Peer review correspondence

European Journal of Immunology

TLR9 and STING agonists synergistically induce innate and adaptive type II IFN

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Handling Executive Committee member: Prof. Marco Colonna

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 – 1 September 2014

Dear Dr. Ishii,

Manuscript ID eji.201445132 entitled "Synergistic activity of TLR9- and STING-agonists in innate and adaptive Type–II IFN induction" which you submitted to the European Journal of Immunology has been reviewed.

The comments of the referee are included at the bottom of this letter. A revised version of your manuscript that takes into account the comments of the referee will be reconsidered for publication.

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 before a decision is rendered.

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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,
Laura Soto Vazquez

On behalf of Prof. Marco Colonna

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Reviewer: 1

Comments to the Author
In this manuscript Temizoz and colleagues investigate the therapeutic potential of using TLR9 and STING agonists in combination to promote type I immunity. The authors found that treatment with CpGK and cGAMP, which are sensed by TLR9 and STING respectively, resulted in strong IFNg production by NK and T cells. This synergism could be inhibited or reduced by blocking IFN-I and IL-12p40. Immunization of mice with OVA plus agonists revealed that the combination of CpGK and cGAMP promoted antibody production and a T cell response indicative of type I immunity. In the EG7 tumor model, the authors found that administration of both agonists inhibited tumor growth.

Although this manuscript is of interest, this reviewer has several concerns. In the tumor model, EG7 cells were injected into mice. Since these cells express OVA, why not investigate OVA-specific responses generated with and without adjuvants? There is no data showing cytokine production (i.e. IFN-I, IL-12, IFNg), specific T cells or cellular infiltration in tumors or draining LN. And, since it is presented here that adjuvant therapy enhances NK and CD8 T cell responses, at least with respect to IFNg production, why not evaluate a tumor model(s) that relies on NK and/or CTL for clearance, as evidence suggests that elimination of EG7 is largely independent of NK cells. Another experiment, perhaps more appropriate, would be to immunize mice with OVA plus agonists then challenge the mice with EG7. Additionally, there

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is no data provided in this manuscript that links adjuvant induced antibody responses or IFN-g production to tumor rejection. Was IFNg ever neutralized after adjuvant treatment in the tumor model? The authors consistently measure IFNg as a read out for type I immunity but there is no data showing the effects of adjuvants on cytolytic capacity or proliferation of NK cells or T cells. Finally, the authors may consider using a tumor model that does not express artificial antigens to evaluate therapeutic potential of these adjuvants.

What are levels of IFN-I and IL-12/23 after CpGK and/or cGAMP stimulation of human PBMC and after treatment with blocking Abs? It is interesting that neither CpGK or cGAMP treatment on its own appears to induce any IFNg production by NK cell/T cell in PBMC. However, the combination of both appears to be very potent. What is the mechanism behind this strong synergism? Accordingly, blocking IFN-I alone had no effect on adjuvant-induced IFN-g production. Is this because IL12 production increases in the absence of IFN-I signaling?

The expression of TLR9 and STING are cell-type specific. Expression of TLR9 is more restricted while STING is more ubiquitous. One would expect that targeting both sensors would have a greater impact or affect a larger pool of cells. Which populations in human PBMC are producing IL12 and IFN-I after treatments?

Use of anti-IL12/23 p40 and IL12p40 KO mice will affect IL23 signaling. Is IL23 production induced by these adjuvants and does it have any impact NK cell activation or T cell responses measured in these studies? This should be discussed.

Did the authors assess frequencies of cytokine producing T cells in spleens by intracellular staining after OVA immunization?

In OVA immunization experiments, restimulation of splenocytes with OVA peptides results in little to no IFNg production with OVA peptide 323. So what are the CD4 T cells doing or making in this model?

In contrast to Figure 3, Figure 4 shows no data for mice immunized with OVA and saline.

It is important to show or comment on viability before and after in vitro stimulation/treatments because reduced levels of cytokines may be related to cell death rather than a defect in production.

First revision – authors' response – 3 November 2014

Response to reviewer's comments:

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In this manuscript Temizoz and colleagues investigate the therapeutic potential of using TLR9 and STING agonists in combination to promote type I immunity. The authors found that treatment with CpGK and cGAMP, which are sensed by TLR9 and STING respectively, resulted in strong IFNg production by NK and T cells. This synergism could be inhibited or reduced by blocking IFN-I and IL-12p40. Immunization of mice with OVA plus agonists revealed that the combination of CpGK and cGAMP promoted antibody production and a T cell response indicative of type I immunity. In the EG7 tumor model, the authors found that administration of both agonists inhibited tumor growth.

The authors thank the reviewer for valuaable comments. According to the comments and suggestions we have performed several additional experiments and modified the paper accordingly.

1- Although this manuscript is of interest, this reviewer has several concerns. In the tumor model, EG7 cells were injected into mice. Since these cells express OVA, why not investigate OVA-specific responses generated with and without adjuvants? There is no data showing cytokine production (i.e. IFN-I, IL-12, IFNg), specific T cells or cellular infiltration in tumors or draining LN.

