c-di-GMP at the same time point. How can that be? Is 4h really the best time point for both those read outs? Peak times for mRNA production and protein secretion should be established using time course analyses for the different CDNs, in case responses are missed.
We appreciate the reviewer's concern and thank very much for the suggestion. We repeated the experiments by harvesting monocytes from more healthy donors (n=10) and detecting the production of IFN mRNA production and protein secretion at both 4 h and 24 h post stimulation. We found that the production of IFN transcripts and protein are correlated **(Fig 1G and H)**. The data have been updated and included in the revised manuscript.

7.) In Fig. 2, the requirements of stimulation with ecGAMP should be compared to lower doses of icGAMP to give a comparable stimulation of IFN production. icGAMP induces responses that are 50-fold more potent at the same dose, so a lack of inhibition could be due to saturation of the signalling pathway. Same for Fig 7c.
We appreciate the reviewer's concern. We compared macrophage responses to different concentration of eCDNs and iCDNs and observed that iCDNs are 1~2 log10 fold more potent than icGAMP **(Fig 2)**. Therefore, we have titrated down the concentration of icGAMP (0.1 μg/ml) to normalize it with extracellular cGAMP (5 μg/ml) in terms of IFN induction and repeated corresponding experiments. The data have been updated in the revised manuscript.

8.) poly(dA-dT) is not suitable as a control for DNA sensing, as it also activates the RNA sensing pathway via RNA pol III (Ablasser et al., 2009; Chiu et al., 2009). Any other source of DNA (ISD, herring testis DNA, plasmid etc). would be far preferable as DNA sensing control, as that would be entirely dependent on STING and cGAS. This is particularly important in Fig. 4, where the absence of cGAS only causes a reduction in ecGAMP-induced responses, and poly(dA-dT) also still induces IFNb production (Fig. 4B) and IRF3 phosphorylation (Fig. 4F). Inclusion of a more appropriate DNA sensing control would show whether the cGAS ko is not complete, or whether cGAS acts to boost the ecGAMP response, rather than being completely essential. This should be clarified in the text.
We appreciate the reviewer's comments and advice a lot. We have included ISD as a control and repeated related experiments. The data indicate that cGAS KO is complete **(Fig 5C-G)** and cGAS acts to boost the ecGAMP response, rather than being essential. We have rephrased the text accordingly in the revised manuscript **(p. 9, line 8; p. 10, line 6-7)**.

9.) In Fig. 4F and 4G, total IRF3 levels should be shown as well. Similarly, total protein levels for ULK1 in Fig. 4J, and total TBK1 in S2E. Phospho-STING (Ser366) can also be detected more directly using a commercially available antibody, which may be more convincing than the faint upper band in Fig. 4J. This would also be useful in Fig. 4G.
We appreciate the reviewer's comments. We have included the immunoblotting of the total protein in the updated data **(Fig 4F and 5G)**. Moreover, considering that the data of previous **Fig. 4J** is of lower relevance, we have removed these data in the revised manuscript.

10.) More cGAS ko THP1 cell clones should be tested, as elevated STING protein levels have not previously been reported. The higher STING levels in the ko could confound any additional effect of cGAS deletion on the sensing of intracellular CDNs.
We appreciate the reviewer's concern. We repeated the experiment with 2 clones of cGAS KO THP-1 cells and consistently found that the deficiency of cGAS led to a reduction of the type I IFN response to eCDNs but not to iCDNs **(Fig 5A and B, 5E)**. The observation is similar in cGAS KO mBMDM cells **(Fig 5F)**. Of note, STING expression is not changed in primary cGAS KO mBMDMs **(Fig 5G)**. Therefore, the differential responses to eCDNs and iCDNs in cGAS KO cells do not result from varied STING expression. The updated data have been included in the revised manuscript.

