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Listeria monocytogenes induce IFN-beta expression through an IFI16, cGAS, and STING dependent pathway

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1st Editorial Decision 15 February 2014

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three experts and their comments are provided below.

As you can see the referees find the analysis interesting and are overall supportive. However, they also raise a number of important issues that should be resolved for publication here. The issues raised are clearly outline below and I will just mention a few of them here. Referee #1 would like to see more support for that you actually have a bacteria infection inside the cells and wants quantification to support that. Referee #2 echoes a similar point. Referee #3 finds that the analysis should be extended to either another human cell line or primary hMDMs to validate the findings reported. I realize that a lot of work is need, but the referees provide constructive comments and you might have some of the requested experiments done already. Should you be able to revise the manuscript along the lines indicated by the referees then we would like to see a revised version. I should point out that it is EMBO Journal policy to allow for one round of revision and that it is there important to resolve the major concerns at this stage.

If you have any further questions regarding the requested experiments just contact me.

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://emboj.embopress.org/about#Transparent_Process

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We generally allow three months as standard revision time. As a matter of policy, competing manuscripts published during this period will not negatively impact on our assessment of the conceptual advance presented by your study. However, please contact me as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this three-month deadline, please let me know and I can extend the deadline.

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

REFEREE REPORTS

Referee #1:

The manuscript by Hansen et. al., represents a potentially important paper that examines how human myeloid cells respond to Listeria and find that the response is fundamentally different than in mouse myeloid cells. They take advantage of a Listeria strain that induces high levels of type I IFN in mouse cells due to the over-secretion of c-di-AMP. They confirm that this is the case in mouse bone marrow derived macrophages, but not in human cells. In human cells, they conclude that the type I IFN stimulating ligand is DNA. There main evidence for this is that both STING and cGAS knockdowns block the response. Surprisingly, they also find that IFI16 is required. The latter is a very provocative result.

1. My major issue with this paper is that there is no data presented that allows me to appreciate the bacterial infections. In addition, their materials and methods fail to describe how they quantitate the infections other than stating an MOI. Did they wash after infection? Did they add gentamicin, as is standard? I would need to see bacterial growth curves in the cell and ideally micrographs showing how many bacteria per cell. In Fig 6 they show DAPI staining to highlight DNA, but this should also reveal bacteria in the cells, but I cannot see any. My fear is that bacterial DNA is being introduced into their cells via the listeriolysin O-dependent pores from the outside. This would be an artifact.

2. One of there major arguments that the ligand is not c-di-AMP is that the L028 strain doesn't induce more IFN. However, the other strain (10403S) induces way more IFN in the human cells which is really difficult to explain.

3. If bacterial DNA is the ligand, then why are they not activating cell death via the AIM2 pathway.

Referee #2:

The manuscript by Hansen et al. describes their work to characterize the molecular mechanisms of IFN-b production during infection of human macrophages by the bacterial pathogen L. monocytogenes. IFN-b induction by this bacterium has been reported to occur through two cytosolic immune sensing pathways, one relying on the detection of bacterial DNA, the other on the detection of the bacterial cyclic di-nucleotide, c-di-AMP. The authors utilize a combination of bacterial strains known to hyper secrete c-di-AMP and a number of host cell knockdowns to discriminate between activation of the two pathways during infection. Their findings support the conclusions that the induction of IFN-b during Listeria infection of human macrophages is independent of c-di-AMP sensing and instead relies on the detection of bacterial DNA. These findings are likely to be of general interest as an increasing number of bacteria known to stimulate these pathways emerge. Furthermore, these findings illustrate the importance for considering the model used for deciphering the innate immune response to infection.

Although I am generally positive to the findings and interpretation of the data I do have a couple of concerns that should be addressed.

1. The conclusion that DNA is a major trigger of IFN-b during Listeria infection is somewhat misleading. First, levels of IFN-b induced in human cells are really not very striking in comparison

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to the mouse macrophages, at least based on the transcriptional data in Figure 2. This suggests that this really might not be a "major" pathway triggered by Listeria in human cells. In fact, given that some human alleles have lost the ability to detect c-di-AMP, perhaps humans have evolved away from activating IFN in response to this infection. Along this line of reasoning, it seems important to establish that the DNA being sensed during infection is actually a relevant observation. DNA is both ubiquitous and sticky. During culture, bacterial lysis can lead to DNA release. This could then be carried into the cytosol with infecting Listeria. Why weren't bacteria washed prior to infection? Is IFN induction still observed if cells are washed with PBS before infection?

2. The authors show that bacteria with enhanced c-di-AMP secretion do not induce enhanced interferon in human cells, have the authors considered using strains of Listeria reported to undergo enhanced lysis (See Sauer et al. Cell Host Microbe. 2010 May 20;7(5):412-9)? This would provide further evidence in favor of the proposed model.

