European Journal of Immunology

Peer review correspondence

Type I IFN signaling triggers immunopathology in tuberculosis-susceptible mice by modulating lung phagocyte dynamics

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Handling Executive Committee member: Prof. Iain McInnes

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

First Editorial Decision – 17 December 2013

Dear Prof. Kaufmann,

Please accept my sincere apologies for the prolonged delay in processing the review of your manuscript ID eji.201344219 entitled "Type I IFN signaling triggers immunopathology in tuberculosis-susceptible hosts by modulating lung phagocyte dynamics" which you submitted to the European Journal of Immunology. There was a difference of opinion among the original reviews and additional advice was sought. All opinions have now been assessed by the Executive Editor and the comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. You will see that referee 1 felt that the strain-specific nature of your findings significantly lowers the impact of your work and should know that this referee felt that on this basis your manuscript should be rejected. Although we do not agree with this recommendation we do strongly

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encourage you to fully address the concerns of this referee, as well as those of the other referees, in your revised submission.

You should also pay close attention to the editorial comments included below. *In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.*

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,
Karen Chu

On behalf of Prof. Iain McInnes

Dr. Karen Chu
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Reviewer: 1

Comments to the Author
In this manuscript by Dorhai et al, is from a well established group, describing that Type I signaling triggers immunopathology in a TB-susceptible strain of mouse by impacting lung phagocyte dynamics.

The study itself is well designed and executed and the findings interesting. However, the major drawback of the study is that the effect of Type I signaling appears to be dominant only in the 129 background of a TB-susceptible mouse strain. Previous work by Desvignes et al, 2012 (and data shown in this paper)

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demonstrate that in a TB-resistant strain of mouse such as B6, the impact of deletion of IFNAR is minimal and does not impact survival in response to Mtb infection. No data are shown for pathology in this model, either in the published study or this study. Thus, it appears that the current study too broadly interprets the results seen in the 129 strain as resembling “active TB”, and that it is possible that Type 1 signaling only has an effect on this particular background strain of mouse. Accordingly, the title needs to be changed to “ in a tuberculosis susceptible mouse strain”.

Reviewer: 2

Comments to the Author
This is a well-conducted and through study demonstrating the important role of Type I interferons in immunopathology following TB infection. I have the following recommendations:

1. Immunohistochemistry is used to show changes in inflammation (Fig 1C, and S1 B and D), NOS expression (Fig 1D), MPO levels (Fig 4B and 5B). Although the differences seem clear, they should be quantified, by some scoring system.
2. Fig S2. The background of the IFNar1-/- animals needs to be stated explicitly.
3. The explanation for the role of neutrophils in dissemination of infection needs to be discussed in more depth (p13)
4. A number of the legend on figure axes seem very small and need to be checked before final submission

Reviewer: 3

Comments to the Author
Type I IFN signaling triggers immunopathology in tuberculosis-susceptible hosts by modulating lung phagocyte dynamics
eji.201344219 (Dec 2013)

The study by Dorhoi and colleagues put forward a new hypothesis regarding the role of type I IFN in the immune response, and survival of highly susceptible mice strain 129S, to Mtb. The difference of the outcome of infection in 129S WT or IFNAR-/- is very pronounced and highly convincing. Absence of IFNAR protected 129S susceptible mice from infection with Mtb, observed by: (i) increased survival, (ii) reduced bacterial burden, and (iii) reduced immunopathology. The data is interesting as the study adds to the role of type I IFN during Mtb infection, which is still controversial. The main novelty introduced by the data presented herein was the increase rate of cell death in the lungs of WT Mtb infected mice, when compared to IFNAR-/- mice. The authors hypothesize that this early death events promote a higher

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recruitment of PMN cells that appear to be detrimental to the survival of mice. Indeed, WT mice survived the Mtb infection when PMN cells were depleted early during infection. The authors went further to show that WT mice had increased levels of Mtb-infected alveolar macrophages, suggesting enhanced replication of Mtb in WT alveolar macrophages. Despite a similar percentage of Mtb-infected PMNs in both strains of mice, the enhanced cell death and higher recruitment of PMNs into the lungs of WT mice resulted in an elevated number of infected PMNs in WT mice, suggesting that Mtb spreads more severely from alveolar macrophages to recruited PMNs in WT mice when compared to IFNAR-/- mice. Thus, this paper provides a mechanistic basis for the data published by O'Garra and colleagues that show a PMN associated type I IFN gene signature in TB active patients, not observed in controls.

There are however several issues that need to be addressed. Overall, the data are very correlative and the massive amount of data presented make the paper a bit heavy and hard to follow at times.

Specifically:

