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Cyclic-diGMP and cyclic-diAMP activate the NLRP3 Inflammasome

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Transaction Report:

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

1st Editorial Decision 28 January 2013

Thank you for the submission of your manuscript to EMBO reports. Please accept my sincere apologies for the delay in the review process of your study. We have now received the full set of reports on it, which I copy below.

As you will see, all referees agree that the study is potentially interesting and novel. Overall, although the referees are positive about the work they each point to additional experiments that will need to be addressed to strengthen the conclusions. Referees #2 and #3 both suggest further experiments to investigate the in vivo significance of NLRP3 inflammasome activation by c-di-GMP. Referee #2 suggests a number of additional controls and indicates that in order to help elucidate this novel pathway other candidates known to affect the NLRP3 pathway should also be investigated. Furthermore, referee #3 notes that the hypothesis that c-di-GMP might directly bind to NLRP3 should be tested.

Given the potential interest of the novel findings and considering that all referees provide

constructive suggestions on how to move the study forward, I would like to give you the opportunity to revise the manuscript, with the understanding that the main referee concerns, including those mentioned above, have to be fully addressed. Acceptance of the manuscript would entail a second round of review. I would like to point out that it is EMBO reports policy to allow a single round of revision only, and that thus acceptance or rejection of the manuscript will depend on the outcome of the next final round of peer-review.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. If you feel that this period is insufficient to address the referees' concerns I can potentially extend this period slightly. Also, the length of the revised manuscript should not exceed roughly 30,000 characters (including spaces). Should you find the length constraints to be a problem, you may consider including some peripheral data in the form of Supplementary information. However, materials and methods essential for the understanding of the key experiments should be described in the main body of the text and may not be displayed as supplemental information only.

We have also started encouraging authors to submit the raw data for western blots (i.e. original scans) to our editorial office. These data will be published online as part of the supplementary information. This is voluntary at the moment, but if you agree that this would be useful for readers I would like to invite you to supply these files when submitting the revised version of your study.

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

You are able to opt out of this by letting the editorial office know (emboreports@embo.org). If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

We also welcome the submission of cover suggestions or motifs that might be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1

In the manuscript entitled Cyclic-diGMP and cyclic-diAMP activate the NLRP3 Inflammasome, Abdul-Sater et al. find that the bacterial secondary messengers c-di-GMP and c-di-AMP activate the inflammasome via NLRP3. Using chemical inhibitors that block K+ or Ca++ mobilization, the authors further show that cyclic dinucleotide mediated activation of the inflammasome and the interferon response occur via differing mechanisms. Interestingly, they find that the potential c-dinucleotide sensor, STING is dispensable for c-di-GMP mediated activation of the inflammasome. The authors also find that c-di-GMP does not require NLRP3 or the ASC1 adaptor to activate the IFN response, lending toward a separate pathway in the activations of the IFN and inflammasome responses.
The understanding of how different inflammasomes undergo activation is certainly of importance. This work identifies the bacterial PAMPs c-di-GMP and c-di-AMP as ligands that activate the inflammasome and further attempts to characterize and differentiate the mechanisms by which c-dinucleotides activate the inflammasome and the IFN response. As such this work is of considerable interest. However, to further strengthen the conclusions in the manuscript, it is suggested that the authors:
1. Test if c-di-GMP also requires NLRP1 or AIM2 to activate the inflammasome and IFN responses.
2. Determine if NLRP3 and other inflammasomes are required by c-di-GMP to produce IL-18.

Referee #2

General comments

Abdul-Sater et al. describe an intriguing set of experiments showing Nlrp3 inflammasome activation by the bacterially-derived cyclic dinucleotides, di-cGMP and di-cAMP. To date, most scientific emphasis has focused on the induction of type I IFN by these chemical species, so the current manuscript reveals a divergent pathway downstream of dinucleotide detection, one in which the canonical sensor, MPYS/STING, does not appear to be involved. Additionally, Nlrp3 activation appears independent of mitochondrial ROS or membrane potential that have been implicated in this response by other groups (Zhou et al. Nature 469(7329):221-5, 2011; Nakahira et al., Nat. Immunol. 12(3):222-30, 2011). This points the existence of MPYS/STING- and mitochondria-independent pathways for Nlrp3 inflammasome activation by di-cGMP and di-cAMP. Both are novel findings but the manuscript tends to end abruptly at this point. No identification of the sensor or at least some newer protein component of this pathway is pursued further. This is disappointing from the perspective that several recent candidates have been uncovered (eg. DDX41, PKR, GBP5, BRCC3 or even cyclic GMP-AMP Synthase which produces cGAMP) (Parvatiyar et al., Nat. immunol. 13(12):1155-61, 2012; Lu et al., Nature 488(7413):670-4, 2012; Shenoy et al., Science 336(6080):481-5, 2012; Sun et al., Science 2012 Dec 20. [Epub ahead of print]). Likewise, Abdul-Sater and colleagues recently found potential binding partners on biotin-c-diGMP affinity matrices (Abdul-Sater et al. Microbes Infect. 4(2):188-97, 2012) that could also be examined together with those above to help elucidate this novel pathway. As such the manuscript would benefit from significant revision that includes these latter controls.

