THE
EMBO
JOURNAL

Manuscript EMBO-2012-80790

RIG-I detects infection with live *Listeria* by sensing secreted bacterial nucleic acids

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Review timeline:

Submission date:	19 January 2012
Editorial Decision:	27 February 2012
Revision received:	09 July 2012
Editorial Decision:	9 August 2012
Revision received:	30 August 2012
Accepted:	03 September 2012

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 27 February 2012

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

As you can see while there is an interest in the study, significant concerns are also raised with the key findings. In particular, the role of IPS-1 in the process is brought up also considering that other labs have reported different results. There are also concerns raised regarding the role of SecA2. Important controls and information for how some of the experiments were carried out are also missing. Further data to look at the IFN-beta levels in vivo is also needed. In other words, the referees raise significant hesitation regarding many of the conclusions and major work needs to be carried out to support the key findings. I don't know if you will be able to address the concerns raised in full, but that is what it would take for publication in the EMBO Journal. Should you be able to add more data that would resolve the issues raised then we would be willing to look at a revised version. Please note that as it is unclear at this stage if the proposed revisions will be able to address the concerns raised, that the outcome of a re-review is uncertain and you should be aware of this up front. I would also like to add that it is EMBO Journal policy to allow a single major round of revision only and it is therefore important to address the concerns raised 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

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Should you have any further questions please don't hesitate to contact me.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS

Referee #1

This manuscript investigates receptors and pathways leading to IFN-beta and IL-1beta production in macrophages infected with Listeria monocytogenes (Lm). The authors propose the SecA2-dependent release of nucleic acids by intracellular Lm. Secreted RNA molecules are more potent stimulators of cytokine production than their intracellular counterparts. Both RNA and DNA employ the molecules Rig I (Mda5), IPS-1 and Sting to stimulate IFN-b production, whereas IL-1b release requires Rig I/Mda5, Card9 and the inflammasome adapter Asc. The authors claim this occurs without an impact on caspase 1 cleavage.

Comments:
1. The authors claim superior ability of secreted RNA to stimulate the Rig-I pathway. This is done based on RNA amounts, rather than numbers of molecules. The superior immune stimulation may simply be due to a smaller average size of the secreted RNA. Alternatively total RNA may contain species that actively suppress signal transduction. One way to test this is adding increasing amounts of total RNA to the secreted RNA.
2. ELISA is used as a read-out of signaling to the IFN-b gene. The authors cannot rule out effects of the signaling paths subsequent to gene induction. mRNA data should be shown.
3. The reason for the discrepancy to the Sun et al. and Soulat et al. papers regarding the involvement of IPS-1 remains somewhat of a mystery. The authors try to explain this by showing that the IPS-1 requirement for IFN-b induction is only seen at low to moderate MOI. Sun et al. tested MOIs of 10 and 100, Soulat et al. used an MOI of 10. Both studies should have detected the IPS-1 involvement according to figure 1D of this manuscript. Unlike IPS-1, the authors report an involvement of Rig-I also at high MOIs. The IPS-1-independent pathway operating at high MOI remains unexplained.
4. SecA2 mutant Lm are defective regarding cell-to-cell spreading. Can the authors rule out that this influences their results? Is it not possible that SecA2 mutants infect a lower proportion of cells over time, but, due to their inability to spread to neighbouring cells, replicate to higher numbers in those cells that are infected? This notion is supported by figure 4D showing higher numbers of actin clouds in SecA2 mutants. In the same figure it appears that Hly mutants are also covered by actin. Is this a technical problem?
5. There is no normalization control for figure 4C regarding the impact of the SecA2 translocon on secretion. A secreted protein not affected by SecA2 should be shown as well as a protein secreted by SecA2.
6. figure 4G shows that upon infection of RigI-/- macrophages with SecA2 mutants about 20% IFN-b production remains. How is this explained?
7. The authors state that 'the CARD9 pathway also mediates IL-1b activation in response to cytosolic Listeria without affecting caspase-1 cleavage'. To my opinion there is much less cleaved Caspase 1 in CARD9-/- cells in figure S5C.
8. The manuscript contains a significant amount of recently published data (e.g. figure 1A) without providing appropriate references: O'Connell R, M. et al. JI 174, 1602 (2005); O'Riordan et al. PNAS 99, 13861 (2002); Stetson and Medzhitov, Immunity. 24:93 (2006); Stockinger, S. et al. JI 169 (2002); Stockinger, S. et al. JI 173, 7416 (2004).

Referee #2

This manuscript by Abdullah, et al, investigates the role of the nucleic acid sensor, RIG-I, in detection of bacterial nucleic acids during Listeria monocytogenes infection. The authors show evidence that a source of bacterial nucleic acids during infection is secreted RNA and secreted

DNA, which appears to be released through the auxiliary secretion system, SecA2.

Recent studies have documented the participation of nucleic acid sensors in the innate immune response to bacterial pathogens. However, the mechanisms by which bacterial nucleic acids are exposed to these sensors are not fully defined. The authors have provided evidence for a novel and interesting SecA2-mediated mechanism that can explain how bacterial nucleic acids enter the cytosol in the absence of bacteriolysis. Although it still unclear what the function of secreted bacterial nucleic acids, and why they are secreted through a secretion mechanism strongly associated with pathogenesis, the data shown in general support the stated conclusions, and the experiments are well executed.

