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The innate immune receptor MDA5 Limits Rotavirus Infection but Promotes Cell Death and Pancreatic Inflammation

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Transaction Report:

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

1st Editorial Decision 25 January 2017

Thank you for submitting your manuscript to The EMBO Journal. I am sorry for the delay in getting back to you, but I have now received the comments back from the three referees.

As you can see from the comments below, the referees find the analysis interesting and insightful. They raise a number of constructive comments that I would like to ask you to address in a revised version. In particular they find that further analysis regarding the role of RIG-I in the process and what type of cell death pathway is triggered is needed. I think the point raised by referee #2 to look at glycemia is a good one. Do you have any data on this?

Should you be able to address the raised concerns then I would like to invite a revised version. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to address the raised concerns at this stage. Let me know if we need to discuss anything further

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

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, we request that you contact the editor 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 us know in advance and we may be able to grant an extension.

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

REFEREE REPORTS

Referee #1:

Sadler and colleagues have investigated the role of cytosolic RNA sensor Mda5 in the immune response to enteric rotavirus (RV) infection, and have also examined the potential association of RV with type 1 diabetes. The authors demonstrate that Mda5 activity not limits RV infection through the induction of IFN and pro-inflammatory cytokines as well as by promoting cell death. It was interesting that, the Mda5-dependent antiviral response was strong in the pancreas of RV-infected mice, thereby suggesting relationship with autoimmunity in T1D. The authors conclude that MDA5-induced cell death and inflammation in the pancreas may predispose to autoimmune destruction of β-cells.

This is an interesting study, for the most part the experiments are well-conceived and conducted. A number of issues should be addressed by the authors:

1. The authors have not ruled out the involvement of RIG-I per se, or TLR3 in the response to RV infection, an important aspect that may temper their conclusions.
2. Fig. 3F is difficult to interpret - could the authors improve their description of these results.
3. Fig. 4 - the IRF3 translocation is really not clear - a phospho-IRF3 analysis would be more informative here.
Why would the levels of IkBa be different between WT and Ifih-/- cells?
4. Although TNFa and IL-6 levels modulate depending on Mda5 function (Fig. 4C and D), there is no evidence of processing on pro-IL1B, one of the measures of immunogenic cell death. Why is no processing observed in these cells, whereas in vivo IL1B is produced and quantified in the in vivo model.
5. Could the authors lay out Fig. 5 in a manner that presents the data more clearly? Its quite confusing as is.
6. The experiments with the &946, V923 and X627 versions of Mda5 are interesting and indicate that mutation of Mda5 alters IFNB, p56 and NF-kB induction. However a crucial experiment seems to be missing. Expression of these variant proteins in ifih1-/- cells should have a dramatic impact on inflammation markers and cell survival if the concept of this paper is correct. Have the authors attempted such an experiment?

Referee #2:

- general summary and opinion about the principle significance of the study, its questions and findings

The authors describe the key role of MDA5 in the control of rotavirus (RV) infection and inflammation in pancreas comparing WT and IFIT1-/- mice. Using IFNAR1-/- mice and IFNR blocking antibody, they demonstrate that MDA5 antiviral response involves both IFN-dependent and -independent pathways and that MDA5 exerts an NFκB-dependent pro-apoptotic activity. They further observed in vitro that 3 minor alleles of IFIT1 associated with protection against T1D present lower antiviral, pro-apoptotic and pro-inflammatory properties than the major allele in RV infected HEK293 cells.

This is an interesting issue because little is known about the mechanism(s) leading to beta cell lost during acute RV infection and its potential relevance in further T1D related autoimmunity and T1 diabetes.
The novelty of this work is that the authors describe an MDA5 dependent antiviral response

particular to the pancreas of RV-infected mice, observation that they carefully see as a "consonance" in autoimmunity in T1D.
Their interpretation is in agreement with several other articles describing the key role of type 1 IFN in the primary events leading to autoimmunity and T1D.

- specific major concerns essential to be addressed to support the conclusions

The authors observe that the absence of MDA5 lead to increased viral production and conclude this is "due to the altered survival of the different MEFs" (line 107). This observation is just a correlation so this conclusion is abusive and should be modulated.

The role for MAD5 in the control of RV (Boquet et al, J Immunol. 186(3):1618-26, 2011) and the importance of pathways involving MAVS in rotavirus induced innate immune response (Di Fiore et al, Virus Research 208, 89-98, 2015) have been described previously. These papers should be quoted.

Figure S4 reveals the pathogenicity of RV infection in the context of the expression of the different MDA5 alleles. These data are erroneously related to the measure of RV titers Figure 7I in both the legend of Figure S4 (line 819) and in the text (line 267).

The authors claim that their observations of a key role in MDA5 in the control of RV infection and innate immune response argue against the proposition that persistent (Coxsackie)viral infection induces autoimmunity. To my view it is just an alternative source of local type 1 IFN production and further inflammation in the pancreas, an early event triggering autoimmunity. As observed in RV infected pancreas, SNPs in IFIH1 associated with reduced risk for T1D would induce less pro-inflammatory signals than the major IFIH1 allele in CVB persistently infected islets. Could the authors clarify their view about this?

