European Journal of Immunology

Type I IFN signaling facilitates the development of IL-10-producing effector CD8+ T cells during murine influenza virus infection

Li Jiang, Shuyu Yao, Su Huang, Jeffrey Wright, Thomas J. Braciale and Jie Sun

Correspondence: Dr. Jie Sun, Department of Pediatrics, Herman B. Wells Center for Pediatric Research, Indiana University School of Medicine, Indianapolis

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Review Timeline:	Submission date:	31-May-2016
	First Editorial decision:	30-Jun-2016
	Revisions received:	17-Aug-2016
	Second Editorial decision:	22-Sep-2016
	Accepted:	30-Sep-2016

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

30-Jun-2016

Dear Dr. Sun,

Manuscript ID eji.201646548 entitled "Type I interferon signaling facilitates the development of IL-10-producing effector CD8+ T cells during influenza virus infection" 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.

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 refereesâ€™ 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 referee(s) 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 referee(s) 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,
Nadja Bakocevic

On behalf of
Prof. Annette Oxenius

Dr. Nadja Bakocevic
Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com
www.eji-journal.eu

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Reviewer: 1

Comments to the Author

Jiang et al describe a novel function of Type I interferon (IFN) on IL-10 production by effector CD8+ T cells. This study suggests that IFN enhances IL-27 production by antigen presenting cells (APCs) which can increase IL-10 expression from CD8+ T cells. They also suggest that this pathway involves IRF4 and Blimp-1.
Main concerns:
The central hypothesis of the paper is the enhancement in IL-27 production by lung APCs in response to IFN, In the abstract, the authors state: We find that tpe I IFNs can enhance IL-27 production by lung

antigen presenting cells, thereby facilitating IL-10-producing CD8+ T cell development through a CD8+ T cell non-autonomous way• (*NOTE, type• is misspelled) However, in Fig. 3, the source of IL-27 is unclear. The authors made no attempt to distinguish lung epithelial/stromal cells from actual immune APCs. It is possible that there might be additional sources of IL-27 in lungs contributing to this phenotype. Lung APCs should be analyzed in isolation. Additionally, the conclusion of this figure Type I IFN signaling is required for the production of IL-27• is perhaps overstated considering that some p28 is still produced. If the authorsâhypothesis is correct that IFN signaling promotes IL-27 to drive IL-10 secretion in CD8+ T cells, then the authors should complete this pathway experimentally by testing whether administration of IL-27 to Ifnar1 deficient animals during influenza infection restores IL-10 production by effector CD8+ T cells.

Additional concerns regarding the figures and figure legends are listed below (not requiring additional experiments):

Figure legends should be clarified; statistics and number of animals used for each study should be explained.

Sources of certain mouse strains (Vert-X and CD8-specific conditional KO line) should be listed.

Figure 1. The authors need to clarify whether stimulation of influenza specific T cells is achieved by WT or KO dendritic cells. Additionally, influenza specific T cells are still observed in the absence of IFNAR and STAT2, their statement in figure legend is an over-interpretation of the data.

Figure s2. Sources of neutrophils, macrophages, dendritic cells and details of the stimulation (time and amount of IFN) should be listed. In the absence of statistics, authors conclusions on promotion of IL-27 gene expression does not hold. There are some instances (e.g. Panel A) where addition of IFN seems to reduce IL-27B expression. Protein quantification should be added to this figure.

Figure 2g/h. Authors conclusion of the deficiency of IFNAR1 only moderately decreased IL-10/eGFP expression by CD4 T cells• are not supported by the data. The panel suggests that there is no significant difference between two strains. p value should be reported if such claims will be made.

Figure 4. Please add the amount of influenza and the route of infection. Also, it is important to show that blood and lungs of these animals are reconstituted equally (1:1) by both donor cell populations. For Figure 4b., ELISA results for secretion of IL-10 and IFN-gamma should be shown.

Figure 5/6. Figures do not distinguish the role of IL-2 and IL-27 for IL-10 induction. Panels that assess the role of IL-2 and IL-27 in the absence of presence of IFN is crucial for the interpretation of the data.