1. The authors should show the RIG-I dependent stimulatory activity of the secreted nucleic acids fraction treated with both RNase and DNase to determine whether that completely abrogates Type I IFN induction. This is a key control. Although the removal of the 5' phosphate from the seRNA did strongly decrease activity, CIAP is not specific for RNA and could possibly be acting on other substrates in the sample.
2. The authors should include in each figure legend, how many times each set of experiments was repeated. This information was only included for a few experiments.
3. The authors show in Fig. 4C that the Listeria secA2 mutant secretes less nucleic acid by providing an image of an agarose gel. Although the data shown support their conclusion, this is not really a quantitative method, nor does it show the magnitude of the defect over several experiments. This experiment should be done quantitatively using OD 260/260 or by using fluorescence probes that bind, e.g. ds DNA to give a better measure of the defect.
4. In Fig. 4D, the low magnification of the fluorescence images makes it difficult to appreciate the data supporting equivalent cytosolic residence of the secA2 mutant, since these images are meant to be an indication of cytosolic infection. Readers would benefit from seeing images at higher magnification.
5. In Supplemental Fig. 5B and C, the authors still observe caspase-1 cleavage even in absence of RIG-I or CARD-9. The data shown in this figure are important for their model and should be included in the main body of the manuscript, with loading controls (e.g., the full length caspase-1 or a separate protein control like GAPDH). Although the authors speculate that the caspase-1 cleavage still observed during infection by the secA2 mutant may come from AIM2 or NLRP3, this could be tested more directly by transfecting in the secreted nucleic acid fraction with or without RNase+DNase treatment. Presumably in this case (unlike during infection with the SecA2 mutant), caspase-1 activation would be absent in the RIG-I deficient macrophages.

Referee #3

In this manuscript, Abdullah and collaborators focus on two of the most studied innate immune pathways (Pathway leading to type I IFN production and the inflammasome pathway) using one of the most studied host-pathogen system: Listeria monocytogenes infection of bone marrow-derived macrophages. They show that the RIG-I/IPS-1 pathway is important to trigger IFN-β production in response to Listeria infection, that transfection of macrophages with secreted RNA from L. monocytogenes can recapitulate this finding, that a secA2 mutant is leading to a decrease type I IFN induction, which correlates with less nucleic acid secretion in vitro. Furthermore, they show that RIG-I is important for inflammasome activation in response to L. monocytogenes and that infection with the secA2 mutant triggers less inflammasome activation than infection with WT L. monocytogenes. The authors conclude on a model in which specific nucleic acids are secreted by cytosolic L. monocytogenes via the secA2 pathway and very sensitively detected by RIG-I to trigger type I IFN induction and inflammasome activation.

While the results are potentially very exciting, they are highly controversial since
-it has been established by three majors groups in the field that type I IFN sensing during L. monocytogenes infection was independent of MAVS/ IPS-1.
-Inflammasome activation in response to L. monocytogenes infection has been shown to be dependent on three sensing systems: i) Aim2-mediated sensing of cytosolic DNA ii) Naip5/Ipaf-mediated sensing of flagellin iii) NLRP3-mediated sensing of LLO.
-The secA2 mutant has been extensively characterized in the Portnoy laboratory but they never involved the secA2 pathway in type I IFN induction or inflammasome activation despite looking

extensively at these pathways.

The results need thus to strongly reinforce in order to be fully convincing in regard of the current literature on the subject and on the fact that MAVSKO macrophages have been characterized by numerous people in the field, which have missed the phenotype. The specific comments for type I IFN induction, the inflammasome pathway and secA2 mutant are presented below.

Unfortunately, this manuscript is lacking some pieces of information that would have been key to judge the experiments and the data (e.g., what do the authors call secreted nucleic acids and how they purify it?). Several inconsistencies are present in the manuscript (e.g., Fig 3D or in vivo infections in the material and methods section). The material and methods section needs to be extended at least in supplemental material and methods. The figure legends need to be more detailed.

Finally, this manuscript is lacking in vivo data showing IFN-b level (by qRT-PCR for example) at different times post-infection in WT, STINGKO and MAVSKO or RIG-IKO to validate the role of this novel pathway in vivo during Listeria infections.

IFN induction:
1) Only one time point is shown. Kinetics of IFN induction are lacking and need to be shown in WT, RIG-I and STINGKO to better understand if the RIG-I pathway is important as a primary pathway or possibly a secondary pathway (amplification?).
2) Most of the work on RIG-I or inflammasome pathway has been done using C57BL6 macrophages, it would be helpful to check your phenotype with macrophages on a B6 background and not a CD1 background.
3) How do you explain in Fig. 1D that IFN-b induction is dependent on RIG-I but independent on IPS-1/MAVS? More experiments with MAVSKO need to be performed and presented to be able to compare side-by side the relative contribution of the STING and the RIG-I/MAVS-signalling pathway.

Inflammasome activation:
1) Kinetics of IL-1b release and cell death need to be shown in order to understand what stage of the infection we are looking at.
2) Is RIG-I acting directly to control inflammasome activation? Type I IFN has been shown to control inflammasome activation during Francisella and Listeria infection and Aim2 is an IFN-inducible gene. The effect could thus be indirect and mediated by RIG-I-mediated IFN control. The authors could prestimulate macrophages with type I IFN to induce Aim2 before infection and see if inflammasome activation is still RIG-I dependent. In addition, the authors could look at AIM2 levels in WT and RIG-IKO macrophages.
3) Most of the group working on inflammasome activation have used RIG-I KO on a C57B6 background. The authors need to get those macrophages to double-check the RIG-I-dependency of their phenotypes.
4) Caspase-1 activation cannot be compared on different gels as presented in Figure S5B. Therefore I don't think the authors can conclude on differences in caspase-1 activation between WT, MDA5 KO or WT-CD1 and RIG-I KO.
5) Fig S5C To me it looks like CARD-9 controls Casp1 processing. The authors need to show proIL-1b levels for all the genotypes to clearly discriminate effect on proIL-1b levels from direct effect on the inflammasome complex itself.
6) "Line 270-271: Consistent with these results we observed inflammasome activation upon transfection of secreted Listeria DNA or RNA into macrophages that was also RIG-I dependent (not shown)."
Transfection of DNA into the host cytosol triggers AIM2 inflammasome independently of the DNA species. The authors need to show this piece of data and control for Aim2 levels in the different macrophages.