3. It seems the response to c-di-AMP in different knockdowns should have been characterized as it was for different stimuli in Fig. 4. The authors show that IFN induction during Lm infection correlates with the level of IFI16 in the cells. Furthermore, STING does not vary among these cell types. If bacterial CDNs are not stimulating the response then the presence and absence of IFI16 and cGAS should have no effect on IFN in response to this molecule, while both STING and DDX41 (as the authors showed) should be required. This would certainly further strengthen the claim for DNA as the Listeria inducing ligand.

4. Human STING can vary in responsiveness to bacterial cyclic di-nucleotides as described by the authors. However, there are different alleles of this gene and it has been shown that the protein from THP-1 cells actually is quite responsive to bacterial CDNs (See Diner et al. Cell Reports, Volume 3, Issue 5, 1355-1361). In light of this, it is quite surprising that there is not a dependence on c-di-AMP responsiveness in human cells certainly to the LO28 strain. Consistent with c-di-AMP responsiveness, the fluorescence microscopy shows significant activation of STING following c-di-AMP stimulation. Does STING relocalization occur in response to these bacteria independent of cGAS and IFI16? If these are shown to be dispensable for IFN in response to c-di-AMP (as recommended above), then STING activation (i.e. foci formation) should also not be observed in response to Lm.

As an aside, one interesting finding here might be the observation of STING activation in response to Lm in the absence of cGAS and IFI16. There have been other pathways described that are induced by STING separate from IFN, including STAT6 . Might another pathway be activated in response to the bacterial nucleotide?

Minor points:
Figure 1 panels E and F are mixed up compared to the legend.
How much cdA and DNA were in panel G.

Fig 2:
Panel A should the strain be 10403s?
Convert the d to Δ

Why are hMDM responding at such a high level in response to 10403s relative to LO28? Not to be offensive but please double check that the strains were not mixed up. If they were reversed the pattern would look very similar to the mBMMs and correlate well with c-di-AMP secretion.

Fig. 4
Panels in the figure don't match what is listed in the legend. A-D and E-H are swapped

Fig 5
Why was the DDX41 knockdown left out of this experiment? One would expect TBK1 to phosphorylation to not change, is this the case?

Fig 6
Co-localise should be co-localize

Line 371-cyclic-di-GMP-AMP should be cyclic-GMP-AMP. Also is this the 2x(3',5') (i.e. bacterial) or the 2',5'-3',5' (i.e. eukaryotic) nucleotide?

Referee #3:

Sensing of nucleic acids and cyclic di-nucleotide is a very important aspect of the innate immune responses to both viral and bacterial pathogens. Listeria have been historically used as a model to study the innate immune response to bacterial pathogens. While the pathway leading to type I IFN induction in murine macrophages has been extensively characterized, the pathway in human cells remains largely uncharacterized. In addition, recent data in the literature suggest that the STING-dependent pathways might be different between human and mice. In this manuscript, Hansen et al. investigate the signaling pathway leading to type I IFN induction in human macrophages infected with Listeria. Importantly, they demonstrate that the pathway leading to type I IFN is different in human macrophages as compared to murine macrophages. Particularly, they show that while in murine macrophages, bacterial cyclic-di-AMP is responsible for triggering the STING-mediated IFN induction, in human macrophages the cGAS/STING pathway detecting cytosolic bacterial DNA is major to elicit type I IFN secretion. In addition, they demonstrate a role for Ifi16 but no role for Ddx41.
This is a potentially very important study to understand the sensing mechanism leading to the type I IFN induction in human cells and weight the relevance of the previous published studies performed in mice.
However, the authors need to strengthen their data in order to be fully convincing. This is particularly important in this field since numerous (sometimes redundant) pathways have been described in the literature, most of them (although obtained in mice) in contradiction with the pathway identified by the authors.

Major comments:

1) One of the key results is the result presented in Fig.2 that is clearly suggesting a different sensing mechanism between murine bone marrow derived macrophages (100 fold higher IFN with LO28 strain as compared to 14030s) and human monocyte-derived macrophages (7 fold higher IFN with 10403s strain as compared to LO28). However the difference between the different strains LO28 and 14030s does not seem to be so important in THP-1 macrophages (2 fold higher IFN with 10403s strain as compared to LO28). As all the mechanistic insights of this study were obtained in the THP-1 macrophages, this raises the question to know whether PMA-differentiated THP-1 macrophages are a good model to learn what is the signaling pathway in human primary macrophages? For example, STING and Ddx41 protein levels seem quite different in PMA-differentiated macrophages and hMDMs. How does hMDM respond to cdiAMP and other CDN as compared to THP-1cells (see Fig. 1E)? The authors should try to validate their findings using siRNA in primary hMDMs. Alternatively, they should use another human cell line such as U937 to validate their findings.