Figure 6. Authors conclusion on Blimp-1 is required for the production of IL-10 by CD8+ T cells upon type I IFN signaling• is not a correct interpretation of the results. Blimp-1 deficient cells are unable to respond to IL-2 and IL-27 stimulation. There is not enough evidence for the role of IFNs in this setting.

Reviewer: 2

European Journal of Immunology

Comments to the Author

The paper of Jiang et al describes that type I IFNs are required for the development of IL-10 producing CD8 T cells by a CD8 T cell non-autonomous way and by direct signalling to CD8 T cells.

The reviewer has the following major concerns:

1. The paper lacks novelty. The effect of type I IFNs on IL-10 production in the lung after infection with influenza A virus has been demonstrated by Arimori et al (Antiviral Research 99; 2013:230).

2. The analysis of the local immune response in the lung is rather thin.
- which are the IL-10 producing cell populations?
- kinetics of IL-10 producing CD8 and CD4 T cells are not given.
- absolute number of IL-10 producing cells are lacking.
- is there a different susceptibility of wt and IFNAR-/- mice to influenza infection? Score / body weight? Are the virus titers in the lung different?

3. To demonstrate direct type I IFN signalling for IL-10 production of CD8 T cells the authors generated wt/IFNAR-/- mixed bone marrow chimeras.
- The FACS plots given in Fig. 4 look rather strange (there seems to be a technical problem).
- Information about the chimerism before and after infection is missing.
- No absolute cell numbers for wt and IFNAR-/- cell populations are given.
- To draw this central conclusion a more accurate analysis is needed.

4. The paper from Arimori et al (Type I interferon plays opposing roles in cytotoxicity and interferon-Î³ production by natural killer and CD8 T cells after influenza A virus infection in mice. J Innate Immun. 2014;6(4):456-66) is not cited and discussed.

First Revision – authors' response

17-Aug-2016

Reviewer 1:

We appreciate the reviewer's comment that we described " a novel function of type I IFN on IL-10 production by effector CD8+ T cells" in our manuscript. The specific responses to the reviewer's concerns are listed below.

European Journal of Immunology

Major concerns:

1. *"However, in Fig. 3, the source of IL-27 is unclear. The authors made no attempt to distinguish lung epithelial/stroma cells from actual immune APCs. Additionally, the conclusion of this figure "Type I IFN signaling is required for the production of IL-27" is perhaps overstated considering that some p28 is still produced".*

   In this revision, we have now included new data in which we compared IL-27 subunits Ebi3 and p28 expression levels between lung immune APCs (DC, macrophage and neutrophils) and lung epithelial/stroma cells (CD45 negative cells). We found that lung immune APCs express much higher levels of IL-27 than lung epithelial/stroma cells (particularly p28 subunit), which supports our claim that "IL-27 is mainly produced in lung immune APCs". These new data are shown in new Fig3 A (line 274-277).

   In response to the reviewer's "perhaps overstated" comment, we have rephrased the sentence to "Type I IFN signaling is required for the maximal production of IL-27" (line 271).

1. *"If the authors' hypothesis is correct that IFN signaling promotes IL-27 to drive IL-10 secretion in CD8+ T cells, then the authors should complete this pathway experimentally by testing whether administration of IL-27 to Ifnar1 deficient animals during influenza infection to restore IL-10 production".*

   We are unable to perform this experiment due to the recent move and current transition of our lab from the Indiana University to the Mayo Clinic. As shown in Figure 4 to 6, in addition to their effects in promoting IL-27 production to drive IL-10 production by CD8+ T cells, direct type I IFN signaling in CD8+ T cells is also important for the optimal development of IL-10 producing CD8+ T cells through the promotion of Blimp-1 and IRF4 expression in CD8+ T cells. Therefore, we expect that the administration of IL-27 to Ifnar1 deficient mice will not be able to fully restore IL-10 production due to the absence of direct type I IFN signaling in CD8+ T cells in this situation, and so the proposed experiment, we believe, is not critical to draw our main conclusion in the manuscript.