Secreted nucleic acids and secA2 mutant:
1) Unfortunately, the procedure leading to the purification of secreted DNA or RNA is not described in the material and methods section (the reference is a preview in Immunity). It is thus very difficult to evaluate the findings related to the potency of these nucleic acids to be detected by the innate immune system.

Based on Fig. 2A I still suspect that you have some lysis occurring within your culture since there is a clear band of genomic DNA stuck at the top of the well. I don't see how live bacteria could secrete in such amount gDNA. Furthermore as you see the two main ribosomal RNA bands, it looks to me that the pattern of "secreted nucleic acids" is not so different from the pattern observed after lysing bacteria. A side-by-side comparison of secreted nucleic acid versus total nucleic acids (agarose electrophoresis) is needed.

2) The differences observed between IFN-b induction in response to secreted nucleic acids as compared to total nucleic acids are very reminiscent of the increase in IFN-b induction observed when adding Nod-1 ligand to L. monocytogenes DNA (Leber JH et al, Plos Pathogens). You could thus have another PAMP in your secreted nuclei acids, which would be lost during the preparation of total nucleic acids. You should look at the role of NOD1 using NOD1KO macrophages or control your secreted nucleic acids for Nod1 ligand contamination.

3) "Where not indicated otherwise, cells were transfected for 16 h with 20 µg/ml of total Listeria nucleic acids"
Concentrations of 1ug/ml are routinely used to trigger AIM2 inflammasome activation and lower concentrations are used to induce type I IFN. Can the authors comment on this 20 µg/ml dose, which seems very high to me?

4) Complementation of the secA2 mutant would be a plus.

5) The secA2 mutant has a cell-to-cell spread defect. If less cells are infected, less cells will produced IFN-b and IFN-b should be lower. Why did you rule out this possibility?

6) The secA2 pathway is known to control autolysin secretion and is thus likely to control secretion of Nod1-2 ligands, which are important for IFN-b induction. Furthermore, autolysins could be responsible for spontaneous bacterial death and thus release of nucleic acids. You should at least discuss more extensively those results.

Minor comments:

The authors need to include the nucleic acid quantity in each figure panel.

Line 227: Heat-killed bacteria lack key PAMPs (Vita-PAMPs) such as RNA and possibly diAMPc and diGMPc (sander 2011), a bacterium dying within the host cytosol and delivering Vita-PAMPs into the host cytosol cannot be compared to delivery of dead bacteria into the host cytosol.

1st Revision - authors' response 09 July 2012

Referee #1

This manuscript investigates receptors and pathways leading to IFN-beta and IL-1beta production in macrophages infected with Listeria monocytogenes (Lm). The authors propose the SecA2-dependent release of nucleic acids by intracellular Lm. Secreted RNA molecules are more potent stimulators of cytokine production than their intracellular counterparts. Both RNA and DNA employ the molecules Rig I (Mda5), IPS-1 and Sting to stimulate IFN-b production, whereas IL-1b release requires Rig I/Mda5, Card9 and the inflammasome adapter Asc. The authors claim this occurs without an impact on caspase 1 cleavage.

Comments:
Question 1. *The authors claim superior ability of secreted RNA to stimulate the Rig-I pathway. This is done based on RNA amounts, rather than numbers of molecules. The superior immune stimulation may simply be due to a smaller average size of the secreted RNA. Alternatively total RNA may contain species that actively suppress signal transduction. One way to test this is adding increasing amounts of total RNA to the secreted RNA.*
Answer: We have performed the experiment suggested by the reviewer. We did not observe any inhibitory effect of RNA isolated from Listeria lysates on the induction of IFN by secreted RNA.

This is now shown in Fig. S2B. Therefore, it is more likely that secreted RNA contains more RIG-I ligands than RNA isolated from *Listeria* lysates.

Question 2. *ELISA is used as a read-out of signaling to the IFN-b gene. The authors cannot rule out effects of the signaling paths subsequent to gene induction. mRNA data should be shown.*
Answer: We have repeated all experiments and now provide mRNA data for IFN induction for all relevant experiments. These data are included in the supplementary figures. We reproduced ALL findings for proteins with the novel mRNA data.

Question 3. *The reason for the discrepancy to the Sun et al. and Soulat et al. papers regarding the involvement of IPS-1 remains somewhat of a mystery. The authors try to explain this by showing that the IPS-1 requirement for IFN-b induction is only seen at low to moderate MOI. Sun et al. tested MOIs of 10 and 100, Soulat et al. used an MOI of 10. Both studies should have detected the IPS-1 involvement according to figure 1D of this manuscript. Unlike IPS-1, the authors report an involvement of Rig-I also at high MOIs. The IPS-1-independent pathway operating at high MOI remains unexplained.*
Answer: We have decided to remove the data on IPS1 from our manuscript because at present it is not clear from the literature how our data compare to the data that was previously published.
There are important methodological differences between our results and the results reported by the groups mentioned by the reviewer. Soulat et al. used RAW cell lines over-expressing MAVS, used siRNA-mediated knockdown (that was not complete) and infected with an MOI 10 for 4 hrs. Sun et al. used mouse embryonic fibroblasts and peritoneal as well as bone marrow-derived macrophages. In their publication it is unclear whether infections with *Listeria* were performed at MOI of 10 or 100 for which cell type. In the figure legend also it was not mentioned with which MOI those results were obtained. In our study, we used bone-marrow derived macrophages throughout the study, only used genetic knockout models and always infected with an MOI of 10.
It is possible that adaptor molecules other than IPS-1 are involved in the signaling downstream of RIG-I. The molecule(s) that links RIG-I to downstream signaling pathways remains to be identified.

