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Supporting Information

for

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Nadine Honke, Namir Shaabani, Katja Merches, Asmae Gassa, Anke Kraft, Katrin Ehrhardt, Dieter Häussinger, Max Löhning, Ulf Dittmer, Hartmut Hengel, Mike Recher, Philipp A. Lang and Karl S. Lang

Immunoactivation induced by chronic viral infection inhibits viral replication and drives immunosuppression through sustained IFN-I responses

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Review Timeline:

Submission date:	28-Apr-2015
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Handling Executive Committee member: Prof. Annette Oxenius

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- 27-May-2015

Dear Dr. Lang,

Manuscript ID eji.201545765 entitled "Chronic viral infection leads to immunosuppression by inhibiting enforced viral replication through sustained IFN-I responses" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter. All reviewers like your study but found some gaps that need to be explained.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication. Should you disagree with any of the referees' concerns, you should address this in your point-by-point response and provide solid scientific reasons for why you will not make the requested changes.

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,
Katharina Schmidt

On behalf of
Prof. Annette Oxenius

Dr. Katharina Schmidt
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Reviewer: 1

Comments to the Author

In this study the authors investigate how a pre-existing chronic LCMV infection may interfere with the adaptive immune response to a superinfecting virus. Based on a previous study showing that the induction of an adaptive immune response to VSV relies on enforced replication in CD169+ macrophages, they now go on to show that a chronic viral infection also impairs the B cell response to VSV, and that this is also associated with blunted virus replication in the chronically LCMV infected hosts. In an attempt to rule out competing mechanisms they go on to demonstrate that perforin mediated immunopathology is not involved - this has previously been suggested to play a central role in LCMV-induced immunomodulation. Through studies of mice neonatally infected with LCMV, the authors also try to rule out other CD8+ T cell mediated

mechanisms. By demonstrating the foot-print of type I IFN synthesis in chronically infected mice followed by studies in IFNAR and IRF3xIRF7 deficient mice, the authors reach the conclusion that prolonged production of type I IFN in chronically LCMV infected mice prevents enforced viral replication of new viruses, thus markedly limiting the humoral immune response, which would otherwise have been induced.

Major criticisms:

Overall, an interesting story revealing a previously unappreciated way in which a pre-existing viral infection may impair the immune response to a superinfection. While persuasive, there are a number of logical shortcuts that need to be exchanged with hard data, before the suggested interpretation can be unequivocally accepted.

Thus, first of all the presence of type I IFN itself is never demonstrated; only down-stream markers for its presence are detected. Regarding the interpretation of the underlying mechanism(s), the authors rely on two models for chronic LCMV infection assuming that they are equivalent; however, while some phenomena are repeated, they are not identical, e.g. cf. the T cell tolerance in neonatally infected mice. Extrapolating from one situation to the other therefore requires direct and clear confirmation regarding the similarity with respect to key elements in the interpretation. Consequently, the authors need to document the presence of type I IFN also in neonatally infected mice, establishing the similarity of the situation regardless of the mode in which the chronic infection is established. Without this info the data from neonatally infected mice cannot be used as a tool to analyze the role of CD8+ T cells in suppression the B cell response in mice becoming chronically infected as adults. Under these conditions the evidence for a lack of CD8+ T cell involvement only hinges on the data from perforin deficient mice, which does not rule out other T cell mediated mechanisms than cell killing.

Minor comments:

I think the title is an unfortunate choice given that the authors in end of the Discussion states: “…the failure of immune response was not due to immunosuppression.. “ Also that immunosuppression is not always immunosuppression is exactly the interesting point of this paper.

In several places the authors imply that the data from perforin deficient mice exclude a role for CD8+ T cells, however, without the data from neonatally infected mice, a key role for CD8+ T cells cannot be ruled out , cf. my comments above. The phrasing should therefore be modified to make this clear.

The authors need to ascertain the absence of type I IFN production in IRF3xIRF7 deficient mice

The authors needs to address if changes unrelated to type I IFN could affect the results in IFNAR and IRF3xIRF7 deficient mice; the course of LCMV infection in these mice is likely quite different from that in WT mice, and this might impact the immune system beyond the absence of IFN.

In the beginning of the Discussion the authors state that “they demonstrate that chronic LCMV infection leads to prolonged activity of type I IFN”• . However, this is not a new observation; the group of Ray Welsh published data to this end more than 10 years ago.