Specific points

Figure 1. The concentrations of di-cGMP and di-cAMP (10nmol) used in Fig. 1(A) and (C) are quite high and probably above the level that is normally bioavailable. The authors should include a titration curve to get a sense of how meaningful this activation may be in vivo, especially since these nucelotides only appear to be weak agonists compared to the other canonical Nlrp3 stimuli. The IFN-β qPCR (fig. S1C) could be added to this panel as the unaffected pathway control rather than hide it in the Supplementary section.

For Fig 1(A), it would be helpful to see the whole blot that also contains the uncleaved caspase-1 p45 subunits. Likewise, a supernatant loading control is needed for both the p10 and p17immunoblots to evaluate differences. Hmgb1 could also be shown as another secreted inflammasome-related marker.

For Fig 1(B) and (C), these results can be combined as a single panel.

For Fig. 1(C), the corresponding amounts of IL-1β secreted in response to c-diAMP (and attendant channel/cation inhibitors) needs to shown as well, especially given its emphasis in the title of the manuscript.

Figure 2. For Fig 2(B) and (D), please indicate units (even if arbitrary) alongside the ordinate scale.

For panels 2A-E, the response to c-diAMP also needs to be included throughout.

Figure 3. As for Fig 1(A), it would be helpful to see the whole blot that also contains the uncleaved caspase-1 p45 subunits and a supernatant loading control to again evaluate differences, especially where lower amounts of the p10 are found in THP-1 cells (3C). c-diAMP should also be examined under these conditions.

Figure 4. Nice experiment and well-controlled. Convincing evidence that c-diGMP converges on the Nlrp3 inflammasome in vivo although whether this effect is direct or indirect is unknown. c-diAMP should similarly be tested in the peritonitis model and FACs profiles of Gr1+ neutrophils or CD11b+ phagocytes shown alongside the secondarily-derived histogram data. Does a caspase-1

inhibitor (eg. YVAD) also block this elicitation?

Page 9. In the brief discussion section, the authors suggest the existence of new cyclic dinucleotide recognition pathways and allude to preliminary studies that have largely excluded a role for GBP5 [reference 40]. Yet no data are provided. Preferably this should be added or at the very least cited as unpublished results. Likewise, the examination of DDX41, PKR, or BRCC3 should also be undertaken since these are all known to affect the NLRP3 pathway. A reluctance to pursue these new candidates is the weakest part of manuscript and could genuinely help move the field forward if undertaken.

Referee #3

Cyclic-diGMP (cdG) and cyclic-diAMP (cdA) are important second messangers in bacteria.
The work by Abdul-Sater et al. shows that cdG and - to a lesser extent - cdA activate the inflammasome, as assessed in THP1 and BMMs by cleavage of caspase-1 and IL-1b. cdG-driven inflammasome activation requires mobilization of calcium and potassium ions. By using BMMs derived from different knockout mouse strains, this work further demonstrates that the Nlrp3 inflammasome is involved in cdG sensing. Finally, injection of cdG induced an Nlrp3-dependent recruitment of cells to the peritoneal cavity.

This work nicely shows that cdG is an activator of the Nlrp3 inflammasome and that this activation requires calcium and potassium mobilization. However, as for now, the relevance of this activator, as compared to other bacterial components known to activate different inflammasomes (or at least the Nlrp3 inflammasome) is not addressed.

Major concerns are :

1) The list of (Nlrp3) inflammasome activators is already very long, both for activators of sterile and microbial origin. The authors should address the contribution of this activator to bacterial-induced inflammasome activation. One possibility would be to fractionate bacterial lysates, identify fractions with inflammasome-activating capacity and their content (see Woodward et al., Science, June 2010). Another possibility would be to take advantage of bacterial enzymes specifically modifying cdG content, express - or not - such enzymes in cells, infect them with bacteria and measure inflammasome activity. This would indicate the contribution by cdG to global inflammasome activation by bacteria (Burdette et al. Nature, October 2011).
2) The authors clearly show that Nlrp3 is responsible for sensing cdG, leading to inflammasome activation. However, the interesting possibility that this nucleotide might directly bind Nlrp3 is never mentioned, nor experimentally addressed. As Nlrp3 possesses a nucleotide binding domain and it was recently shown to directly bind DNA (Shimada et al. Immunity 2012), the authors should address this hypothesis (as far as technically possible).

Minor concerns :

1) The effects of cdA as inflammasome activator are shown in Figure 1A and Suppl. Figure 1A, in BMM only. Is this true also in THP1? Are the underlying mechansims the same as the ones proposed for cdG? The title refers to both cdG and cdA as Nlrp3-inflammasome activators, though this is not supported by experimental data.
2) Is it strictly required to transfect cdG?
3) Figure 2 and most of Figure 4 are showing negative results. Though these are important pieces of information, at least the results presented in Figure 2 should be moved to Supplementary.

Response to Reviewer # 1

We are grateful that Reviewer #1 felt that our study was of considerable interest and have made every effort to address his/her two major concerns.

"*1. Test if c-di-GMP also requires NLRP1 or AIM2 to activate the inflammasome and IFN responses.*"

To determine whether NLRP1b or AIM2 are required for c-diGMP and c-diAMP mediated inflammasome activation, BMMs from NLRP1 and AIM2 knockout mice, along with corresponding will type controls were prepared and evaluated.