Question 4a. *SecA2 mutant Lm are defective regarding cell-to-cell spreading. Can the authors rule out that this influences their results? Is it not possible that SecA2 mutants infect a lower proportion of cells over time, but, due to their inability to spread to neighbouring cells, replicate to higher numbers in those cells that are infected? This notion is supported by figure 4D showing higher numbers of actin clouds in SecA2 mutants. In the same figure it appears that Hly mutants are also covered by actin. Is this a technical problem?*
Answer: We developed an assay system to quantify macrophage infection by *Listeria*. To this end, we have setup a flow cytometry-based approach that detects infection by intracellular antibody-based staining for *Listeria*-membrane constituents. These studies demonstrated that a similar number of macrophages were initially infected by wildtype *Listeria* or D*secA2 (Fig. S4E)*. We also provide evidence that *Listeria* proliferation is not significantly different between wildtype *Listeria* or D*secA2*. However, it is not possible to formally exclude that the spreading defect has consequences for the induction of IFN. Yet, D*ActA2* also have a cell-to-cell spreading defect. Unlike D*secA2,* all secretion systems are intact in D*ActA2*. Induction of IFN and IL-1b was even higher after infection with D*ActA2* compared to wildtype *Listeria* and was far higher compared to D*secA2* infection (with the same MOI for all *Listeria* strains)(Data shown to referees, but removed from RPF file). These data imply that the cell-spreading defect is unlikely to affect the recognition of D*secA2* by the cytosolic receptors.

We have improved the quality of the confocal microscopy pictures now showing cells at higher resolution, which allows to clearly identify individual *Listeria* and that show that only the wild type and secA2 mutant listeria are able to polymerize actin in the cells.

Question 5. *There is no normalization control for figure 4C regarding the impact of the SecA2 translocon on secretion. A secreted protein not affected by SecA2 should be shown as well as a protein secreted by SecA2.*
Answer: We performed this control as suggested by the reviewer (shown below).
However, as we cannot provide formal evidence for the role of SecA2 in the secretion of small size *Listeria* nucleic acids that are relevant for the activation of cytosolic immune sensory receptors, we have decided to remove the SecA2 from the title and the abstract. In the results section we only refer

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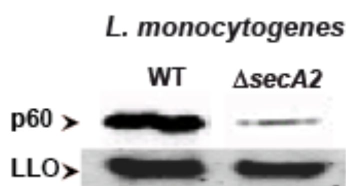
to data generated with the SecA2 mutant as a possible transport mechanism to shuttle bacterial nucleic acids into the cytosol of infected macrophages.



Question 6. *figure 4G shows that upon infection of RigI-/- macrophages with SecA2 mutants about 20% IFN-b production remains. How is this explained?*
Answer: This might be due to the recognition of ci-di-AMP/GMP or *Listeria* DNA that is released in the cytosol from *Listeria* that undergo spontaneous lysis in the cytosol or are secreted in a SecA2-independent manner into the cytosol. We have included this statement in the results section.

7. The authors state that 'the CARD9 pathway also mediates IL-1b activation in response to cytosolic Listeria without affecting caspase-1 cleavage'. To my opinion there is much less cleaved Caspase 1 in CARD9-/- cells in figure S5C.
Answer: We agree with the reviewer that the results shown in the originally submitted manuscript were not convincing. We have repeated the experiment three more times and now provide unequivocal evidence that the level of cleaved caspase 1 is not altered in CARD9-/- macrophages. In contrast, there is a decreased expression of pro-IL1beta.

Question 8. *The manuscript contains a significant amount of recently published data (e.g. figure 1A) without providing appropriate references: O'Connell R, M. et al. JI 174, 1602 (2005); O'Riordan et al. PNAS 99, 13861 (2002); Stetson and Medzhitov, Immunity. 24:93 (2006); Stockinger, S. et al. JI 169 (2002); Stockinger, S. et al. JI 173, 7416 (2004).*
Answer: We have included these references. The experiments were included because they provided important controls and did not intend to raise the impression that novel results were generated.

Referee #2

Although it still unclear what the function of secreted bacterial nucleic acids, and why they are secreted through a secretion mechanism strongly associated with pathogenesis, the data shown in general support the stated conclusions, and the experiments are well executed.

Question 1a. *The authors should show the RIG-I dependent stimulatory activity of the secreted nucleic acids fraction treated with both RNase and DNase to determine whether that completely abrogates Type I IFN induction. This is a key control.*
Answer: We have performed these experiments. The results clearly show that RNAse and DNAse treatment abrogate RIG-I mediated recognition of secreted RNA and secreted DNA. This also excludes a contamination with NOD1 or NOD2 ligands. The data are included as new Fig. S2I.
We agree with the reviewer`s comment that CIAP is not specific for RNA, but it is the only reagent to study RIG-I recognition of RNA and have rephrased the text in the results section.

Question 1b. *Although the removal of the 5' phosphate from the seRNA did strongly decrease activity, CIAP is not specific for RNA and could possibly be acting on other substrates in the sample.*
Answer: we have rephrased the sentence in the results part to make it clear that CIAP is not specifically acting on RNA. However, transfection of CIAP-treated secreted RNA, total RNA and secreted DNA into RIG-I deficient macrophages induces the same level of IFN as the non-treated samples, i.e. the ability of the treated nucleic acids to induce STING or MDA5 dependent IFN production is not affected by the CIAP treatment.

Question 2. *The authors should include in each figure legend, how many times each set of experiments was repeated. This information was only included for a few experiments.*
Answer: This information is now included in the figure legends.

Question 3. *The authors show in Fig. 4C that the Listeria secA2 mutant secretes less nucleic acid by providing an image of an agarose gel. Although the data shown support their conclusion, this is not really a quantitative method, nor does it show the magnitude of the defect over several experiments. This experiment should be done quantitatively using OD 260/260 or by using fluorescence probes that bind, e.g. ds DNA to give a better measure of the defect.*
Answer: We agree with the reviewer that the agarose gel is not very informative. Because of the many additional figures and supplementary figures that are included in the revised manuscript we decided to remove the agarose gel and replace it with a table on the photometric quantification of nucleic acids.