11.) Co-localisation of ecGAMP with cGAS (Fig. 5c) is not very convincing. Can this be quantified, and compared with intracellular cGAMP?
We have detected colocalization of both ecGAMP and icGAMP with cGAS, and observed a significant colocalization of cGAS with ecGAMP but not icGAMP **(Fig. S6C)**.

12.) The pull-down in Fig. 6c needs a control that doesn't interact with the resin, e.g. GST and a different His-tagged protein.
We have repeated the experiment including GST as a control. The data demonstrate that cGAMP does not interact with GST **(Fig.7D)** and this has been included in the revised manuscript.

13.) It would be interesting to see whether the cGAS and STING interaction also occurs after treatment with intracellular cGAMP.
We thank the reviewer for the question. We compared the formation of the cGAS/STING complex in response to ecGAMP and icGAMP in parallel. The results demonstrate that icGAMP interacts with STING at an earlier time point (2 h post stimulation) but does not induce the formation of a cGAMP/cGAS/STING complex **(Fig 7F)**, which may explain why cGAS is not required for iCDNs sensing by STING.

14.) The authors show that cGAS with mutations in its catalytic domain cannot rescue the response to ecGAMP in cGAS ko THP1 cells, implying that the production of cGAMP may be important in the amplification of the response by cGAS. This could be tested, e.g. using in vitro cGAS activity assays in the presence of low doses of other CDNs (similar to those in Fig S8), or by stimulating cells with an extracellular CDN such as c-di-AMP, and then measuring the endogenous cGAMP produced in response by LC-MS.
We appreciate the reviewer's concern and suggestion. To determine whether cGAMP synthesized by cGAS upon CDN binding is involved in eCDNs sensing, we reconstituted STING KO RAW264.7 cells with mouse STING R231A mutant (mSTING^{R231A}) **(Appendix Fig S7A)**. The cells still can initiate responses to dsDNA by sensing noncanonical 2'3'-cGAMP generated by cGAS while lacking responsiveness to canonical CDNs **(Burdette, Monroe et al. 2011)**. As expected, stimulation of extracellular c-di-GMP failed to induce a type I IFN response in STING KO cells **(Appendix Fig S7B and C)**. Of note, reconstitution with mSTING^{R231A} did not rescue the c-di-GMP-induced type I IFN response, but restored the responses to 2'3'-cGAMP and ISD in STING KO cells **(Appendix Fig S7B and C)**. Therefore, the binding of eCDNs to cGAS and its subsequent dimerization apparently does not lead to production of cGAMP. These data indicate that the failure in restoration of the impaired type I IFN response in cGAS KO THP-1 cells by complementation with enzymatically inactive cGAS$^{E225A\ D227A}$ **(Appendix Fig S7E and F)** may be because the amino acids E225A D227A themselves rather than the enzyme activity of cGAS are critical for the sensing of eCDNs. We therefore did not further perform the in vitro cGAS activity assays as suggested by the reviewer.

Minor points:

1.) In Fig. 1A, is the NT data the same for all three CDNs? If so, only show once.
The data has been updated accordingly in the revised manuscript.

2.) Data points are not shown in Fig 4D cGAS KO.
We thank the reviewer for the correction. The missing data points have been included in the updated data of the revised manuscript **(Fig R1)**.

3.) In Fig. 6D, should the GST-cGAS and His-STING labels on the right be swapped?
We thank the reviewer for the correction and have amended it in the revised manuscript.

References

Ablasser, A., M. Goldeck, T. Cavlar, T. Deimling, G. Witte, I. Rohl, K. P. Hopfner, J. Ludwig and V. Hornung (2013). "cGAS produces a 2'-5'-linked cyclic dinucleotide second messenger that activates STING." Nature **498**(7454): 380-384.
Bridgeman, A., J. Maelfait, T. Davenne, T. Partridge, Y. Peng, A. Mayer, T. Dong, V. Kaever, P. Borrow and J. Rehwinkel (2015). "Viruses transfer the antiviral second messenger cGAMP between cells." Science **349**(6253): 1228-1232.
Burdette, D. L., K. M. Monroe, K. Sotelo-Troha, J. S. Iwig, B. Eckert, M. Hyodo, Y. Hayakawa and R. E. Vance (2011). "STING is a direct innate immune sensor of cyclic di-GMP." Nature **478**(7370): 515-518.

