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Molecular dissection of plasmacytoid dendritic cell activation in vivo during a viral infection

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Editorial Correspondence 7th Feb 18

Thanks for submitting your manuscript to The EMBO Journal. Your study has now been seen three referees and I have provided the comments below. As you can see from the comments, the referees express interest in the paper but they also raise substantial concerns with different aspects of the paper (microarray experiments, the use of PDCA-1 as a marker to sort pDCs and the conclusiveness regarding the key conclusions).

As many concerns are raised and as it is unclear if they can be resolved, I would like to ask you for a point-by-point response to see what can be done to address the concerns raised within a 3-6 months timeframe before taking a decision on the paper. Please note that the use of the pDC marker is a key issue.

Happy to discuss further and let me know if you have any questions regarding this.

REFEREE COMMENTS

Referee #1

Molecular dissection of plasmacytoid dendritic cell activation in vivo during a viral infection

Tomasello et al. explore the molecular determinants leading to plasmacytoid dendritic cell (pDC) activation in vivo following murine cytomegalovirus (MCMV) infection. The authors aim to decipher the precise role of the TLR and type I IFN signaling pathways in pDC activation using a model of mixed (WT and mutant) bone marrow chimera mice (MBMC). Following reconstitution and MCMV infection, pDC are isolated from the spleen by flow cytometry and subjected to microarray analysis, qPCR or phenotyping by flow cytometry.
Analysis of the micro-array data reveals the pathways involved in pDC activation and especially their differential dependence on IFN-I and/or TLR signaling. The authors convincingly show that

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IFNa production by pDC upon MCMV infection depends on the TLR9/7-Myd88-IKKb-IRF7 pathway without the need of positive feedback from type I IFN signaling, nor from AP3 expression.

Major concerns:

Overall, the paper remains very difficult to read even after several readings. It gives the impression to go in many directions rather than to decipher a particular mechanism. The experiments are well conceived, performed and controlled. However, I have concerns about some of the interpretations proposed. There is clearly a need for rewriting and simplification. The presentation of the microarray data is very difficult to follow and it remains unclear how all these data are connected with the rest of the study.

The authors evaluate whether MCMV infection of pDC is necessary for IFNa production using a MCMV stain expressing GFP. However, sensing of MCMV by pDC can occur independently of viral replication, e.g. after fusion following MCMV infection. Thus, the experiment with the GFP MCMV does not support the conclusion drawn by the authors (Page 14 line 6).
In addition, the rational for using IFNAR blockade in MBMC mice is not well explained (Page 14 line 15). How blocking IFNAR would shift the molecular mechanism and promote IFNa production that is already abrogated in Myd88-KO pDC?

The authors confirmed that AP3, which is an adaptor complex involved in the recruitment of IRF7, is dispensable for pDC activation in MCMV-infected mice (Fig3E), as already shown (Del Prete 2015). Del Prete et al. additionally showed that in contrast to in vivo infection, pDC lacking AP3 do not produce IFNa in response to MCMV exposure in vitro. Thus, it remains to be established the precise molecular pathways responsible for IFNa production following MCMV infection in vivo. The results reported here by Tomassello et al. comparing MCMV infection and CpG injection, may support the hypothesis of a second signal, AP3- and IFNAR-independent, leading to the IKKb-IRF7-IFNa signaling pathway activation. The authors did not decipher how MCMV infection selectively leads to IKKb activation.

Minor concerns:

The results on IRF7 regulation by Myd88 and IFNAR signaling are new and of interest. However, how IRF7 expression levels relate to IFNa production remains to be determined. Indeed, IFNAR-KO mice display higher levels of IFNa+ pDC after MCMV infection as compared to WT mice (Fig4I), whereas pDC from IFNAR-KO MBMC mice have lower expression of IRF7 (Fig5B). The authors should comment this result. Does IFNAR signaling induce a negative feedback on IFNa production in this model?

The authors previously showed that during MCMV infection, pDC maturation was dependent on IFNAR signaling, but the production of IFNa was not tested at the time (Dalod et al. 2003). Here using the MBMC mice, the pDC maturation (as judged by CD86 expression) depended on IFNAR signaling whereas IFNa production was not affected by the absence of IFNAR. These interesting results point to the possible requirement of an additional signal for IFNa production by pDC during MCMV infection.

As for AP-3, the authors confirm previous results regarding the involvement of LFA1 in pDC activation (Fig7). Saitoh et al. recently showed that Itgal-KO pDC have a defect in TLR-7 trafficking (Saitoh et al. 2017). It remains to be determined whether Igtal-KO pDC have a similar defect in response to TLR-9 stimulation due to an impairment of TLR trafficking. However, no difference was observed between MCMV infection and CpG injection in Itgal-KO pDC from MBMC mice (Fig7). Since IFNa production by pDC remains dependent of Myd88, these results suggest that LFA1 is not the factor involved in IFNa production during MCMV infection in IFNAR-KO pDC.

SEM needs to be added to most of the panels from Fig3, Fig4 and Fig6.

Referee #2

In this manuscript, the authors suggest that during an immune response to MCMV, pDCs produce IFN-a mostly through cell-cell contact with infected cells, rather than themselves being infected but their response still depend on the TLR9/MyD88/IRF7 pathway. They also claim that the in vivo response by pDCs to a virus is very different at the molecular level to the one in response to CpG. They also show that the IFN response during MCMV infection does not require signaling by the IFNR.