Question 4. *In Fig. 4D, the low magnification of the fluorescence images makes it difficult to appreciate the data supporting equivalent cytosolic residence of the secA2 mutant, since these images are meant to be an indication of cytosolic infection. Readers would benefit from seeing images at higher magnification.*
Answer: We have followed the reviewer`s suggestion and now provide figures with higher magnification.

Question 5. *In Supplemental Fig. 5B and C, the authors still observe caspase-1 cleavage even in absence of RIG-I or CARD-9. The data shown in this figure are important for their model and should be included in the main body of the manuscript, with loading controls (e.g., the full length caspase-1 or a separate protein control like GAPDH)*
Answer: We now provide full length caspase 1 as controls in Immunoblots.

Question 6. *Although the authors speculate that the caspase-1 cleavage still observed during infection by the secA2 mutant may come from AIM2 or NLRP3, this could be tested more directly by transfecting in the secreted nucleic acid fraction with or without RNase + DNase treatment. Presumably in this case (unlike during infection with the SecA2 mutant), caspase-1 activation would be absent in the RIG-I deficient macrophages.*
Answer: We have followed the reviewer´s suggestion and performed these experiments. The results are now shown in Figure 6D. They demonstrate that caspase 1 cleavage is absent in RIG-I deficient macrophages.

Referee #3

Unfortunately, this manuscript is lacking some pieces of information that would have been key to judge the experiments and the data (e.g., what do the authors call secreted nucleic acids and how they purify it?
Answer: We apologize for not having provided more information on this relevant issue in the originally submitted manuscript. We now provide a detailed description on how Listeria nucleic acids were isolated and purified from supernatants or cell lysates.

Question: *Several inconsistencies are present in the manuscript (e.g., Fig 3D*
Answer: We have repeated the experiment under question several times and discovered that DMSO used for bringing the RNA polymerase inhibitor into solution influences the response of macrophages to transfected nucleic acids.
We now used lower DMSO concentrations that did not affect the response of macrophages to transfected nucleid acids, and analyzed mRNA expression of IFN to shorten the time period macrophages are exposed to DMSO. These results demonstrate that the inhibitor only prevented IFN induction following transfection of seDNA but not seRNA. These data are now shown as new Fig. 3D.

Question: *in vivo infections in the material and methods*
Answer: we apologize for this. These parts have been removed.

Question: *The material and methods section needs to be extended at least in supplemental material and methods*
Answer: we now provide additional information on the method for isolation of secreted nucleic acids.

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Question: *The figure legends need to be more detailed.*
Answer: We now provide more concise information in the figure legends that helps to understand the experiments shown.

Question: *Finally, this manuscript is lacking in vivo data showing IFN-b level (by qRT-PCR for example) at different times post-infection in WT, STINGKO and MAVSKO or RIG-IKO to validate the role of this novel pathway in vivo during Listeria infections.*
Answer: We have been able to obtain macrophages from the various knockout mice in a collaboration, but it was not possible to transfer STING knockout animals to Bonn. Moreover, it would take many months to bring them via embryo transfer into our animal facility, which would be required to breed enough animals for experiments. It is out of scope of this manuscript, which reports on a new molecular mechanisms of *Listeria* recognition in the cytosol of infected cells.

IFN induction:
1) Only one time point is shown. Kinetics of IFN induction are lacking and need to be shown in WT, RIG-I and STINGKO to better understand if the RIG-I pathway is important as a primary pathway or possibly a secondary pathway (amplification?).
Answer: we have done the experiment as suggested by the reviewer and found that there was no IFNb mRNA at 4 hrs in STING ko macrophages, suggesting that STING is important for initiating IFNb induction. This data is now shown as new Fig. 1C.

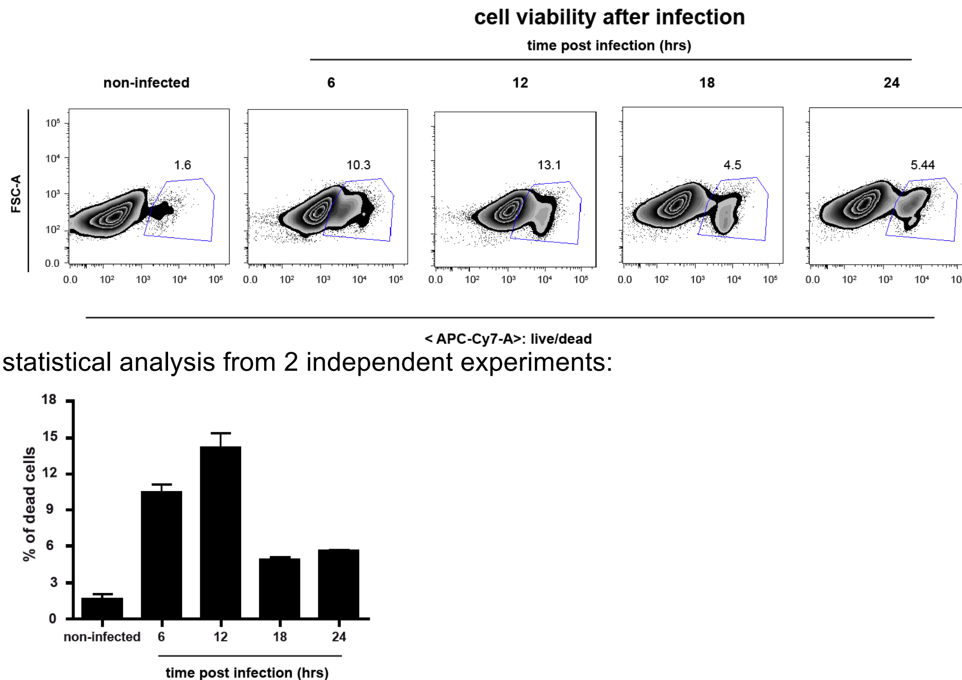
Question 2) *Most of the work on RIG-I or inflammasome pathway has been done using C57BL6 macrophages, it would be helpful to check your phenotype with macrophages on a B6 background and not a CD1 background.*
Answer: In the original publication by Akira it had been already mentioned that the RIG-I knockout mice on a C57Bl/6 background breed very badly. There is no RIG-I knockout line on a C57Bl/6 background available in entire Europe. We do not see a clear scientific reason to assume that CD1 mice are different from C57Bl/6 mice, because these mice responded in an identical fashion to Listeria infection or transfection with Listeria nucleic acids, as we show in the controls of our experiments.