- The amount of data in the paper is massive. Perhaps some of the figures could become supplementary data, like figure 3 that is something not central for the message of the paper. Furthermore, the array data is not very useful and does not bring anything new to the paper, at least in its current form. The real time RT-PCR data is sufficient to support the hypotheses of the authors.
- Most of the figures show one experiment representative of the 2 or 3 performed. The average of the 2 or 3 experiments should be plotted and the statistical analysis performed on these results.
- High susceptibility of WT 129S2 mice to TB seems to be dependent on type I IFN signaling since absence of IFNAR improved survival and protection to infection. This pathogenic role of type I IFN appears to be much more severe in 129S2 mice than C56BL/6 mice. It would be important to show whether susceptibility versus resistance of 129S2 versus C56BL/6 mice correlates with high versus low levels of type I IFN induced after Mtb infection. Also, the CFU differences between BL6 WT or IFNAR-/- are shown for day 40 post-infection, a lot later than what was observed in 129S background. Do the authors consider that the same mechanism is involved, ie, early enhanced neutrophil responses? Still in regard to the comparison C57BL6 WT or IFNAR-/-, it is not clear why two different doses of infection were used in Figure S2A and S2B. What happens to the survival % of BL6 or IFNAR-/- if a high dose of innoculum is used?
- In figure 4A and in lines 147-8, the authors show that IFNAR-/- mice present higher frequencies of AMs both at 14 and 21 days post-infection. This should be discussed. Also, the authors state that IFNAR regulated resident AM populations, but do not discuss the mechanisms that may be involved in this observation.
- In figure 5 the authors use chimeras to understand whether the effects were due to hematopoietic or radioresistant or both cells. The survival of KO->WT or WT->KO is intermediate to that observed upon infection of WT or IFNAR-/- mice (Fig. 5A). However, in Fig. 5B it is shown that the amount of cytokines and chemokines in KO->WT is actually similar to that observed for infected KO. Since the authors suggest

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that the death is associated to high inflammation, why are KO->WT not as resistant as IFNAR-/-? In the same line, the amount of neutrophils in WT->KO appears to be higher than that in KO->WT (Fig. 5B) and yet the survival profile is the same.

- In figure 8, the authors show an increased number of PMN cells infected with Mtb and in figure 6 an elevated cell death in the BAL of WT mice, when compared to IFNAR-/- mice. The paper would benefit greatly if the cell populations that are dying in WT mice were determined and also if a mechanistic insight on the drivers of cell death were clarified. It is likely that the elevated cell death observed in WT mice is just a consequence of elevated PMN recruitment, as these are short lived cells. Also it is not very clear on how the changes in myeloid populations impact the survival of WT versus IFNRA-/- mice. In this regard, PMN cells have been shown to have protective roles during early infection with Mtb. It would be interesting to show whether PMNs from IFNRA-/- where more efficient at killing Mtb that WT PMNs.

Overall the paper is well written but it is not straight forward to follow the data. Despite this, the data is interesting and of interest to the field, as it puts forward a new exciting hypothesis regarding the role of type I IFN during TB. The paper only lacks for the correlative nature of the data.

Minor points:

- Figure 1 shows death of 129S WT infected animals in app 30 days, but the text refers that the animals died within 40 days post-infection.
- Figure 2 shows lower levels of IL-1 in infected IFNAR-/- than WT lungs suggesting that type I IFN signaling enhances IL-1 production during Mtb infection of susceptible 129S2 mice, contrary to what has been described (Mayer-Barber KD et al., Immunity 2011; Novikov A et al., J Immunol 2011). The authors should discuss these differences.
- Figure 3: Do the data show frequencies of IFN-g+TNF-a+ cells (as stated in the legend of the figure) or IFN-g+ cells (as stated in the graph axis)? Plus, CD4-gated FACS profiles do not seem representative of the data plotted in the graph.
- The sudden use of BMDMs and BMDCs is confusing. With regard to BMDMs, for example, the analysis of chemokine production (Fig. 6A) does not fit the in vivo observations (Fig. 2B).
- Figure 7: Why different methods to assess Mtb growth were used in figure 7A and B? For the experiment of figure 7B the authors should show that the difference observed at 48h post-infection was not due to different infection rate.
- Figure 8: the authors mention that the approaches followed in 8A and B versus 8C are complementary. However, in what regards data on the % of GFP or AFB + among BAL PMNs, the two approached lead to different results. The authors should comment on this.
- Supporting Information Figure 1: the legend states that “(E) Serum concentrations of lactate dehydrogenase at day 21 p.i. in WT mice (129S2 and C57BL76) …” but the figure only shows the data for WT (129S2).
- Certain panels of the figures are misplaced (for example, Fig. 1E is referred in the text before Fig. 1D; the same for FIG. 5C/B and 5E/D, and Fig.S1F/D).

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- Lines 106-7: The authors state that «The milder pathology in Ifnar-/- animals was accompanied by a lower serum lactate dehydrogenase concentration». However, data in Figure S1E show similar levels of LDH. Is the observed difference statistically significant?
- Lines 193-4: “Frequencies of PMNs were increased in BALF of Ifnar1-/- mice (Figure 6D).” – But figure 6D shows a significantly decrease in PMNs frequency among BAL cells.

First revision – authors’ response – 17 March 2014

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Dear Editors, Dear Dr McInnes,

Attached please find our revised manuscript “Type I IFN signaling triggers immunopathology in tuberculosis-susceptible mice by modulating lung phagocyte dynamics” by Dorhoi, Yeremeev, et al, for resubmission to the *European Journal of Immunology*.

We thank you and the reviewers for the helpful comments and suggestions, which have helped us improve the quality of our manuscript. We have performed new experiments (Figs. 5F and 6B), additional data analyses (Figs. 1D&E, 3B, and 4C) and modified the manuscript to include and discuss the new results. These additional investigations support and strengthen the main conclusion of our work. We trust our manuscript has been significantly improved and is of central interest for the journal and the scientific community focusing on immunology in infection and inflammation.

In the editorial letter, you cited concerns regarding the quality of figures. We have carefully addressed this point and prepared figures and figure legends closely following journal standards. Full gating strategies were provided for FACS analysis (Figs. S3A, S4A, and S5). Changes in the manuscript text are indicated by underlined text.

In the following, please find our point-by-point reply to the questions raised by the reviewers.