2) Polymorphims in human STING have been shown to greatly influence the response to cyclic dinucleotides (Yi G et al Plos One, 2013). The THP-1 STING-endocding cDNA should be sequenced to decipher if the loss of response to Listeria-secreted cyclic-diAMP might be due to a cdiAMP-non-responding polymorphism (e.g. the R232H or the R293Q alleles which are largely represented in the human population-respective frequencies 13.7% and 1.5%). Similarly, in the experiment presented Fig2, the number of blood donors is unknown. Results of at least three independent donors should be shown ideally with the STING genotype associated.

3) All the mechanistic is demonstrated thanks to the use of THP-1 cell lines expressing ShRNA. The Materials and Methods section is very limited for these key reagents. The authors should give the clone number for each ShRNA used. How long do the authors keep the cells? Does it correspond to single cell clones or to a polyclonal population. The efficiency of the knock-down needs to be shown by western blotting. In addition, for the key findings (Ifi16 and cGAS), the authors need to show at least two independent ShRNA cell lines (or rescue experiments).

4) In most of these sensing pathways, there is a lot of redundancy with some pathway acting earlier than others. While the kinetic of IFN-b induction is shown, it would be important to see whether early on (4h PI), the induction is also dependent of ifi16, cGAS and independent on Ddx41. Indeed,

while the DNA sensing pathway might be predominant at 6h PI, at an early time points if there is no lysis of Listeria within the host cytosol, the c-diAMP sensing pathway might dominate. In addition, ifi16 is supposed to be IFN-inducible in contrast to Ddx41 (Parvatiyar K et al. Nat immunol 2012), Ddx41 could thus play a role in Listeria sensing early on.

5) The immunofluorescence for STING and Ifi16 in Listeria-infected cells is not the most convincing. Fixation with PFA (Fig. 6A, B) gave much lower background than the Methanol fixation (Fig. 6C) and should be used. It would be nice to see quantification of the number of STING specks in WT, ifi16 and cGAS ShRNA THP-1 cells.

6) The most convincing experiments are the one presented in Fig. 4I, J, K, L. Those experiments represent the core of the paper and should be further controlled by showing that TNF-a levels are not affected in the different ShRNA cell lines upon infection. In addition, control with cGAMP transfection and polyI:C treatment should be shown to demonstrate the specificity of the knock-down in regards to Listeria infection.

7) Fig5: Controls with cGAMP transfection should be shown to demonstrate the specificity of the shRNA knock-down in regards to Listeria/DNA sensing. In addition, you should be comprehensive and include the two other cell lines ShDdx41 and STING.

Minor comments:
1-Line 131: double check the DNA concentration 0.125 mg/ml or 0.125 ug/ml as indicated in the figure?
2-Line 224: The immunofluorescence is showing a perinuclear localisation which is consistent with an ER localisation but not directly the latter localisation.
3-A lot of experimental details are lacking (e.g. concentrations in Fig. 1G, the name of the Listeria strain and the MOI need to be indicated for each figure...).
4-Fig.6: 2 or 4h post treatment, the text does not jibe with the figure legend.
5-Fig.6: does DAPI in your condition stain all bacteria or only dead bacteria?
6-BMDC are presented in the materials and methods section, not in the results.
7-Did the author look at LRRFIP1, which has been shown to be involved in Listeria sensing (Yang P et al, nat immunol 2010)? This work should be at least cited.
8-I don't think the part with the Listeria extract is the most relevant even if such techniques are widely used. The stability of CDN in this extract should be discussed.

1st Revision - authors' response 13 May 2014

Referee #1:

1. My major issue with this paper is that there is no data presented that allows me to appreciate the bacterial infections. In addition, their materials and methods fail to describe how they quantitate the infections other than stating an MOI. Did they wash after infection? Did they add gentamicin, as is standard? I would need to see bacterial growth curves in the cell and ideally micrographs showing how many bacteria per cell. In Fig 6 they show DAPI staining to highlight DNA, but this should also reveal bacteria in the cells, but I cannot see any. My fear is that bacterial DNA is being introduced into their cells via the listeriolysin O-dependent pores from the outside. This would be an artifact.

RE: In the revised manuscript we provide data from two approaches where we have addressed the question on whether the bacteria is actually able to infect the cells. First, we have fixed infected cells and performed electron microscopy. This data demonstrate that *L. monocytogenes* does indeed enter into the cells and is found both in vacuolar compartments and in the cytoplasm (Figure S1D). Second, we have performed a classical bacterial growth experiment on lysates from cells infected for different time intervals (Figure S1E). These data demonstrate that *L. monocytogenes* does productively infect the PMA-differentiated THP1 cells used for many experiments in this study.
In the revised manuscript we also present data from an experiment where the cells were washed in PBS after infection, and demonstrate that introduction of this procedure does not affect the IFNbeta expression by the infected cells (Figure S1B). Finally, cells were treated with gentamycin 1 h after infection. This information has been added to the Methods section.

