EMBO reports

Bacteria-instructed B cells cross-prime naïve CD8$^+$T cells triggering effective cytotoxic responses

Raquel García-Ferreras, Jesús Osuna-Pérez, Guillermo Ramírez-Santiago, Almudena Méndez-Pérez, Andrés Acosta-Moreno, Lara del Campo, Maria Jose Gomez-Sanchez, Marta Iborra, Beatriz Herrero-Fernández, José María González-Granado, Francisco Sánchez-Madrid, Yolanda Carrasco, Patricia Boya, Nuria Martinez-Martin, and Esteban Veiga
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Corresponding author(s): Esteban Veiga (eveiga@cnb.csic.es)

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Transaction Report:

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1st Editorial Decision **28th Oct 2022**

Dear Dr. Veiga

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting, but they also raise a number of concerns and have suggestions on how to further strengthen your study, which should be addressed.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (January 29th, 2023). Please discuss the revision progress ahead of this time with me if you require more time to complete the revisions.

Moreover, please contact me if you wish to discuss the revisions further in a Zoom meeting or if you have any questions.

You can either publish the study as a short report or as a full article. For short reports, the revised manuscript should not exceed 27,000 characters (including spaces but excluding materials & methods and references) and 5 main plus 5 expanded view figures. The results and discussion sections must further be combined, which will help to shorten the manuscript text by eliminating some redundancy that is inevitable when discussing the same experiments twice. For a normal article there are no length limitations, but it should have more than 5 main figures and the results and discussion sections must be separate. In both cases, the entire materials and methods must be included in the main manuscript file.

*****IMPORTANT NOTE:
We perform an initial quality control of all revised manuscripts before re-review. Your manuscript will FAIL this control and the handling will be DELAYED if the following APPLIES:

1) A data availability section providing access to data deposited in public databases is missing. If you have not deposited any data, please add a sentence to the data availability section that explains that.

2) Your manuscript contains statistics and error bars based on n=2. Please use scatter blots in these cases. No statistics should be calculated if n=2.

When submitting your revised manuscript, please carefully review the instructions that follow below. Failure to include requested items will delay the evaluation of your revision.*****

When submitting your revised manuscript, we will require:

1) a .docx formatted version of the manuscript text (including legends for main figures, EV figures and tables). Please make sure that the changes are highlighted to be clearly visible.

2) individual production quality figure files as .eps, .tif, .jpg (one file per figure).
Please download our Figure Preparation Guidelines (figure preparation pdf) from our Author Guidelines pages https://www.embopress.org/page/journal/14693178/authorguide for more info on how to prepare your figures.

3) a .docx formatted letter INCLUDING the reviewers' reports and your detailed point-by-point responses to their comments. As part of the EMBO Press transparent editorial process, the point-by-point response is part of the Review Process File (RPF), which will be published alongside your paper.

4) a complete author checklist, which you can download from our author guidelines (). Please insert information in the checklist that is also reflected in the manuscript. The completed author checklist will also be part of the RPF.

5) Please note that all corresponding authors are required to supply an ORCID ID for their name upon submission of a revised manuscript (). Please find instructions on how to link your ORCID ID to your account in our manuscript tracking system in our Author guidelines
()

6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

- For the figures that you do NOT wish to display as Expanded View figures, they should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content. Appendix figures should be referred to in the main text as: "Appendix Figure S1, Appendix Figure S2" etc. See detailed instructions regarding expanded view here:

- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) At EMBO Press we ask authors to provide source data for the main and EV figures. Our source data coordinator will contact you to discuss which figure panels we would need source data for and will also provide you with helpful tips on how to upload and organize the files.

Additional information on source data and instruction on how to label the files are available .

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10) Figure legends and data quantification:
The following points must be specified in each figure legend:

- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
- the nature of the bars and error bars (s.d., s.e.m.)
- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.

Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.

See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat

- Please also include scale bars in all microscopy images.