Yours sincerely,

Stefan H.E. Kaufmann

Reviewer 1

In this manuscript by Dorhoi et al, is from a well established group, describing that Type I signaling triggers immunopathology in a TB-susceptible strain of mouse by impacting lung phagocyte dynamics.

The study itself is well designed and executed and the findings interesting. However, the major drawback of the study is that the effect of Type I signaling appears to be dominant only in the 129 background of a TB-susceptible mouse

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strain. Previous work by Desvignes et al, 2012 (and data shown in this paper) demonstrate that in a TB-resistant strain of mouse such as B6, the impact of deletion of IFNAR is minimal and does not impact survival in response to Mtb infection. No data are shown for pathology in this model, either in the published study or this study. Thus, it appears that the current study too broadly interprets the results seen in the 129 strain as resembling "active TB", and that it is possible that Type 1 signaling only has an effect on this particular background strain of mouse. Accordingly, the title needs to be changed to " in a tuberculosis susceptible mouse strain".

Author (AU): We thank this Reviewer for reading our paper and for the recommendation to modify the title. We have modified the title to more specifically focus on our findings. As reported in this manuscript and by others [1;2] deletion of IFNAR1 in the C57BL/6 mouse strain, which is resistant to tuberculosis (TB) and the most widely used mouse strain for laboratory investigations, impacts on bacterial replication. As some of the mentioned papers use low-dose infection and a resistant mouse it is counterintuitive and impossible to observe differences in survival, particularly when the mutant mice control bacterial replication better. We did not observe major differences in pathology (extent of lesions) or immunostaining for iNOS. These data are presented for the Reviewer in Fig. 1 below.

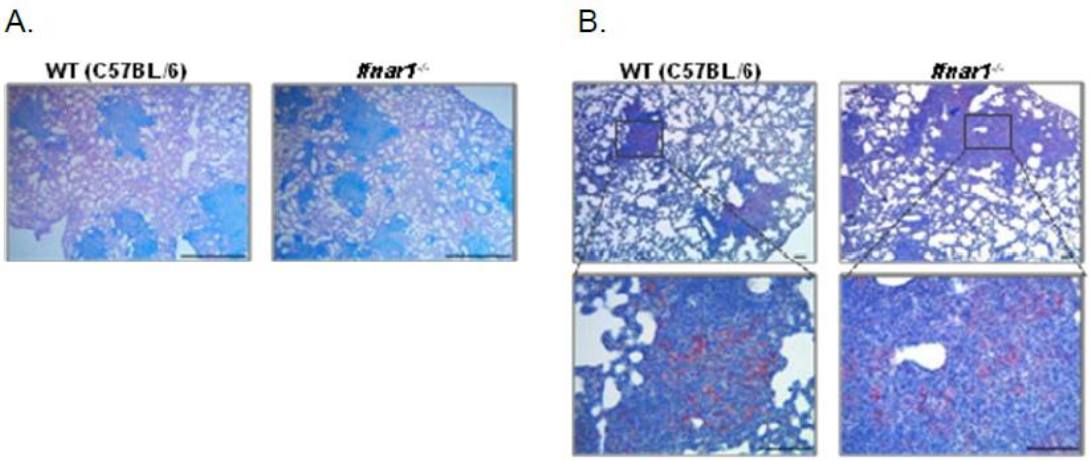

Figure 1. Lung pathology and immunostaining for iNOS in TB-resistant C57BL/6 mice (WT) and their IFNAR1-deleted counterparts. Mice were infected with ~ 200 CFUs of *M. tuberculosis* H37Rv and lung tissue was collected and processed for histology at day 21 post-infection. (A) Giemsa staining (scale bar, 1000 µm; n=5). (B) Immunohistochemistry for iNOS (scale bar, 100 µm; n=5).

We partially understand the concerns of the Reviewer and are also aware that experimental mouse TB is one available model to study this infection, with advantages

and drawbacks. We would like to recall that billions of people are latently infected with Mtb, while only about 10% of exposed individuals develop active disease. Thus one might presume that most of these 10% are genetically susceptible to TB. Mice in general (and most inbred laboratory mouse strains) are much more resistant to TB than humans. Important experimental data have also been obtained with guinea pigs, an extremely susceptible animal model. So it is reasonable to use susceptible mouse strains for TB experiments. I addition, we want to underline that resistant mouse strains do not mimic a "primary progressive" infection, as no clinical signs (weights loss, impairment of lung function) are detectable following low-dose infection. Accordingly, findings in these strains likely do not (or incompletely) apply to disease processes accompanying primary progressive TB. As such, the vast majority of studies performed with C57BL/6 mice could be "an effect of this particular strain", as the reviewer noted, with limited relevance to primary progressive TB. We consider careful assessment of different mouse models and their suitability for given questions a priority in TB. Multiple studies currently recommend usage of susceptible mouse strains (e.g. C3HeB/Fe/J) to understand TB pathology [3;4], immune processes [5] and evaluation of drug efficacy [6]. We are confident that our TB-susceptible mouse model substantially adds to understanding IFNAR1-mediated immune processes in primary progressive TB.

Reviewer 2

AU: We thank the Reviewer for emphasizing the complexity of our studies and relevance for understanding immunopathology in TB. We are grateful for the recommendations and feel that we could address them by providing new data in the manuscript and more in-depth discussions.

This is a well-conducted and through study demonstrating the important role of Type I interferons in immunopathology following TB infection. I have the following recommendations:

1. Immunohistochemistry is used to show changes in inflammation (Fig 1C, and S1 B and D), NOS expression (Fig 1D), MPO levels (Fig 4B and 5B). Although the differences seem clear, they should be quantified, by some scoring system.