Diner, E. J., D. L. Burdette, S. C. Wilson, K. M. Monroe, C. A. Kellenberger, M. Hyodo, Y. Hayakawa, M. C. Hammond and R. E. Vance (2013). "The innate immune DNA sensor cGAS produces a noncanonical cyclic dinucleotide that activates human STING." Cell Rep **3**(5): 1355-1361.
Lau, L., E. E. Gray, R. L. Brunette and D. B. Stetson (2015). "DNA tumor virus oncogenes antagonize the cGAS-STING DNA-sensing pathway." Science **350**(6260): 568-571.
Marina-Garcia, N., L. Franchi, Y. G. Kim, Y. Hu, D. E. Smith, G. J. Boons and G. Nunez (2009). "Clathrin- and dynamin-dependent endocytic pathway regulates muramyl dipeptide internalization and NOD2 activation." Journal of immunology **182**(7): 4321-4327.
McFarland, A. P., S. Luo, F. Ahmed-Qadri, M. Zuck, E. F. Thayer, Y. A. Goo, K. Hybiske, L. Tong and J. J. Woodward (2017). "Sensing of Bacterial Cyclic Dinucleotides by the Oxidoreductase RECON Promotes NF-kappaB Activation and Shapes a Proinflammatory Antibacterial State." Immunity **46**(3): 433-445.
Monroe, K. M., S. M. McWhirter and R. E. Vance (2010). "Induction of type I interferons by bacteria." Cell Microbiol **12**(7): 881-890.
Morchikh, M., A. Cribier, R. Raffel, S. Amraoui, J. Cau, D. Severac, E. Dubois, O. Schwartz, Y. Bennasser and M. Benkirane (2017). "HEXIM1 and NEAT1 Long Non-coding RNA Form a Multi-subunit Complex that Regulates DNA-Mediated Innate Immune Response." Mol Cell **67**(3): 387-399 e385.
Parvatiyar, K., Z. Zhang, R. M. Teles, S. Ouyang, Y. Jiang, S. S. Iyer, S. A. Zaver, M. Schenk, S. Zeng, W. Zhong, Z. J. Liu, R. L. Modlin, Y. J. Liu and G. Cheng (2012). "The helicase DDX41 recognizes the bacterial secondary messengers cyclic di-GMP and cyclic di-AMP to activate a type I interferon immune response." Nature immunology **13**(12): 1155-1161.
Wassermann, R., M. F. Gulen, C. Sala, S. G. Perin, Y. Lou, J. Rybniker, J. L. Schmid-Burgk, T. Schmidt, V. Hornung, S. T. Cole and A. Ablasser (2015). "Mycobacterium tuberculosis Differentially Activates cGAS- and Inflammasome-Dependent Intracellular Immune Responses through ESX-1." Cell host & microbe **17**(6): 799-810.
Xia, P., S. Wang, Z. Xiong, X. Zhu, B. Ye, Y. Du, S. Meng, Y. Qu, J. Liu, G. Gao, Y. Tian and Z. Fan (2018). "The ER membrane adaptor ERAdP senses the bacterial second messenger c-di-AMP and initiates anti-bacterial immunity." Nat Immunol **19**(2): 141-150.

2nd Editorial Decision 17 December 2018

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 manuscript in EMBO reports. However, referees #1 and #3 have some remaining concerns or further suggestions we ask you to address in a final revised version of your manuscript.