Question 3) *How do you explain in Fig. 1D that IFN-b induction is dependent on RIG-I but independent on IPS-1/MAVS? More experiments with MAVSKO need to be performed and presented to be able to compare side-by side the relative contribution of the STING and the RIG-I/MAVS-signalling pathway.*
Answer: We have decided to remove the data on IPS1 from our manuscript because at present it is not clear from the literature how our data compare to the data that was previously published.
There are important methodological differences between our results and the results reported by the groups mentioned by the reviewer. Soulat et al. used RAW cell lines over-expressing MAVS, used siRNA-mediated knockdown (that was not complete) and infected with an MOI 10 for 4 hrs. Sun et al. used mouse embryonic fibroblasts and peritoneal as well as bone marrow-derived macrophages. In their publication it is unclear whether infections with *Listeria* were performed at MOI of 10 or 100 for which cell type. In the figure legend also it was not mentioned with which MOI those results were obtained. In our study, we used bone-marrow derived macrophages throughout the study, only used genetic knockout models and always infected with an MOI of 10.
It is possible that adaptor molecules other than IPS-1 are involved in the signaling downstream of RIG-I. The molecule(s) that links RIG-I to downstream signaling pathways remains to be identified.

Inflammasome activation:
1) Kinetics of IL-1b release and cell death need to be shown in order to understand what stage of the infection we are looking at. Cell death as suppl. Figure, live dead stain by flow cytometry, 6, 18,24 hours
Answer: We have done a time-kinetic analysis of IL-1b release (Data shown to referees, but removed from RPF file). As so many additional figures have already been added to the revised manuscript we decided not to include this particular set of information in the manuscript, but to show it in the point-by-point reply.

IL-1b from cell culture supernatant at indicated time points after infection with wildtype Listeria.

Also, we provide a detailed analysis of macrophage cell death during the conditions of the experiment, shown below.



2) Is RIG-I acting directly to control inflammasome activation?
Answer: Yes, we have previously shown that RIG-I interacts with ASC in a MAVS-independent manner to induce caspase-1 activation and IL-1beta release (J. Ruland 2010 Nat. Immunol).

Question: *Type I IFN has been shown to control inflammasome activation during Francisella and Listeria infection and Aim2 is an IFN-inducible gene. The effect could thus be indirect and mediated by RIG-I-mediated IFN control. The authors could prestimulate macrophages with type I IFN to induce Aim2 before infection and see if inflammasome activation is still RIG-I dependent. In addition, the authors could look at AIM2 levels in WT and RIG-IKO macrophages.*
Answer: We thank the reviewer for this very good suggestion. We have performed the experiments as suggested and found that IFN pre-treatment increased the generation of IL-1b released by RIG-I knockout macrophages (new Fig. 5). However, the difference in IL-1b production between wildtype and RIG-I knockout macrophages persisted despite treatment with IFN. These results suggest that RIG-I on the one hand contributed to efficient inflammasome activation by inducing early IFN production. On the other hand, they also indicate that there is a direct contribution of RIG-I to inflammasome activation (as previously published) that cannot be replaced by early IFN production. The experiments further demonstrated that IFN production did not influence AIM2 expression. These data are consistent with previously published reports by Alnemri et al 2010 and Monack et al. 2007.

Question 3) *Most of the group working on inflammasome activation have used RIG-I KO on a C57B6 background. The authors need to get those macrophages to double-check the RIG-I-dependency of their phenotypes.*
Answer: In the original publication by Akira it had been already mentioned that the RIG-I knockout mice on a C57Bl/6 background breed very badly. There is no RIG-I knockout line available in entire Europe on a C57Bl/6 background. We do not see a clear scientific reason to assume that CD1 mice are different from C57Bl/6 mice, because these mice responded in an identical fashion to Listeria infection or transfection with Listeria nucleic acids. Furthermore, also other researchers in the field used exactly these mice for their studies, such as Sander et al (Nature 2011).

4) Caspase-1 activation cannot be compared on different gels as presented in Figure S5B. Therefore I don't think the authors can conclude on differences in caspase-1 activation between WT, MDA5 KO or WT-CD1 and RIG-I KO.

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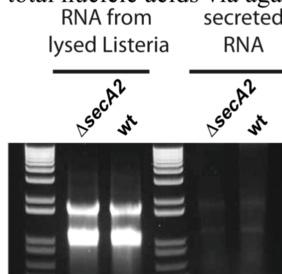
Answer: We have repeated all these experiments and how show western blots where the analysis is performed simultaneously on the same gel.

Question 5) *Fig S5C To me it looks like CARD-9 controls Casp1 processing. The authors need to show proIL-1b levels for all the genotypes to clearly discriminate effect on proIL-1b levels from direct effect on the inflammasome complex itself.*
Answer: We agree with the reviewer. We have repeated the experiment three more times and now provide unequivocal evidence that the level of cleaved caspase 1 is not altered in CARD9-/- macrophages. In contrast, there is decreased expression of pro-IL1beta.