- minor concerns that should be addressed

The failure of rotavirus infected macrophage to produce IL-1β has been described previously (Di Fiore et al, Virus Res 208 :89-97, 2015), in this paper the authors do not detect induction of IL-1β expression. Interestingly the results presented here on rotavirus infected peritoneal macrophages reveal the absence of processing of pro-IL-1β, suggesting that rotavirus interferes with the activation of caspase 1/inflammasome in infected macrophages. This observation deserves to be discussed in regards of the results of Di Fiore and collaborators.

Line 320: The reference related to "IFNλs have been demonstrated to be essential to control RV infection" (Pott et al, PNAS, 108(19): 7944-9, 2011) should be quoted.

The authors should explain the rationale for using 3 different methods to evaluate cell survival: crystal violet staining, codetection of Annexin V and 7-AAD by flow cytometry and YO-PRO staining.

MEF survival 24h post infection by RV is detected by crystal violet in both Figure 1D and figure 3 E. Why to put twice this data? Moreover in Figure 3E, mortality in IFNAR1-/- MEF is 20% higher than in WT MEF while it is 50% higher in Figure 1D. If there is a difference which is it? Please clarify this point.

Viral titers are expressed in FFU/ml or FCFU/ml, the units should be harmonized.

The review quoted to illustrate the proposed role for virus infections as potential primary agents in the initiation of T1D is more than 10 years old and should be replaced by one of the excellent recent reviews discussing this point.

Some figures are difficult to understand and should be better explained in the text and/or legend:
Figure S4 C: The legend should be improved to explain that data present cellular viability in both transfected (GFP+ cells) and non transfected cells (GFP- cells).
Figure 7 :
. (A) The time points for the images presenting association between MDA5-MAVS and MAVS-

MAVS are missing in the legend.
. (F) This figure is entitled NFκB activity while in the text is mentioned as reporter assay reflecting IL8 promoter activity. This should be clarified.

The review: de Beeck AO, Eizirik DL (2016) Viral infections in type 1 diabetes mellitus--why the beta cells? Nat Rev Endocrinol 12: 263-73 should be corrected: Op de Beeck A, Eizirik DL (2016) Viral infections in type 1 diabetes mellitus--why the beta cells? Nat Rev Endocrinol 12: 263-73

- any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

It was published by others that RV infection induces transient hyperglycemia, pancreatic cell destruction and reduction of islets size in C57/B6 mice (Honeyman et al, PLOS ONE 9, e106560, 2014). In order to validate the authors' hypothesis that the absence of MDA5 protects beta cells from RV induced islet destruction and local inflammation, the authors could compare glycemia, islet aspect and potential pancreatic inflammation after RV infection in WT and IFITH1-/-.

Referee #3:

The manuscript describes a study that aims to address the role of MDA5, one of hte RIG_I-like receptors (RLRs), in rotavirus (RV) infection control. MDA5 variants are differentially associated with type 1 diabetes (T1D), and the authors hypothesize that RV infection of the pancreas , and differential control of this infection, can lead to a T1D outcome. The data shows that MDA5 can signal cell death pathways to drive the death of RV-infected cells and that this occurs through MAVS interaction. The MAVS interactions appears to be variable among MDA5 mutants differentially associated with T1D. These differences also link to differential level of IFN expression, and expression of proinflammatory cytokines, and importantly MDA5 is required for control of RV infection of the pancreas, as shown in mice.

Overall the data are solid to reveal a strong role for MDA5 in driving innate immune defenses against RV, and bring forward a new concept that the host response to this infection as mediated by MDA5 is the driver of the T1D outcome.

Specific comments:

1. The use of proper nomenclature. The innate immunity field now used RIG-I like receptor (RLR) instead of RIG-I-like helicase (RLH). This nomenclature was adopted to reflect RIG-I and MDA5 as members of the pathogen recognition receptor family that includes TLR, NLR, CLR, and now the RLRs. Please use the modern nomenclature RLR).
2. How does RIG-I perform in RV infection, a comparison of the RIG-I vs. MDA5 phenotype should be examined. Similarly, MDA5 signaling was shown by Reise Sousa lab to be dependent on LGP2. The role of LGP2 in RV sensing and infection should be addressed in order to fully define the how MDA5 and RLRs in general impact and are regulated by RV. Currently the study does not address specific of MDA5 over other pathogen recognition receptors, including TLRs and RLRs, and others, in the sensing of RV and triggering of the host response to RV infection. Specificity is a highly important item to address considering that the conclusion is that the host response driven by MDA5 (vs. some other pathogen recognition receptor) is responsible for the T1D outcome. Note that in figure 2A only about 50% of the IFN is reduced in the MDA5 ko cells. Thus, additional pathogen recognition receptors must contribute to this response. Is MDA5 dominant here or just another player?
3. Cell death driven by MDA5 in RV infection is the major outcome that links with a probable T1D phenotype via destruction of pancreas cells. This is shown clearly in Fig 3. Thus, what is the cell death pathway that we are dealing with here, is it caspase-dependent (presumably it is), and if so, what caspases are being engaged here by MDA5? These experiments require for probing of caspase/cell death pathways to reveal the point of interaction with MDA5. Is the death signaling MAVS-dependent?
4. Differential MAVS interaction of MDA5 variants is of high interest here. The fluorescence analysis shown in 7A indicates MAVS interaction among the MDA5 snp mutants is different. How this differential impacts the strength of MAVS interaction is not revealed however and how it play

out for downstream signaling to IRF3 and cell death responses is not clear. The authors should include a biochemical (coimmunoprecipitation of MDA5 variants with MAVS, immunoblot of total vs. phospho-iRF3 abundance, caspase activation/signaling of cell death) for each MDA5 variant to best validate each mutant linked with innate immune and cell death responses.