AU: We have performed quantifications of the IHC/IF images and these are now provided in Figs. 1D,E; 3B; 4C. Lung morphometry indicates that the extent of lesions was significantly higher in WT (129S2) mice compared to animals lacking IFNAR1, despite similar numbers of granulomatous infiltrates (Fig. 1C). We now provide relative mean fluorescence values for quantification of iNOs and MPO staining (Figs. 1E; 3B; 4C). These new results are presented and discussed on pages 5 and 7.

2. Fig S2. The background of the IFNar1-/- animals needs to be stated explicitly.

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AU: We have now provided additional information in the text, which underlines the TBresistant C57BL/6 background of the *Ifnar1–/–* animals in this particular experiment.

3. The explanation for the role of neutrophils in dissemination of infection needs to be discussed in more depth (p13)

AU: Neutrophils (PMNs) were repeatedly reported to interact with mycobacteria [7]. While their mycobactericidal role is still a matter of debate, there are multiple lines of evidence that PMNs secrete immune mediators upon Mtb phagocytosis and subsequently modify the inflammatory milieu. By releasing cytokines and chemokines, notably TNF-α [8] and CCL3, CXCL2 [9], PMNs could foster accumulation of phagocytes, which serve as habitat for Mtb and indirectly facilitate bacterial dissemination. PMNs can migrate and directly promote spread of mycobacteria to the draining lymph nodes [10]. This information is presented in the Discussion, on page 15.

4. A number of the legend on figure axes seem very small and need to be checked before final submission

AU: We have now prepared the figures at higher resolution.

Reviewer 3

AU: We are grateful to this Reviewer for carefully reading our manuscript and for the detailed critical comments, which have helped us to improve our manuscript. The Reviewer notes the novelty of this work, which provides mechanistic evidence for clinical data. We feel that we have addressed the constructive criticisms and that the newly provided data allow substantiating our conclusion on the critical role of IFN I in modulating inflammation in TB (see pages 9, 10, 14; Fig. 5).

The study by Dorhoi and colleagues put forward a new hypothesis regarding the role of type I IFN in the immune response, and survival of highly susceptible mice strain 129S, to Mtb. The difference of the outcome of infection in 129S WT or IFNAR-/- is very pronounced and highly convincing. Absence of IFNAR protected 129S susceptible mice from infection with Mtb, observed by: (i) increased survival, (ii) reduced bacterial burden, and (iii) reduced immunopathology. The data is interesting as the study adds to the role of type I IFN during Mtb infection, which is still controversial. The main novelty introduced by the data presented herein was the increase rate of cell death in the lungs of WT Mtb infected mice, when compared to IFNAR-/- mice. The authors hypothesize that this early death events promote a higher recruitment of PMN cells that appear to be detrimental to the survival of mice. Indeed, WT mice survived the Mtb infection when PMN cells were depleted early during infection. The authors went further to show that WT mice had increased levels of Mtb-infected alveolar macrophages, suggesting

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enhanced replication of Mtb in WT alveolar macrophages. Despite a similar percentage of Mtb-infected PMNs in both strains of mice, the enhanced cell death and higher recruitment of PMNs into the lungs of WT mice resulted in an elevated number of infected PMNs in WT mice, suggesting that Mtb spreads more severely from alveolar macrophages to recruited PMNs in WT mice when compared to IFNAR-/- mice. Thus, this paper provides a mechanistic basis for the data published by O'Garra and colleagues that show a PMN associated type I IFN gene signature in TB active patients, not observed in controls.

There are however several issues that need to be addressed. Overall, the data are very correlative and the massive amount of data presented make the paper a bit heavy and hard to follow at times.

Specifically:

- The amount of data in the paper is massive. Perhaps some of the figures could become supplementary data, like figure 3 that is something not central for the message of the paper. Furthermore, the array data is not very useful and does not bring anything new to the paper, at least in its current form. The real time RT-PCR data is sufficient to support the hypotheses of the authors.

AU: We have moved Fig. 3 to the supplementary material (new Fig. S3) following the Reviewer's suggestion.

The array data prompted us to investigate early events at day 14, when bacterial burdens were similar. This information was central for investigations on chemokines and cellular events within the bronchoalveolar space. Generally microarrays are scientific tools that generate novel hypotheses, and this was the case in our study. Moreover, this approach allowed us to obtain information on biologic processes in an unbiased manner. The strong biostatistics analysis, reflected in the plots we provide, enabled us to further investigate cell recruitment. We consider these data important for the paper in mirroring the logical flow of experimental work, which allowed us to dissect the role of IFN I in early inflammatory events in TB. Unless the Editor decides to the contrary, we would, therefore, prefer to leave the array data in the paper.

- Most of the figures show one experiment representative of the 2 or 3 performed. The average of the 2 or 3 experiments should be plotted and the statistical analysis performed on these results.

AU: We have followed this Reviewer's recommendations. All *in vivo* data are now plotted and analyzed by pooling results from multiple independent experiments. This information is presented in detail in the figure legends, namely, numbers of independent experiments performed and total number of mice (npooled). We consider *in vitro* data is most appropriately presented as representative experiment.