I have the following concerns:

Major concerns:
- The authors are presenting microarray data on splenic pDCs isolated from mixed BM chimeras using different combination of KO mice infected with MCMV. I have 2 majors concerns about this experiment. First, there are multiple complex experimental steps involving BM reconstitution, infection by a virus, cell sorting and microarrays. Because of the inherent variables in conducting such an experiment, it is not adequate to perform these analyses with just 2 mice per group. Furthermore, the authors have decided to use 2 fold as a criteria to consider a gene as regulated. This seems not very stringent (again with just 2 mice) and the authors should give some rationale with statistical backup as to why this is an acceptable threshold to use.
The second concern is that the marker PDCA-1 used to isolate pDCs from the spleens is known to be a classical interferon-inducible gene and is inadequate to sort pDCs, in particular in a model where IFN is a key element of the response. This is an issue across the entire manuscript as it questions that the authors are looking at a pure population of pDCs.
- One of the main claims of this study is that the requirements for IFN production by pDCs are different between MCMV infection and CpG injection. This is based on the data presented on Fig 3. It is well known that the kinetic of the IFN response is tightly regulated in pDCs and the profile of gene expression is changing drastically overtime. In this experiment, the authors are comparing the expression profile in pDCs at 36h post MCMV versus 7h post CpG. This might be the optimal timing for these 2 stimuli to induce IFN but this cannot be used the compare the response by pDCs to these 2 conditions. It is thus difficult to draw the conclusion made by the authors in this experimental setting.

Minor concerns:
- The authors show that GFP (meaning MCMV infected) positive cells are not preferentially IFN-producing cells suggesting that infection of the pDCs by the virus is not required to produce IFN although this still requires the TLR9/MyD88/IRF7 pathway. The data suggest that this is due to cell-cell contact involving infected cells. Although the data are very interesting, it is difficult to reconcile with the fluorescent data in Fig 7 suggesting that many YFP (IFN-b producing) positive cells are infected by the virus. This should be clarified.

Non-essential suggestions:
- The authors may want to make sure that they clearly identify in their introduction what refers to human versus mouse pDCs. Although both mouse and human pDCs shares multiple parameters, some are different and the literature cited should better match this.
- Last paragraph of the introduction should not be used to repeat the findings already described in the abstract.

Referee #3

It has been thought that IFN signaling through IFNAR is responsible for the dramatic high expression of IFN by plasmacytoid dendritic cells (pDCs), and that this pathway depends on high levels of IRF7, which PDCs naturally have. The going hypothesis is that the natural high levels of IRF7 in pDCs is due to constitutive response to homeostatic levels of IFN. However, this feedback loop's role in in vivo viral infection is unclear.

This manuscript explores the details of how viral infection induces type I IFN in pDCs. To address this question, they performed mixed bone marrow chimeras and analyzed pDC gene expression.

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This approach controls for potential compensatory gene expression changes when full knockouts are used, thus it is an exciting approach. The analysis is extensive and shows clearly that many viral infection-induced genes are independent of INFAR, including IFN itself. Their data show that IFN is induced similarly in WT and IFNAR KO pDC suggesting that a feedback loop via INFAR is not required for high levels of IFN production by pDCs. Importantly, they showed that the high level of IFN production by pDC in response to CpG DNA (TLR9) did require IFNAR expression. Furthermore, not all responses were intact in IFNAR KO pDCs, they did not upregulate CD86, IRF7, Mx1 and several other genes showing that the IFN effect was specific.

They go on to show that AP3 is not required for either CD86 or IFN in response to viral infection, but is required for IFN, but not CD86, responses to CpG DNA. The response to MCMV viral infection occurs via the TLR9/MyD88 pathway not via cytosolic sensors, which would not require the IFNAR amplification pathway, as demonstrated by the fact that IFN production was not restricted to infected cells.

Although the topic is important, and the data are generally well presented, logical, and robust, a few things need to be addressed:

1) In figure 5, the authors should show the overlay of WT and IRF7KO pDC stained with anti-IRF7 antibody. That control is much more important than isotype controls. The way the figures are presented in 5A, it is difficult to tell the staining patterns in the histograms.

2) Throughout much of the text, the authors refer to the cytokine as IFN-I, but on page 16 they use IFNβ. The nomenclature should be unified.

3) The descriptions for use of IFNAR blocking antibodies to rescue certain responses (eg IFN production in IRF7KO cells) needs to be better described. The logic, as presented, for interpreting these experiments is confusing.

4) Details about some experiments and staining need to be added. One example is the description of the data obtained from the IKKa mutant mice in Figure 6 are lacking, as well as description of MOMA-1 being a marker for marginal zone macrophages.

5) Figure 7B should include an overlay of the YFP and MCMV, there is a wide open white space in the figure for this. It would significantly increase the ability to see adjacent infected and IFN producing cells. The overlay with the red makes it extremely difficult to distinguish the green YFP cells and the blue infected cells. The individual color images would be better as black and white for ease of viewing the individual channels.

6) The data in figure 7B are not sufficient to conclude cell contacts are important, thus the connection to integrins is weak. Also, in the figure legend, the authors need to indicate what the arrows and asterisks indicate. The data on integrins seem disconnected from the rest of the paper and do not add much to the conclusions.

7) The authors should consider, and include in the discussion, the possibility that MCMV infection triggers more than one TLR and that is in part responsible for the differences between the dependence of MCMV infection and CpG DNA-induced responses on IFN feedback and AP3.

1st Editorial Decision 21st Feb 18

Thank you for sending me the point-by-point response. I have now had a chance to take a careful look at it. I appreciate that you can address many of the concerns raised. The one issue that I think will be important to address is to add some more insight is the mechanism of IFN-I production following MCMV infection in vivo. We don't need the full mechanism but some insight would clearly strengthen the paper.

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I am open to consider a revised manuscript and I will send it back to the original referees. However, at this stage I can't provide any guarantees on the outcome of the review process. I do need strong support from the referees for consideration here.

1st Revision - authors' response 16th May 18te

Point-by-point answer

Referee#1
Major points.
There is clearly a need for rewriting and simplification. The presentation of the microarray data is very difficult to follow and it remains unclear how all these data are connected with the rest of the study.
We thank the reviewer for helping us to make our manuscript clearer and crisper.
We have simplified the microarray data analysis and better integrated it to the rest of the study.
We have reorganized and rewritten part of the manuscript according to reviewer's advice (pages 8-11).