Additional minor concerns as stated by the reviewers that "not requiring additional experiments"

1. *"Figure legends should be clarified."*

   We have modified our figure legends to minimize confusion and clarify the conditions used.

2. *"Statistics and numbers of animals should be explained".*
   We have added animal numbers and statistics to our figure legends.

3. *"Source of mouse strains (Vert-X and CD8-specific conditional KO line" should be listed.*
   We have added the information in Methods section indicating sources of mouse strains (line 129-135).

4. *"Figure 1. The authors need to clarify whether stimulation of influenza specific T cells is achieved by WT or KO dendritic cells. Additionally, influenza specific T cells are still observed in the absence of IFNAR and STAT2, their statement in figure legend is an over-interpretation of the data".*

   We have clarified that the stimulation of influenza specific T cells is achieved by WT DCs. We have also modified our figure legends to "Type I IFN signaling is required for the optimal in vivo expression of IL-10 by $CD8^+$ T cells" to avoid potential over-interpretation of the data (line 590).

5. *"Figure s2. Sources of neutrophils, macrophages, dendritic cells and details of the stimulation (time and amount of IFN) should be listed. In the absence of statistics, author's conclusions on "promotion of IL-27 gene expression" does not hold. There are some instances (e.g. Panel A) where addition of IFN seems to reduce IL-27B expression. Protein quantification should be added to this figure".*
   We have added the sources of the cells in supplementary figure legends (in red fonts). We were unable to perform statistically analysis because we have pooled the cells isolated from individual mice (usually at least 2 mice) and performed real-time PCR experiments. As stated, all experiments were repeated with similar findings. In addition, IL-27 Ebi3 subunit is constitutive expressed in many cell types and the p28 is rather the rate-limited subunit of IL-27. According to Figure S2, type I IFN greatly promoted p28 expression in all three immune APCs. Therefore, we believe it is sound that "type I IFNs promotes IL-27 expression in immune APCs". Nevertheless we have modified the figure legends to "Type I IFN signaling promotes IL-27 p28 gene expression by APCs" (in red font). We did not perform protein measurement and so is unable to provide protein quantification in this in vitro experiment. However, our in vivo protein quantification in WT and Ifnar1-deficient mice suggest that type I IFN is required for the optimal IL-27 production in vivo (Fig 3C).

6. *"Figure 2g/h. Authors conclusion of "the deficiency of IFNAR1 only moderately decreased IL-10/eGFP expression by CD4 T cells" are not supported by the data. The panel suggests that there is no significant difference between two strains. p value should be reported if such claims will be made".*

   In response to the reviewer's comment, we have added "n.s., non-significant" to the figure. We have modified the sentence to "the deficiency of IFNAR1 did not significantly decrease IL-10/eGFP expression by CD4 T cells" in the text (line 266).

7. *"Figure 4. Please add the amount of influenza and the route of infection. Also, it is important to show that blood and lungs of these animals are reconstituted equally (1:1)*

by both donor cell populations. For Figure 4b., ELISA results for secretion of IL-10 and IFN-gamma should be shown".

We have added the amount of influenza and the route of infection in the Method section (lines 139, 140). We are sorry for the overlook. As far as reconstitution, we have included data showing reconstitution rate in the lung T cell compartments after infection (Figure S3 A) (lines 297, 298). The reconstitution rate is roughly 40%:60% (KO vs WT) in CD8+ T cell compartment. We did not check reconstitution rate before infection. In this experiment, we compared IFN-g and IL-10 expression specifically in gated WT (CD45.1+) or Ifnar1 KO (CD45.2+) CD8 T cells. Furthermore, we have normalized IL-10 production to IFN-g positive antigen-specific cells and found IFNAR1 direct signaling is required for optimal IL-10 production in this situation. Therefore, we felt it is not essential to have absolute equal (50%:50%) reconstitution.