1st Revision - authors' response 26 May 2017

Referee #1:

Sadler and colleagues have investigated the role of cytosolic RNA sensor Mda5 in the immune response to enteric rotavirus (RV) infection, and have also examined the potential association of RV with type 1 diabetes. The authors demonstrate that Mda5 activity not limits RV infection through the induction of IFN and pro-inflammatory cytokines as well as by promoting cell death. It was interesting that, the Mda5-dependent antiviral response was strong in the pancreas of RV-infected mice, thereby suggesting relationship with autoimmunity in T1D. The authors conclude that MDA5-induced cell death and inflammation in the pancreas may predispose to autoimmune destruction of β-cells.

This is an interesting study, for the most part the experiments are well-conceived and conducted. A number of issues should be addressed by the authors:

1. The authors have not ruled out the involvement of RIG-I per se, or TLR3 in the response to RV infection, an important aspect that may temper their conclusions.

Our data demonstrates an immune impairment in animals and cells that express all innate immune factors apart from MDA5. Accordingly, the immune impairment that was detected is evidence that other innate immune proteins, such as RIG1 and TLR3, are unable to fully compensate for the loss of MDA5. Importantly, the data shows that, although it is critical in the pancreas, Mda5 is less active or, alternatively, its activity can be compensated for by other innate immune factors in other tissues, such as the colon. This tissue specificity is a major point of the study.

We include additional data in the revised manuscript that compares the induction of the IFNβ promoter in rotavirus infected cells that express the different RLR. This data show that MDA5 is not more sensitive to rotavirus than RIG1 in this cell context (Fig 1F). The description of this data has been added to the legend of Figure 1 and in the manuscript (ln 110-117).

2. Fig. 3F is difficult to interpret - could the authors improve their description of these results.

We altered the labelling of the treatments in this figure. We recolour the bars of the graph to distinguish cells transfected with the control, MDA5 or MAVS constructs. The figure legend is edited to read 'A quantification of apoptosis in HEK293 cell co-transfected with empty control (C), MDA5 or MAVS expressing constructs and either control (C), IκBα or IRF3ΔN constructs to repress the activity of NFκB or IRF3, respectively. These cells were either left untreated (-) or transfected (+) with FuGene:pIC for 10 h, then apoptosis was assessed by measuring the nuclear accumulation of YO-PRO by co-localisation with the nuclear Hoechst stain (see also S3 Fig).'.

Also, the text is edited to read 'These data show that suppressing NFκB but not IRF3 activity by expressing Iκbα or a dominant-negative IRF3 construct (IRF3ΔN), respectively, reduced MDA5 or MAVS-dependent cell death (Fig3F and S2 Fig). Accordingly, MDA5 appears to induce apoptosis in HEK293 cells by activating NFκB via MAVS.' (ln 168-172).

3. Fig. 4 - the IRF3 translocation is really not clear - a phospho-IRF3 analysis would be more informative here.

We moved this figure to the supplementary material (S3 Fig). The text and figure legend has been edited to account for this change.

Why would the levels of IkBa be different between WT and Ifih-/- cells?

A regulatory loop controls IκBα expression through NFκB activity. As MDA5 activates NFκB, the *Ifih1-/-* cells demonstrate reduced NFκB activity, and so, lower levels of IκBα. This explanation was in the manuscript and so we make no change to the text.

4. Although TNFa and IL-6 levels modulate depending on Mda5 function (Fig. 4C and D), there is no evidence of processing on pro-IL1B, one of the measures of immunogenic cell death. Why is no processing observed in these cells, whereas in vivo IL1B is produced and quantified in the in vivo model?

Production of mature IL1β requires induction and then caspase-1 processing by the inflammasome complex. These two steps are induced by separate stimuli. The data shows only induction of pro-IL-1β, with other inflammatory cytokines IL6 and TNFα, in macrophage cells *in vitro*. Accordingly, secondary signals required to form the inflammasome are either repressed or are absent. The production of IL-1β *in vivo* suggests that these stimuli are provided extrinsically to macrophages or, alternatively, IL-1β is being produced by another cell type, such as neutrophils. This explanation has been added to the text (ln 200-202 and 214-216).

5. Could the authors lay out Fig. 5 in a manner that presents the data more clearly?

We have labelled the proteins/transcripts at the top of the figure above the measures in the selected tissues to try to make the interpretation of his data simpler. Fig 6 has been reformatted in the same way.