Sensing of MCMV by pDC can occur independently of viral replication, e.g. after fusion following MCMV infection. Thus, the experiment with the GFP MCMV does not support the conclusion drawn.
We agree that GFP expression likely identifies only pDC that are replicating the virus, not pDC that have been abortively infected upon viral fusion. We have modified our interpretation accordingly (page 15), which does not change the message of the paper. Indeed, we can conclude that the pDC producing IFN-I are not replicating the virus, and, reciprocally, that most pDC replicating the virus are not producing IFN-I. In addition, since we show that type I IFN production by pDC is strictly dependent on TLR9 and MyD88, even in IFNAR-KO cells, this proves that sensing of MCMV in pDC for this function occurs in endosomes and not in the cytosol, as stated by Referee#3.

The rational for using IFNAR blockade in MBMC mice is not well explained.
We thank this reviewer as well as Referee #3 for pointing out that the rationale for these experiments is not explained clearly enough. We have edited the manuscript accordingly (page 15).

The authors confirmed that AP3, which is an adaptor complex involved in the recruitment of IRF7, is dispensable for pDC activation in MCMV-infected mice (Fig3E), as already shown (Del Prete 2015). Del Prete et al. additionally showed that in contrast to in vivo infection, pDC lacking AP3 do not produce IFNa in response to MCMV exposure in vitro.
We respectfully disagree with the phrasing of the referee that we "confirmed" the conclusion from Del Prete et al. that AP3 is dispensable for pDC activation in MCMV-infected mice. Del Prete et al. did not investigate pDC type I IFN production in vivo in AP3-deficient mice infected with MCMV. As correctly stated by the referee, they showed that pDC lacking AP3 do not produce IFN-alpha in response to MCMV exposure in vitro. However, they did not observe any decrease in systemic levels of type I IFN in MCMV-infected AP3-deficient mice. Then, extrapolating the mechanisms of pDC activation by MCMV in vitro to the in vivo situation, they concluded: "these observations suggest that AP-3 deficiency abolishes the TLR9-mediated IFN-α response of pDC during MCMV infection", "it is therefore conceivable that in pearl mice, other hematopoietic and nonhematopoietic cells (ie, lymphoid-tissue stromal cells) could contribute to the overall in vivo production of IFN-α" and "the AP-3-deficient mouse model [confirms] previous results obtained with different experimental models on the limited role of pDC in contributing to the overall response to MCMV infection". In other words, Del Prete et al. assume that pDC fail to produce type I IFN in vivo in MCMV-infected AP3-deficient mice. Thus, they conclude that their data show that "Interferon-α Production by Plasmacytoid Dendritic Cells Is Dispensable for an Effective Anti-Cytomegalovirus Response in Adaptor Protein-3-Deficient Mice" as the title of their paper states. We demonstrate here that this interpretation is incorrect. Contrary to what is inferred by Del Prete et al. from their in vitro data but actually not experimentally tested, we show here that in AP3-deficient mice pDC production of type I IFN upon MCMV-infection is not affected (page 13, Fig 3E). Thus, we thank the referee for this comment, because it reinforces the main message of our paper: the conclusions drawn from in vitro experiments should only be extrapolated to in vivo conditions with caution (page 22). Actually, it is essential to experimentally test how immune responses are orchestrated in

vivo rather than stopping at inferring conclusions to in vivo conditions from reductionist in vitro experiments.

The precise molecular pathways responsible for IFN-I production following MCMV infection in vivo [remain to be established]. The results […] may support the hypothesis of a second signal, AP3- and IFNAR-independent, leading to [IKKb-IRF7-IFNa activation]. […] LFA1 is not the factor involved […].
As this referee understood well, it was with the initial aim to decipher how MCMV infection selectively promotes pDC IFN-I production in the absence of AP3 or IFNAR that tested the role of LFA1. However, we agree that it is not per se the factor involved since it is also required for the response to CpG. However, its requirement shows involvement of cell-cell contacts in promoting pDC IFN-I production both during CpG stimulation and MCMV infection. It is likely that the exact nature of these cell contacts differ between these two stimulation condition and that precise characterization of the cell types and molecules involved will help understanding how MCMV infection selectively promotes pDC IFN-I production in the absence of AP3 or IFNAR. We have rewritten the corresponding section of the results (page 19) and discussion (page 23-24) accordingly. Moreover, we have made additional major efforts to test additional candidate molecular mechanisms promoting pDC IFN-I production during MCMV infection in the absence of IFN-I positive feedback signaling, namely i) compensation of IFN-I by other types of IFN as assessed by testing pDC IFN-I production in infected Stat1-KO MBMC (page 12, Fig EV2A-C), ii) putative compensation by IRF1 of IRF7 decrease, as assessed by testing pDC IFN-I production in infected Irf1-KO MBMC treated or not with anti-IFNAR1 neutralizing antibodies (page 16-17, Fig EV4C-E), and iii) alternative intracellular routing of viral material and TLR9 by an ATG5-dependant LC3-associated phagocytosis rather than through AP3, as assessed in Siglech-iCre;Atg5$^{fl/fl}$ MBMC (page 13-14, Fig EV3F-I). pDC IFN-I production was not impaired in any of these conditions. Hence, we have further ruled out three candidate mechanisms. As discussed at the end of the manuscript, deciphering the nature of the enigmatic second signal mentioned by the referee is currently hampered by the lack of proper mutant mouse models to specifically track and genetically manipulate pDC in vivo. Several teams including our are currently putting a major effort to overcome this roadblock. Achieving it will require a considerable amount of time, resources and efforts making it beyond the scope of the present study. Both referees #1 and #3 emphasized that our study already brings novel and interesting knowledge to the field.