- High susceptibility of WT 129S2 mice to TB seems to be dependent on type I IFN

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signaling since absence of IFNAR improved survival and protection to infection. This pathogenic role of type I IFN appears to be much more severe in 129S2 mice than C56BL/6 mice. It would be important to show whether susceptibility versus resistance of 129S2 versus C56BL/6 mice correlates with high versus low levels of type I IFN induced after Mtb infection. Also, the CFU differences between BL6 WT or IFNAR-/- are shown for day 40 post-infection, a lot later than what was observed in 129S background. Do the authors consider that the same mechanism is involved, ie, early enhanced neutrophil responses? Still in regard to the comparison C57BL6 WT or IFNAR-/-, it is not clear why two different doses of infection were used in Figure S2A and S2B. What happens to the survival % of BL6 or IFNAR-/- if a high dose of innoculum is used?

AU: Currently, we are more closely investigating differences between C57BL/6 (TBresistant) and 129S2 (TB-susceptible) mice. Regarding signaling through IFNAR1, we have tested whether the IFN I and IFN I-associated genes are differentially expressed in these WT strains using the ROAST algorithm implemented in the mroast function from the limma R package [11] (see Fig. 2 below). The set of genes involved in the IFN I response was significantly higher expressed in the 129S2 strain on day 14 ($p=0.039$) (at this time-point similar lung CFUs) and day 25 ($p= 0.009$) (higher CFUs in 129S2).

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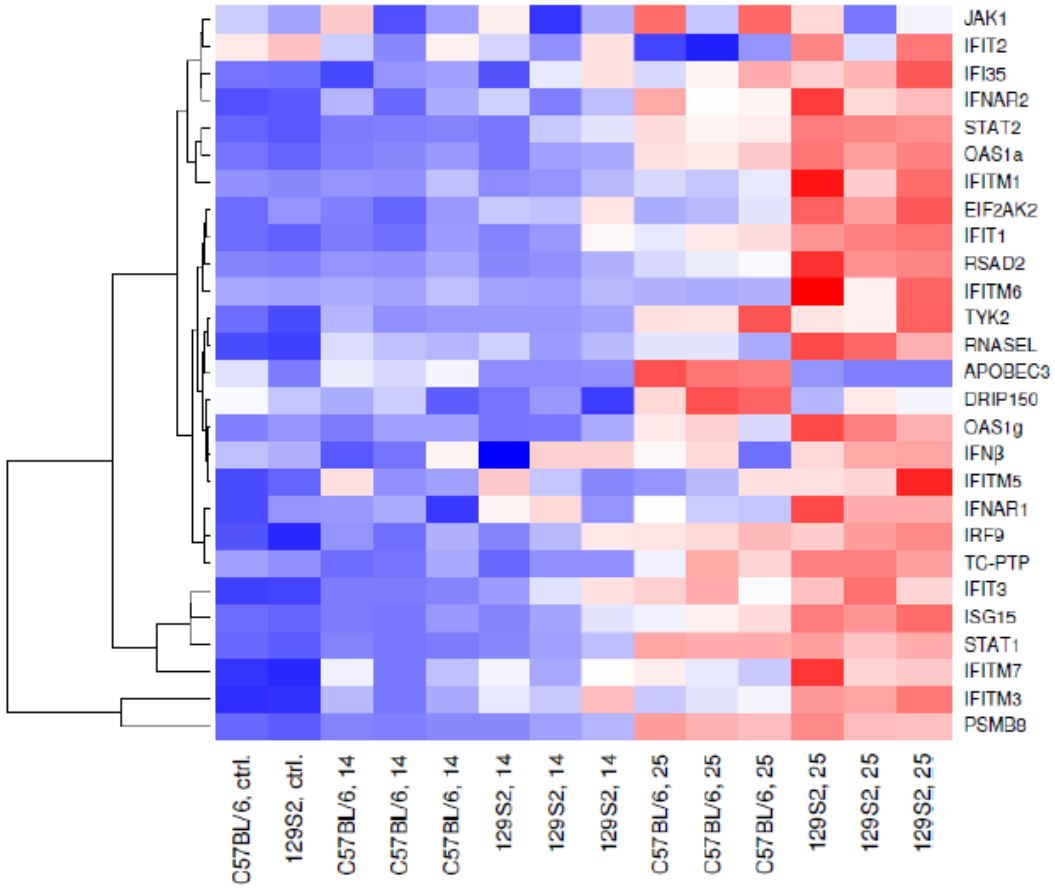

Figure 2. Heatmap showing relative gene expression for genes involved in type I IFN response in lung homogenates collected at different time points following low-dose infection with Mtb in WT mouse strains (C57BL/6 and 129S2). Expression levels have been calculated by averaging the technical replicates, and gene-wise normalized (three independent experiments with five mice per time-point in each experiment). White corresponds to average gene expression, blue corresponds to below average, and red corresponds to above average gene expression.

Based on these findings we conclude that 129S2 mice mount a stronger IFN I response compared to C57BL/6 mice. As mentioned above, a more detailed investigation, including this result will be within the scope of a follow-up study.

We observed that frequencies of PMNs recruited in the bronchoalveolar space early upon infection are heightened in TB-susceptible WT animals [current manuscript 129S2, [12], C57BL/6] and thus it appears that multiple mechanisms may be responsible for susceptibility. Even when we challenged the *Ifnar1–/–* mice on C57BL/6 background with 500 CFUs, we did not fully reproduce the results we observed with the 129S2 animals in terms of differences in recruited PMNs within airways. A trend was observed, but no significant differences (Fig. S4D). Please note we detect ca. 40% PMNs among leukocytes in BAL fluid in 129S2 mice at 14 days p.i. and even at higher Mtb infection

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dose only 10% in C57BL/6 mice (see also [12]).