2. One of their major arguments that the ligand is not c-di-AMP is that the L028 strain doesn't induce more IFN. However, the other strain (10403S) induces way more IFN in the human cells which is really difficult to explain.
RE: As the reviewer correctly points out, the finding that L028 does not induce more IFN than 10403S in human cells represents an argument against c-di-AMP being the major ligand inducing IFN expression. At this stage we cannot explain why 10403S in fact induces more IFN than L028 in hMDMs and to a lesser extent also in THP1 cells. Nevertheless, these data do illustrate a key difference between human and murine myeloid cells in responsiveness to Listeria infection, and demonstrate that IFN induction does not correlate with the activity of MdrT in human cells. In the revised manuscript we further include data with the human monocytic cell line U937, in which – like in MDMs and THP1s – Listeria-induced IFN-beta expression does not correlate with the activity of MdrT (Figure 2D).

3. If bacterial DNA is the ligand, then why are they not activating cell death via the AIM2 pathway.
RE: During the revision of the manuscript, we have generated a THP1-derived AIM2 shRNA knockdown cell line with more than 80% knock-down. However, this cell line still induced IL-1b in response to synthetic DNA (poly-dAdT) in LPS-pretreated cells, thus preventing us from using it for assessment of AIM2-dependent inflammasome activation. In the infected cultures we do observe cell death starting from between 18 and 24 hrs p.i. However, since all data are based on samples isolated before this time point (e.g. standard set-up for RNA isolation was 6 h p.i.), we do not think this has impacted on our results. In the revised manuscript we have included data demonstrating that the IL- 1b response to Listeria infection is dependent on bacterial escape into the cytoplasm, thus providing some hint for the involvement of the AIM2 pathway in inflammasome activation during Listeria infection as reported by others (Cell Host Microbe. 2010 May 20;7(5):412-9). These data are presented as Figure 1C in the revised manuscript.

Referee #2:
1. The conclusion that DNA is a major trigger of IFN-b during Listeria infection is somewhat misleading. First, levels of IFN-b induced in human cells are really not very striking in comparison to the mouse macrophages, at least based on the transcriptional data in Figure 2. This suggests that this really might not be a "major" pathway triggered by Listeria in human cells. In fact, given that some human alleles have lost the ability to detect c-di-AMP, perhaps humans have evolved away from activating IFN in response to this infection. Along this line of reasoning, it seems important to establish that the DNA being sensed during infection is actually a relevant observation. DNA is both ubiquitous and sticky. During culture, bacterial lysis can lead to DNA release. This could then be carried into the cytosol with infecting Listeria. Why weren't bacteria washed prior to infection? Is IFN induction still observed if cells are washed with PBS before infection?
RE: This reviewer raises some concern about DNA being the main trigger, and request data from experiments where the bacteria are washed (to remove most of the extracellular DNA) prior to infection of the macrophages. We have now performed experiments where the bacteria have been washed three times in PBS prior to infection. These data show no role for extracellular DNA (or other substances washed away by PBS) in the IFN response induced after treatment with Listeria. The data are presented as Figure S1B and described in the revised manuscript.

2. The authors show that bacteria with enhanced c-di-AMP secretion do not induce enhanced interferon in human cells, have the authors considered using strains of Listeria reported to undergo enhanced lysis (See Sauer et al. Cell Host Microbe. 2010 May 20;7(5):412-9)? This would provide further evidence in favor of the proposed model.
RE: This is a very good point and we have therefore taken two approaches to examine for induction of IFNb expression under conditions of enhanced bacteriolysis. First, we have treated macrophages with increasing concentrations of ampicillin 2 hours after infection and isolated total RNA 4 hours later. Second, the cells were infected with a Listeria mutant reported to have a higher tendency to undergo lysis (Sauer et al. Cell Host Microbe. 2010 May 20;7(5):412-9). In both cases did we observe elevated IFNb expression under conditions of enhanced bacteriolysis. These data are shown as Figure 2E and 2F in the revised manuscript.

3. It seems the response to c-di-AMP in different knockdowns should have been characterized as it was for different stimuli in Fig. 4. The authors show that IFN induction during Lm infection correlates with the level of IFI16 in the cells. Furthermore, STING does not vary among these cell

types. If bacterial CDNs are not stimulating the response then the presence and absence of IFI16 and cGAS should have no effect on IFN in response to this molecule, while both STING and DDX41 (as the authors showed) should be required. This would certainly further strengthen the claim for DNA as the Listeria inducing ligand.
RE: In the revised manuscript, we have extended Figure S6 to also include data with cell lines with KD of cGAS, IFI16, and STING. The data demonstrate – as expected – that lack of IFI16 or cGAS does not affect the responsiveness of the cells to c-di-AMP.