We were unable to provide ELISA data since it was mixed bone marrow chimera experiment. ELISA data would not distinguish IL-10/IFN-g produced by WT or Ifnar1-deficient T cells in a mixed cell population. Nevertheless, our ELISA data in whole Ifnar1-deficient animals suggested IFNAR1 signaling in vivo is specifically required for IL-10 but not IFN-g production (Figure 1 E, F).

8. *"Figure 5/6. Figures do not distinguish the role of IL-2 and IL-27 for IL-10 induction. Panels that assess the role of IL-2 and IL-27 in the absence of presence of IFN is crucial for the interpretation of the data".*

In our previous publication (Sun et al, Nat Immunol. **2011** Apr;12(4):327-34), we have concluded that IL-2 or IL-27 alone minimally induce IL-10 production by CD8 T cells. The production of IL-10 by CD8 T cells requires the action/presence of both IL-2 and IL-27. In this manuscript, we mainly want to determine whether type I IFNs further promote IL-10 production in addition to the effects of IL-2 plus IL-27. We agree with the reviewer that, in order to dissect the mechanisms of action of type I IFNs, it would be interesting in the future to look at the cooperative effects of type I IFNs with IL-2 or IL-27. Nevertheless, to avoid confusion, we have modified our figure legends and subtitle in the text to "Type I IFNs further promote IL-10 production by CD8+ T cells in the presence of IL-2 plus IL-27" to avoid the confusion (lines 308, 664).

9. *"Figure 6. Authors' conclusion on "Blimp-1 is required for the production of IL-10 by CD8+ T cells upon type I IFN signaling" is not a correct interpretation of the results. Blimp-1 deficient cells are unable to respond to IL-2 and IL-27 stimulation. There is not enough evidence for the role of IFNs in this setting".*

We agree it could be possible that Blimp-1 deficient cells are unable to respond to IL-2 and IL-27 stimulation. However, we found that type I IFNs signaling could further promote Blimp1 expression in CD8 T cells. To avoid confusion, we have modified our figure legends and the text to "Blimp-1 and IRF4 are required for the development of IL-10-producing effector $CD8^+$ T cells induced by type I IFNs plus IL-2 and IL-27" (lines 323, 678, 679).

European Journal of Immunology

Reviewer 2

We thank the reviewer for the comments. Below are the specific responses to the comment.

1. *"The paper lacks novelty. The effect of type I IFNs on IL-10 production in the lung after infection with influenza A virus has been demonstrated by Arimori et al (Antiviral Research 99; 2013:230)."*

   We respectfully disagree with the reviewer's opinion that our manuscript lacks novelty. As we have cited, Arimori et al showed that type I IFNs promote IL-10 production during influenza infection. Our data supported their findings. However, the cellular and molecular mechanisms by which type I IFNs promote IL-10 production were not identified by the paper or by any other previous paper in the field. Our manuscript found that type I IFNs greatly promote IL-10 production specifically in CD8+ T cells during influenza infection. Furthermore, we found that type I IFNs drive IL-10 production by CD8+ T cells through two pathways; promoting IL-27 production by immune APCs and directly signaling to CD8+ T cells to upregulate Blimp-1 and IRF4. Thus, as noted by reviewer 1, we believe our data compliment and further extend the data observed by Arimori et al by describing a novel function and underlying mechanisms of type I IFNs on IL-10 production by effector CD8+ T cells.

2. *"The analysis of the local immune response in the lung is rather thin. which are the IL-10 producing cell populations? kinetics of IL-10 producing CD8 and CD4 T cells are not given. absolute number of IL-10 producing cells are lacking. is there a different susceptibility of wt and IFNAR-/- mice to influenza infection? Score / body weight? Are the virus titers in the lung different?"*