We applied a higher dose (~500 CFUs) in C57BL/6 mice to induce a situation resembling the inflammatory milieu observed in the susceptible mice. We did not detect any deaths of WT or *Ifnar1–/–* mice with this infection dose. Higher infection, i.e. 1,000 CFUs H37Rv, will result in dramatic death within 30 days in C57BL/6 mice and likely result in profound differences in early cell kinetics inside alveoli.

- In figure 4A and in lines 147-8, the authors show that IFNAR-/- mice present higher frequencies of AMs both at 14 and 21 days post-infection. This should be discussed. Also, the authors state that IFNAR regulated resident AM populations, but do not discuss the mechanisms that may be involved in this observation.

AU: We have performed experiments aiming at clarifying the fate of AMs upon infection, in presence or absence of IFNAR1. The reduction of AMs in WT compared to KO mice as early as day 14 p.i. (Fig. 3) was due to incremental cell death, specifically in this lung population (Fig. 5F). These novel data are now presented and discussed on pages 9 and 14.

- In figure 5 the authors use chimeras to understand whether the effects were due to hematopoietic or radioresistant or both cells. The survival of KO->WT or WT->KO is intermediate to that observed upon infection of WT or IFNAR-/- mice (Fig. 5A). However, in Fig. 5B it is shown that the amount of cytokines and chemokines in KO->WT is actually similar to that observed for infected KO. Since the authors suggest that the death is associated to high inflammation, why are KO->WT not as resistant as IFNAR-/-? In the same line, the amount of neutrophils in WT->KO appears to be higher than that in KO->WT (Fig. 5B) and yet the survival profile is the same.

AU: We now provide pooled survival curves with chimeras including WT>WT and KO>KO, next to WT>KO and KO>WT (Fig. 4A). In view of current information regarding ontogeny of AMs [13;14] and repopulation after radiation-induced cytoablation we consider these novel data more appropriate. According to the above-mentioned reports AMs arise from circulating monocytes, mature shortly after birth and are maintained locally by proliferation. Because radiation targets dividing cells, the results detailed above imply that radiation may not result in deletion of non- or slow-dividing AM pool and that the replenished AM pool may differ compared with cells originally seeding the lungs after birth. In the 129S2 background it is not currently possible to dissect these events due to lack of CD45.1 and CD45.2 tracking modalities. With this information in mind, our novel chimera results are, however, similar to previous results and indicate that both hematopoietic and radioresistant cells are responsible for the observed phenotype. The fact that KO>WT do not show the phenotype of KO>KO could be due to differences in activation and subsequently function of radioresistant cells. Although AMs

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and PMNs (of KO phenotype) do not support Mtb replication as WT cells do, they release mediators and may activate lung-resident cells, e.g., pneumocytes, to release chemokines. Abundance of inflammatory mediators of epithelial cell origin, e.g. CXCL5 is regulated by bacterial sensing along with sensing inflammatory cytokines and both processes could be modulated by IFN I [12]. We measured concentrations of inflammatory mediators in lung tissue and estimated abundance of PMNs at 21 days p.i., however lethality of WT>KO and KO>WT occurred at later times, perhaps reflecting protracted inflammation and the fact that signaling in both the hematopoietic and radioresistant compartment imprinted the inflammatory phenotype of the WT 129S2 mice. Quantification of PMNs in lung specimens from chimeric mice (Fig. 4C) indicates that although IFNAR1 on hematopoietic cells is the main driver of PMN recruitment, signaling in radioresistant cells contributes also significantly to this process.

- In figure 8, the authors show an increased number of PMN cells infected with Mtb and in figure 6 an elevated cell death in the BAL of WT mice, when compared to IFNAR-/- mice. The paper would benefit greatly if the cell populations that are dying in WT mice were determined and also if a mechanistic insight on the drivers of cell death were clarified. It is likely that the elevated cell death observed in WT mice is just a consequence of elevated PMN recruitment, as these are short lived cells. Also it is not very clear on how the changes in myeloid populations impact the survival of WT versus IFNRA-/- mice. In this regard, PMN cells have been shown to have protective roles during early infection with Mtb. It would be interesting to show whether PMNs from IFNRA-/- where more efficient at killing Mtb that WT PMNs.

AU: We thank the Reviewer for this valuable suggestion. We have performed these experiments and confirmed that BAL leukocytes in WT mice present elevated cell death compared to KO animals (Fig. 5F). Increased cell death observed in WT mice was not a consequence of elevated PMN numbers, in fact we detected lower frequencies of annexinV/7AAD+ PMNs in WT mice. However, the great majority of cells within alveoli are represented by AMs (10× more compared to PMNs). We observed significantly increased frequencies of dead (late apoptotic/necrotic) AMs in WT animals (Fig. 5F). Further studies will clarify the modalities of cell death controlled by IFN I.

Regarding propensity of PMNs to kill Mtb, our detailed analysis of AFB per PMN does not support this scenario. The number of Mtb detected in PMNs is relatively low (mostly 1–3 bacteria per phagocyte) and this is similar in WT and KO mice. Using BM-purified PMNs to investigate killing capacity would not fully recapitulate the properties of tissuerecruited PMNs, as these cells are able to modulate their ROS levels upon migration and local activation by cytokines.

Overall the paper is well written but it is not straight forward to follow the data.

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Despite this, the data is interesting and of interest to the field, as it puts forward a new exciting hypothesis regarding the role of type I IFN during TB. The paper only lacks for the correlative nature of the data.