Question 6) *"Line 270-271: Consistent with these results we observed inflammasome activation upon transfection of secreted Listeria DNA or RNA into macrophages that was also RIG-I dependent (not shown)." Transfection of DNA into the host cytosol triggers AIM2 inflammasome independently of the DNA species. The authors need to show this piece of data and control for Aim2 levels in the different macrophages.*
Answer: There is no difference in AIM2 expression in wildtype and RIG-I knockout macrophages before and after *Listeria* infection (new Fig. S5A).

Secreted nucleic acids and secA2 mutant:
Question 1a) *Unfortunately, the procedure leading to the purification of secreted DNA or RNA is not described in the material and methods section (the reference is a preview in Immunity). It is thus very difficult to evaluate the findings related to the potency of these nucleic acids to be detected by the innate immune system.*
Answer: We apologize for not having provided more information on this relevant issue in the originally submitted manuscript. We now provide a detailed description on how Listeria nucleic acids were isolated and purified from supernatants or cell lysates.

Question 1b: *Based on Fig. 2A I still suspect that you have some lysis occurring within your culture since there is a clear band of genomic DNA stuck at the top of the well. I don't see how live bacteria could secrete in such amount gDNA. Furthermore as you see the two main ribosomal RNA bands, it looks to me that the pattern of "secreted nucleic acids" is not so different from the pattern observed after lysing bacteria. A side-by-side comparison of secreted nucleic acid versus total nucleic acids (agarose electrophoresis) is needed.*
Answer: As requested we now provided a side-by-side comparison of secreted nucleic acid versus total nucleic acids via agarose electrophoresis.



However, as we cannot provide formal evidence for the role of SecA2 in the secretion of small size *Listeria* nucleic acids that are relevant for the activation of cytosolic immune sensory receptors, we have decided to remove the SecA2 from the title and the abstract. In the results section we only refer to data generated with the SecA2 mutant as a possible transport mechanism to shuttle bacterial nucleic acids into the cytosol of infected macrophages.

Question 2) *The differences observed between IFN-b induction in response to secreted nucleic acids as compared to total nucleic acids are very reminiscent of the increase in IFN-b induction observed when adding Nod-1 ligand to L. monocytogenes DNA (Leber JH et al, Plos Pathogens). You could thus have another PAMP in your secreted nuclei acids, which would be lost during the preparation of total nucleic acids. You should look at the role of NOD1 using NOD1KO macrophages or control your secreted nucleic acids for Nod1 ligand contamination.*
Answer: To assess whether differences in induction of IFN in response to total and secreted nucleic acids is due to contamination with NOD1 or NOD2 ligands in the secreted nucleic acids, we transfected macrophages from NOD1-deficient, NOD2-deficient or wild type mice with secreted nucleic acids treated with RNase, DNase or both and then determined induction of IFN mRNA by qRT-PCR. We did not observe any differences in the induction of IFN in wild type compared to

NOD1-deficient or NOD2-deficient macrophages. This rules out a contamination of secreted Listeria nucleic acids with NOD1 or NOD2-ligands. The data are shown as new Fig. S2I.

Question 3) *"Where not indicated otherwise, cells were transfected for 16 h with 20 µg/ml of total Listeria nucleic acids" Concentrations of 1ug/ml are routinely used to trigger AIM2 inflammasome activation and lower concentrations are used to induce type I IFN. Can the authors comment on this 20 µg/ml dose, which seems very high to me?*

Answer: We apologize for this typo, it meant 2 µg for 10^5 macrophages. We have now clearly indicated in ALL figure legends the nucleic acid concentrations used for transfection.

Nevertheless, high concentrations of nucleic acids for transfection were successfully used by us in the past (Schlee et al. Immunity 2009), but also by Sander et al used also 20µg/ml of total E.coli RNA for 10^6 cells (Nature 2011). Also Vijay et al Nature Immunology 2010 used up to 3 µg of polydAdT, a ligand for AIM2, Sauer et al (Infect. Immun. 2011) used 500 µg/ml of poly(dAT:dTA) or poly(I:C) for thetransfection of BMDM for the induction of type I IFN in their experiments.

4) Complementation of the secA2 mutant would be a plus.

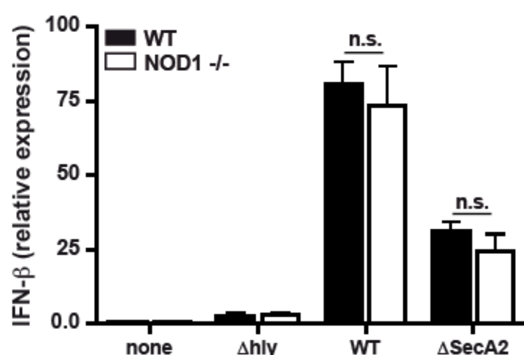
Answer: Unfortunately, we do not have access to a re-complemented strain of deltaSecA2.

Question 5) *The secA2 mutant has a cell-to-cell spread defect. If less cells are infected, less cells will produced IFN-b and IFN-b should be lower. Why did you rule out this possibility?*

Answer: We developed an assay system to quantify macrophage infection by *Listeria*. To this end, we have setup a flow cytometry-based approach that detects infection by intracellular antibody-based staining for *Listeria*-membrane constituents. These studies demonstrated that a similar number of macrophages were initially infected by wildtype *Listeria* or D*secA2 (Fig. S4E)*. We also provide evidence that *Listeria* proliferation is not significantly different between wildtype *Listeria* or D*secA2*. However, it is not possible to formally exclude that the spreading defect has consequences for the induction of IFN. Yet, D*ActA2* also have a cell-to-cell spreading defect. Unlike D*secA2,* all secretion systems are intact in D*ActA2*. Induction of IFN and IL-1b was even higher after infection with D*ActA2* compared to wildtype *Listeria* and was far higher compared to D*secA2* infection (with the same MOI for all *Listeria* strains)(Data shown to referees, but removed from RPF file). These data imply that the cell-spreading defect is unlikely to affect the recognition of D*secA2* by the cytosolic receptors.