Minor points.
IFNAR-KO mice display higher levels of IFN-I+ pDC after MCMV infection […], whereas pDC from IFNAR-KO MBMC mice have lower expression of IRF7. The authors should comment this result. Does IFNAR signaling induce a negative feedback on IFN-I production in this model?
IFNAR-KO pDC display higher IFN-I production only in plain IFNAR-KO mice but not in IFNAR-KO MBMC mice. This thus shows that enhanced IFN-I production in plain IFNAR-KO mice is not due to cell-intrinsic IFN-I effects in pDC, but to modification in the environment of pDC in complete mutant mice. In other words, IFNAR signaling does not induce a cell-intrinsic negative feedback on pDC IFN-I production during MCMV infection. However, IFNAR signaling strongly decreases viral replication in vivo and our previously published experiments with different doses of viral inoculum or with mutant viruses which replication can be controlled by drug administration have shown that the intensity of pDC activation during MCMV infection increases with the intensity of early viral replication (Robbins SH … Dalod M. PLoS Pathog. 2007;3:e123. PMID: 17722980). The objective of the experiment shown in Fig4I is to show that, in plain IFNAR-KO animals, pDC type I IFN production still strictly depends on MyD88 and thus on endosomal virus sensing through TLR9, even though it is strongly enhanced due to increased viral replication in the mice and occurs with only low levels of IRF7 expression.

The authors confirm previous results regarding the involvement of LFA1 in pDC activation. Saitoh et al. recently showed that Itgal-KO pDC have a defect in TLR-7 trafficking (Saitoh et al. 2017). It remains to be determined whether Itgal-KO pDC have a similar defect in [TLR9 trafficking].
We do not only confirm previous results regarding the involvement of LFA1 in pDC activation, but significantly extend this knowledge. For the first time to our knowledge, we show that cell-intrinsic LFA1 functions strongly promote pDC IFN-I production in vivo, and not only in response to a synthetic TLR9 ligand but also to a physiological viral infection (pages 18-19). Addressing the underlying mechanism, including whether it involves impaired TLR9 trafficking, is beyond the

scope of the present manuscript. Indeed, our study focuses on contrasting the molecular requirements for TLR9-dependent pDC IFN-I production in vivo in response to MCMV infection as opposed to administration of CpG, and, as this referee pointed out, our results show that LFA1 is required under both conditions.

SEM needs to be added to most of the panels from Fig3, Fig4 and Fig6.
We have added SEM to these panels.

Referee #2

Major concerns
It is not adequate to perform [microarray] analyses with just 2 mice per group [using] 2 fold as a criteria [for gene regulation]. Give some rationale with statistical backup as to why this is [acceptable].
We have changed accordingly the method for identification of the genes differentially expressed between conditions. All WT samples for each condition can be considered as biological replicates, which gives 14 biological replicates for WT pDC from uninfected animals and the same number for infected mice. This allowed robust statistical analyses using Limma to identify the genes differentially expressed between these 2 conditions, with use of statistical threshold of 0.05 which is very stringent since it is based on adjusted p values including a false discovery rate correction (page 30, Fig 1 and 2). Whereas there are only two replicates for mutant pDC from infected mice, they can be compared to six replicates of WT pDC from the same experiments, which allows using Limma again for the identification of differentially expressed genes. A 2-fold change threshold is commonly used in many gene expression analysis papers, such as those from ImmGen, a leader in the field (Miller JC … Merad M. Nat Immunol. 2012;13:888-99. PMID: 22797772; Robinette ML … Colonna M. Nat Immunol. 2015;16:306-17. PMID: 25621825), including in their Cell paper comparing the responses of different immune cell types from mouse spleen to subcutaneous injection of IFN-I where only duplicate data were used (Mostafavi S … Benoist C. Cell. 2016;164:564-78. PMID: 26824662).

[The] marker PDCA-1 […] is inadequate to sort pDCs [… it questions the purity of the pDC populations].
We thank the reviewer for making us aware that we did not explain clearly enough our gating strategy. Of course, we agree that the marker PDCA-1 is a classical interferon-inducible gene and is inadequate by itself to identify pDC. However, we want to stress out that we did not purify or gate pDC merely as PDCA1+ cells. We used a much more complex and precise phenotypic characterization of these cells, as illustrated on Fig. 1B, to ensure of their proper identification/purity. Specifically, within the gate of singlet live cells, pDC were defined as negative for lineage markers expressed on neutrophils (Ly6G), NK and NK T cells (NK1.1), B cells (CD19) and T cells (TcRb), as well as negative for CD11b, but expressing intermediate levels of CD11c and high levels of PDCA-1. This has now been written explicitly in the legend of Figure 1, page 33.

Importantly, contrary to the situation in humans, no specific marker has yet been identified for mouse pDC. In particular, SiglecH is not sufficient per se to identify pDC, since it is expressed on some populations of spleen macrophages as well as on pre-cDC (Swiecki M … Colonna M. J Immunol. 2014;192:4409-16. PMID: 24683186; Schlitzer A … Ginhoux F. Nat Immunol. 2015;16(7):718-28. PMID: 26054720). Moreover, SiglecH expression is decreased on activated pDC, including during MCMV infection (Zucchini … Dalod M. Int Immunol. 2008;20:45-56. PMID: 18000008; Puttur F et al. PLoS Pathog. 2013;9:e1003648. PMID: 24086137), especially on those producing type I IFN (Zucchini … Dalod M. Int Immunol. 2008;20:45-56. PMID: 18000008). This prevents using SiglecH to identify pDC in infected mice, since it would lead to missing most of the IFN-I-producing pDC.