For one study BMMs were prepared from NLRP1b knockout mice on the 129 background from Beverly Koller's laboratory (*Kovarova et al., 2012, JI*), since only this background is sensitive to the NLRP1-PAMP, Lethal Toxin (*Boyden et al., 2006, Nat Gen.*). As anticipated, the NLRP1[-/-] BMMs were highly resistant to stimulation with lethal toxin, but fully responsive to stimulation with either c-diAMP or c-diGMP. These observations are now discussed on page 7 and detailed in Supplementary Figure 2C.

For the other study, BMMs were prepared from AIM2[-/-] mice from Kate Fitzgerald's laboratory (*Rathinam, et al., 2010, Nat Imm.*). These studies were carried out in the C57Bl/6J background. As previously reported, AIM2[-/-] BMMs were defective in their response to stimulation with poly(dA:dT), but fully responsive to stimulation with either c-diAMP or c-diGMP. These observations are now discussed on page 7 and detailed in Supplementary Figure 2B.

"*2. Determine if NLRP3 and other inflammasomes are required by c-di-GMP to produce IL-18.*"

We had never considered evaluating IL-18 since we assumed they phenocopied the IL1β response. While this in fact was the case when BMMs were stimulated with ATP, the response to LT was proportionately more robust and to cyclic dinucleotides proportionately more modest. A review of the recent literature revealed that other groups have observed intriguing differences between NLRP3 inflammasome dependent IL-1β and IL-18 secretion (*e.g., Schmidt et al., 2012, PLoS One*). These observations are now discussed on page 7 and detailed in Supplementary Figure 2D.

Response to Reviewer # 2

We are grateful that Reviewer #2 felt that our observations on STING and ROS independent stimulation of the NLRP3 inflammasome by cyclic dinucleotides were novel. Our effort to address the reviewers insightful concerns, including evaluating additional candidate "receptor" molecules, is detailed below.

"*Likewise, the examination of DDX41, PKR, or BRCC3 should also be undertaken since these are all known to affect the NLRP3 pathway. . . . could genuinely help move the field forward if undertaken.*"

Identifying the host "receptor" for c-diGMP remains an important and ongoing, full-time effort in the laboratory. Some of our extensive negative efforts, including those requested by the reviewer are detailed below. We are currently setting up new screens, but we feel at this point they are beyond the scope of current manuscript. Of note, other than studies suggesting that NLRP3 directly binds nucleic acids, evidence that NLPR3 directly binds or how it is activated by other PAMPs is still an active area of investigation.

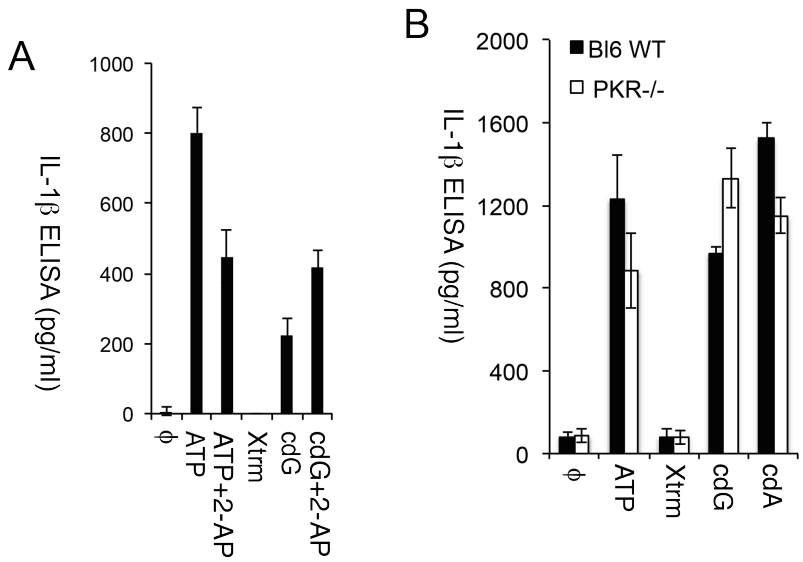
Our initial efforts entailed the collaborative development of a biotinylated analog of c-diGMP (*Abdul-Sater et al., 2012, Microbes & Infection*). Biochemical fractionation identified ~ 50 candidate-binding proteins, but most of them were resident, well-

characterized nuclear RNA binding proteins. Disappointingly, analysis of knockdowns or knockouts (when available) of remaining candidate c-diGMP binding proteins failed to yield any significant phenotypes.

We also extended these studies to include **DDX41** and **GBP5**, for the same reasons the reviewer highlighted them. Despite considerable effort, with a total of 6 shRNA constructs, we were unable to achieve a stable knockout down of DDX41, contrasting what Liu and Cheng reported (*Parvatiyar, et al., 2012, Nat. Imm.*). We note that the knockout has not yet been generated. We discussed our essentially negative collaborative experiments with the MacMicking laboratory on **GBP5** in our initial manuscript. The data was not included because John MacMicking felt that it was important to fully backcross this knockout onto a single genetic background (i.e., C57Bl/J) prior to carrying out any additional studies.