Further, I have these editorial requests:

- As I mentioned in my previous letter, the Expanded View format, which will be displayed in the main HTML of the paper in a collapsible format, has replaced the Supplementary information. Thus, please select up to 5 images from the Appendix file to be displayed 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. Please select up to 5 figures as EV figures, name them accordingly, upload these as single files, and provide their legend in the main manuscript text. The remaining supplementary material should then be supplied as a single pdf labeled Appendix (see below). For more details please refer to our guide to authors:
http://embor.embopress.org/authorguide#manuscriptpreparation

- Please add page numbers to the Appendix, and the TOC (table of contents), and name the file 'Appendix'. Please provide a more detailed TOC which lists all the items contained in the Appendix. Please use the nomenclature Appendix Figure Sx or Appendix Table Sx for Appendix items, and change the callouts accordingly throughout the manuscript text.

- Please move the 'List of Abbreviations' from the Appendix to the main manuscript test.

- It seems figures 2G and 2H are presently not called out. Please add their callouts to the manuscript text.

- As they are significantly cropped, could you provide the source data for the entire Western blots shown in the manuscript (including the EV figures and the Appendix figures)? The source data will be published in separate source data files online along with the accepted manuscript and will be linked to the relevant figures. Please submit scans of entire gels or blots 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.

- Please also show the WBs with images with equal contrast and background, as unmodified as possible.

- Could statistical testing be done (shown) for the diagrams in Fig. 1, S1 and S6H?

- Please add the information for the deposition of the crystallographic data (database, accession numbers) to the methods section.

- Please fill in also field B5 in the author checklist.

- Please submit 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 with your revised manuscript.

- Please provide an ORCID for co-corresponding author Dorhoi, and link it to her author account.

When submitting your revised manuscript, we will require:
- a Microsoft Word file (.doc) of the final revised manuscript text
- editable TIFF or EPS-formatted figure files (main figures and EV figures) in high resolution (of those with adjusted panels or labels).
- The revised Appendix.
- The synopsis image.

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.

REFEREE REPORTS

Referee #1:

Overall the authors have done a great job at addressing all my comments and those of the other referees.
Critical additions to the manuscript are the following experimental validation that:
1) cGAS engagement by eCDNs does seem to activate cGAMP formation - which supports the observations that cGAS would in this context rather act to bring CDNs to STING.
2) the effect seen is not due to a contamination of eCDNs (as seen with the phosphodiesterase experiment and linear cGAMP).
3) the fact that HEK STING cells restored with cGAS are able to engage in sensing the eCDNs (as anticipated by the reviewers).
4) Biological significance of this engagement of cGAS in potentiation of HSV-1 infection.
5) the revised line of thought is much clearer.
6) Added correlation between binding of mouse cGAS and cDNs and activities.

However there are a few points which need to be revised.

1) The data with the capacity to eCDNs to increase ISD-induced responses is problematic (Figure 8). Indeed, ISD is transfected with lipofectamine and since eCDNs are added at the same time there

is a strong chance that they are also able to piggy back on the transfection to get inside the cells - similar to iCDNs. So as such, the use of this data to support biological significance is questionable. However the rest of the figure with HSV-1 infection is much more significant regarding the role of eCDNs in the context of infection.

2) There is still a logical leap regarding why the role of cGAS was investigated (Page 9 line 10). The hypothesis that cGAS would be involved here is odd. It would make more sense to state that for instance: "Surprisingly, unlike HEK293T STING cells, HEK cGAS/STING expressing cells were sensitive to eCGAMP, indicating a role for cGAS expression in eCDNs sensing by STING".

3) The use of "representative" in the figure legends is confusing. Usually, people use representative when showing data from 1 experiment representative of a group of independent experiments. But here the authors appear to use "representative" instead of averaged : e.g. "Data are representative of 3 independent experiments" (when the rest indicates that the points are actually aggregates from 3 experiments). Same is seen when using "Data are means+SD representative of at least 3 independent experiments performed with biological triplicates. Each symbol represents the mean of biological triplicates"... Please use "averaged" instead of "representative" (assuming the reviewer is correct about what is actually meant).

