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Species-specific detection of the antiviral small molecule compound CMA by STING

Taner Cavlar, Tobias Deimling, Andrea Ablasser, Karl-Peter Hopfner and Veit Hornung

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(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.)

Editor: Karin Dumstrei

1st Editorial Decision 05 December 2012

Thank you for submitting your manuscript to the EMBO journal. Your study has now been seen by three referees and their comments are provided below.

As you can see the referees find the analysis interesting. Referees #2 and 3 raise relative minor concerns that should not involve too much additional work to address. However, referee #1 raises more significant issues that have to be resolved before further consideration here. Should you be able to address the raised concerns then I would like to invite you to submit a suitably revised version for our consideration. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to address the raised concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFEREE REPORTS

Referee #1

Species-specific detection of 10-carboxymethyl-9-acridanone by STING
By Cavlar et. al.

General

Production of type I IFN offers protection against pathogens. Small molecules have been shown to

elicit potent IFN production in murine models. For example, CMA can induce production of type I IFN and this ability of CMA has been linked to its anti-viral properties. However, the anti-viral properties of CMA observed in murine models have not been successfully translated to humans. The question of why CMA (and possibly many other small molecules) fails to offer protection against viruses in humans remained unanswered until recently. Cavlar et al. now show that CMA exerts its effect by modulating the newly discovered innate immune protein STING. Using a murine macrophage model the authors first provide compelling evidence for the involvement of CMA in eliciting an innate immune response. They show that TLRs and RIG-I or MDA5 do not participate in CMA-mediated response. Instead, STING seems to be sufficient for CMA-mediated activation of immune response. Although a sensor for CMA could not be identified, binding of CMA to STING activates the STING-TBK1-IRF3 pathway resulting in production of type I IFN. CMA could bind murine STING-LBD, but not human STING-LBD. Cellular response to CMA could be restored by introducing murine STING or swapping human STING-LBD with its murine counterpart. Using thermal shift assays, the authors show that CMA binds murine STING but not human STING. Based on these results, the authors conclude that the inability of CMA to illicit IFN production in humans is because it cannot bind human STING. The authors further model and dock CMA on murine STING to depict its mode of binding. However, the model docked with the ligand fails to explain the mechanism of activation of STING by CMA and does not provide structural basis for why CMA cannot bind human STING. In addition, the conformation of the template of STING used for docking studies is different than 4 other structures deposited in PDB.

Comments

The authors have attempted to answer an important, long standing question of why some small molecules (example CMA) that have anti-viral properties in murine models fail to confer anti-viral protection in humans. Such studies are a reminder for exerting caution while extrapolating results from murine models to humans. Although the species are highly similar, the benefits may not always translate. It also brings out the pitfalls and limitations of the currently available model systems for evaluating therapeutic effects. In this study, the authors show that CMA exerts its therapeutic potential by activating STING-mediated immune responses. The cell-based functional experiments performed by the authors clearly suggest a role for the STING-TBK1-IRF3 axis in CMA-induced anti-viral protection in murine model. In addition, it seems that CMA is unable to induce such a response in presence of human STING. These results as such are interesting. However, the authors fail to provide -

1. A conclusive proof for direct binding/non binding of CMA to mSTING/hSTING; for example, via a structural view of murine STING bound with CMA. Thermal shift assays (TSA) can provide some guidance on the binding, but there have been many instances where compounds have shown no shift in TSA, but were highly active in functional assays. Besides, CMA does not possess the 2-fold symmetry of c di-GMP and may not be able to bind STING in a similar manner and increase the Tm.

2. Because CMA is structurally very different than c di-GMP (primarily it does not have 2-fold symmetry as c di-GMP), results of the docking studies performed by the authors to infer the mode of binding of CMA are not convincing. Mutagenesis and functional effect of the mutagenesis in cell-based systems would be necessary to support inferences of the modeling studies. The template of STING used for building the model of murine STING has distinctly different conformation than the other structures deposited in PDB. Did the authors attempt modeling and subsequent docking using other templates of STING? Was there any significant difference in the results?