Question 6) *The secA2 pathway is known to control autolysin secretion and is thus likely to control secretion of Nod1-2 ligands, which are important for IFN-b induction. Furthermore, autolysins could be responsible for spontaneous bacterial death and thus release of nucleic acids. You should at least discuss more extensively those results.*

Answer: There is no reduction of IFN induction following wildtype Listeria infection of macrophages deficient for NOD1 or NOD2 (Girardin PNAS 2007; Decker JI 2004) making it unlikely that NOD1/2-ligands contribute to IFN induction during Listeria infection. We also did not find any involvement of NOD1 or NOD2 for induction of IFN in response to Listeria infection using macrophages from the respective knockout mice.



We cannot formally exclude that the lack of autolysin release in secA2 mutants may also lead to less autolysis. We have discussed this issue in more detail in the results and discussion sections.

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Minor comments:
The authors need to include the nucleic acid quantity in each figure panel.
Answer: We have done as suggested by the reviewer.

Line 227: Heat-killed bacteria lack key PAMPs (Vita-PAMPs) such as RNA and possibly diAMPc and diGMPc (sander 2011), a bacterium dying within the host cytosol and delivering Vita-PAMPs into the host cytosol cannot be compared to delivery of dead bacteria into the host cytosol
Answer: We fully agree with the reviewer. The main point of our manuscript is that secretion of nucleic acids followed by RIG-I-mediated recognition of such secreted RNA and DNA serves as a Vita-PAMP.

2nd Editorial Decision — 9 August 2012

Thank you for submitting your manuscript to the EMBO Journal. I asked the three original referees to review the paper and referees #2 and 3 were able to do so. I have now received their comments, which are provided below. While referee #2 appreciates that you have added data to strengthen the findings, s/he also finds that the lack of definitive support for the secretion mechanism involved in microbial nucleic acid secretion limits the impact of the analysis. Referee #3 is more positive and support publication here. I have also asked referee #3 to comment on the issue raised by referee #2. While referee #3 is in agreement with referee #2 that the secretion mechanism is less clear at this stage, s/he nevertheless finds the study interesting and suitable for publication here. I understand the points that referee #2 is making, but given the overall comments on your paper we have come to the decision to accept the paper for publication here. There are just a few issues to resolve before final acceptance here.

1. Please take a look at fig 5F. The caspase-1 blot looks very exposed and the resolution is not very crisp. Do you have another blot to add to this figure?

2. We also now encourage publication of source data, particularly for electrophoretic gels and blots. Would you be willing to provide a PDF file per figure that contains the original, uncropped and unprocessed scans of all or key gels used in the figures 5 and 6? The PDF files should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation could be useful but is not essential. The PDF files will be published online with the article as supplementary "Source Data" files. If you have any questions regarding this just contact me.

That should be all. You can send us the requested data by email.

Yours sincerely

Editor
The EMBO Journal

REFEREE REPORTS

Referee #2

In this revised manuscript, Abdullah, et al, propose a model whereby live Listeria monocytogenes secretes RNA and DNA into the extracellular environment. In macrophages, the environment outside the bacterium would be the cytosol, where secreted RNA or DNA could be detected by nucleic acid sensors, such as RIG-I, leading to Type I IFN induction.

The authors provide an exciting hypothesis and tried to strengthen their support for it by including additional controls in this version of the study. A key aspect of the original hypothesis was the idea that the secreted nucleic acids were secreted through the SecA auxiliary secretion mechanism. The authors were not able to show convincingly in the last manuscript that there was less nucleic acid secretion by the secA2 mutant. Since those data are not shown here either, and the authors focus less on SecA2 in the title and abstract, one is left to infer that the secA2 mutant secreted just as much

nucleic acid into cultures as WT (WT data shown in Table S1). If that is so, then it is unclear why the secA2 mutant stimulates less Type I IFN in WT macrophages and suggests that this mutant (which is quite altered in morphology and cell wall structure) may activate RIG-I less because of a mechanism that is distinct from nucleic acid secretion.

While the authors may very well have identified an important new molecular signal for innate immune sensing, in their current form the data supporting a distinct population of "secreted RNA" are not compelling. In the absence of a discrete secretion mechanism, it is difficult to rule out the possibility of nucleic acids being released due to bacterial lysis or damage, which undermines their hypothesis and their interpretation of all the data. Release of nucleic acids during natural lysis might well result in different ligands than during experimental lysis (in the same way mammalian DNA processed during apoptosis is distinct from DNA released from experimental cell lysis), leading to differential activation of RIG-I by "seRNA". Without a clear role for SecA2 or another secretion mechanism, the remaining primary conclusion is that somehow microbial RNA activates RIG-I, which is not particularly novel.

Referee #3

The authors have answered most of my comments. How the secreted nucleic acids differ from nucleic acids is still unclear (referee 1 question 1 and referee 3 question 1b). Furthermore the complementation of the secA2 mutant is still lacking. Despite these two caveats, the authors have performed an extensive amount of work of very good technical quality to dissect a highly redundant pathway. The new data fully support their conclusions and should be of broad interest to the community working on the innate immunity to pathogens.

2nd Revision - authors' response 30 August 2012

We have included a quantification of small size nucleic acids secreted from wildtype or mutant Listeria in Figure 4C. In line 239-45 we describe the results in the results section. The figure legend and the numbering of the subfigures were changed accordingly. In a new Fig. S6E we provide a evidence that there is indeed less small size nucleic acids whereas genomic nucleic acids remain unchanged. In line 258 we now mention that “our data indicate” a role for SecA2 in nucleic acid transport.

These are minor changes but they meet the reviewer´s notion that we should provide direct evidence for less secretion of nucleic acid in the secA2 mutant.

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