Indeed, there exist a substantial body of older data on LCMV â€“induced immunosuppression , which it

might to relevant to cite, particularly as the T-cell immunopathology model is only one of several models previously suggested.

Reviewer: 2

Comments to the Author
The study by Honke et al claims that chronic viral infection leads to immunosuppression by inhibiting enforced viral replication through sustained IFN-I responses. These claims are, however, not really supported by the data. The question the authors address is a complex one and the experiments they perform do not address this complexity in a sufficient manner.

1) The major claim is that chronic infection with LCMV reduces responses to VSV by restricting it`s replication due to increased levels of type I interferon. If this was the case, antibody responses induced by inactivated VSV should be normal. This experiment should be done.
2) Immunopathology in perforin-deficient mice is extensive after infection with LCMV and their claim that VSV-specific antibody responses in perforin deficient mice are reduced despite absence of immunopathology is wrong. Indeed, figure 2B shows that the CD169+ macrophages are largely absent in LCMV-infected perforin-deficient mice.
Likely, antibody responses are therefore reduced due to immunopathology.
Fig 3) IgM responses in neonates are normal. It would be interesting to see whether type I IFN and responsive genes are indeed unregulated in these mice.
Fig 4B) VSV obviously replicates better in Type I IFNR deficient mice; with or without infection with LCMV. Hence, it is not a surprise that antibody responses also go up. Presumably, LCMV-infected Type IFNR-deficient mice even die after infection with VSV because of extensive viral replication.

Reviewer: 3

Comments to the Author
This study addresses the role Type 1 interferon (IFN-I) plays during persistent LCMV infection in inhibiting enforced virus replication in CD169+ macrophages. The current study demonstrates that sustained IFN-I signaling prevents enforced virus replication in CD169+ macrophages and results in inadequate priming of adaptive humoral responses during co-infection with VSV. The work is interesting and continues previously published work by this group (Honke, et al., 2012. Nature Immunology) by extending the concept to understanding mechanisms of immune suppression during persistent virus infection. However, I have one major concern regarding the authors' conclusions.

VSV is know to induce IFN-I at early time-points during infection. In fact, the authors demonstrated in their 2012 NI paper that the reason for the enforced virus replication specifically in the CD169+ cells is their expression of the Usp18 gene which inhibits IFN-I signaling by competing with Jak1. Why is it that IFN-I generated during persistent LCMV infection does not have the same effect as IFN-I generated early during VSV infection?

What are the levels of Usp18 expression in CD169+ macrophages in LCMV docile infected animals?

How do the authors explain the above discrepancy?

First Revision – authors' response - 16-Sep-2015

Point to point reply
Reviewer: 1

Comments to the Author
In this study the authors investigate how a pre-existing chronic LCMV infection may interfere with the adaptive immune response to a superinfecting virus. Based on a previous study showing that the induction of an adaptive immune response to VSV relies on enforced replication in CD169+ macrophages, they now go on to show that a chronic viral infection also impairs the B cell response to VSV, and that this is also associated with blunted virus replication in the chronically LCMV
infected hosts. In an attempt to rule out competing mechanisms they go on to demonstrate that perforin mediated immunopathology is not involved, which has previously been suggested to play a central role in LCMV-induced immunomodulation. Through studies of mice neonatally infected with LCMV, the authors also try to rule out other CD8+ T cell mediated mechanisms. By demonstrating the foot-print of type I IFN synthesis in chronically infected mice followed by studies in IFNAR and
IRF3xIRF7 deficient mice, the authors reach the conclusion that prolonged production of type I IFN in chronically LCMV infected mice prevents enforced viral replication of new viruses, thus markedly limiting the humoral immune response, which would otherwise have been induced.

Major criticisms:
• Overall, an interesting story revealing a previously unappreciated way in which a pre-existing viral infection may impair the immune response to a superinfection. While persuasive, there are a number of logical shortcuts that need to be exchanged with hard data, before the suggested interpretation can be unequivocally accepted. Thus, first of all the presence of type I IFN itself is never demonstrated, only down-stream markers for its presence is detected.

We found that the expression of IFN-a4 and IFN-β1 mRNA in the spleen was highly upregulated in chronically infected mice. We have included these findings in Figure 4A.