3. Inhibition of the STING response by addition of 2 bromo groups to CMA further complicates and actually weakens the argument of a role for STING in the CMA-response. From the docking results shown in Figure 7D, it seems that the bromides would have no effect on the binding.

The part where CMA activates STING mediated response, while human STING is unable to stimulate a similar response is exciting. I suggest the authors tone down the thermal shift discussion and re-work the modeling studies part.

Specific comments
1. Title - suggest change to "Activation of STING-mediated signaling by 10-carboxymethyl-9-

acridanone is species-specific" since the authors have not provided enough evidence for STING functioning as a sensor or detector for CMA.

2. Abstract - "CMA directly binds to STING and triggers a strong antiviral response through the TBK1/IRF3 route". There isn't enough evidence for CMA binding STING and eliciting an anti-viral response. Cell based experiments where labeled CMA is introduced in the cell eliciting a response followed by extraction of STING from the cells with labeled CMA (similar to those experiments performed for demonstration of c di-GMP binding ability of STING by Brunette et. al.) and/or a structural view of STING bound with CMA would be necessary to claim this. Suggest change sentence to "CMA triggers antiviral response through the STING-TBK1-IRF3 route".

3. Abstract - change "... two CMA molecules bind to the central c-diGMP..." to "... two CMA molecules may bind to the central c-diGMP..."

4. Introduction - Last sentence and other places in the manuscript - "the species-specific recognition of a novel class of STING ligands" - CMA can induce STING-mediated response. To claim detection, recognition, sensing of novel ligand by STING additional evidence is required. Suggest re-word similar sentences to ".... stimulation or induction of STING-mediated responses ..."

5. Results - CMA does not bind the human STING LBD - Why does the protein show two Tms in the denaturation curves (Figure 5 B iii)? Does CMA bind TBK1 or IRF3?

6. 2, 7-dibromo-CMA inhibits STING activation - Evidence connecting 2, 7-di-bromo CMA's ability to abrogate IFN production via inhibition of STING is lacking. Was the inhibition of STING a consequence of its inability to bind 2, 7-di-bromo CMA? How did this compound perform in the thermal shift assays? Again does this compound bind TBK1 or IRF3?

7. Homology modeling and docking .... The docking studies are very speculative. CMA is very small and aromatic. It could sit anywhere in the protein and not necessarily at the position where c di-GMP binds. The 2-fold symmetry of c di-GMP is well suited for binding into a similar symmetrical binding pocket. CMA is structurally very different than c di-GMP. Therefore, it probably would not bind at the same position as STING.
The fact that R231A mutation had no effect on CMA-mediated activation of STING (it abolished c di-GMP-mediated activation) further suggests that the CMA binding site predicted by docking may not be correct. R231A mutation did not affect the response to DNA because DNA is a large molecule and a single alanine scanning mutation (R231A) is unlikely to affect the activation of STING. Besides STING may not be directly sensing DNA. IFI16 has been implicated for this role. Can the model of murine STING docked with the ligand explain why 2, 7 - di-bromo CMA inhibited STING activation?

8. Supplemental Figure S4. This figure is important to show the species specific difference between murine and human STING implicated for differential response of STING to CMA. Suggest move Figure S4 to main text. Clearly mark or highlight with a different background color the amino acids forming the V-shaped interface of STING. Within this region, mark amino acids directly contacting c di-GMP.

9. Figure 5 A iii and S3 iii - Is the increase in Tm in presence of CMA significant?

Referee #2

Hornung and colleagues present the interesting finding that 10-carboxymethyl-9-acridanone (CMA), a known anti-viral small molecule, activates type I IFNs via direct binding to STING, a recently revealed important central adaptor protein in mediating nucleic acid signalling to IFN induction. Furthermore they show that CMA acts via mouse, but not human STING, which is consistent with previous studies suggesting that CMA mainly induces anti-viral responses in mice and not humans. They also present a novel STING inhibitor (2,7-dibromo-CMA) which will be og great interest to the innate immunity community.