Referee #2:

The authors made a significant effort in replying to this reviewer's comments and the manuscript is now greatly improved. The authors did not address our main point nb.1 regarding the mechanism by which extracellular cGAMP is signalling, and how this could be different from the traditional DNA/cGAS/cGAMP/STING sensing pathway (the same question was also raised by reviewer 3). However, this reviewer appreciates the amount of data that is already present in the manuscript and feels that this could be addressed in a separate, future study. The manuscript therefore is a good candidate for EMBO Reports, provided that the authors address these minor comments:

1. In the new Figure 3 B-E, the authors use a lower dose of icGAMP and it becomes apparent that dynasore treatment impairs sensing of both ecGAMP and icGAMP. Moreover, in Figure S2D, the internalization of FITC-icGAMP was done using a different dose and can therefore not be used for interpretation of Fig 3 B-E (1ug/ml in Fig S2 vs 0.1ug/ml in Fig 3). For these reasons, the conclusion about requirement for clathrin-dependent endocytosis of ecGAMP is overstated and should be removed from the manuscript, including the abstract.

2. In the new Figure 4 G-H, the authors try to show in HEK293T that express STING but not cGAS that STING is not sufficient for ecGAMP sensing. They then try complementing these cells with cGAS in Figure S5B to show that cGAS is required. However, the IFN response to ecGAMP in Fig S5B is extremely low and this reviewer is wondering if this could be due to a dose effect? Maybe HEK293T, even overexpressing cGAS and STING, need a higher amount of ecGAMP to produce IFN? The authors should at least indicate which dose of icGAMP was used in Fig 4 G-H and move Fig S5B to Fig 4 (as a new panel I) because this is a very important result.

3. In Fig 8, the effects of ISD and ecGAMP appear to be additive, rather than synergistic.

Referee #3:

The authors have significantly improved the manuscript with further experiments and additional controls and clarifications. Even though this work raises further questions about the detailed mechanism and physiological relevance, the observations reported here are convincing and novel, and provide an interesting advance in the field. My specific concerns have been addressed in this revision.

2nd Revision - authors' response 17 January 2019

Referee #1:

Overall the authors have done a great job at addressing all my comments and those of the other referees.
Critical additions to the manuscript are the following experimental validation that:
1) cGAS engagement by eCDNs does seem to activate cGAMP formation - which supports the observations that cGAS would in this context rather act to bring CDNs to STING.
2) the effect seen is not due to a contamination of eCDNs (as seen with the phosphodiesterase experiment and linear cGAMP).
3) the fact that HEK STING cells restored with cGAS are able to engage in sensing the eCDNs (as anticipated by the reviewers).
4) Biological significance of this engagement of cGAS in potentiation of HSV-1 infection.
5) the revised line of thought is much clearer.
6) Added correlation between binding of mouse cGAS and cDNs and activities.

We thank the reviewer for the appreciation of our efforts on improving the manuscript.

However there are a few points which need to be revised.

1) The data with the capacity to eCDNs to increase ISD-induced responses is problematic (Figure 8). Indeed, ISD is transfected with lipofectamine and since eCDNs are added at the same time there is a strong chance that they are also able to piggy back on the transfection to get inside the cells - similar to iCDNs. So as such, the use of this data to support biological significance is questionable. However, the rest of the figure with HSV-1 infection is much more significant regarding the role of eCDNs in the context of infection.
We appreciate the reviewer's concern and highly agree with the point that eCDNs in our experimental condition will get inside the cells upon lipofectamine transfection and hence will function as iCDNs. We have therefore removed previous Figure 8A-C in the revised manuscript.