We also made an effort to evaluate several candidate proteins suggested by the reviewer. To determine whether the deubiquitinase **BRCC3** might be involved in c-diGMP stimulated inflammasome activation we employed the isopeptidase inhibitor G5 (3,5-bis[(4-Nitrophenyl)methylene]-1,1-dioxide,tetrahydro-4H-thiopyran-4-one), employed by Py et al. (*2013, Mol. Cell*) to highlight a role for BRCC3 in NLRP3 inflammasome activation. We found that like YVAD, G5 potently inhibited activation of the NLRP3 inflammasome by multiple "ligands" including c-diGMP and c-diAMP, suggesting this affect is general and not specific for cyclic dinucleotides. These observations are now discussed on page 8 and detailed in Supplementary Figure 4B.

We also evaluated a potential role for **PKR**, reported last year to regulate NLRP3 inflammasome activation by the Tracey laboratory (*Lu et al., 2012, Nat*). Initial studies, carried out right after this study was published, with 2 amino purine (a PKR inhibitor) and failed to replicate the published data (see below). To more thoroughly address the reviewer's concern, BMMs were generated from PKR[-/-] and control mice from the Koromilas laboratory. Again we found no significant defect on inflammasome activation, consistent with a recent report from the Nunez laboratory (*Lu et al. Nature 2012; He et al. Eur. J. Immunol 2013*). These observations are now discussed on



page 8.

Figure R1 – Cyclic dinucleotide stimulated inflammasome activation is independent of PKR

(**A**) IL-1β levels were measured by ELISA in LPS primed BMMs were pretreated with 2 amino purine (2-AP; 1 mM, 30 min) prior to stimulation with ATP (5 mM; 30 min) or transfection (X-tremegene HP; Xtrm) with c-diGMP (10 nmol; 6h). (**B**) IL-1β levels were measured by ELISA in LPS primed WT C57Bl/6J or PKR[-/-] BMMs treated with

ATP (5 mM; 30 min) or transfection (X-tremegene HP; Xtrm) with c-diGMP (10 nmol; 6h) or c-diAMP (10 nmol; 6h).

We think it is unlikely that **cGAS**, a DNA responsive cyclase that directs the formation of the cyclic dinucleotide c-diAGMP, will function as a "sensor" upstream of the NLRP3 inflammasome. Rather, we wonder whether c-diAGMP will also activate the inflammasome. Our collaborator is currently synthesizing this compound for us.

"*Specific points . . Figure 1. The concentrations of c-diGMP and c-diAMP . . are quite high . . The authors should include a titration curve . . . The IFN-b qPCR should be added . . .*"

The requested dose-response studies were performed and are included in Figure 1B. These new results are discussed on page 5. The IFN-β qPCR was moved from Figure S1C into revised Figure 2. (*We opted to split the original Figure 1 into 2 figures to accommodate requests for additional data; and the suggestion to move the data from original Figure 2 to supplemental material*).

"*For Fig 1(A), . . helpful to see the whole blot . . (with) uncleaved caspase-1 p45 subunits. Likewise, . . loading control . . for both the p10 and p17 immunoblots . . . Hmgb1 could also be shown as another secreted inflammasome-related marker.*"

We modified Figure 1A (as well as Figures 3 & 4) to include data on the immature forms of caspase-1 and pro-IL-1β, as requested. However, we are unaware of a widely accepted loading control for proteins secreted in the supernatant. Rather, like many other investigators we employed an equivalent number of cells in each sample. Of note, in most cases immunoblotting data was independently validated by IL-1β ELISA. As requested, we also measured HMGB1 release into the supernatant. Consistent with reports suggesting HMGB1 release may not be a reliable general measure of NLRP3 inflammasome activation (*Willingham et al., 2007 Cell Host & Microbe*), we found that ATP, but neither c-diAMP nor c-diGMP significantly stimulated HMGB1 release. These observations are now discussed on page 8 and detailed in Supplementary Figure 4A.

"*For Fig 1(B) and (C), these results can be combined as a single panel.*"

This data has been combined into a single panel in Revised Figure 2.

"*For Fig. 1(C), the corresponding amounts of IL-1β secreted in response to c-diAMP (and attendant channel/cation inhibitors) needs to shown as well . . . .*"

The requested experiment evaluating responses to c-diAMP stimulation has been included in Figure 2A. These new results are discussed on page 6.

"*Figure 2. For Fig 2(B) & (D), . . indicate units (even if arbitrary) alongside the ordinate scale.*"

These labels have been added in a revised figure, as requested. However, these data have been moved to Supplementary Figure 3, as requested by reviewer #3.

"*For panels 2A-E, the response to c-diAMP also needs to be included throughout.*"

Since these are essentially negative results, and they have been moved to supplementary material, we opted not to repeat these studies.

"*Figure 3 . . . it would be helpful to see the whole . . uncleaved caspase-1 p45 subunits . . (3C). c-diAMP should also be examined under these conditions.*"

We modified Figure 3 to include data on the immature forms of caspase-1 and pro-IL-1β, as requested. Please refer to our previous discussion regarding loading controls.

We agree with the reviewer that including treatment with c-diAMP makes sense, and we did so for all new experiments that were carried out. However, repeating the studies in Figure 3 with c-diAMP would have required a significant amount of additional effort (i.e., preparing BMMs from 3 knockout mice, as well as growing 4 THP-1 lines). We hope that the reviewer feels that the data provided in revised Figures 1 and 2

makes a sufficiently compelling case that these two cyclic dinucleotides are functionally similar, with c-diAMP being less potent, as has been reported in several studies.