There are some minor issues to address, and some further controls to perform:

1. The authors state (first paragraph in Intro) that conserved microbial patterns are commonly referred to as MAMPs. However these are COMMONLY referred to as PAMPs (pathogen associated molecular patterns) and RARELY referred to as MAMPs.
2. Although the authors focus on the type I IFN induction pathway, they never actually measure type I IFN production / secretion (e.g. IFNbeta or IFNalpha by ELISA or bioassay). For example, in Fig 1D and E, the graphs are titled 'IFNbeta production' but what is actually measured is luciferase protein under the control of an IFNbeta promoter. At least some data should be presented showing that CMA causes endogenous type I IFN production in mouse and not human cells.
3. In most cases, increasing the concentration of CMA actually gives less of a response (Figs S2A, B, 5B). IS this a bell-shaped response? So do lower concentrations than those used here show a dose-dependent increase in responses?
4. Fig 1E - please specify the units of time on the graph or in the legend.
5. IT has recently been shown that DDX41, as well as STING, senses and mediates responses to cyclic-di-nucleotides (Parvatiyar et al, NAture Immunology, 2012). In fact it is claimed there that DDX41 is more important for sensing than STING. Here, the authors should include or exclude a role for DDX41 in responding to CMA, which should be straightforward using siRNA.
6. Results section, third section, it is stated that CMA has been reported to induce IFN I in human cells. Please reference this statement and explain which cells? Was this cell type tested by the authors here? Perhaps there are cell-type differences, as well as species differences in the response to CMA?
7. Can the authors measure any real anti-viral effects of CMA (e.g. reduced viral replication) in their cell culture models with and without mouse STING? It is still unclear from the paper whether STING actually mediates an anti-viral response to CMA (as opposed to cytokine induction).
8. Fig 2 is negative data which should be combined with Fig S1, either as a main, or supplemental, figure.
9. Figure 5 - please show the expression levels of the four different STING constructs, in order that the reader can correlate this with the functional readouts

Referee #3

This manuscript demonstrates that CMA induces type I IFN via activating STING through the TBK1/IRF3 route in a species-specific manner. CMA-induced cytokine production depends on murine STING, and is independent of TLR and MAVS signals. The C-terminal ligand-binding domain (LBD) of STING determines this species-specificity CMA activity. Differential scanning fluorimetry (DFS) shows CMA bind LBD of murine STING. 2.7-dibromo-CMA inhibits murine STING activated by CMA and c-diGMP etc., functions as STING inhibitor. C-diGMP and CMA binding appears to be supported by π-π stacking between Y166 of murine STING and the acridone ring, and hydrogen bonding between R237 of murine STING and the carboxy group of CMA. The authors concluded that small molecule based on the CMA scaffold interfere STING activated by DNA sensing. Despite of the high homology of the human and murine LBD, human cells do not respond to CMA. DFS displays CMA does not bind the LBD of human STING shown unlike c-diGMP.

Overall, the findings are novel and important in this field. The conclusion is supported by the data, and the manuscript is concisely written.

Specific comment
Given that the occupying binding pocket of STING interferes STING activation, the authors had better check whether 2,7-dibromo-CMA inhibits human STING activated by c-diGMP.

Minor comment
In Figure 1D, the authors show that the IFNb promoter is activated in response to CMA, but not to poly I:C, though they show that poly I:C induced Ifnb gene. Although this is a control experiment, the authors should check the data if this is correctly shown.