AU: We have now reorganized the data to improve clarity as suggested by the Reviewer, and provide novel results to support our hypothesis. We hope that our efforts have improved the manuscript by adding mechanistic insights into the roles of type I IFN in TB.

Minor points:

- Figure 1 shows death of 129S WT infected animals in app 30 days, but the text refers that the animals died within 40 days post-infection.

AU: We have now pooled data from multiple experiments (Fig. 1A). In all of these experiments, one 129S2 mouse survived and the rest died within 27–34 days p.i. thus enabling us to state that the mice died within 40 days p.i. Please note the same pattern of survival was reported in two additional experiments using control IgG (Fig. 6G) (death within 27–40 days p.i.).

- Figure 2 shows lower levels of IL-1 in infected IFNAR-/- than WT lungs suggesting that type I IFN signaling enhances IL-1 production during Mtb infection of susceptible 129S2 mice, contrary to what has been described (Mayer-Barber KD et al., Immunity 2011; Novikov A et al., J Immunol 2011). The authors should discuss these differences.

AU: The Reviewer is correct and this is the subject of a follow-up study. We have decided not to emphasize these differences until an adequate explanation can be found. These findings are the subject of independent studies in our laboratory.

- Figure 3: Do the data show frequencies of IFN-g+TNF-a+ cells (as stated in the legend of the figure) or IFN-g+ cells (as stated in the graph axis)? Plus, CD4-gated FACS profiles do not seem representative of the data plotted in the graph.

AU: The data show frequencies of IFN-γ+ cells and this has now been amended for clarity. The profiles (FACS dot plots) are representative. Please note we have subtracted medium value as background from PPD values.

- The sudden use of BMDMs and BMDCs is confusing. With regard to BMDMs, for example, the analysis of chemokine production (Fig. 6A) does not fit the in vivo observations (Fig. 2B).

AU: We have used BMDMs and BMDCs as models for myeloid cells interacting with Mtb. We have moved these data to the supplement to reduce potential confusion and focus on AMs by leaving these data in the main figures.

The chemokine production in BMDMs (Fig. 5A in revised manuscript) partially fit the *in vivo* observations (Fig. 2B). While certain chemokines were directly influenced by IFN I

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signaling in macrophages (e.g. CCL2; CCL5), abundance of other members *in vivo* likely represented the cumulative effect of IFN I and differential bacterial burden.

- Figure 7: Why different methods to assess Mtb growth were used in figure 7A and B? For the experiment of figure 7B the authors should show that the difference observed at 48h post-infection was not due to different infection rate.

AU: We have used 3H-Uracil to assess Mtb replication over an extended time (5 days). This also allowed us, to a certain extent, to monitor cellular and extracellular Mtb growth (metabolic activity). We have now performed experiments to validate the finding that phagocytosis did not differ between the tested mouse strains. These data are provided in Fig. 6B and discussed on page 10.

In addition we have measured uptake of GFP-BCG at 4 h p.i. and have not found a difference between WT and IFNAR1-deficient cells (see Fig. 3 below).

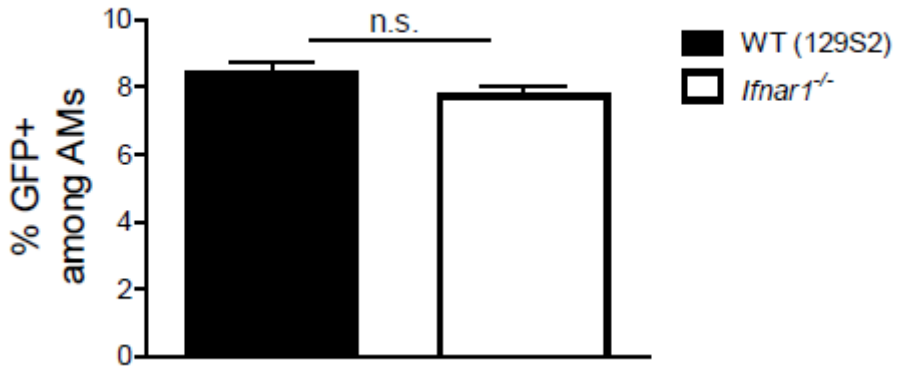

Figure 3. Phagocytosis of GFP-BCG by AMs. AMs were obtained from BALF by adherence to tissue culture treated plates (purity > 98% CD11c+ cells) and infected with GFP-BCG at MOI 10. Phagocytosis of bacteria was estimated at 4 h p.i. (n=3), n.s. not significant, Student's t test.

- Figure 8: The authors mention that the approaches followed in 8A and B versus 8C are complementary. However, in what regards data on the % of GFP or AFB + among BAL PMNs, the two approached lead to different results. The authors should comment on this.

AU: We have employed reporter bacteria (GFP-Mtb) to estimate the Mtb content in BALF phagocytes. As described by others and presented in our gating strategy (Fig. S5B), a population shift allows detection of positive cells. Due to the limited numbers of bacteria in PMNs and the possibility that flow cytometry would miss low signal events we decided to validate this information using AFB staining of BAL cells. Using this approach we observed that indeed most PMNs contain few Mtb (Fig. 6F), and thus we may have detected primarily strong signal events with FACS. Please note we set our gate for GFP+ signal most stringently to avoid interference with myeloid cell autofluorescence (Fig.

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S5B). We do not consider these results discrepant, especially because number-wise the difference was obvious also using FACS technology (Fig. 6D).

- Supporting Information Figure 1: the legend states that "(E) Serum concentrations of lactate dehydrogenase at day 21 p.i. in WT mice (129S2 and C57BL76) …" but the figure only shows the data for WT (129S2).