In order to investigate OVA-specific responses generated by the adjuvants, on day 23, we stimulated the spleen cells of the immunized mice with either OVA protein or class I/II specific peptides (OVA257) and measured IFNg production. In response to stimulation with the MHC class I specific peptide (OVA257), significantly higher amounts of IFNg, compared to the control treatment group, were induced only in the mice that have taken the combination treatment. However, this was not the case for OVA or OVA 323 stimulation (data not shown), suggesting that CD8+ T cells that are induced by combination treatment could be responsible for tumor suppression (Fig. S4A). In addition, we confirmed that the anti-tumor effect of the combination, in the EG-7 tumor model, was dependent on the CD8+ T cell activity, rather than the NK cell activity, as the combination failed to suppress the tumor growth in the RAG2 KO mice, which is deficient for T cells, but not for NK cells (Fig. S4B).

2- And, since it is presented here that adjuvant therapy enhances NK and CD8 T cell responses, at least with respect to IFNg production, why not evaluate a tumor model(s) that relies on NK and/or CTL for clearance, as evidence suggests that elimination of EG7 is largely independent of NK cells. Another experiment, perhaps more appropriate, would be to immunize mice with OVA plus agonists then challenge the mice with EG7. Finally, the authors may consider using a tumor model that does not express artificial antigens to evaluate therapeutic potential of these adjuvants.

As the reviewer indicated, since we identified NK cells as the main players in the IFNg synergy in our in vitro human PBMC studies, we also investigated the anti-tumor effect of our combination in the B16 F10 mouse melanoma tumor model, which relies on NK cells for clearance (Chen S. et al, Journal of Experimental Medicine: 202, 1679-1689 (2005)) and does not express artificial antigens. Although cGAMP, itself, could significantly suppress the tumor growth compared to the control group, combination had the strongest anti-tumor effect, resulting in almost complete tumor elimination (Fig. 5B). Thus, our

combination acts a strong anti-tumor agent, capable of suppressing tumors that relies not only on CD8+ T cells, but also on NK cells for clearance.

Moreover, in our tumor models, because we wanted to show immunotherapeutic potential of the combination as an antigen-free anti-cancer agent, rather than its adjuvant activity, instead of challenging the mice after immunization, we treated the tumor bearing mice with intra-tumor injections of the combination without antigen. Thus, our tumor suppression model shows that combination can work as an antigen-free immunotherapeutic agent, in addition to being a potent type 1 adjuvant as shown in our immunization studies.

3- Additionally, there is no data provided in this manuscript that links adjuvant induced antibody responses or IFN-g production to tumor rejection. Was IFNg ever neutralized after adjuvant treatment in the tumor model? The authors consistently measure IFNg as a read out for type I immunity but there is no data showing the effects of adjuvants on cytolytic capacity or proliferation of NK cells or T cells.

According to our tumor suppression experiments in EG-7 tumor model, there is no correlation between the OVA-specific antibody responses and tumor suppression (data not shown), but as significantly higher IFNg levels, compared to control treatment group, were detected only in the spleen cells of the combination treated mice, after OVA257 stimulation (Fig. S4A), it is possible that IFNg, produced by CD8+ T cells, is involved in the mechanisms mediating anti-tumor immune responses that are generated by combination treatment. Furthermore, our in vivo CTL cytotoxicity assay revealed that compared to the PBS, K3 CpG, or cGAMP immunization groups, only combination of K3 CpG and cGAMP could induce strong antigen-specific CD8+ CTL cytotoxicity (Fig. S1B).

4- What are levels of IFN-I and IL-12/23 after CpGK and/or cGAMP stimulation of human PBMC and after treatment with blocking Abs? Accordingly, blocking IFN-I alone had no effect on adjuvant-induced IFN-g production. Is this because IL12 production increases in the absence of IFN-I signaling?

As the reviewer mentioned, the reason why type I IFN does not have any effect on the adjuvant-induced IFNg production could be the increase in the IL-12/23 production after the antibody treatment, as we have showed in the Fig. S6B.

5- It is interesting that neither CpGK or cGAMP treatment on its own appears to induce any IFNg production by NK cell/T cell in PBMC. However, the combination of both appears to be very potent. What is the mechanism behind this strong synergism? The expression of TLR9 and STING are cell-type specific. Expression of TLR9 is more restricted while STING is more ubiquitous. One would expect that targeting both sensors would have a greater impact or affect a larger pool of cells. Which populations in human PBMC are producing IL12 and IFN-I after treatments?