During MCMV infection, PDCA-1 is further induced on pDC, including those producing cytokines, to levels higher than those reached by the other cell types responding to type I IFN (Zucchini … Dalod M. Int Immunol. 2008;20:45-56. PMID: 18000008). Thus, the use of a Bst2/PDCA1(high) gating strategy further ensures lack of contamination by other cell types, since pDC express higher levels of this marker even under conditions of IFN-I induction where other cells types up-regulate it but always to lower levels than those expressed on pDC. Actually, especially when the use of SiglecH is not possible, our gating strategy for pDC identification represents the current state of the

art for that matter, as attested by the use of very similar gating strategies in recent papers published by leaders in the field (Dallari S … Zuniga EI. Nat Commun. 2017;8:14830. PMID: 28368000; Grajkowska LT … Reizis B. Immunity. 2017;46:65-77. PMID: 27986456; Bao M … Liu YJ. J Exp Med. 2016;213:2383-2398. PMID: 27697837; Scott CL … Lambrecht BN, Berx G. J Exp Med. 2016;213:897-911. PMID: 27185854; Yun TJ … Cheong C. Cell Metab. 2016;23:852-66. PMID: 27166946).

In any case, we have now added to the manuscript a microarray meta-analysis of a more complete dataset, encompassing other mononuclear phagocyte types sorted from the spleen of the same C57BL/6 control or MCMV-infected mice than the pDC included in the manuscript, and further combined with Immgen data including many immune cells types (Figures EV1 and S1). This meta-analysis clearly shows that our pDC cluster with those of Immgen and lack any detectable expression of key control genes specific of other cell types.

The kinetic of the IFN response is tightly regulated in pDCs and the profile of gene expression is changing drastically overtime. [The authors cannot] compare the responses by pDCs at 36h post MCMV versus 7h post CpG […].
We thank the reviewer for raising this issue, thereby making us ware that we needed to better explain the rational of this comparison and why it is fair and relevant. Synthetic TLR ligands have been widely used as surrogate stimuli to dissect immune responses to viral infections. Such studies have been most often performed without any comparison with in vivo viral infections. These studies have led to the prevailing model of how pDC respond to viral infections as represented in all textbook and reviews on this subject, including the most recent ones. Hence, de facto, the research community is using stimulation of pDC by synthetic TLR ligand as a driver model to advance knowledge on molecular and cellular mechanisms driving the responses of these cells to viral infections, irrespective of the different kinetics of pDC responses in these different settings. Thus, it is actually of utmost importance to perform such an experimental side-by-side comparison as we did, in order to determine to which extent the knowledge on the molecular regulation of pDC responses to viral-type stimuli acquired by using synthetic TLR ligands as surrogate models does apply to a real viral infection. This is the main issue addressed here.

In any case, we agree that the kinetic of the IFN-I response is tightly regulated in pDCs and that the profile of gene expression is changing drastically overtime. This is exactly why we compared pDC at the respective times of their peak IFN-I production for each of the two stimulations tested in vivo, namely MCMV infection versus CpG stimulation. MCMV is a slow replicating virus which takes 24 to 30 hours to complete its first infection cycle. Hence, viral replication is not detected in vivo before 24 to 30 hours after infection. Accordingly, pDC activation is not detected before 30 hours post-infection but sharply peaks at 36 hours, around 12 hours after completion of the first cycle of viral replication in vivo, whereas it is almost entirely gone by 48 hours after infection, as we and others have documented (Delale T … Trinchieri G, Brière F. J Immunol. 2005;175:6723-32. PMID: 16272328; Krug A … Colonna M. Immunity. 2004;21:107-19. PMID: 15345224; Zucchini … Dalod M. Int Immunol. 2008;20:45-56. PMID: 18000008; Swiecki M … Colonna M. Immunity. 2010;33:955-66. PMID: 21130004). Thus, taking into account the time it takes for MCMV to replicate in vivo which is critical to promote pDC activation during the infection (Robbins SH … Dalod M. PLoS Pathog. 2007;3:e123. PMID: 17722980), the kinetic of pDC activation after MCMV infection rather resembles that following CpG injection, i.e. peaking 6 to 12 hours following initial sensing of the virus-type stimuli by the pDC. This has been explicitly written in the revised main text of the manuscript (pages 11-12, Fig S2).

Moreover, we have now mapped on publicly available kinetics studies of splenic pDC activation by CpG in vitro the gene modules differentially expressed by splenic pDC at 36 hours after MCMV infection in vivo. This analysis has been added to the paper (Figure S2). It confirms that the profile of gene expression in pDC is changing overtime during CpG stimulation, and shows a striking parallel between the gene expression changes induced in pDC between between 4 and 12 hours after in vitro stimulation with CpG as compared to at 36 hours after in vivo MCMV infection. Hence, this analysis demonstrates that it is rigorous to compare the molecular mechanisms controlling pDC activation at 7 hours after CpG stimulation versus 36 hours after MCMV infection, since the gene expression profiles of pDC in these two conditions are very similar.

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Minor concerns:
The authors show that [MCMV infected] cells are not preferentially IFN-producing cells […] it is difficult to reconcile with the fluorescent data in Fig 7 suggesting that many [IFN-b producing] cells are infected by the virus. This should be clarified.
On the immunohistofluorescence images, the YFP signal correspond to IFN-b+ cells whereas MCMV-infected cells were detected with an antibody. We did not use a GFP-expressing virus for this experiment. The vast majority of YFP+ cells were not infected, and reciprocally, the vast majority of infected cells were not positive for EYFP. In any case, we agree that reading the previous Figure was difficult, as emphasized also by referee#3 in his 5th point. This was due in part to acquisition of the images at insufficient magnification, as well as to use of colors that were too similar for IFN-b+ (EYFP+) cells shown in green, and MCMV-infected cells shown in light blue. To solve this issue, we have taken novel immunohistofluorescence images, including at a higher optical magnification, and we have changed the color used in order to facilitate discrimination between IFN-b+ (EYFP+) cells still shown in green, and MCMV-infected cells now shown in red (Fig 7D-F). In accordance with the suggestion from referee#3, we have also included as supplemental Figure the individual images in grayscale (Fig EV5B).as well as an overlay of the YFP and MCMV without the MOMA1 signal (Fig 7E).