"*Figure 4. Nice experiment . . . . c-diAMP should similarly be tested . . . FACs profiles of Gr1+ neutrophils or CD11b+ phagocytes shown alongside the secondarily-derived histogram data. Does a caspase-1 inhibitor (e.g., YVAD) also block this elicitation? "*

These studies, which required large quantities of c-diGMP, were performed by Dr. Lei Jin while he was a fellow in the Cambier laboratory. He has since moved to the U. of Albany making these experiments difficult to repeat. However, we did carryout an additional ex vivo study to demonstrate that YVAD blocks the c-diGMP and c-diAMP stimulated IL-1β production (Fig. S4B).

The requested FACS plots for Ly6G+ neutrophils and F4/80+ macrophages have been included in Supplementary Figure S5A. Please note, these FACS plots represent percentile distributions. None-the-less, it was surprising that despite an equivalent peritoneal recruitment of leukocytes 15 hours post c-diGMP treatment, that there was a significant difference in ratio of recruited macrophages vs. Ly6G+ neutrophils in WT and MPYS/Sting KO mice. We surmise that this actually reflects a change in the kinetics of PMN recruitment, which has been reported to be significantly impeded by IFN-α/β production (*e.g., Jablonska et al., 2010, JCI; Shahangian et al., 2009, JCI; Navarini et al., 2006, PNAS*). Thus, absent IFN- α/β production in the MPYS/Sting KO mice there is likely to be a more rapid (i.e., earlier) recruitment of PMNs, followed by the subsequent, obligate, well-described recruitment of macrophages. Dr. Lei Jin plans to explore this hypothesis in his own laboratory.

"*Page 9. In the brief discussion section, . . .a role for GBP5 . . . DDX41, PKR, or BRCC3 . . ."*

We have carried out additional experiments, as detailed at the beginning of the response to reviewer #2) and discuss each of these proteins in the text.

Response to Reviewer # 3

We are grateful that Reviewer #3 felt that our work nicely demonstrated that c-diGMP activates the NLRP3 inflammasome through a process that is dependent on calcium and potassium mobilization. Our effort to address the reviewers insightful concerns, notably including placing these results in a more physiological context are detailed below.

"*1) The list of (Nlrp3) inflammasome activators is already very long, . . . The authors should address the contribution of this activator to bacterial-induced inflammasome activation. One possibility . . . fractionate bacterial lysates , . . (see Woodward et al., Science, June 2010). Another possibility . . . enzymes specifically modifying cdG content, express . . measure inflammasome activity. This would indicate the contribution by cdG to global inflammasome activation by bacteria (Burdette et al. Nature, October 2011).*"

We had thought about this while assembling data for the original manuscript, and once again at the reviewer's urging. We had considered employing a mutant of Listeria that is defective in c-diAMP production, but were quickly disabused of this notion since Listeria expresses several additional diguanylate cyclases. Another important issue is that the inflammasome appears to be triggered by multiple PAMPs during a bacterial infection. Finally, the relationship between pyroptosis, autophagy and apoptosis is complicated.

We felt that transfecting low molecular extracts from bacteria into macrophages was not particularly physiological. Likewise, as suggested by the reviewer, we considered ectopically expressing a bacterial phosphodiesterase in macrophages, but set this aside as a bit nonphysiological.

Rather, we decided to exploit the same series of *Legionella pneumophila* cdg strains (*Levi et al., 2011, mBio*) we had employed to identify c-diGMP as a candidate PAMP in stimulating IFN-β production (*Abdul-Sater et al., 2012, M&I*). However, we needed to

generate these lines in a flagellin deficient background so that the inflammasome would not be activated via the Naip5-NLRC4 axis (*Lightfield et al., 2008, Nat Imm.*). Assaf Levi generated 3 mutants and the corresponding *fla-* control. Specifically, this included: *fla-,cdgS3*, in which IPTG dependent induction of the *cdgS3* cyclase leads to 3-4 fold increase in c-diGMP levels; *fla-,cdgS3B*, a point mutant of *cdgS3* that is defective in c-diGMP feedback inhibition; and *fla-,cdgS4*, in which IPTG dependent induction of the *cdgS4* phosphodiesterase leads to 3-4 fold decrease in the basal level of c-diGMP. Although far from perfect, since loss of fla only reduced IL-1β production by 50%, the *cdgS3* and *cdgSB3* lines were both associated with an increase in IL-1β secretion and *cdgS4* with a very modest reduction. We have now included this data in Figure 1C.