6. The experiments with the &946, V923 and X627 versions of Mda5 are interesting and indicate that mutation of Mda5 alters IFNB, p56 and NF-kB induction. However a crucial experiment seems to be missing. Expression of these variant proteins in ifih1-/- cells should have a dramatic impact on inflammation markers and cell survival if the concept of this paper is correct. Have the authors attempted such an experiment?

This was our original intention. However, only a subset of *Ifih1-/-* MEFs could be forced to express the transgene. This meant that there was no co-ordinated response to the transgene, which prevented assessment of MDA5-activity. Attempts to enriched MDA5 expressing MEFs (by FACS) were unsuccessful due to cell death induced by MDA5 activity. This forced us to resorted to transient expression experiments in alternative cell types. A number of different cell lines were screened to isolate one that responded to MDA5 expression. We used HEK293 cells as these didn't have detectable expression of MDA5 and demonstrated MDA5-dependent cell signalling when transformed with MDA5-expression constructs (Figure S5). Importantly, the high transfectability of these cells allows a co-ordinated response to the transgene. As this explanation was already given (ln 270-274) we have made no change to the manuscript.

Referee #2:

- general summary and opinion about the principle significance of the study, its questions and findings

The authors describe the key role of MDA5 in the control of rotavirus (RV) infection and inflammation in pancreas comparing WT and IFIT1-/- mice. Using IFNAR1-/- mice and IFNR blocking antibody, they demonstrate that MDA5 antiviral response involves both IFN-dependent and -independent pathways and that MDA5 exerts an NFκB-dependent pro-apoptotic activity. They further observed in vitro that 3 minor alleles of IFIT1 associated with protection against T1D present lower antiviral, pro-apoptotic and pro-inflammatory properties than the major allele in RV infected HEK293 cells.

This is an interesting issue because little is known about the mechanism(s) leading to beta cell lost during acute RV infection and its potential relevance in further T1D related autoimmunity and T1 diabetes. The novelty of this work is that the authors describe an MDA5 dependent antiviral response particular to the pancreas of RV-infected mice, observation that they carefully see as a "consonance" in autoimmunity in T1D. Their interpretation is in agreement with several other

articles describing the key role of type 1 IFN in the primary events leading to autoimmunity and T1D.

- specific major concerns essential to be addressed to support the conclusions

The authors observe that the absence of MDA5 lead to increased viral production and conclude this is "due to the altered survival of the different MEFs" (line 107). This observation is just a correlation so this conclusion is abusive and should be modulated.

The quoted sentence discusses a difference in the measures of virus replication and is preceded by the qualifier 'appears to be' '. We replace the word 'appears' with the words 'was supposed' to better reflect the speculative nature of our explanation in the revised manuscript (ln 105).

The role for MAD5 in the control of RV (Boquet et al, J Immunol. 186(3):1618-26, 2011) and the importance of pathways involving MAVS in rotavirus induced innate immune response (Di Fiore et al, Virus Research 208, 89-98, 2015) have been described previously. These papers should be quoted.

Broquet et al was already cited. Di Fiore et al is now also cited in the revised manuscript (ln 76).

Figure S4 (now coded S5) *reveals the pathogenicity of RV infection in the context of the expression of the different MDA5 alleles. These data are erroneously related to the measure of RV titers Figure 7I in both the legend of Figure S4 (line 819) and in the text (line 267).*

This has been corrected by replacing the reference to Fig 7I in the legend of Fig S5 to the intended Fig 7G in the revised manuscript (ln 933). The reference to Fig S5 has been removed from the text.

The authors claim that their observations of a key role in MDA5 in the control of RV infection and innate immune response argue against the proposition that persistent (Coxsackie)viral infection induces autoimmunity. To my view it is just an alternative source of local type 1 IFN production and further inflammation in the pancreas, an early event triggering autoimmunity. As observed in RV infected pancreas, SNPs in IFIH1 associated with reduced risk for T1D would induce less pro-inflammatory signals than the major IFIH1 allele in CVB persistently infected islets. Could the authors clarify their view about this?

Reports have demonstrated that persistent virus infection, for instance with LCMV, SIV, HIV, HBV, HCV and HTLV1, inhibit cytotoxic T cells as they become exhausted from continuous T cell receptor stimulation from persistent antigen. The presence of exhausted T cells in patients with autoimmune diseases correlates with a more favourable prognosis. Type I IFN themselves have not been demonstrated to rescue exhausted T cells during persistent virus infection. In fact, the major immune checkpoint protein PD-1 is an IFN-inducible gene. Accordingly, persistent virus infection with heightened IFN signalling would not be expected to promote autoimmunity. We argue that autoimmunity is not a consequence of an impaired antiviral response, as is inferred by a failure to clear persistent viral infections, but of an excessive innate immune response, which would be anticipated to reduce viral persistence. Notably, it was observed that specifically ablating Mdam5 expression protected β-cells in the NOD mouse model, while ablating type I IFN signalling did not. Part of this discussion is added to the reviewed manuscript. (ln 304-307 and 327-328).