2) There is still a logical leap regarding why the role of cGAS was investigated (Page 9 line 10). The hypothesis that cGAS would be involved here is odd. It would make more sense to state that for instance: "Surprisingly, unlike HEK293T STING cells, HEK cGAS/STING expressing cells were sensitive to eCGAMP, indicating a role for cGAS expression in eCDNs sensing by STING".
We highly appreciate the reviewer's excellent advice and have revised the manuscript accordingly (line 11-14 page 10).

3) The use of "representative" in the figure legends is confusing. Usually, people use representative when showing data from 1 experiment representative of a group of independent experiments. But here the authors appear to use "representative" instead of averaged : e.g. "Data are representative of 3 independent experiments" (when the rest indicates that the points are actually aggregates from 3 experiments). Same is seen when using "Data are means+SD representative of at least 3 independent experiments performed with biological triplicates. Each symbol represents the mean of biological triplicates"... Please use "averaged" instead of "representative" (assuming the reviewer is correct about what is actually meant).
We thank the reviewer for the comment. The data are averaged from a group of independent experiments. We have corrected this accordingly throughout the manuscript.

Referee #2:

The authors made a significant effort in replying to this reviewer's comments and the manuscript is now greatly improved. The authors did not address our main point nb.1 regarding the mechanism by which extracellular cGAMP is signaling, and how this could be different from the traditional DNA/cGAS/cGAMP/STING sensing pathway (the same question was also raised by reviewer 3). However, this reviewer appreciates the amount of data that is already present in the manuscript and feels that this could be addressed in a separate, future study. The manuscript therefore is a good candidate for EMBO Reports, provided that the authors address these minor comments:

We thank the reviewer for the pertinent comments. We fully agree that the detailed mechanism(s)/signaling cascades underlying the differential response to eCDNs and canonical DNA/cGAS/cGAMP/STING pathway need to be addressed by future investigations.

1. In the new Figure 3 B-E, the authors use a lower dose of icGAMP and it becomes apparent that dynasore treatment impairs sensing of both ecGAMP and icGAMP. Moreover, in Figure S2D, the internalization of FITC-icGAMP was done using a different dose and can therefore not be used for interpretation of Fig 3 B-E (1ug/ml in Fig S2 vs 0.1ug/ml in Fig 3). For these reasons, the conclusion about requirement for clathrin-dependent endocytosis of ecGAMP is overstated and should be removed from the manuscript, including the abstract.

We thank the reviewer for the correction. We are sorry for the typo regarding the concentration in Fig S2. The internalization of FITC-icGAMP was done using the same dose at the concentration of 0.1 μg/ml as that in Fig 3 B-E. Our data demonstrate that the dynasore treatment impairs sensing of both ecGAMP and icGAMP. Dynasore treatment dramatically reduces the internalization of eCDNs **(Fig 3A and Appendix Fig S2C)** while leaving uptake of FITC-icGAMP unchanged **(Appendix Fig S2D).** This indicates that clathrin-dependent endocytosis is at least partly involved in sensing of eCDNs, but not of iCDNs. Moreover, out data demonstrate that maturation of early endosomes is required for sensing of eCDNs **(Fig. 3F-K)**, favoring involvement of endocytosis in eCDNs sensing. Yet, we fully agree with the reviewer's comments and we have removed "clathrin-dependent" in the revised manuscript including the abstract to tone down our statement.

2. In the new Figure 4 G-H, the authors try to show in HEK293T that express STING but not cGAS that STING is not sufficient for ecGAMP sensing. They then try complementing these cells with cGAS in Figure S5B to show that cGAS is required. However, the IFN response to ecGAMP in Fig S5B is extremely low and this reviewer is wondering if this could be due to a dose effect? Maybe HEK293T, even overexpressing cGAS and STING, need a higher amount of ecGAMP to produce IFN? The authors should at least indicate which dose of icGAMP was used in Fig 4 G-H and move Fig S5B to Fig 4 (as a new panel I) because this is a very important result.