4. Human STING can vary in responsiveness to bacterial cyclic di-nucleotides as described by the authors. However, there are different alleles of this gene and it has been shown that the protein from THP-1 cells actually is quite responsive to bacterial CDNs (See Diner et al. Cell Reports, Volume 3, Issue 5, 1355-1361). In light of this, it is quite surprising that there is not a dependence on c-diAMP responsiveness in human cells certainly to the LO28 strain. Consistent with c-di-AMP responsiveness, the fluorescence microscopy shows significant activation of STING following c-di-AMP stimulation. Does STING relocalization occur in response to these bacteria independent of cGAS and IFI16? If these are shown to be dispensable for IFN in response to c-di-AMP (as recommended above), then STING activation (i.e. foci formation) should also not be observed in response to Lm.
RE: In the revised manuscript we demonstrate that IFI16, cGAS, and STING relocalize after DNA transfection and Listeria infection but only STING relocalizes after c-di-AMP transfection (Figure 6 and new Figure S7). These data are consistent with the functional data presented in Figure 4 and S6. During the revision, we have examined for STING foci formation in IFI16 and cGAS KD cells, and do indeed observe a clear tendency towards fewer STING foci. However, the data are not conclusive,and the authors do not think the shRNA KD system is optimal for microscpopy-based analyses. We would prefer to use CRISPR/Cas-generated KO cells, and are now in the process of generating these cells. Therefore, in the revised manuscript we do not include data on DNA/Listeria-induced STING formation IFI16 and cGAS KD cell lines.

Minor points:

Figure 1 panels E and F are mixed up compared to the legend.
How much cdA and DNA were in panel G.
RE: The Legend for figure 1 has been corrected.

Fig 2: Panel A should the strain be 10403s? Convert the d to Δ
RE: Done!

Why are hMDM responding at such a high level in response to 10403s relative to LO28? Not to be offensive but please double check that the strains were not mixed up. If they were reversed the pattern would look very similar to the mBMMs and correlate well with c-di-AMP secretion.
RE: This is of course an important point, and we have hence repeated the experiment. Again we see that 10304s induces more than LO28 in hMDMs but not in mBMMb. This observation we cannot explain at this stage, but it supports the conclusion that IFN-induction by L.monocytogenes does not correlate with expression of the efflux pumps MdrT.

Fig. 4. Panels in the figure don't match what is listed in the legend. A-D and E-H are swapped.
RE: The Legend for figure 4 has been updated.

Fig 5. Why was the DDX41 knockdown left out of this experiment? One would expect TBK1 to phosphorylation to not change, is this the case?
RE: This experiment has been performed included in the revised manuscript (Figure 5 right panel).

Fig 6. Co-localise should be co-localize
RE: Corrected!

Line 371-cyclic-di-GMP-AMP should be cyclic-GMP-AMP. Also is this the 2x(3',5') (i.e. bacterial) or the 2',5'-3',5' (i.e. eukaryotic) nucleotide?
RE: We used the 3',5'-3',5' cGAMP (since the 2',5'-3',5' was not commercially available when these experiments were initiated). The text has been updated.

Referee #3:

Major comments:

1) One of the key results is the result presented in Fig.2 that is clearly suggesting a different sensing mechanism between murine bone marrow derived macrophages (100 fold higher IFN with LO28 strain as compared to 14030s) and human monocyte-derived macrophages (7 fold higher IFN with 10403s strain as compared to LO28). However the difference between the different strains LO28 and 14030s does not seem to be so important in THP-1 macrophages (2 fold higher IFN with 10403s strain as compared to LO28). As all the mechanistic insights of this study were obtained in the THP-1 macrophages, this raises the question to know whether PMA-differentiated THP-1 macrophages are a good model to learn what is the signaling pathway in human primary macrophages?
For example, STING and Ddx41 protein levels seem quite different in PMA-differentiated macrophages and hMDMs. How does hMDM respond to cdiAMP and other CDN as compared to THP-1cells (see Fig. 1E)? The authors should try to validate their findings using siRNA in primary hMDMs. Alternatively, they should use another human cell line such as U937 to validate their findings.
RE: This reviewer raises some concern about the much more potent induction of IFN by 10403s in MDMs but not in THP1s. The main point from this data is that unlike in murine cells, for all the human cells we observe that 10403s induces IFN expression to the same extent or more than LO28. This suggests that the expression of the multidrug efflux pump MdrT does not correlate with IFN induction in human cells. This is further supported by new data from U937 included in the revised manuscript (Figure 2D). At this stage we cannot explain why 10403s is a more potent inducer of IFN expression than LO28 in MDMs, which is not seen to the same extent in THP1 cells. One possibility is that, this bacteria strain undergoes more bacteriolysis than LO28 in the MDMs. It should also be noted, that although we see the same phenomenon for donor 2 and 3 (Figure S3), this is not as pronounced as for donor 1 (Figure 2B).