- minor concerns that should be addressed

The failure of rotavirus infected macrophage to produce IL-1β has been described previously (Di Fiore et al, Virus Res 208 :89-97, 2015), in this paper the authors do not detect induction of IL-1β expression. Interestingly the results presented here on rotavirus infected peritoneal macrophages reveal the absence of processing of pro-IL-1β, suggesting that rotavirus interferes with the activation of caspase 1/inflammasome in infected macrophages. This observation deserves to be discussed in regards of the results of Di Fiore and collaborators.

See our response to Reviewer 1, point #4.

Line 320: The reference related to "IFNλs have been demonstrated to be essential to control RV infection" (Pott et al, PNAS, 108(19): 7944-9, 2011) should be quoted.

This reference is cited in the reviewed manuscript (ln 337-338).

The authors should explain the rationale for using 3 different methods to evaluate cell survival: crystal violet staining, codetection of Annexin V and 7-AAD by flow cytometry and YO-PRO staining.

Directly counting cells or estimating by staining with crystal violet captures the total cell number. The different dyes distinguish viable from dying cells, thereby confirming that the reduced cell number is due to death rather than, for instance, altered proliferation. The relative uptake of the different dyes has been used to indicate the mechanism of cell death. The uptake of Annexin V without 7-AAD and rapid nuclear staining with YO-PRO distinguish apoptotic cells. Fig 3F shows rapid YO-PRO uptake suggesting MDA5 induces apoptosis. However, Fig 3A shows co-incident staining with Annexin V and 7-AAD staining, which is more indicative of necroptosis. Because of this ambiguity we avoid speculating on the mechanism by which MDA5 induces cell death in these experiments. See the additional discussion on this below (in response to Reviewer 3, point #3). Part of this explanation has been added to the revised manuscript (ln 501-506).

MEF survival 24h post infection by RV is detected by crystal violet in both Figure 1D and figure 3 E. Why to put twice this data? Moreover in Figure 3E, mortality in IFNAR1-/- MEF is 20% higher than in WT MEF while it is 50% higher in Figure 1D. If there is a difference which is it? Please clarify this point.

The description of Fig 1D was incorrect (it described data that had been moved to 3E). The legend has been corrected to describe that the data shows a direct count of cells (ln 789-790). The different measures in 1D and 3E show the same response trend, although the degree of the difference is greater as assessed by directly counting cells as opposed to estimating the cell number by staining with crystal violet. This presumably reflects an under estimated by crystal violet staining because of its limited dynamic range.

Viral titers are expressed in FFU/ml or FCFU/ml, the units should be harmonized.

The misuse of FFU has been corrected to FCFU (ln 420).

The review quoted to illustrate the proposed role for virus infections as potential primary agents in the initiation of T1D is more than 10 years old and should be replaced by one of the excellent recent reviews discussing this point.

We replaced Peng & Hagopian 2006 with the slightly more recent Hober et al 2010 (ln 68).

*Some figures are difficult to understand and should be better explained in the text and/or legend: Figure S4 C (*S5 C in the revised manuscript*): The legend should be improved to explain that data present cellular viability in both transfected (GFP+ cells) and non transfected cells (GFP- cells).*

This change has been made as suggested (ln 931-933).

Figure 7 (A) The time points for the images presenting association between MDA5-MAVS and MAVS-MAVS are missing in the legend.

The time points are now given in the figure legend (ln 863 & 865).

Figure 7 (F) This figure is entitled NFκB activity while in the text is mentioned as reporter assay reflecting IL8 promoter activity. This should be clarified.

The *IL-8* promoter report is NFκB responsive, so both descriptions are accurate. To be consistent, we have changed the title of Fig 7F to read 'IL-8 induction', and edited the legend to read *IL-8*-promoter (from 'NFκB-responsive'). We also changed our use of the protein titles (IFNβ- and P56-)

to use the gene titles for the other two reporter constructs (*IFNB1*- and *IFIT1-)* in the figure legend (ln 871-872).