In order to explain our proposed mechanisms for the innate IFNg synergy, we added the Fig S6A and also tried to clarify the mechanisms of this synergy in discussion as below:

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According to our findings, NK cells are the major IFNg-producing cells in the hPBMC culture following combination stimulation (Fig. 1B). On the other hand, previous reports have shown that although NK cells express low levels of TLR9, cells that respond to CpG stimulation are the TLR9-expressing pDCs and B cells in hPBMCs (Hornung V. et al, Journal of Immunoogy: 168, 4531–4537 (2002)). Also, IL-12 and type I IFNs have been reported to regulate IFNg production and cytotoxicity in NK cells (Nguyen KB. et al, Journal of Immunology: 16, 4279–4287 (2002) and Hornung V. et al, Journal of Immunoogy: 168, 4531–4537 (2002)). Given those reports and our in vitro data, our proposed mechanism for the synergistic induction of innate IFNg is that mainly pDCs may respond K3 CpG while, together with pDCs, other cells, such as cDCs or macrophages, may respond to cGAMP to produce high amounts of type I IFNs and IL-12, which then synergize to induce IFNg production in NK cells, by signaling through IL-12 and type I IFN receptors (Fig S6A). So, we also agree with you about that targeting both sensors would have a greater impact by affecting a larger pool of cells, which will contribute to this IFNg synergy.

6- Use of anti-IL12/23 p40 and IL12p40 KO mice will affect IL23 signaling. Is IL23 production induced by these adjuvants and does it have any impact NK cell activation or T cell responses measured in these studies? This should be discussed.

We have discussed this issue in the discussion as below:

The use of anti-IL-12/23p40 neutralizing antibodies in our in vitro studies and IL-12p40 mice in the in vivo studies can't rule out the possible involvement of IL-23 in the mechanisms of innate or adaptive IFNg synergy, as IL-23 signaling, which was shown to affect NK cell activation and T cell responses, will be defective in both experimental designs. Our studies regarding this issue showed that although no antigen-specific IL-17 was detected in the spleen cell cultures of the immunized mice as an indirect indicator of in vivo IL-23 induction, and no IL-23 was induced in mouse PBMCs by combination stimulation, IL-23 is induced in the FL-DCs only by combination stimulation (please see the figure below), but not by cGAMP or K3 CpG stimulations, suggesting a possible role for IL-23 in the mechanisms of innate or adaptive IFNg synergy, which needs further investigation.

7- Did the authors assess frequencies of cytokine producing T cells in spleens by intracellular staining after OVA immunization?

We tried to detect IFNg- and IL-13-producing cells in the spleen cells of the immunized mice after 44 h of stimulation with the antigen. Below, please find the results of one of our experiments, in which frequency of IL-13-/IFNg-producing cells are shown. However, probably because the number of IFN-/IL-13-producing cells are very low, we couldn't observe a strong synergy in the IFNg-producing CD8+ T cells, as observed in the ELISA. But in the combination immunization group, we could observe a significantly higher frequency of IFNg-producing CD4+ T cells, compared to the other groups. These results may also suggest that combination increases the capacity of CD8+ T cells to produce more IFNg, rather than increasing the number of IFNg-producing cells. Also, significantly higher frequencies of IL-13-producing CD8/CD4+ T

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cells were detected in the cGAMP-immunized group, compared to the other groups, similar to our ELISA results.

8- In OVA immunization experiments, restimulation of splenocytes with OVA peptides results in little to no IFNg production with OVA peptide 323. So what are the CD4 T cells doing or making in this model?

Because restimulation of splenocytes with the OVA peptide 323 resulted in little or no IFNg production in spite of the robust CD8+ T cell and Th1-type antibody responses, we suspected the possibility that OVA peptide 323 may not be working properly (degraded/damaged). Thus, in order to understand the functionality of our OVA peptide, we tried stimulating spleen cells of OT-II mouse. As you can see in the figure below, strong IFNg production was induced by OVA323, but not by OVA257, suggesting that the OVA pepride 323 that we use is functioning, properly.

Therefore, we concluded that our combination induces strong CD8+ T cell responses and CD4+ T cell responses that may recognize some other CD4 T cell epitope rather than OVA323. So, we think that the CD4+ T cell responses that are induced by the combination are aiding in the generation of antigen-specific CD8+ T cell and B cell responses.

9- In contrast to Figure 3, Figure 4 shows no data for mice immunized with OVA and saline.

The reason why we didn not have the OVA-only immunization groups in this figure is the availability of the KO mice at the time of the experiments. Also, we didn't think that there would be a significant difference between the OVA-only immunization groups of the WT and KO mice. However, as the reviewer has asked, we performed another experiment, in which we immunized WT or KO mice with the OVA + combination, or OVA-only, using the available KO mice (MyD88 KO and STING mutant), and included the data as Fig. S7, which shows that there is no significant differences between the OVA-only immunization groups of the WT and KO mice.

10- It is important to show or comment on viability before and after in vitro stimulation/treatments because reduced levels of cytokines may be related to cell death rather than a defect in production.

We have included our in vitro cytotoxicity assay results in Fig S5 and commented in the discussion section as below:

Importantly, combination stimulation does not affect cell viability (Fig S5), which may affect cytokine production.

Second Editorial Decision – 5 December 2014

Dear Dr. Ishii,

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It is a pleasure to provisionally accept your manuscript entitled "Synergistic activity of TLR9- and STING-agonists in innate and adaptive Type–II IFN induction" 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,
Karen Chu

on behalf of Prof. Marco Colonna

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