"*2) The . . . Nlrp3 is responsible for sensing cdG, . . inflammasome activation. . . .the possibility that this nucleotide might directly bind Nlrp3 is never mentioned, nor experimentally addressed. . . Nlrp3 possesses a nucleotide binding domain . . . directly bind DNA (Shimada et al. Immunity 2012), the authors should address this hypothesis . . .."*

We had not originally considered this possibility because we were a bit skeptical that DNA was a bona fide physiological ligand for NLRP3 (*We note that based on the growing number of in vitro NLRP3 binding proteins that* ***NLRP3 may be quite sticky***). However, encouraged by the reviewer we explored exploiting the 2' biotinylated c-diGMP derivative that the Beaucage laboratory had previously synthesized for us (*Grajkowski et al.,* 2008, *Bioconj Chem*), along with a UV crosslinking assay we (see *Abdul-Sater et al., 2012*). Unfortunately, there have been some technical difficulties in generating additional 2'-biotin-c-diGMP in a timely manner. Instead, we reprobed some of our older biotin-c-diGMP pulldown blots with a commercially available NLRP3 antibody, but this largely negative data was not sufficiently compelling to include. We would however like to point out that in our purification of c-diGMP binding proteins we did not identify NLRP3 or any member of the NLR family (*Abdul-Sater et al., 2012*). We however discuss of this possibility on page 10.

"Minor concerns: 1) The effects of cdA . . . BMM only. Is this true also in THP1? Are the underlying mechanisms the same as the ones proposed for cdG?"

Reviewer #2 had similar concerns. As discussed above, we carried out a number of additional experiments with c-diAMP, including: a dose response (Fig. 1B), demonstrating c-diAMP stimulated inflammasome activation was sensitive to inhibitors of K+ and Ca++ mobilization (Fig. 2A), as well as to treatment with YVAD (Fig. S1B). We also confirmed the ability of c-diAMP to stimulate IL-1β secretion in THP-1 cells. These results are now included in Fig. S1A (right panel).

"*2) Is it strictly required to transfect cdG?"*

Yes, transfection is required for cyclic dinucleotide stimulated cytokine expression (i.e., IFN-β and IL-1β). We used two different transfection reagents in our study (Lipofectamine and Xtremegene), but digitonin permeablization works as well. In contrast, just adding c-diGMP to the supernatant will not stimulate inflammasome activation. Curiously however, introducing c-diGMP into the peritoneum absent a transfection reagent elicits a robust response, but no one seems to know how this works. This is now more carefully discussed in the text.

"*3) Figure 2 and .. Figure 4 are showing negative results. . . . at least the results presented in Figure 2 should be moved to Supplementary."*

As requested, we have moved the data the used to be in Figure 2 into Supplementary Figure 4.

2nd Editorial Decision 21 June 2013

Thank you for the submission of your revised manuscript to our offices. I have taken over its handling from Alejandra Clark, as she was my maternity leave at the journal and is no longer at the office. I apologize for the time it has taken us to return to you with a decision on your study. Referee 2 was unfortunately unavailable to assess this revised version, and so referees 1 and 3 were asked to assess also your responses to his/her concerns. As you will see in the comments pasted below, although referee 1 now supports publication, referee 3 has several outstanding concerns remaining from the first round of review.

In this case, it is clear that support for publication is high. Nevertheless, EMBO reports places significant emphasis on the physiological relevance of the findings it publishes. Thus, we would be willing to open a last exceptional round of minor revision for you to address some of the remaining concerns in this regard, due to the use of non-physiological concentrations of cyclic-diGMP and problems in the peritonitis model. I would be interested in discussing what you think you could do to address these remaining issues without unreasonably delaying publication.

I look forward to hearing from you.

REFEREE REPORTS:

Referee #1:

In their manuscript entitled Cyclic-diGMP and cyclic-diAMP activate the NLRP3 Inflammasome, the authors performed additional experiments to strengthen their data on cyclic dinucleotides in their role of activating the NLRP3 inflammasome. Although numerous activators of the NLRP3 inflammasome have been identified, these authors delineated how cyclic dinucleotides (which were previously demonstrated to serve as PAMPs) can also activate the inflammasome via a separate mechanism not involving STING. They also went on to show that cyclic dinucleotides activate IL-1b in a manner different than polydA:dT. Importantly, the authors also utilized bacteria that overexpressed cyclases as well as phosphodiesterases to show cyclic dinucleotides were physiologically relevant in activating the inflammasome.

Referee #3:

The finding is novel and of interest to the inflammasome field. However, revisions failed to successfully answer some important points raised by the reviewers. I therefore think that some concerns should be more adequately addressed.

Major concerns:

1) A main concern was to show the physiological relevance of cdG-induced inflammasome activation. The authors explain that «an issue is that the inflammasome is triggered by multiple PAMPs during bacterial infection», rendering therefore difficult to assess the contribution by cdG. But if other PAMPs are responsible for most inflammasome activation, the physiological relevance of this new pathway is only minor.

To address this point, an experiment is shown in new Figure 1C: the authors generated flagellin-deficient L. pneumophila strains expressing more or less cdG. The strains expressing more cdG correlate to increased IL1b release, but tell us little on physiological levels of cdG. When comparing IL-1b induced by flagellin-deficient control strain to the flagellin-deficient strain cdgS4 no significant difference is observed. Although the comment in the text is: "...IL-1β secretion was considerably lower after infection with L. pneumophila ectopically expressing cdgS4. These observations support a physiological role for c-diGMP in the activation of the inflammasome during a bacterial infection" the results rather contradict a role for bacterial cdG at endogenous amounts.