1st Revision - authors' response 28 February 2013

Referee #1

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General

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could not be identified, binding of CMA to STING activates the STING-TBK1-IRF3 pathway resulting in production of type I IFN. CMA could bind murine STING-LBD, but not human STING-LBD. Cellular response to CMA could be restored by introducing murine STING or swapping human STING-LBD with its murine counterpart. Using thermal shift assays, the authors show that CMA binds murine STING but not human STING. Based on these results, the authors conclude that the inability of CMA to illicit IFN production in humans is because it cannot bind human STING. The authors further model and dock CMA on murine STING to depict its mode of binding. However, the model docked with the ligand fails to explain the mechanism of activation of STING by CMA and does not provide structural basis for why CMA cannot bind human STING. In addition, the conformation of the template of STING used for docking studies is different than 4 other structures deposited in PDB.

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We responded to the above raised concerns in the specific comments section below.

Specific comments

1. Title - suggest change to "Activation of STING-mediated signaling by 10-carboxymethyl-9-acridanone is species-specific" since the authors have not provided enough evidence for STING functioning as a sensor or detector for CMA.

Please refer to point 4 below.

2. Abstract - "CMA directly binds to STING and triggers a strong antiviral response through the TBK1/IRF3 route". There isn't enough evidence for CMA binding STING and eliciting an anti-viral response. Cell based experiments where labeled CMA is introduced in the cell eliciting a response followed by extraction of STING from the cells with labeled CMA (similar to those experiments performed for demonstration of c di-GMP binding ability of STING by Brunette et. al.) and/or a structural view of STING bound with CMA would be necessary to claim this. Suggest change sentence to "CMA triggers antiviral response through the STING-TBK1-IRF3 route".

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While this manuscript was in review we managed to grow crystals of mSTING in complex with CMA and determine the crystals structure. These experimental data replace the modeling studies and directly reveal how mSTING binds CMA. In particular, we now show that two CMA molecules directly bind to the preformed STING dimer in the c-diGMP binding pocket. Moreover, we now show that CMA triggers an antiviral response via STING. As such, we believe that the above-cited conclusions (STING being a direct receptor for two CMA molecules, CMA inducing antiviral activity in a STING dependent fashion) are correct.

5. Results - CMA does not bind the human STING LBD - Why does the protein show two Tms in the denaturation curves (Figure 5 B iii)? Does CMA bind TBK1 or IRF3?

Differential scanning fluorimetry indirectly measures protein conformation using a fluorescent dye (SYPRO orange) that binds to hydrophobic amino acids. Hydrophobic parts of a protein are usually not accessible to this dye under physiological conditions, yet upon denaturation (e.g. heat-dependent denaturation) fluorescence increases as more and more hydrophobic amino acids gain access to this dye. Measuring fluorescence as a function of temperature using DSF reveals a protein-specific gain in fluorescence whereas, compounds that are binding to a protein can increase the stability of a protein and therefore shift the gain in fluorescence signal to higher temperature.
In our assays, the LBD of hSTING shows a higher thermal stability in the presence of cyclic diGMP or cyclic diAMP, but not in the presence of CMA. As such we conclude that CMA does not stabilize hSTING (as opposed to mSTING). This can either be due to a lack of binding, or CMA fails to e.g. fold the lids in hSTING as opposed to mSTING. These two possibilities are discussed. For all conditions tested, the LBD of our human hSTING construct used in these studies reproducibly shows a small "shoulder" in its temperature profile at lower temperature whereas this is not seen for mSTING. This phenomenon indicates that hSTING unfolds as function of temperature in two steps. The "shoulder" would correspond to an intermediate conformation (e.g. unfolding of some larger loop regions). Many proteins do not denature (or vice versa fold) in a single step, so the observed curve is not unusual.

At this point there is no evidence that CMA would bind to TBK1 or IRF3, and as such we have not addressed this hypothesis.

6. 2, 7-dibromo-CMA inhibits STING activation - Evidence connecting 2, 7-di-bromo CMA's ability to abrogate IFN production via inhibition of STING is lacking. Was the inhibition of STING a consequence of its inability to bind 2, 7-di-bromo CMA? How did this compound perform in the thermal shift assays? Again does this compound bind TBK1 or IRF3?