   Our main point here in the manuscript is to describe the effects and mechanisms of type I IFNs on the development of IL-10 producing effector CD8 T cells. The questions asked by the reviewer are very interesting questions and have been already addressed by our previous two publications (Sun et al, Nat Med. **2009** Mar;15(3):277-84 and Sun et al, Nat Immunol. **2011** Apr;12(4):327-34), in which we have carefully analyzed IL-10 producing cell populations, kinetics of IL-10 producing CD4 and CD8 T cells, absolute cell numbers and their functions etc. Other publications including the paper by Arimori et al. in Antiviral Research have already addressed important role of type I IFN in influenza infection. We therefore intended not to repeat these previous publications in this manuscript but to focus on our efforts on identifying the mechanism by which type I IFNs promote IL-10 producing CD8+ effector T cells. In the current format, the manuscript has already included 6 multiple panel figures and 4 supplementary figures, which we believe that it has sufficient data to support our main claim in the paper.

3. *To demonstrate direct type I IFN signalling for IL-10 production of CD8 T cells the authors generated wt/IFNAR-/- mixed bone marrow chimeras. The FACS plots given in Fig. 4 look rather*

strange (there seems to be a technical problem). Information about the chimerism before and after infection is missing. No absolute cell numbers for wt and IFNAR-/- cell populations are given. To draw this central conclusion a more accurate analysis is needed.

We are sorry for the confusion of the data. We think the so-called "strange FACS plots" referred by the reviewer are probably due to the high basal X-axis (IFN-g) levels caused by the specific settings of the FACS machine during acquisition. We have included the FACS plots showing the isotype Ab staining we used for gating (Figure 3 B). We have also changed FACS display from "contour plots" to "dot plots" in this figure to further minimize the confusion. We have considerable experiences in FACS analysis following intracellular staining of IFN-g and IL-10 as supported by extensive previous publications (Sun et al, Nat Med. 2009 Mar;15(3):277-84; Sun et al, Nat Immunol. 2011 Apr;12(4):327-34 and Yao et al Mucosal Immunol. 2015 Jul;8(4):746-59). We hope that we made it more clear in this revision that direct type I IFN signaling is required for the optimal IL-10 production by CD8+ T cells in vivo. Furthermore, these in vivo data are supported by our in vitro co-culture experiments in which we have shown direct type I IFN signaling could promote CD8 T cells to produce IL-10 in the presence of IL-2 and IL-27.

As far as reconstitution, we have included data showing reconstitution rate in the lung T cell compartments after infection (Figure S3 A) (lines 297, 298). The reconstitution rate is roughly 40%:60% (KO vs WT) in CD8+ T cell compartment. We did not check reconstitution rate before infection. In this experiment, we examined IFN-g and IL-10 expression specifically in gated WT (CD45.1+) or Ifnar1 KO (CD45.2+) CD8 T cells. We felt it is not essential to examine cell numbers of IL-10 producing-cells as these are mixed bone marrow chimeras and our main point is to determine the relative development (ratio) of IL-10+ cells compared to antigen specific IFN-g+ CD8 T cells in WT or Ifnar1-deficient CD8 T cell population. As shown in Figure 4 C, we found IL-10 producing cells is relative diminished in the absence of IFNAR1 direct signaling when we normalized IL-10 producing cells to IFN-g positive antigen-specific cells.

4. *The paper from Arimori et al (Type I interferon plays opposing roles in cytotoxicity and interferon-γ production by natural killer and CD8 T cells after influenza A virus infection in mice. J Innate Immun. 2014;6(4):456-66) is not cited and discussed.*

   We are sorry for the overlook of the paper. We have cited and discussed the paper in the revision of the manuscript (lines 98, 245-247).

Second Editorial Decision

22-Sep-2016

Dear Dr. Sun,

We are sorry for a slight delay in the peer review of your manuscript. It is a pleasure to provisionally accept your manuscript entitled "Type I interferon signaling facilitates the development of IL-10-producing effector CD8+ T cells during influenza virus infection" 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,
Nadja Bakocevic

on behalf of
Prof. Annette Oxenius

Dr. Nadja Bakocevic
Editorial Office
European Journal of Immunology
e-mail: ejied@wiley.com
www.eji-journal.eu