The authors should perform additional experiments (please refer to the suggestions in the first revision round) to address this point. If these alternative approaches would fall short of supporting a role for physiological cdG concentrations, this should be shown and discussed appropriately in the text; in which range are cdG concentrations in bacteria, how do these compare to concentrations used in the experiments?; are there species/conditions where this compound might gain importance over other bacterial PAMPs?

2) The FACS data shown in Suppl. Figure 5 raise reasonable doubts on the interpretation of Figure 4C. In fact, peritonitis models are usually poorly affected by the absence of NLRP3 (but fully dependent on IL-1R deficiency, due to IL-1a) and in agreement with this notion the primary graphs show neutrophil recruitment to the peritoneal cavity of NLRP3ko mice. Moreover, Sting deficiency strongly affects neutrophil recruitment at this time-point; the interpretation by the authors is that in Stingko this recruitment has taken place with faster kinetics. However, an alternative interpretation could be that in vivo the upstream sensor is Sting. The authors should address this point performing peritonitis experiments at earlier time points (5-6 h is the commonly used time) looking at neutrophils.

Minor concerns:

1) As asked by reviewer 2, it is essential to examine also c-diAMP in the conditions shown in Figure 3 (at least 3A).

2) The blots added in Figure 3 should be aligned (or even better; if the same Ab detects both proIL1 and IL-1 bands, a unique blot could be shown); it is not even clear which band corresponds to proIL1b.

3) Data on PKRko should be shown as supplementary results.

2nd Revision - authors' response 26 July 2013

Thank you for your many helpful comments and the opportunity to improve our manuscript. Please find enclosed a newly revised version of our manuscript, "Cyclic-diAMP and cyclic-diGMP activate the NLRP3 inflammasome", which address concerns raised by you and the reviewers.

As per your suggestions the text has been revised, shortened and requested additional / revised panels have been added to Figures 3, 4 and S2. This includes: revised panels in Figures 3 and 4 that reveal both pro-IL-1β and IL-1β, as well as pro-caspase-1 and caspase-1 bands in the same blot (increasing the size of each figure !); and the addition of our negative data on PKR to Figure S2D. The most significant changes to the text include moving some methodological details to supplementary material, eliminating 10 references and substantially revising text that discusses physiological levels of cyclic dinucleotides and the divergent roles STING plays in cultured macrophages versus our in vivo peritonitis model. The revised paragraphs follow below (new text is in blue).

. . . . . Remarkably, transfection of c-diGMP or cdiAMP, at doses previously shown to promote the MPYS/STING dependent IFN–β expression [21, 22], was associated with an analogous robust and dose dependent increase in caspase-1 and IL-1β production (Fig. 1B). Importantly, these doses were similar to those of transfected poly(dA:dT) required to stimulate the AIM2 inflammasome [33, 34]. . . . . .

To explore a physiological role for c-diGMP in inflammasome activation, L. pneumophila cdgS strains previously exploited to identify a role for c-diGMP in IFN-I induction, were evaluated [21, 35]. Specifically, these studies examined L. pneumophila strains ectopically expressing: cdgS4 (lpg0156), a phosphodiesterase associated with ≥ 3 fold reduction in c-diGMP levels (i.e., from 8.3 to 2.4 fmol/OD of bacteria); cdgS3 (lpg0155), a diguanylate cyclase associated with ≥ 3 fold increase c-diGMP levels (i.e., from 8.3 to 24 fmol/OD); and cdgS3B, a point mutant that is resistant to c-diGMP feedback inhibition [35]. Of note, these levels correspond closely with endogenous levels of c-diAMP previously reported for intracellular L. monocytogenes infections [23]. Additionally, since L. pneumophila flagellin is also known to activate the Naip5-NLRC4 inflammasome [36], it was important to carry out these studies in flagellin-deficient strains. As expected, loss of flagellin led to a ~2-fold reduction in IL-1β secretion (see Fig. 1C). Consistent with changes in c-diGMP levels, IL-1β secretion was significantly increased in BMMs infected with the flagellin null L. pneumophila ectopically expressing cdgS3 or cdgS3B. However, the reduction in IL-1β secretion in L. pneumophila ectopically expressing cdgS4 was not significant (Fig. 1C; [35]).

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Subsequent studies explored the ability of c-diGMP to stimulate inflammasome activation in vivo, absent a priming response (i.e., signal 1), as has been reported for MSU, silica, alum and hemozoin, [47-49][50]. Peritoneal instillation of c-diGMP was associated with the robust accumulation of leukocytes (PMNs and macrophages) in wild type mice (Fig. 4C and S5A [31]). As anticipated, pretreatment of mice with an IL-1β blocking antibody prevented this response (Fig. S5B). Consistent with our in vitro analysis, the IL-1β mediated recruitment of leukocytes was dependent on NLRP3. Even though the overall peritoneal response was only minimally affected by the loss of MPYS/STING, more detailed analysis revealed this was associated with an altered distribution in accumulating leukocytes (Fig. 4C and S5A). The recruitment of PMNs was significantly reduced, likely reflecting a loss in IL-1β production. These findings suggest that MPYS/STING functions upstream of NLRP3 in vivo. This contrasts our observation in cultured macrophages in which both signal 1 (i.e., PRR dependent NF-κB activation) and signal 2 (i.e., c-diGMP, ATP, nigericin, MSU, poly(dA:dT), etc.) are required to activate the inflammasome independent of MPYS/STING*. Consistent with this, c-diGMP is able to directly activate physiologically relevant levels of NF-κB (Lei Jin, manuscript in preparation*). These observations implicate STING in diGMP dependent NF-κB activation and may explain why some NLRP3 "ligands" are direct inflammasome activation in vivo absent signal 1.*

* I was not sure how to reference this since Dr. Lei Jin is a co-author.