Our data suggest that 2, 7-dibromo-CMA competes with CMA for STING binding.
Nevertheless, we decided to remove the data on 2, 7-dibromo-CMA inhibiting STING, as we felt that it would go beyond the scope of this manuscript to elucidate its mechanism of inhibition.

7. Homology modeling and docking .... The docking studies are very speculative. CMA is very small and aromatic. It could sit anywhere in the protein and not necessarily at the position where c di-GMP binds. The 2-fold symmetry of c di-GMP is well suited for binding into a similar symmetrical binding pocket. CMA is structurally very different than c di-GMP. Therefore, it probably would not bind at the same position as STING.
The fact that R231A mutation had no effect on CMA-mediated activation of STING (it abolished c di-GMP-mediated activation) further suggests that the CMA binding site predicted by docking may not be correct. R231A mutation did not affect the response to DNA because DNA is a large molecule and a single alanine scanning mutation (R231A) is unlikely to affect the activation of STING. Besides STING may not be directly sensing DNA. IFI16 has been implicated for this role.
Can the model of murine STING docked with the ligand explain why 2, 7 - di-bromo CMA inhibited STING activation?

Our crystal structure data now clearly show that two CMA molecules directly bind in a rotational symmetry to the preformed STING dimer. In contrast to our docking studies, the carboxymethyl groups of the CMA molecules face the lid region and not the pocket of the STING dimer. Nevertheless, as predicted, the crystal structure shows that two CMA molecules are stacked in a

similar fashion as the symmetric c-di-GMP molecule.
As revealed by the crystal structure, R231 is not involved in the recognition of CMA, which nicely fits to the point mutation data obtained with the R231A mutant. Based on these data, we would predict that cGAMP also does not require R231 for STING binding.

8. Supplemental Figure S4. This figure is important to show the species specific difference between murine and human STING implicated for differential response of STING to CMA. Suggest move Figure S4 to main text. Clearly mark or highlight with a different background color the amino acids forming the V-shaped interface of STING. Within this region, mark amino acids directly contacting c di-GMP.

This figure was removed given the fact that we now present new crystallographic data on murine STING binding to CMA. A new figure showing the superposition of mouse STING, bound to two CMA molecules with human STING is now presented in Fig. S7.

9. Figure 5 A iii and S3 iii - Is the increase in Tm in presence of CMA significant?

These experiments were repeated twice with highly similar results, as such we did not provide a statistical analysis. However, we believe that by providing melting curves (multiple data points) the reader will appreciate the robustness of these findings.

Referee #2

Hornung and colleagues present the interesting finding that 10-carboxymethyl-9-acridanone (CMA), a known anti-viral small molecule, activates type I IFNs via direct binding to STING, a recently revealed important central adaptor protein in mediating nucleic acid signalling to IFN induction. Furthermore they show that CMA acts via mouse, but not human STING, which is consistent with previous studies suggesting that CMA mainly induces anti-viral responses in mice and not humans. They also present a novel STING inhibitor (2,7-dibromo-CMA) which will be og great interest to the innate immunity community.

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1. The authors state (first paragraph in Intro) that conserved microbial patterns are commonly referred to as MAMPs. However these are COMMONLY referred to as PAMPs (pathogen associated molecular patterns) and RARELY referred to as MAMPs.

The reviewer is correct. We are sorry for this misnomer. We now changed the wording of this part.

2. Although the authors focus on the type I IFN induction pathway, they never actually measure type I IFN production / secretion (e.g. IFNbeta or IFNalpha by ELISA or bioassay). For example, in Fig 1D and E, the graphs are titled 'IFNbeta production' but what is actually measured is luciferase protein under the control of an IFNbeta promoter. At least some data should be presented showing that CMA causes endogenous type I IFN production in mouse and not human cells.