We appreciate the reviewer's concern. As the reviewer pointed out, ecGAMP (5 μg/ml) induced much less type I IFN response than icGAMP (0.1 μg/ml) in HA-STING HEK293T cells reconstituted with cGAS **(Fig. 4I)**, though eCDNs and iCDNs are used at the indicated concentration of equivalent potency to induce type I IFN response in macrophages. Moreover, though eCDNs induced type I IFN responses in HA-STING HEK293T cells reconstituted with cGAS in a dose-dependent manner, the saturated eCDNs are still less potent than iCDNs **(Fig. R1)**. These results indicate that additional factor(s) other than cGAS are involved in the differential responses to eCDNs and iCDNs.

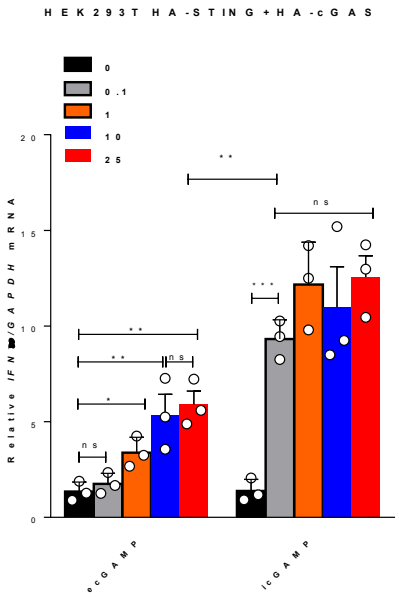

Figure R1. qRT-PCR detection of IFNβ mRNA in HEK293T cells stably transfected with both HA-STING and HA-cGAS stimulated with either ecGAMP or icGAMP at the indicated concentrations (μg/ml) for 24 h.

As per the reviewer's suggestion, the doses of icGAMP and ecGAMP used in Fig 4G-H have been indicated and Fig S5B has been moved to Fig 4 as new panel I in the revised manuscript.

3. In Fig 8, the effects of ISD and ecGAMP appear to be additive, rather than synergistic.
We appreciate the reviewer's concern. As reviewer #1 pointed out that the data with the capacity to eCDNs to increase ISD-induced responses in Figure 8A-C is problematic because eCDNs may get inside the cells upon lipofectamine transfection. We therefore removed corresponding data in the revised manuscript.

Referee #3:

The authors have significantly improved the manuscript with further experiments and additional controls and clarifications. Even though this work raises further questions about the detailed mechanism and physiological relevance, the observations reported here are convincing and novel, and provide an interesting advance in the field. My specific concerns have been addressed in this revision.
We greatly thank the reviewer for appreciations on our efforts to improve our manuscript as well as the encouraging comments.

2nd Editorial Decision 4 February 2019

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the report from referee #2 who now fully supports the publication of your manuscript in EMBO reports. However, s/he has a further suggestion (see below), i.e. to include the figure you showed in your point-by-point response (Fig. R1) as EV Figure (or Appendix Figure) in the manuscript. I support this, and thus ask you to do this in a final revised version of the paper. Please add the figure either to one of the EV figures or to the Appendix, and mention this data in the results section with appropriate call outs.

Further, I have these final editorial requests:

- Please add the information for the deposition of the crystallographic data (database, accession numbers) to the methods part of the manuscript in a section called 'data availability'.

- In the source data file for Fig. 4 the data for 4H shows up twice. Please remove the duplications.

- Please format the references according to EMBO reports style. 'et al.' should be used if there are more than 10 authors, but the first 10 names should always be shown. See: http://embor.embopress.org/authorguide#referencesformat

- Please submit a schematic summary figure with higher resolution and bigger fonts (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels). Presently (see the attached file in the size it would be shown online) this is all a bit small, especially the text.

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.