Non-essential suggestions:
- The authors [could] identify in their introduction what refers to human versus mouse pDCs.
We have edited the introduction accordingly (pages 3-6).
- Last paragraph of the introduction should not […] repeat the abstract.
We have shortened accordingly the last paragraph of the introduction (page 6).

Referee #3.

1) In figure 5, the authors should show the overlay of WT and IRF7KO pDC stained with anti-IRF7 antibody.
We have edited the Figure accordingly (page 16, Fig 5A).

2) The nomenclature should be unified [with consistent use of] IFN-I.
We willingly used IFN-b one time in the main text, because MoDC and macrophages stimulated with CpG were reported to specifically produce IFN-b but not IFN-a (Schmitz F … Wagner H. Interferon-regulatory-factor 1 controls Toll-like receptor 9-mediated IFN-beta production in myeloid dendritic cells. Eur J Immunol. 2007;37:315-27. PMID: 17273999).

3) The descriptions for use of IFNAR blocking antibodies needs to be better described.
We thank this reviewer as well as Referee #1 for pointing out that the rationale for these experiments is not explained clearly enough. We have edited the manuscript accordingly (page 15).

4) Details about some experiments and staining need to be added.
We thank the reviewer for pointing this out. We have edited the manuscript accordingly.

5) Figure 7B should include an overlay of the YFP and MCMV. The individual color images would be better as black and white for ease of viewing the individual channels. Also, in the figure legend, the authors need to indicate what the arrows and asterisks indicate.
We thank the referee for his suggestions to help us improve readability of that Figure. Indeed, we agree that reading the previous Figure was difficult, as emphasized also by referee#2. This was due in part to acquisition of the images at insufficient magnification, as well as to use of colors that were too similar for IFN-b+ (EYFP+) cells shown in green, and MCMV-infected cells shown in light blue. To solve this issue, we have taken novel immunohistofluorescence images, including at a higher optical magnification, and we have changed the color used in order to facilitate discrimination between IFN-b+ (EYFP+) cells still shown in green, and MCMV-infected cells now shown in red (Fig 7D-F). In accordance with the referee suggestion, we have also included as supplemental Figure the individual images in grayscale (Fig EV5B) as well as an overlay of the YFP and MCMV without the MOMA1 signal (Fig 7E).

6) The data in figure 7B are not sufficient to conclude cell contacts are important, thus the connection to integrins is weak. The data on integrins seem disconnected from the rest of the paper and do not add much to the conclusions.

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We thank the reviewer for pointing out that we did not explain well enough the rationale of testing LFA1 role and what conclusion we can draw from it in connection with the rest of the paper. It was with the initial aim to decipher how MCMV infection selectively promotes pDC IFN-I production in the absence of AP3 or IFNAR that we tested the role of LFA1. However, we agree that it is not per se the factor involved since it is also required for the response to CpG. However, its requirement shows involvement of cell-cell contacts in promoting pDC IFN-I production both during CpG stimulation and MCMV infection. It is likely that the exact nature of these cell contacts differ between these two stimulation condition and that precise characterization of the cell types and molecules involved will help understanding how MCMV infection selectively promotes pDC IFN-I production in the absence of AP3 or IFNAR. We have rewritten the corresponding section of the results (pages 18-19) and discussion (page 24) accordingly.

7) The authors should consider, and include in the discussion, the possibility that MCMV infection triggers more than one TLR and that is in part responsible for the differences between the dependence of MCMV infection and CpG DNA-induced responses on IFN feedback and AP3.
Accordingly to the referee hypothesis, TLR9 signaling would be strongly impaired in pDC KO for Ifnar1 or AP3 in response both to CpG and MCMV stimulation, but in the latter case it would be compensated by engagement of another TLR. Indeed, we have reported that TLR7 can partly compensate loss of TLR9 for pDC sensing of MCMV in vivo (Zucchini N … Dalod M. Cutting edge: Overlapping functions of TLR7 and TLR9 for innate defense against a herpesvirus infection. J Immunol. 2008;180:5799-803. PMID: 18424698). If this cooperation can override the need of IFN-I positive feedback, we would expect that pDC IFN-I production should be abrogated in TLR7KO mice treated with IFNAR1 blocking antibodies. However, this is not the case (Figure 4L). Hence, it is unlikely that the main mechanism explaining maintenance of IFN-I production in Ifnar1-unresponsive pDC upon MCMV infection in contrast to CpG stimulation is due to engagement of TLR7 in the former activation condition. Moreover, loss of TLR9 leads to a strong decrease in the percentage of pDC expressing IFN-I during MCMV infection, as we confirm again in the present study (Figure 4L). Since IFN-I production is not restored in TLR9-KO pDC upon IFNAR1 blockade, it shows that TLR9 remains a critical sensor to promote pDC IFN-I production during MCMV infection even in the absence of IFN-I positive feedback loop. However, we cannot exclude that other pathogen sensor can cooperate with TLR9 to promote pDC IFN-I production during MCMV infection in the absence of IFN-I positive feedback signaling.

2nd Editorial Decision — 16th Jul 18

Thank you for submitting your revised manuscript to the EMBO Journal. I am sorry for the delay in getting back to you with a decision but it took a bit longer than anticipated to receive the full input back on your study.