2) Polymorphims in human STING have been shown to greatly influence the response to cyclic dinucleotides (Yi G et al Plos One, 2013). The THP-1 STING-encoding cDNA should be sequenced to decipher if the loss of response to Listeria-secreted cyclic-diAMP might be due to a cdiAMP-nonresponding polymorphism (e.g. the R232H or the R293Q alleles which are largely represented in the human population-respective frequencies 13.7% and 1.5%). Similarly, in the experiment presented Fig2, the number of blood donors is unknown. Results of at least three independent donors should be shown ideally with the STING genotype associated.
The data presented in Figure 2B are from one donor. In the revised manuscript we additionally show data from two other independent donors. These data are presented in a new Figure S3.

3) All the mechanistic is demonstrated thanks to the use of THP-1 cell lines expressing ShRNA. The Materials and Methods section is very limited for these key reagents. The authors should give the clone number for each ShRNA used. How long do the authors keep the cells? Does it correspond to single cell clones or to a polyclonal population. The efficiency of the knock-down needs to be shown by western blotting. In addition, for the key findings (Ifi16 and cGAS), the authors need to show at least two independent ShRNA cell lines (or rescue experiments).
RE: In the revised manuscript we provide clone number for each shRNA used, and also information on how long the cells are kept in culture, as well as single versus poly clonicity of the populations. The cell lines used were recently described by this group (PNAS. 110(48):E4571; J Immunol. 190(5):2311), and the original descriptions included demonstration of degree of knock-down by Western blotting. In addition, our previous work with cell lines with shRNA-mediated KD of factors in the DNA sensing pathway included use of independent cell lines – all data supporting the conclusion that the observed phenomena in the DNA-sensing pathway were not due to shRNA-mediated offtarget effects (PNAS 110(48):E4571). All these informations have been added to the revised manuscript.

4) In most of these sensing pathways, there is a lot of redundancy with some pathway acting earlier than others. While the kinetic of IFN-b induction is shown, it would be important to see whether early on (4h PI), the induction is also dependent of ifi16, cGAS and independent on Ddx41. Indeed, while the DNA sensing pathway might be predominant at 6h PI, at an early time points if there is no lysis of Listeria within the host cytosol, the c-diAMP sensing pathway might dominate. In addition,

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ifi16 is supposed to be IFN-inducible in contrast to Ddx41 (Parvatiyar K et al. Nat immunol 2012), Ddx41 could thus play a role in Listeria sensing early on.
RE: We have now performed the proposed kinetics experiments using Ctrl shRNA, sh cGAS, sh IFI16, and sh STING. The cells were stimulated with DNA or infected with Listeria. The data demonstrate that cGAS and STING are required for induction of IFN expression by both DNA and Listeria at alltime points tested. By contrast, IFI16 was not essential for IFNb expression at the early time points, potentially suggesting a role for this protein in maintenance rather than initiation of DNA-driven IFN expression. The data are presented as figure 4M and 4N in the revised manuscript and described/discussed in the text. This series of experiments were done as the very last experiments in the revision process. Unfortunately, the routine check for knock-down of expression revealed that the sh DDX41 cells had lost the knock-down, for which reason, we were not able to generate data from these cells.

5) The immunofluorescence for STING and Ifi16 in Listeria-infected cells is not the most convincing. Fixation with PFA (Fig. 6A, B) gave much lower background than the Methanol fixation (Fig. 6C) and should be used. It would be nice to see quantification of the number of STING specks in WT, ifi16 and cGAS ShRNA THP-1 cells.
RE: As described in the response to point 4 of reviewer #2 we have examined for STING foci formation in IFI16 and cGAS KD cells, and do indeed observe a clear tendency towards fewer STING foci. However, since the data are not conclusive (most likely due to between 15 and 20 % remaining expression in the KD cell lines), the authors do not think the shRNA KD system is optimal for microscpopy-based quantitative analyses. In the revised manuscript we do however provide data on the percentage of cells with STING foci formation above the back-ground level (dsDNA: 50%; c-di- AMP: 75%; *L.monocytogenes*: 30%. This information has been added to the text in the revised manuscript.

6) The most convincing experiments are the one presented in Fig. 4I, J, K, L. Those experiments represent the core of the paper and should be further controlled by showing that TNF-a levels are not affected in the different ShRNA cell lines upon infection. In addition, control with cGAMP transfection and polyI:C treatment should be shown to demonstrate the specificity of the knockdown in regards to Listeria infection.
RE: The shRNA cell lines were thoroughly controlled for specificity for the DNA sensing pathway in Jakobsen et al (PNAS, 2013). In the revised manuscript, we have included a data demonstrating that Listeria-induced TNF-a expression in human myeloid cells is independent of a cytosolic pathway (Fig 1D). Therefore, one additional control, of relevance for the present work would be to test for Listeria-induced TNF-a expression in the shRNA cell lines. The prediction would be that TNF-a induction should be independent of components on the DNA sensing pathway. We have in fact performed such experiments at a preliminary level for some of the cell lines, and do indeed find this. However, since the cell lines were already well characterized for specificity, the authors have not prioritized the experiment proposed examining for TNF-a expression after Listeria infection. However, if, the reviewer insists on this point, the authors shall be pleased to complete this experiment to a conclusive stage. In another part of this point, reviewer #3 proposes to control the cells for responsiveness to cyclic-di-nucleotides (cGAMP). The authors have complied with this point by examining for IFNb induction by c-di-AMP in the full panel of shRNA cell lines. The data demonstrate as expected that DDX41 and STING, but not cGAS and IFI16, are not required to achieve full responsiveness to ci-di-AMP (Figure S6).