• Regarding the interpretation of the underlying mechanism(s), the authors rely on two models for chronic LCMV infection assuming that they are equivalent; however, while some phenomena are repeated, they are not identical, e.g. cf. the T cell tolerance in neonatally infected mice. Extrapolating from one situation to the other therefore requires direct and clear confirmation regarding the similarity with respect to key elements in the interpretation. Consequently, the authors need to document the presence of type I IFN also in neonatally infected mice, establishing the similarity of the situation regardless of the mode in
which the chronic infection is established. Without this info the data from neonatally infected mice cannot be used as a tool to analyze the role of CD8+ T cells in suppression the B cell response in mice becoming chronically infected as adults. Under these conditions the evidence for a lack of CD8+ T cell involvement only hinges on the data from perforin deficient mice, which does not rule out other T cell mediated mechanisms than cell killing.
We used these two models to examine the impact of CD8+ T cells on enforced viral replication. As suggested by the reviewer, we used perforin-deficient mice to rule out T cell–mediated killing, whereas we used mice infected as neonates to show that T-cell tolerance did not influence enforced viral replication. Additionally, we used another mouse model (MHC-I deficient mice) to study the role of CD8+ T cells in immunosuppression during chronic infection, we included the results in Figure 3C and 3D.
We also investigated IFN signaling and IFN expression in neonatally infected mice and included our findings in Figure 4B.

Minor comments:
• I think the title is an unfortunate choice given that the authors in end of the Discussion states: "..the failure of immune response was not due to immunosuppression.." Also that immunosuppression is not always immunosuppression is exactly the interesting point of this paper.
We changed the title to more clearly reflect the point of the paper.
• In several places the authors imply that the data from perforin deficient mice exclude a role for CD8+ T cells, however, without the data from neonatally infected mice, a key role for CD8+ T cells cannot be ruled out, cf. my comments above. The phrasing should therefore be modified to make this clear.
We agree that our findings from perforin-deficient mice exclude only the cytotoxic role of CD8+ T cells, we changed the phrase in the manuscript. Additionally, we included the MHC-I deficient mouse model to study the role of CD8+ T cells in immunosuppression during chronic infection (Figure 3C and 3D).
• The authors need to ascertain the absence of type I IFN production in IRF3xIRF7 deficient mice. The authors needs to address if changes unrelated to type I IFN could affect the results in IFNAR and IRF3xIRF7 deficient mice; the course of LCMV infection in these mice is likely quite different from that in WT mice, and this might impact the immune system beyond the absence of IFN.

In a previous work (Lang PA et al, 2009), we found that Irf7-deficient mice do not produce IFN-α after infection with LCMV or VSV. We expect that mice double deficient in Irf3 and Irf7 exhibit a similar defect in IFN-α production.

In order to avoid the difference in LCMV course in IFNAR deficient mice, we infected WT mice with LCMV-Docile and after 30 days, before VSV infection, we stopped IFN-α effect through treatment with anti-IFN-αR1 antibody. As control we used isotype antibody. We included the results in Figure 4C and 4D.

• In the beginning of the Discussion the authors state that “they demonstrate that chronic LCMV infection leads to prolonged activity of type I IFN”. However, this is not a new observation, the group of Ray Welsh published data to this end more than 10 years ago. Indeed, there exist a substantial body of older data on LCMV –induced immunosuppression , which it might to relevant to cite, particularly as the T-cell immunopathology model is only one of several models previously suggested.

We corrected this sentence and we have cited other publications about LCMV-induced immunosuppression.

Reviewer: 2

Comments to the Author
The study by Honke et al claims that chronic viral infection leads to immunosuppression by inhibiting enforced viral replication through sustained IFN-I responses. These claims are, however, not really supported by the data. The question the authors address is a complex one and the experiments they perform do not address this complexity in a sufficient manner.

• 1) The major claim is that chronic infection with LCMV reduces responses to VSV by restricting it`s replication due to increased levels of type I interferon. If this was the case, antibody responses induced by inactivated VSV should be normal. This experiment should be done.
We have measured the concentrations of neutralizing antibodies in naive mice and in mice chronically infected with LCMV after immunization with UV light–inactivated VSV. Both groups exhibited similar antibody titers. We have included the findings in Figure 2C.