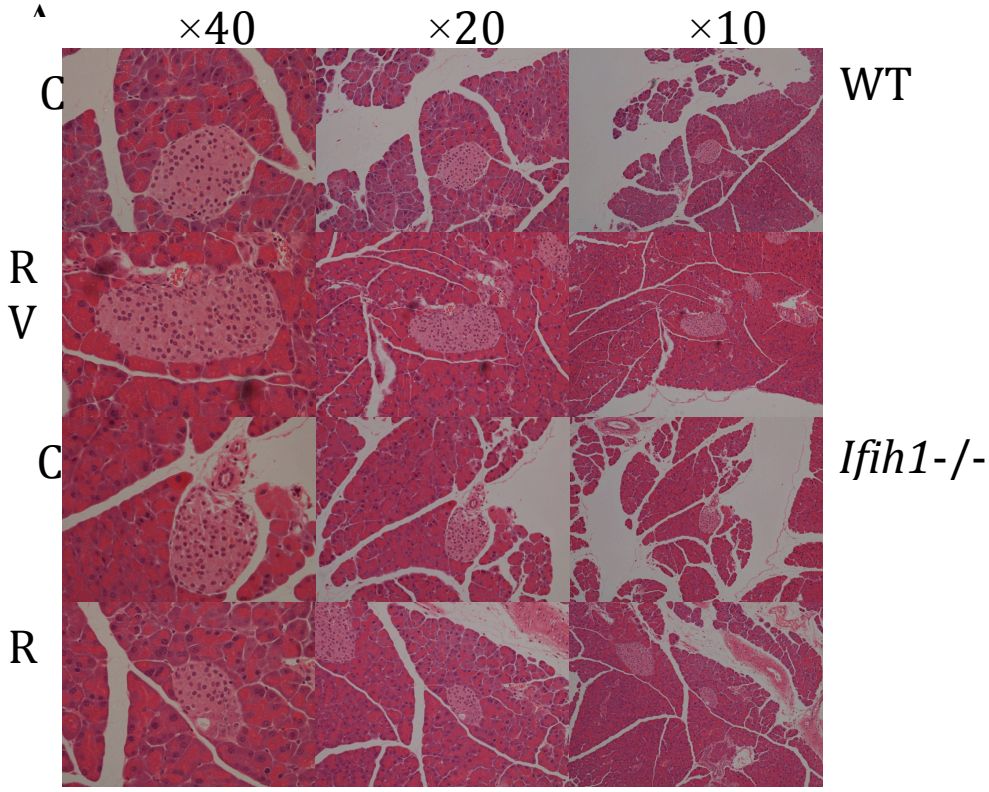
The review: de Beeck AO, Eizirik DL (2016) Viral infections in type 1 diabetes mellitus--why the beta cells? Nat Rev Endocrinol 12: 263-73 should be corrected: Op de Beeck A, Eizirik DL (2016) Viral infections in type 1 diabetes mellitus--why the beta cells? Nat Rev Endocrinol 12: 263-73.

This correction has been made (ln 303 and 690).

- any additional non-essential suggestions for improving the study (which will be at the author's/editor's discretion)

It was published by others that RV infection induces transient hyperglycemia, pancreatic cell destruction and reduction of islets size in C57/B6 mice (Honeyman et al, PLOS ONE 9, e106560, 2014). In order to validate the authors' hypothesis that the absence of MDA5 protects beta cells from RV induced islet destruction and local inflammation, the authors could compare glycemia, islet aspect and potential pancreatic inflammation after RV infection in WT and IFITH1-/-.

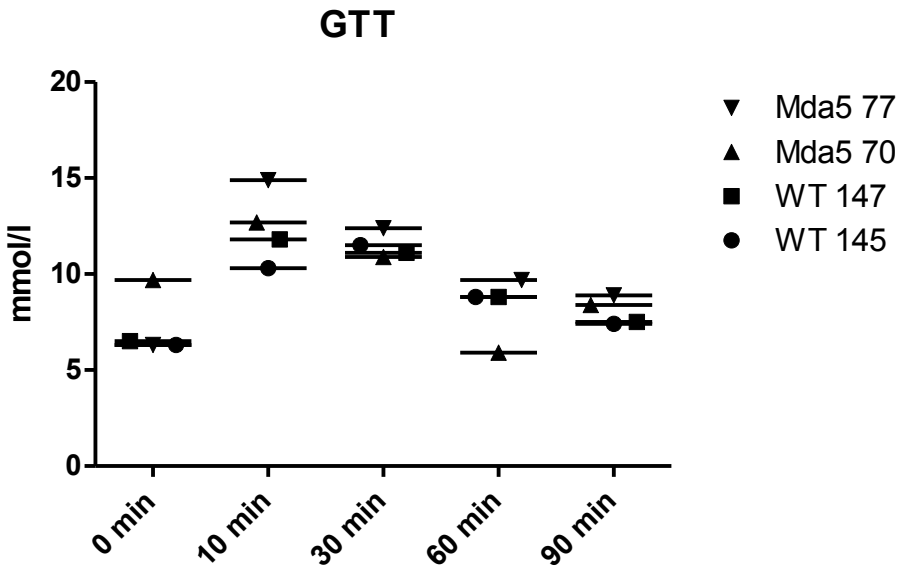
We attempted to replicate the experiments of Honeyman et al in wild-type and *Ifih1-/-* mice. However, in our hands three-week-old mice, as used by Honeyman et al, had very high mortality. Accordingly, we used older (five-week-old) animals. Preliminary testing of blood glucose levels didn't detect any differences. Conversations with Dr Honeyman lead us to believe that the differences they detected were less likely in these older animals. Examination of the pancreas from the mice in our experiments didn't detect any conspicuous difference between the *Ifih1-/-* compared to wild-type mice infected with rotavirus as shown here.



H&E stained sections of the pancreas from mock or rotavirus infected Ifih1-/- and wild-type mice.

It seems unlikely that there would be a conspicuous change in the pancreas of mice in Honeyman's study as the hyperglycemia rapidly resolved, suggesting that the pancreas is not injured by virus infection. This implied to us that the hyperglycemia was a consequence of inflammation. To test this we performed some preliminary experiments whereby we treated *Ifih1-/-* and wild-type mice with

the dsRNA mimetic pIC. This experiment does not replicate rotavirus infection but it simplified the timing of the induction of the inflammatory response to dsRNA with the timing of a glucose tolerance test. As is apparent in the preliminary data shown here there was no major difference between the *Ifih1-/-* and WT mice.