7) Fig5: Controls with cGAMP transfection should be shown to demonstrate the specificity of the shRNA knock-down in regards to Listeria/DNA sensing. In addition, you should be comprehensive and include the two other cell lines ShDdx41 and STING.
RE: In the revised Figure 5 we have included data with shDDX41 and shSTING demonstrating that Listeria-induced TBK1 phosphorylation is reduced in shSTING cells but not shDDX41 cells. Due to lack of time and the necessity to prioritize, we have not performed the requested control experiment using transfection with cGAMP and detection of pTBK1 as readout. The main reason for prioritizing this way is that we feel that the shRNA cells have been well characterized elsewhere in the manuscript as well as in a previous publication from the lab (Jakobsen et al, PNAS, 2013).

Minor comments:
1-Line 131: double check the DNA concentration 0.125 mg/ml or 0.125 ug/ml as indicated in the figure?

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RE: Corrected!

2-Line 224: The immunofluorescence is showing a perinuclear localisation which is consistent with an ER localisation but not directly the latter localisation.
RE: Corrected!

3-A lot of experimental details are lacking (e.g. concentrations in Fig. 1G, the name of the Listeria strain and the MOI need to be indicated for each figure...).
RE: Information added!

4-Fig.6: 2 or 4h post treatment, the text does not jibe with the figure legend.
RE: Thank you for identifying this error – now corrected!

5-Fig.6: does DAPI in your condition stain all bacteria or only dead bacteria?
RE: In this work, we have not distinguished between live or dead bacteria. The issue of how the bacterial DNA is made accessible for cytosolic DNA sensors is very relevant, but the authors find this to be beyond the scope of the present manuscript.

6-BMDC are presented in the materials and methods section, not in the results.
RE: The text has been corrected to describe the generation of murine BMMs.

7-Did the author look at LRRFIP1, which has been shown to be involved in Listeria sensing (Yang P et al, nat immunol 2010)? This work should be at least cited.
RE: We did not look into the potential involvement of LRRFIP1, mainly because this proposed sensor was reported to signal through a b-catenin-acetyltransferase pathway and not the STING-TBK1-IRF3 pathway under investigation in this study. However, the authors agree that this important paper should be cited, and have included it in the revised manuscript (Yang et al Nat Immunol. 2010).

8-I don't think the part with the Listeria extract is the most relevant even if such techniques are widely used. The stability of CDN in this extract should be discussed.
RE: The authors have set up an assay to measure IFN induction by heat-stable small molecules used (in a slightly different form) by the Chen group in the first Science paper describing cGAMP as the second messanger in DNA signaling. Using this system we now demonstrate that the bacterial extracts do not contain significant levels of c-di-AMP. We have included this new data as Figure S5C in the revised manuscript. Together, with the data on IFN stimulation with live L. monocytogenes, they further support the conclusion that bacterial DNA rather than c-di-AMP is the main stimulator of IFNb expression in infected human macrophages.

2nd Editorial Decision	26 May 2014

Thank you for submitting your revised manuscript to The EMBO journal. The three referees have now seen your study and as you can see below they appreciate the introduced changes.

Referees #1 and 2 have a few remaining concerns that can be addressed with appropriate text changes. Once we received the revised version we will proceed with its acceptance for publication here.

REFEREE REPORTS

Referee #1:

The resubmission of the manuscript by Hansen et al., includes a substantial amount of new data which has improved the manuscript and most, but not all of the reviewers comments have been addressed. In my case (Reviewer #1), most of my issues have been well addressed other than explaining in Fig 6 why the DAPI staining fails to identify intact bacteria.

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I also note that the comment from reviewer #3 requesting the authors report on the STING allele in the THP-1 cells was not addressed. This is an important point.

Referee #2:

The authors have done a good job addressing the critiques. The data presented and the analysis both seem to support the premise that DNA is a major source of IFN-b during infection of human myeloid cell lines. I only have minor comments.

1) Line 121: Induction of IL-1β protein expression was also dependent on bacterial escape into the cytoplasm.

Actually induction of proIL-1B is dependent on TLR stimulation. The processing and secretion of IL-1B is dependent upon cytosolic entry. I believe the authors are measuring mature IL-1B. If so, this should be worded to distinguish between the two.