REFEREE REPORTS

Referee #2:

The authors have addressed all remaining questions and the manuscript should now be published in EMBO Reports. The editor and authors may wish to discuss whether Fig R1 in the response letter should be included in the EV Figures. The observation that the saturation point for eCDNs and iCDNs is different hints at differences in mechanism, and thereby nicely justifies a follow-up study.

2nd Revision - authors' response 11 February 2019

Referee #2:

The authors have addressed all remaining questions and the manuscript should now be published in EMBO Reports. The editor and authors may wish to discuss whether Fig R1 in the response letter should be included in the EV Figures. The observation that the saturation point for eCDNs and iCDNs is different hints at differences in mechanism, and thereby nicely justifies a follow-up study.

We thank the reviewer for the encouraging comments and highly appreciate the insightful comments. The data has been included as Figure EV2D in the revised manuscript and mentioned in the results section with appropriate call outs.

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Corresponding Author Name: Stefan HE Kaufmann
Journal Submitted to: EMBO Reports
Manuscript Number: EMBOR-2018-46293V2

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.

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/

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?	No statistical methods were used to predetermine sample sizes. Sample sizes were selected empirically from previous experimental experience with similar assays, and/or from sizes generally employed in the field.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No samples were excluded from the analysis.
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.	NA.
For animal studies, include a statement about randomization even if no randomization was used.	NA.
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.	The investigators were blinded during data collection and analysis when performing immunofluorescence assay and quantification of FITC-cGAMP perinuclear puncta.
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA.
5. For every figure, are statistical tests justified as appropriate?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Kolmogorov-Smirnov test was used for assessing whether the data meet normal distribution with GraphPad Prism 7 Software.
Is there an estimate of variation within each group of data?	Yes.
Is the variance similar between the groups that are being statistically compared?	Yes.

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).	The informaiton of antibodies including catalog number and/or clone number has been provided in the Materials and Methods section of the manuscript.cGAS and STING antibodies showed no detectable signal in knockout cells. The commercial antibodies are well used and reported in lots of previous publications.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No commonly misidentified cell lines were used in this study. All cells were mycoplasma-free with regular checks performed by a LookOut Mycoplasma PCR (i.e., polymerase chain reaction) Detection Kit (MP0035, Sigma-Aldrich).

* 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.	The information has been included in the Materials and Methods Section of the manuscript.Mice were 6–12 weeks of age for all experiments, matched for age and sex, and kept under specific pathogen-free (SPF) conditions at the Max Planck Institute for Infection Biology in Berlin, Germany and at the Tongji University, China. Sting–/– mice on C57BL/6 background were kindly provided by Lei Jin (Albany Medical Center, New York, USA) through Bastian Opitz (Charite Medical University, Berlin, Germany). Cgas–/–mice on C57BL/6 background were originally from The Jackson Laboratory and kindly provided by Skip Virgin (Washington University School of Medicine in St. Louis, MO, USA). Sting–/– and cgas–/–mice were also obtained from The Jackson Laboratory and kept under specific pathogen-free (SPF) conditions at Tongji University. C57BL/6 mice were purchased from Charles River, Germany or Shanghai Laboratory Animal Center, CAS, China and used as WT control.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All animal experiments were performed according to institutional guidelines approved by the local ethics committees of the German authorities (Landesamtes für Gesundheit und Soziales Berlin; Landesamtes für Verbraucherschutz und Lebensmittelsicherheit, Animal Application T0087/13, T0157/15) and of Tongji University.
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.	All animal experiments conformed to these guidelines.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	The buffy coats were purchased from the blood bank of the Shanghai Red Cross. The study encompassed specimens from healthy donors and was approved by the Ethics Committee of the Shanghai Pulmonary Hospital (2018-fk-252).
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.	NA.
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA.
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA.
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA.
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA.

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	The data for the crystallographic data of cGAS and cGAS/cGAMP complex will be deposited in a public database upon the acceptance of the manuscript. The section has been provided at the indicated place of 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).	NA.
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA.
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.	NA.

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.	NA.