We hope that you and the reviewers agree that our revised manuscript, describing for the first time that cyclic di-nucleotides activate the inflammasome will be of significant interest to the readers of *EMBO Reports*. These studies, which comply with all institutional and government guidelines, have neither been published nor are being considered for publication elsewhere. Likewise, each co-author has read and approved the revised manuscript, and has no conflicts of interest to declare.

3rd Editorial Decision 30 July 2013

Thank you for the submission of your revised study. I have now gone through the file once more and believe you have adequately addressed the concerns as outlined in our telephone discussion, including increasing the emphasis on the physiological relevance of the findings and addressing the role of STING in vitro vs in vivo. I am therefore writing with an 'accept in principle' decision, which means that I will be happy to accept your manuscript for publication once a few minor issues/corrections have been addressed, as follows.

In going through the study prior to acceptance, from the figure legends I understand that the values represented in figures 1B,C; 2A,B; 4B,C; S1A,B; S2A,B,C,E,F; and S4B are the mean and standard deviations of triplicates of one out of three independent experiments. Please note that it is not appropriate to present error bars of replicates within a single experiment, and the three independent experiments should be used for their calculation. I would refer you to the following articles for guidance: Vaux et al., EMBO rep 2012; Cumming et al. JCB 2007. I would suggest to either represent the outcome of the analysis as bars or represent each of the individual points in the graph, with the mean and standard deviation of the three independent experiments.

In addition, the label for panel D seems to be missing from supplementary figure 2, and E,F are switched in order.

We now encourage the publication of original source data -particularly for electrophoretic gels and blots, but also for graphs- with the aim of making primary data more accessible and transparent to the reader. If you agree, you would need to provide one PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures and an Excel sheet or similar with the data behind the graphs. The files should be labeled with the appropriate figure/panel number, and the gels should have molecular weight markers; further annotation could be useful but is not essential. The source files will be published online with the article as supplementary "Source Data" files and should be uploaded when you submit your final version. If you have any questions regarding this please contact me.

As a standard procedure, we edit the title and abstract of manuscripts to make them more accessible to a general readership. Please find the (minorly) edited version of the abstract at the end of this email and let me know if you do NOT agree with any of the changes.

Once you have made these minor revisions, please use the following link to submit your corrected manuscript:

Once all remaining corrections have been attended to, you will receive an official decision letter from the journal accepting your manuscript for publication in the next available issue of EMBO reports. This letter will also include details of the further steps you need to take for the prompt inclusion of your manuscript in our next available issue.

Thank you for your contribution to EMBO reports.

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Edited abstract

The cyclic dinucleotides 3'-5' diadenylate (c-diAMP) and 3'-5' diguanylate (c-diGMP) are important bacterial second messengers that have recently been shown to stimulate the secretion of type I Interferons (IFN-Is) through the c-diGMP-binding protein MPYS/STING. Here, we show that physiologically relevant levels of cyclic dinucleotides also stimulate a robust secretion of IL-1β through the NLRP3 inflammasome. Intriguingly, this response is independent of MPYS/STING. Consistent with most NLRP3 inflammasome activators, the response to c-diGMP is dependent on

the mobilization of potassium and calcium ions. However, in contrast to other NLRP3 inflammasome activators, this response is not associated with significant changes in mitochondrial potential or the generation of mitochondrial reactive oxygen species. Thus, cyclic dinucleotides activate the NLRP3 inflammasome through a unique pathway that could have evolved to detect pervasive bacterial PAMPs associated with intracellular infections.

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3rd Revision - authors' response 05 August 2013

Thank you for all of your help with our manuscript and an acceptance in principal pending a few minor issues/corrections. Yesterday afternoon we submitted a revised manuscript in which we addressed each of these concerns. Below please find an itemized list of all of the changes we have made to the manuscript/figures.

1. All ELISA results (Figs. 1B, 1C, 2A, S1A, S1B, S2A, S2B, S2C, S2D, S2E & S4B) and Q-PCR results (Figs. 2B, 4B & S2F) have been replaced with graphs that provide the average of independent experiments with error bars representing standard error.

2. The figure legends of all the figures have been modified to reflect these changes.

3. The abstract has been revised as per your suggestions.

4. A label for panel D in Figure S2 has been added.

5. The order of panels D, E and F in Figure S2 has been revised, as requested.

6. The text referring to the unpublished data by our co-author, Jin Lei, has been revised as per his suggestion to *"Consistent with its response in vivo, MPYS/STING is required for c-diGMPinduced canonical NF-κB activation in myeloid cells (Signal 1) (Jin Lei, manuscript in preparation)"*

7. Labeled (with corresponding figure numbers) source files for all Western Blots (raw scans), the bar graphs (ELISAs) and Q-PCRs have been uploaded as "Related Manuscript Files".

4th Editorial Decision 06 August 2013

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

As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. As you are aware, this File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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