We are sorry for this mislabelling. We now labeled the graphs with “IFN-β reporter activation”. At the same time we have now included IFNβ ELISA measurements in Figure 1D. Moreover, we have performed bioassays using a VSV replicon system (Figure S1 and Figure S3) to show the antiviral activity of CMA-mediated STING activation. All in all these data clearly corroborate the concept of CMA inducing type I IFN via STING in murine cells.
Measuring IFNα in human PBMCs (the most sensitive cell population in our hands) again showed that CMA did not trigger type I IFN production in the human system (Figure 3C).

3. In most cases, increasing the concentration of CMA actually gives less of a response (Figs S2A, B, 5B). IS this a bell-shaped response? So do lower concentrations than those used here show a dose-dependent increase in responses?

The lower response towards CMA in STING-293T cells at higher concentrations is indeed due to a CMA/STING-specific toxicity (untransfected 293T cells remained viable upon CMA challenge). We now included new data to illustrate this fact (Figure S4). Interestingly, macrophages tolerated higher concentrations of CMA (Figure S1).

4. Fig 1E - please specify the units of time on the graph or in the legend.

We are sorry for this omission. The units have now been added to the figure.

5. IT has recently been shown that DDX41, as well as STING, senses and mediates responses to cyclic-di-nucleotides (Parvatiyar et al, NAture Immunology, 2012). In fact it is claimed there that DDX41 is more important for sensing than STING. Here, the authors should include or exclude a role for DDX41 in responding to CMA, which should be straightforward using siRNA.

As suggested by the reviewer, we have performed gain and loss of function experiments to study the role of DDX41 in CMA recognition by STING (please refer to Additional Fig. 1). We overexpressed DDX41 in ascending amounts in HEK293T-mSTING cells and then stimulated the cells with suboptimal ligand concentrations of CMA, c-diGMP, pppRNA and ISD. Overexpression of DDX41 led to an approximately 2-fold enhancement of pppRNA and c-diGMP-dependent transactivation of the IFNβ promoter, however CMA-mediated stimulated was not affected (As expected, ISD stimulation was not active in HEK293T-mSTING cells). On the other hand, performing loss of function experiments in MEFs using siRNA revealed no major impact of DDX41 silencing in the context of RIG-I or STING activation. Altogether, from these data we conclude that DDX41 does not play an important role in STING-dependent c-diGMP, cGAMP or CMA sensing. A similar conclusion can be drawn from the recent publication from the James Chen lab, in which is was shown that silencing DDX41 in MEFs does not impact on DNA sensing by STING.

6. Results section, third section, it is stated that CMA has been reported to induce IFN I in human cells. Please reference this statement and explain which cells? Was this cell type tested by the authors here? Perhaps there are cell-type differences, as well as species differences in the response to CMA?

We now cited Silin et al. in the context of this statement. In this review article several studies are summarized that have shown that CMA induces type I IFN in human cells.

We have taken much effort to identify a human cell type that would respond to CMA (beyond the data shown here, we have tested primary keratinocytes and purified monocytes that nicely respond to DNA stimulation, but show no response towards CMA). We have no explanation how other groups came to the conclusion that CMA induces type I IFN responses in human cells, yet we believe that our data conclusively show that human STING (the target for CMA in in the murine system) does not respond to CMA.

7. Can the authors measure any real anti-viral effects of CMA (e.g. reduced viral replication) in their cell culture models with and without mouse STING? It is still unclear from the paper whether STING actually mediates an anti-viral response to CMA (as opposed to cytokine induction).

As suggested be the reviewer, we have now included data to show that CMA-mediated STING

activation suppresses viral gene expression. To this effect, we have carried out two sets of experiments: (i) We stimulated macrophages with CMA and subsequently infected the cells with a VSV replicon in which viral gene expression is reported by FFLuc expression. These experiments show that CMA inhibits VSV replicon based gene expression in a dose dependent fashion (Suppl. Fig. 1). To prove that the CMA-mediated inhibition of viral gene expression is indeed due to the activation of STING and not mediated by a direct antiviral activity, we carried out experiments in which we transferred supernatant from CMA stimulated cells to cells that we subsequently infected with the VSV replicon (Suppl. Fig. 3). These experiments show that CMA induces antiviral factors in a STING-dependent fashion that can be transferred within the supernatant (most likely type I IFNs).