The relative levels of blood-glucose of wild-type (WT) and Ifih1-/- (MDA5) mice treated by intraperitoneal injection of pIC (25 ug/mouse), then starved for 22 hours before being subjected to a glucose tolerance test.

We also performed some preliminary assessment of caspase-3 cleavage, as a measure of apoptosis, the the pancreas. This showed that rotavirus infection induced a modest (3 fold) increase in the levels of activate caspase-3 in the pancreas as detection by immunohistochemistry. We are not including these measures in the manuscript as, besides further complicating a manuscript that already has a substantial amount of data, our preliminary studies indicate that MDA5 induces cell death by additional mechanism(s). This point is discussed further below.

Referee #3:

The manuscript describes a study that aims to address the role of MDA5, one of hte RIG_I-like receptors (RLRs), in rotavirus (RV) infection control. MDA5 variants are differentially associated with type 1 diabetes (T1D), and the authors hypothesize that RV infection of the pancreas , and differential control of this infection, can lead to a T1D outcome. The data shows that MDA5 can signal cell death pathways to drive the death of RV-infected cells and that this occurs through MAVS interaction. The MAVS interactions appears to be variable among MDA5 mutants differentially associated with T1D. These differences also link to differential level of IFN expression, and expression of proinflammatory cytokines, and importantly MDA5 is required for control of RV infection of the pancreas, as shown in mice.

Overall the data are solid to reveal a strong role for MDA5 in driving innate immune defenses against RV, and bring forward a new concept that the host response to this infection as mediated by MDA5 is the driver of the T1D outcome.

Specific comments:

1. The use of proper nomenclature. The innate immunity field now used RIG-I like receptor (RLR) instead of RIG-I-like helicase (RLH). This nomenclature was adopted to reflect RIG-I and MDA5 as

members of the pathogen recognition receptor family that includes TLR, NLR, CLR, and now the RLRs. Please use the modern nomenclature RLR).

RLH has been replaced with RLR throughout the revised manuscript.

2. How does RIG-I perform in RV infection, a comparison of the RIG-I vs. MDA5 phenotype should be examined. Similarly, MDA5 signaling was shown by Reise Sousa lab to be dependent on LGP2. The role of LGP2 in RV sensing and infection should be addressed in order to fully define the how MDA5 and RLRs in general impact and are regulated by RV. Currently the study does not address specific of MDA5 over other pathogen recognition receptors, including TLRs and RLRs, and others, in the sensing of RV and triggering of the host response to RV infection. Specificity is a highly important item to address considering that the conclusion is that the host response driven by MDA5 (vs. some other pathogen recognition receptor) is responsible for the T1D outcome. Note that in figure 2A only about 50% of the IFN is reduced in the MDA5 ko cells. Thus, additional pathogen recognition receptors must contribute to this response. Is MDA5 dominant here or just another player?

We include additional data of the relative transcriptional response to rotavirus infection that is induced by MDA5, RIG1 and LGP2 (Fig 1F). We have not included data for the co-expressing LGP2 with MDA5 and RIG1. This data shows co-expression of LGP2 with RIG1 or MDA5 represses gene induction. However, this type of experiment has produced misleading results as to the activity of LGP2. The function of LGP2 is still far from resolved and has been a contentious area because of confounding reports.

As discussed above, in response to Reviewer 1's first point, our data confirms an impaired antiviral response in the absence of MDA5 but in the presence of RIG1 and LGP2 and other innate immune factors. Accordingly, we establish MDA5 is an important component of the antiviral response to rotavirus. Our demonstration that there is different dependence upon MDA5 activity in separate tissues is a major finding of this study and the coincidence of the dominance of MDA5 activity and the tissue specific autoimmunity is intriguing.

3. Cell death driven by MDA5 in RV infection is the major outcome that links with a probable T1D phenotype via destruction of pancreas cells. This is shown clearly in Fig 3. Thus, what is the cell death pathway that we are dealing with here, is it caspase-dependent (presumably it is), and if so, what caspases are being engaged here by MDA5? These experiments require for probing of caspase/cell death pathways to reveal the point of interaction with MDA5. Is the death signaling MAVS-dependent?

Although we identify that MDA5 induces cell death partly by apoptosis our data is equivocal on the mechanism(s). Our preliminary data has indicated the MDA5-dependent cell death is complex and appears to involve multiple mechanisms. It is emerging that RLR play a role in necroptosis, which we believe is important in the subsequent development of autoimmunity. Also, a number of very recent reports have identified cell death induced by RLR is mediated by other IFN-regulated factors. Substantial, additional work is required to clarify this cell death pathway. Delineating the mechanism(s) of MDA5-dependent cell death requires additional substantive work that will constitute a subsequent manuscript.