AU: We have amended this error. Thank you for pointing this out.

- Certain panels of the figures are misplaced (for example, Fig. 1E is referred in the text before Fig. 1D; the same for FIG. 5C/B and 5E/D, and Fig.S1F/D).

AU: We have revised the misplaced figure panels accordingly. Thank you for this recommendation.

- Lines 106-7: The authors state that «The milder pathology in Ifnar-/- animals was accompanied by a lower serum lactate dehydrogenase concentration». However, data in Figure S1E show similar levels of LDH. Is the observed difference statistically significant?

AU: Although we observed a trend, the difference is not statistically significant. We have modified the text accordingly (see page 5).

- Lines 193-4: "Frequencies of PMNs were increased in BALF of Ifnar1-/- mice (Figure 6D)." – But figure 6D shows a significantly decrease in PMNs frequency among BAL cells.

AU: We regret this mistake. The text has been revised accordingly, on page 8.

References

1. Desvignes,L., Wolf,A.J., and Ernst,J.D., Dynamic roles of type I and type II IFNs in early infection with *Mycobacterium tuberculosis*. J Immunol 2012. 188: 6205-6215.

2. Stanley,S.A., Johndrow,J.E., Manzanillo,P., and Cox,J.S., The Type I IFN response to infection with *Mycobacterium tuberculosis* requires ESX-1-mediated secretion and contributes to pathogenesis. J Immunol 2007. 178: 3143-3152.

3. Harper,J., Skerry,C., Davis,S.L., Tasneen,R., Weir,M., Kramnik,I., Bishai,W.R. et al., Mouse model of necrotic tuberculosis granulomas develops hypoxic lesions. J.Infect.Dis. 2012. 205: 595-602.

4. Marzo,E., Vilaplana,C., Tapia,G., Diaz,J., Garcia,V., and Cardona,P.J., Damaging role of neutrophilic infiltration in a mouse model of progressive tuberculosis. Tuberculosis.(Edinb.) 2014. 94: 55-64.

5. Commandeur,S., van Meijgaarden,K.E., Prins,C., Pichugin,A.V., Dijkman,K., van den Eeden,S.J., Friggen,A.H. et al., An unbiased genome-wide *Mycobacterium tuberculosis* gene expression approach to discover antigens targeted by human T cells expressed during pulmonary infection. J.Immunol. 2013. 190: 1659-1671.

6. Vilaplana,C., Marzo,E., Tapia,G., Diaz,J., Garcia,V., and Cardona,P.J., Ibuprofen therapy resulted

in significantly decreased tissue bacillary loads and increased survival in a new murine experimental model of active tuberculosis. J.Infect.Dis. 2013. 208: 199-202.

7. Lowe,D.M., Redford,P.S., Wilkinson,R.J., O'Garra,A., and Martineau,A.R., Neutrophils in tuberculosis: friend or foe? Trends Immunol. 2012. 33: 14-25.

8. Dorhoi,A., Desel,C., Yeremeev,V., Pradl,L., Brinkmann,V., Mollenkopf,H.J., Hanke,K. et al., The adaptor molecule CARD9 is essential for tuberculosis control. J Exp.Med 2010. 207: 777-792.

9. Dorhoi,A., Iannaccone,M., Farinacci,M., Fae,K.C., Schreiber,J., Moura-Alves,P., Nouailles,G. et al.,
MicroRNA-223 controls susceptibility to tuberculosis by regulating lung neutrophil recruitment. J.Clin.Invest 2013. 123: 4836-4848.

10. Abadie,V., Badell,E., Douillard,P., Ensergueix,D., Leenen,P.J., Tanguy,M., Fiette,L. et al., Neutrophils rapidly migrate via lymphatics after *Mycobacterium bovis* BCG intradermal vaccination and shuttle live bacilli to the draining lymph nodes. Blood 2005. 106: 1843-1850.

11. Wu,D., Lim,E., Vaillant,F., Asselin-Labat,M.L., Visvader,J.E., and Smyth,G.K., ROAST: rotation gene set tests for complex microarray experiments. Bioinformatics. 2010. 26: 2176-2182.

12. Nouailles,G., Dorhoi,A., Koch,M., Zerrahn,J., Weiner,J., III, Fae,K.C., Arrey,F. et al., CXCL5/LIXsecreting
pulmonary epithelial cells drive destructive neutrophilic inflammation in tuberculosis. J Clin Invest 2014. doi:10.1172/JCI72030 .

13. Guilliams,M., De,K., I, Henri,S., Post,S., Vanhoutte,L., De,P.S., Deswarte,K. et al., Alveolar macrophages develop from fetal monocytes that differentiate into long-lived cells in the first week of life via GM-CSF. J.Exp.Med. 2013. 210: 1977-1992.

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14. Hashimoto,D., Chow,A., Noizat,C., Teo,P., Beasley,M.B., Leboeuf,M., Becker,C.D. et al., Tissueresident
macrophages self-maintain locally throughout adult life with minimal contribution from circulating monocytes. Immunity. 2013. 38: 792-804.

Second Editorial Decision – 11 April 2014

Dear Prof. Kaufmann,

It is a pleasure to provisionally accept your manuscript entitled "Type I IFN signaling triggers immunopathology in tuberculosis-susceptible mice by modulating lung phagocyte dynamics" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Peer review correspondence

European Journal of Immunology

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,
Karen Chu

on behalf of Prof. Iain McInnes

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