8. Fig 2 is negative data which should be combined with Fig S1, either as a main, or supplemental, figure.

We moved Fig 2 to the supplemental section as requested by the reviewer.

9. Figure 5 - please show the expression levels of the four different STING constructs, in order that the reader can correlate this with the functional readouts

The expression levels of the different STING constructs are now shown in the new Fig. 4 (and also in Fig. S5). These data show that all constructs are equally expressed.
Of note, since we did not have the lysates from the old experiments, we repeated these experiments. In this context, we now also used synthetic c-diGMP to stimulate the cells instead of the c-diGMP synthetase.

Referee #3

This manuscript demonstrates that CMA induces type I IFN via activating STING through the TBK1/IRF3 route in a species-specific manner. CMA-induced cytokine production depends on murine STING, and is independent of TLR and MAVS signals. The C-terminal ligand-binding domain (LBD) of STING determines this species-specificity CMA activity. Differential scanning fluorimetry (DFS) shows CMA bind LBD of murine STING. 2.7-dibromo-CMA inhibits murine STING activated by CMA and c-diGMP etc., functions as STING inhibitor. C-diGMP and CMA binding appears to be supported by p-p stacking between Y166 of murine STING and the acridone ring, and hydrogen bonding between R237 of murine STING and the carboxy group of CMA. The authors concluded that small molecule based on the CMA scaffold interfere STING activated by DNA sensing. Despite of the high homology of the human and murine LBD, human cells do not respond to CMA. DFS displays CMA does not bind the LBD of human STING shown
unlike c-diGMP.

Overall, the findings are novel and important in this field. The conclusion is supported by the data, and the manuscript is concisely written.

Specific comment
Given that the occupying binding pocket of STING interferes STING activation, the authors had better check whether 2,7-dibromo-CMA inhibits human STING activated by c-diGMP.

Given the whole new set of data (crystal structure of STING in complex with CMA), we decided to remove the 2,7-dibromo-CMA data, as we feel that it would go beyond the scope of the manuscript to study the inhibition of STING by 2,7-dibromo-CMA.

Minor comment

In Figure 1D, the authors show that the IFNb promoter is activated in response to CMA, but not to poly I:C, though they show that poly I:C induced Ifnb gene. Although this is a control experiment, the authors should check the data if this is correctly shown.

The data are indeed correctly shown. We know that our poly(I:C) preparation very potently inhibits translation in general. As such we assume that even though the message of IFNβ is transcribed, it is poorly translated. Of note, there seems to be a big difference between different poly(I:C) sources, which explains the discrepant reports on antiviral activity elicited by poly(I:C) in the literature.

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Thank you for submitting your revised manuscript to the EMBO Journal. Your manuscript has now been re-reviewed by referees # 1 and 2. As you can see below, both referees appreciate the introduced changes and support publication here. I am therefore very happy to accept the paper.

REFEREE REPORTS

Referee #1

One of concerns was the lack of enough evidence for direct binding of CMA to murine STING (mSTING). The authors now present the binary structure of mSTING bound with CMA. Although the structure does not explain how CMA activates STING nor does it tell why human STING cannot bind CMA, it does help support one of the major findings crucial to the focus of the current study - CMA elicits immune response in murine model via STING by directly binding with STING. As such, this additional data clearly strengthens the interesting novel finding being reported in this study and satisfies my queries.

Referee #2

With extra control experiments and functional assays, and especially with the inclusion of the crystal structure of CMA:STING the authors have significantly strengthened their manuscript and provided further strong evidence for their original claims.