2) I am a bit concerned about the following contradictory statements. Why are you providing new data for these knockdowns in only two of the three proposed experiments? Were the knockdowns checked and good for the western in Fig. 5?

Line 238: As shown in Figure 5, L. monocytogenes-induced phosphorylation of TBK1, which occurs immediately downstream of STING (Tanaka and Chen, 2012), was compromised in cells with reduced expression of IFI16, cGAS or STING but not DDX41.

Figure S6 shows the role of DDX41 in c-di-AMP induced IFN-b using knockdowns.

Yet in the rebuttal the authors state:
Unfortunately, the routine check for knock down of expression revealed that the shDDX41 cells had lost the knock down, for which reason, we were not able to generate data from these cells.

3) Line 445: with cyclic-di-AMP corresponding to a final concentration of 3,6-50 μM.

This description of nucleotide concentration is quite confusing. Is it 3.6-50 or what?

4) Although they have provided one growth curve of Listeria in THP-1 cells (as requested by reviewer #1) it would have been nice to see that levels of bacterial growth in the knockdowns were comparable to control cells to ensure the changes in host response were actually due to host cell signaling not changes in bacterial burden.

With regard to the bacterial growth curve, this is typically plotted on log scale.

Referee #3:

The manuscript has improved thanks to the addition of the temporal requirement of the different players, to the addition of several controls regarding the ShRNA cell lines and the inclusion of primary macrophages from three healthy donors.
The data fully support the conclusions that bacterial DNA and not bacterial cyclic dinucleotide is the major PAMP leading to type I IFN induction in human myeloid cells following Listeria monocytogenes infection.

2nd Revision - authors' response 27 May 2014

Reviewer #1

1. Why does the DAPI staining fail to identify intact bacteria?
RE: At this stage we cannot fully explain this. However, we find it possible that the DAPI

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molecule has restricted access to the DNA of live/intact bacteria as compared to the lysed bacteria. In support of this, we generally do not see mitochondria (ancient bacteria) to stain positive for DAPI in our standard protocol where there is clear nuclear DAPI staining. We have not included this information in the revised manuscript, since we feel that a more through characterization of this would be required before inclusion in a publication.

2. Comment on the STING allele in THP-1 cells.
RE: This is a good point, and we are glad to introduce a brief section in the discussion on the sequence difference between THP1 and reference STING and the potential impact for the present study (line 303).

Referee #2:
1. Line 121: Induction of IL-1β protein expression was also dependent on bacterial escape into the cytoplasm.
RE: In the revised manuscript we have rephrased this sentence to "Accumulation of IL-1β protein in the culture supernatant was also dependent on bacterial escape into the cytoplasm" (line 121).

2a. I am a bit concerned about the following contradictory statements. Why are you providing new data for these knockdowns in only two of the three proposed experiments? Were the knockdowns checked and good for the western in Fig. 5?
RE: As also stated in the first revision, the degree of knock-down was checked by Western blotting. In the first round of revision we provided the requested data on pTBK1 in cells with KD of STING and DDX41. These data confirm the IFNb expression data that Listeria-activated TBK1 activation is dependent on STING but not DDX41. Together with the data presented in the original version of the manuscript, the authors think that the data do support the conclusion that "…L. monocytogenesinduced phosphorylation of TBK1, ……, was compromised in cells with reduced expression of IFI16, cGAS or STING but not DDX41"

2b. Figure S6 shows the role of DDX41 in c-di-AMP induced IFN-b using knockdowns.
RE: The revision proves lasted 3 month and we typically only perform experiments with the shRNA KD cells within one month after thawing (always including a control of KD by WB). The data presented in Figure S6 were performed early in the revision, where the thawed batch of cells exhibited good knock-down. Unfortunately, when we thawed out another batch of DDX41 KD cells (towards the end of the fixed 3 month revision period), with the purpose to perform the requested kinetics experiment, we observed very poor knock-down of DDX41. Therefore, some but not all of the requested data including DDX41 KD cells could be provided.

3. Line 445: with cyclic-di-AMP corresponding to a final concentration of 3,6-50 μM.
RE: We agree with reviewer #2 that the description of the c-di-AMP transfection procedure is not clear, and we have therefore rephrased it in the revised manuscript (line 447).

4a. Although they have provided one growth curve of Listeria in THP-1 cells (as requested by reviewer #1) it would have been nice to see that levels of bacterial growth in the knockdowns were comparable to control cells to ensure the changes in host response were actually due to host cell signaling not changes in bacterial burden.
RE: The authors agree on this point, but did eventually prioritize other parts of the experiments proposed by the reviewers. The main reason for this decision was that IFNβ is believed to play a pathological rather than an antibacterial role in Listeria infection.

4b. With regard to the bacterial growth curve, this is typically plotted on log scale.
RE: In the revised manuscript, the data on bacterial growth are plotted in log scale (Figure S1E).

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