• 2) Immunopathology in perforin-deficient mice is extensive after infection with LCMV and their claim that VSV-specific antibody responses in perforin deficient mice are reduced despite absence of immunopathology is wrong. Indeed, figure 2B shows that the CD169+ macrophages are largely absent in

LCMV-infected perforin-deficient mice. Likely, antibody responses are therefore reduced due to immunopathology.
We have changed the phrase as reviewer suggested. We agree that the numbers of CD169+ macrophages are reduced in perforin-deficient mice, but virus replication disappeared completely even in the existing CD169+ macrophages. This was not the case in MHC-I deficient mice, in which the number of CD169+ macrophages was not reduced, even so there was no enforced viral replication (Figure 3C and 3D).

• Fig 3) IgM responses in neonates are normal. It would be interesting to see whether type I IFN and responsive genes are indeed unregulated in these mice.
Indeed, the IgM response was similar at early time points, but the IgG response was defective in these mice. We measured IFN signaling and IFN expression in neonatally infected mice. We included the results in Figure 4B.

• Fig 4B) VSV obviously replicates better in Type I IFNR deficient mice; with or without infection with LCMV. Hence, it is not a surprise that antibody responses also go up. Presumably, LCMV-infected Type IFNR-deficient mice even die after infection with VSV because of extensive viral replication.
We completely agree with the reviewer. Indeed, IFNAR-deficient mice died after VSV infection, whereas Irf3-/-×Irf7-/- mice exhibited more resistance to VSV infection. For this reason we used this mouse model to show the effect of type I IFN on immunosuppression during chronic infection. We have now mentioned this finding in the manuscript. Moreover, we included a new experiment using anti-IFN-αR1 antibody to show the role of IFN-I in inhibition the enforced virus replication and inducing immunosuppression.

Reviewer: 3

Comments to the Author
This study addresses the role Type 1 interferon (IFN-I) plays during persistent LCMV infection in inhibiting enforced virus replication in CD169+ macrophages. The current study demonstrates that sustained IFN-I signaling prevents enforced virus replication in CD169+ macrophages and results in inadequate priming of adaptive humoral responses during co-infection with VSV. The work is interesting and continues previously published work by this group (Honke, et al., 2012. Nature Immunology) by extending the concept to understanding mechanisms of immune suppression
during persistent virus infection. However, I have one major concern regarding the authors conclusions.

• VSV is known to induce IFN-I at early time-points during infection. In fact, the authors demonstrated in their 2012 NI paper that the reason for the enforced virus replication specifically in the CD169+ cells is their expression of the Usp18 gene which inhibits IFN-I signaling by competing with Jak1. Why is it that IFN-I

generated during persistent LCMV infection does not have the same effect as IFN-I generated early during VSV infection?
This is an interesting point and it was discussed below.

• What are the levels of Usp18 expression in CD169+ macrophages in LCMV docile infected animals?
We sorted the existing CD169+ macrophages with FACS and examined the expression of genes induced by type I IFN. We found that during chronic infection CD169+ macrophages express slightly more Usp18, whereas the expression of genes induced by type I IFN is substantially higher. We have included these findings in Figure 4F.

• How do the authors explain the above discrepancy?
As we have shown in a previous study (NI 2012), the expression of Usp18 in CD169+ macrophages is higher than that in F4/80+ macrophages under naive conditions, whereas the expression of other genes induced by type I IFN is similar in both cell types. The initially high expression of Usp18 can explain why viral replication in CD169+ macrophages is stronger than that in F4/80+ macrophages early during VSV infection.
The present study showed that during chronic LCMV infection the expression of Usp18 in CD169+ macrophages is slightly higher than that in naive CD169+ macrophages (Figure 4F). However, the initial expression of genes induced by type I IFN are already significantly higher in CD169+ macrophages during chronic infection than in naive CD169+ macrophages, a finding that explains the inhibition of enforced viral replication of VSV during superinfection.

References

Lang, P. A., Cervantes-Barragan, L., Verschoor, A., Navarini, A. A., Recher, M., Pellegrini, M., Flatz, L., Bergthaler, A., et al., Hematopoietic cell-derived interferon controls viral replication and virus-induced disease. Blood 2009. 113: 1045-1052.

European Journal of Immunology

Second Editorial Decision - 13-Oct-2015

Dear Dr. Lang,

It is a pleasure to provisionally accept your manuscript entitled "Immunoactivation induced by chronic viral infection inhibits enforced viral replication and leads to immunosuppression through sustained IFN-I responses" 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.

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,
Laura Soto Vazquez

on behalf of
Prof. Annette Oxenius

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