I have now received the comments from all three referees. As you can see below, while referee #2 is still not convinced that PDCA-1 is a good marker to identify pDCs, both referees #1 and #3 are more supportive of the study. I also specifically asked Referee #1 to comment on this particular issue. Referee #1 agrees with referee # 2 that PDCA-1 is found in other cell types and also indicates that one could have done more to check the purity of the pDCs using additional markers. However, s/he also indicates that PDCA-1 is used as a mouse pDC marker and that your microarray data supports that it marks pDCs.

I have looked careful at everything and I agree with referee #1 and 3 that the manuscript provides important new insight. I am therefore pleased to let you know we will publish the manuscript here.

There are still some revisions needed. Have you used additional markers to test the purity of your pDCs if so then please include such data. I would also like you to discuss the issue of using PDCA-1 as a marker for mouse pDCs and clearly discuss the potential limitations. Referee #2's points regarding the number of experiments is also well taken. Please also respond to the other concerns raised by referee #2 and 3 with appropriate text changes.

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REFEREE COMMENTS

Referee #1:

Overall this study brings important and new insights in the field of mouse pDC activation in vivo. Trying to address the critics of referee#2, the authors argue about their gating strategy to identify pDC which is largely based on the use of the PDCA-1 marker (often called Tetherin/BST2). PDCA-1 is indeed expressed at the steady state by mouse pDC. Importantly, since PDCA-1 is an ISG its levels are increased upon activation of pDC. But PDCA-1 can be expressed by many cell types upon exposure to type I IFN. There are no good markers for mouse pDC, such as BDCA-3 (IL3R) for human pDC. I concede that the authors could have further checked the purity of their pDC using additional markers, especially after MCMV infection and CpG-A activation. However, in the field of mouse pDC, PDCA-1 appears as a widely accepted marker for pDC (see recent paper of PF Rodrigues et al in Nature Immunology 2018 https://doi.org/10.1038/s41590-018-0136-9). Moreover, the authors have performed microarrays with their pDC (i.e. using their gating strategy). Compared with the microarrays performed by the Immgen consortium, their patterns look strikingly very similar. This represents to my point of view a solid validation of their approach. Other critics from the referees appear to have been correctly addressed. Therefore, to my point of view, the manuscript deserves to be published.

Minor concerns
The description of the figures should be improved. Figure legends are still not clear and detailed enough.

Referee #2:

To the Authors,

In this new version, the authors have made little changes to address the concerns raised but rather tried to clarify some of the rationale behind the design of their experiments.

I thus have very similar concerns. I do not believe, as most people in the field, that you can use PDCA-1 as a marker to identify pDCs even if you gate out some of the other cells. At minimum, the authors could have repeated one experiment and include additional markers to confirm that their gating strategy was effective but this is not provided. This remains a major concern in my view.

Regarding the number of experiments done, it is unclear how we went from 2 independent replicates to now 14 biological replicates. The figure legend (Fig 1C-D) is still the same and notes that this was done in 2 replicates. This is very confusing.

Finally, as indicated in the original review, TLR9 signaling in pDCs has a critical kinetic component and I don't see a strong significance in comparing the pDC response in vitro at 7h to their response in vivo at 36h. Even though I understand that MCMV is a slow replicating virus, there is nothing that demonstrates that the cells need a full cycle of replication to be activated. I found the author's argument on the need to conduct such studies to be circular.

Referee #3:

The authors have addressed this reviewers concerns about controls, descriptions, experimental details, and data presentation. Specifically, the images are dramatically improved in Figure 7. However, there remains only weak and indirect evidence to support the conclusions that LFA-dependent cell-cell contacts are important for IFN-I production. The authors should not overstate the conclusions that can be made of the data presented.

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2nd Revision - authors' response 23rd Jul 18

Referee #1:
Overall this study brings important and new insights in the field of mouse pDC activation in vivo. Trying to address the critics of referee#2, the authors argue about their gating strategy to identify pDC which is largely based on the use of the PDCA-1 marker (often called Tetherin/BST2). PDCA-1 is indeed expressed at the steady state by mouse pDC. Importantly, since PDCA-1 is an ISG its levels are increased upon activation of pDC. But PDCA-1 can be expressed by many cell types upon exposure to type I IFN. There are no good markers for mouse pDC. I concede that the authors could have further checked the purity of their pDC using additional markers, especially after MCMV infection and CpG-A activation.
We thank the referee for his positive appreciation of our work and for his suggestion. We agree that CD317/PDCA1/Bst2 is not "per se" a marker specific of mouse pDC. However, even under conditions of IFN-I induction leading to its upregulation on a variety of cell types, within CD11c-expressing cells its expression remains higher on pDC. This is the reason why we identified pDC as lineage^{-} CD11b^{-} CD11c$^{low/int}$ CD317^{high}. We already validated this gating strategy in a previous paper (Zucchini et al. Int. Immunol. 2008). We have now added data showing that cells identified by our gating strategy as pDC express high levels of SiglecH, another mouse pDC marker (Fig EV1, page 8). However, MCMV infection leads to a decrease in SiglecH expression on pDC (Fig EV1) (Puttur et al., 2013, Plos Pathog. 2013). Moreover, IFN-I production occured specifically in pDC expressing higher CD317 but lower SiglecH levels than the bulk of the pDC population (Fig EV1, page 12), consistent with our previously published data (Zucchini et al. Int. Immunol. 2008). Therefore, we did not add SiglecH in our pDC gating strategy in order to ensure the best detection of all IFN-I-producing pDC. The lack of any single marker enabling per se the identification of mouse pDC, and the limitations of using CD317 or SiglecH in this regard, are now discussed (pages 25-26).