4. Differential MAVS interaction of MDA5 variants is of high interest here. The fluorescence analysis shown in 7A indicates MAVS interaction among the MDA5 snp mutants is different. How this differential impacts the strength of MAVS interaction is not revealed however and how it play out for downstream signaling to IRF3 and cell death responses is not clear. The authors should include a biochemical (coimmunoprecipitation of MDA5 variants with MAVS, immunoblot of total vs. phospho-iRF3 abundance, caspase activation/signaling of cell death) for each MDA5 variant to best validate each mutant linked with innate immune and cell death responses.

It isn't clear that probing the association between MDA5 and MAVS by co-immunoprecipitation will reveal more than the bimolecular complementation experiments that were performed. Immunoprecipitation and detection of enriched protein complexes is less quantitative than the protocol used. Also, there is already data in the manuscript that demonstrates the impact of the different SNPs for downstream signalling. We assessed the relative activation of IRF3 and NFκB-

responsive reporters. Additionally, the relative induction of cell death by the different MDA5 polymorphs is tested.

2nd Editorial Decision 22 June 2017

Thanks for submitting your revised version to The EMBO journal. Your study has now been re-reviewed by referees # 1 and 2. As you can see from the comments below, the referees appreciate the introduced changes and support publication here.

I am therefore very pleased to accept your manuscript for publication here. Before formal acceptance here there are just a few technical things that have to be resolved.

REFEREE REPORTS

Referee #1:

Sadler and colleagues have responded in detail to the reviewers' comments, and with 3 reviewers and multiple questions, this required modification of Figures, new experiments and extensive clarification. The manuscript is quite convincing and clearly revised.

Referee #2:

The answers of the authors are convincing and the paper is clearer now.
I recommend publication in EMBO Jnl.

EMBO PRESS

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Anthony Sadler
Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2016-96273R

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	The sample size was determined emperically.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	The animal numbers used were predetermined based upon experience that this sample size is sufficient to distinguish differences in the physiological respponses being studied.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	In some instances preliminary experiments were run to determine the response. These preliminary experiments were excluded, otherwise all measures were included in analysis.
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Animals were chosen for experimentation as they were bred.
For animal studies, include a statement about randomization even if no randomization was used.	There was no exclusion/selection criteria for experimentation, besides confirmation of the appropriate genetics and gender.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No
4.b. For animal studies, include a statement about blinding even if no blinding was done	The anlaysis was not blinded
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	Yes
Is there an estimate of variation within each group of data?	Yes
Is the variance similar between the groups that are being statistically compared?	Yes

C- Reagents

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http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Primary antibodies used for Western blots were: mouse anti-Mda5 (Axxora, ALX-210-352), anti-IκBα (Cell Signaling, 9242), mouse anti-Il-1β Abcam, ab9722), anti-β-actin (Abcam, ab8226), mouse anti-P56 (Dr Ganes C Sen, Cleveland Clinic, USA) and anti-GFP (SIGMA). Reagents used for ELSIA were: rat anti-mouse Ifnβ monoclonal antibody 7F-D3 (Abcam, ab24324), rabbit anti-mouse Ifnβ polyclonal antibody (PBL Biomedicals, 32400) and goat anti-rabbit IgG-HRP (Santa Cruz, E2908), rat anti-mouse IL-6 (BD Pharmingen, 554400), biotin rat anti-mouse IL-6 (BD Pharmingen, 554402), HRP-streptavidin conjugate (Invitrogen, 43-4323) and recombinant murine Il 6 standards (BD Phamigen, 554582) and a recombinant murine Ifnβ standard (Professor Paul Hertzog). Tnfα and Il-1β were measured using kits (BD OptEIATM, 558534 and 559603). For microscopic detection of antigen cells were probed with: polyclonal anti-SA11 (Donker et al, 2011), goat anti-rabbit antibody (Alexa® Fluor 594, Invitrogen), Phalloidin (Biotium) and Hoechst (Invitrogen). IRF3 was detected using anti-IRF3 rabbit polyclonal antibody (FL-425, Santa Cruz Biotech) and goat anti-rabbit (Alexa® Fluor 488, Invitrogen).
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The HEK293 and MA104 cell lines was perchased from ATCC. The wild-type and Ifih1-/- MEFs were generated within the study from transgenic mice and were confirmed to display the correct genetics by PCR genotyping and western blot. These cells were not tested for mycoplasma. The Ifnar1-/- mice were a gift from Dr P Hertzog and had been mycoplasma tested. The HEK293FT cells were perchased from Invitrogen.

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	Mouse, C57Bl6J, male, five week wild-type and IFIH1-/-.Mice were bred and housed homogeneously in the Monash Medical Centre Animal Facility under conventional conditions
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All procedures were conducted in accordance with protocols approved by the Monash University Animal Welfare Committee (approval number MMCA/2007/43) under relevant institutional guidelines, the Prevention of Cruelty to Animals Act 1986 and associated regulations, and the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	The practices used for the study are compliant with international animal care guidelines

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	n/a
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	n/a
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n/a
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	n/a
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n/a
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	n/a
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	n/a

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	n/a
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	n/a
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	n/a
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	n/a
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	n/a

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No