However, in the field of mouse pDC, PDCA-1 appears as a widely accepted marker for pDC (see recent paper of PF Rodrigues et al in Nature Immunology 2018 https://doi.org/10.1038/s41590-018-0136-9). Moreover, the authors have performed microarrays with their pDC (i.e. using their gating strategy). Compared with the microarrays performed by the Immgen consortium, their patterns look strikingly very similar. This represents to my point of view a solid validation of their approach. Other critics from the referees appear to have been correctly addressed.
We thank the referee for stating that PDCA1 was successfully used to identify pDC by other laboratories, including in papers recently published in high impact factor journals, and that the comparison of our set of data with the one from Immgen consortium represents a solid validation of our approach to identify pDC. We also appreciate that this referee considers that we have correctly addressed all other criticisms.

Minor concerns
Figure legends are still not clear and detailed enough.
We thank the referee for helping us to further improve our paper. We have added details to the figure legends.

Referee #2:
In this new version, the authors have made little changes to address the concerns raised but rather tried to clarify some of the rationale behind the design of their experiments. I thus have very similar concerns. I do not believe, as most people in the field, that you can use PDCA-1 as a marker to identify pDCs even if you gate out some of the other cells. At minimum, the authors could have repeated one experiment and include additional markers to confirm that their gating strategy was effective but this is not provided. This remains a major concern in my view.
As detailed in response to referee#1, we have now added additional data showing that cells identified by our gating strategy as pDC express high levels of SiglecH (Fig EV1, page 8).

Regarding the number of experiments done, it is unclear how we went from 2 independent replicates to now 14 biological replicates. The figure legend (Fig 1C-D) is still the same and notes that this was done in 2 replicates. This is very confusing.

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We have modified the legend of Fig 1C-D to clarify this point (page 34). "Two independent experiments were performed for each experimental condition (i.e. with Ifnar1-CTR and –TST MBMC on the one hand, versus Myd88-CTR and TST MBMC on the other hand). Samples from uninfected animals are shown as empty symbols and those from MCMV-infected mice as filled symbols. Altogether, this led to 14 biological replicates for WT pDC, irrespective of their CD45 allotypic status and of the type of MBMC they originated from, both for uninfected and MCMV-infected MBMC." We have also clarified the legend of Figure 1E: "Venn diagram showing the overlap between the lists of genes significantly upregulated in WT pDC of infected mice (n=14) as compared to WT pDC of uninfected animals (n=14) (grey circle), or downregulated in Ifnar1-KO (n=2) (red circle) or Myd88-KO (n=2) (green circle) pDC isolated from infected TST MBMC, as compared to WT pDC (n=6) isolated from the same TST or from their matched CTR infected MBMC."

TLR9 signaling in pDCs has a critical kinetic component and I don't see a strong significance in comparing the pDC response in vitro at 7h to their response in vivo at 36h. Even though I understand that MCMV is a slow replicating virus, there is nothing that demonstrates that the cells need a full cycle of replication to be activated.
By combining the use of different mutant viruses and mouse strains, including a virus which replication can be inhibited in vivo upon doxycyclin administration, we have previously demonstrated that pDC IFN-I production during MCMV infection requires completion of at least one cycle of viral replication and is more strongly dependent on in vivo viral replication than on the initial viral inoculum dose (Robbins et al, PLoS Pathog 2007). Indeed, viral replication and pDC IFN-I production reached similarly high levels on a range of inoculum doses in the Balb/C mouse strain, which is naturally devoid of the NK cells receptor Ly49H and thus unable to control early MCMV infection. In contrast, in a congenic mouse strain expressing Ly49H (encoded by the *Klra8* gene), an early control of viral replication was achieved at low to moderate doses of viral inoculum, which correlated with a low pDC activation for IFN-I production, whereas a high pDC IFN-I production was restored in parallel to increased viral replication either upon in vivo NK cell depletion at low inoculum doses or upon injection of high inoculum doses. Finally, treatment of Balb/c with doxycyclin 20 hours after infection very strongly reduced viral replication as well as serum IFN-I titers.

Referee #3:
The authors have addressed this reviewers concerns about controls, descriptions, experimental details, and data presentation. Specifically, the images are dramatically improved in Figure 7. However, there remains only weak and indirect evidence to support the conclusions that LFA-dependent cell-cell contacts are important for IFN-I production. The authors should not overstate the conclusions that can be made of the data presented.
We thank the referee #3 for his appreciation that we have appropriately addressed all her/his concerns about our manuscript, especially for the Fig 7. According to the referee's suggestion, we have further modulated the interpretation (pages 19-20) and discussion (page 25) of our results regarding the role of LFA-1 in cell-cell contacts between pDC and MCMV-infected cells.

3rd Editorial Decision — 25th Jul 18

Thank you for sending me the revised manuscript.

I appreciate the introduced changes and I am happy to accept the manuscript for publication here.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Marc Dalod
Journal Submitted to: EMBO Journal
Manuscript Number: 2017-98836

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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 χ2 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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

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B- Statistics and general methods

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

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Sample size was not predefined, but we performed pilot experiments showing that pool of at least 5 mice was an adequate size to detect a specific effect
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	Please refer to 1.a. We did not perform any statistical analysis to predefine the sample size
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	No animals were excluded
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.	No randomization was used
For animal studies, include a statement about randomization even if no randomization was used.	No randomization was used
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 blinding was used
4.b. For animal studies, include a statement about blinding even if no blinding was done	No blinding was used
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.	We used exclusively non parametric tests for our statistical analysis.
Is there an estimate of variation within each group of data?	Yes. Data were presented as the mean +/- SEM.
Is the variance similar between the groups that are being statistically compared?	Since only non-parametric test were used, this type of comparison was not relevant

C- Reagents

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).	Species, antigen, clone number for all the antibodies have been reported in materials and methods
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

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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.	All these information have been reported in materials and methods, page 25
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	All these information have been reported in materials and methods, page 25
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.	We confirm compliance to ARRIVE guidelines

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
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.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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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.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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	All the information about microarray data submission to GEO database have been reported in materials and methods, page 29
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).	We have deposited datasets (Table EV1) as ZIP file
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).	NA
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G- Dual use research of concern

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