11) Our journal encourages inclusion of *data citations in the reference list* to directly cite datasets that were re-used and obtained from public databases. Data citations in the article text are distinct from normal bibliographical citations and should directly link to the database records from which the data can be accessed. In the main text, data citations are formatted as follows: "Data ref: Smith et al, 2001" or "Data ref: NCBI Sequence Read Archive PRJNA342805, 2017". In the Reference list, data citations must be labeled with "[DATASET]". A data reference must provide the database name, accession number/identifiers and a resolvable link to the landing page from which the data can be accessed at the end of the reference. Further instructions are available at .

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We would also welcome the submission of cover suggestions, or motifs to be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready and please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

This is an interesting report about B cells that are able to cross-prime CD8 T cells after taking up Listeria from DCs and presenting antigenic peptides on MHC I. The authors call the process they have found as "bacteria-trained B cells", a term which I find misleading and it should be avoided. They define this term as "bacterial encounter that trains B cells that acquire antigen presentation abilities". However, B cells always have antigen presentation abilities towards antigens which they take up by the BCR and present as peptides on MHC II. This is mixed up throughout the manuscript. The new thing here is the cross-priming of bacterial antigens and subsequent T cell activation. I would strongly suggest to avoid the term "bacteria-trained B cells", as it is completely unclear. Besides this, the study is interesting and mostly well done and contains many interesting data. Some other points should be addressed, as well:

1) In Fig. 1 B, C and movie EV1 I do not see the intracellular location of Listeria in B cells very well. It looks as they would stick to the membrane, in actin-rich regions. If so, can the authors exclude that what they see in Fig.1G (labels missing) is just loss of Listeria from the membrane of B cells, in contrast to HELA cells? Killing of bacteria is not shown.

2) Most other figures are well done and well controlled. In Fig. 2 A-D, what happens without bacteria ? Here a control is missing. The CD80 and CD86 up regulation could just be a consequence of DC-B cell interaction in the MD4-HEL system.

3) Fig.5. Is there any significant difference in the survival curves detected? Please indicate clearly.

Referee #2:

Manuscript Nr.: EMBOR-2022-56131V1
Garcia-Ferreras et al., "Bacteria-trained B cells cross-prime naïve CD8+ T cells triggering effective cytotoxic responses"

The authors demonstrate that Listeria bacteria can enter B cells through contact with infected bone-marrow derived dendritic cells (DCs). This transfer, however, was performed with hen egg lysozyme (HEL) coated DCs and HEL specific B cell receptor (BCR) transgenic B cells. While bacteria were also transferred without decorating the infected DCs with HEL, BCR engagement via antigen on the DC surface increased bacterial transfer 3fold. B cells that had taken up transgenic bacteria expressing ovalbumin (OVA) were able to form mature immunological synapses with OT-1 CD8+ T cells. Accordingly, they were able to stimulate OT-1 proliferation and activation marker up-regulation (CD25). Listeria cross-presentation was unaltered when the DCs were H2 class I mismatched but reduced when the B cells were TAP deficient. Furthermore, listeriolysin deficient bacteria were less well cross-presented. Interestingly, Atg16L1 deficient B cells were also less able to perform this cross-presentation. Finally, the authors transferred different B cell populations with and without naïve CD8+ T cells into Rag1 deficient mice for challenge by Listeria infection or OVA expressing tumor cell transfer. They observed that only antigen presenting B cells in the presence of CD8+ T cells somewhat controlled Listeria infection and tumor growth. From these findings the authors conclude that bacteria cross-presenting B cells can efficiently prime protective CD8+ T cell responses.

The reported findings are interesting but some of the underlying cell biology and the efficacy of cross-presentation in B cells compared to DCs need to be clarified.

Major comments:
1. The reported in vivo experiments suggest that B cell can significantly contribute to protective anti-Listeria and anti-melanoma CD8+ T cell responses in vivo. However, it remains unclear how their cross-presentation capacity compares to DCs or if they compensate a diminished cross-presentation capacity by higher numbers. Therefore, the authors should perform normalized experiments in which both DCs and B cells capture similar amounts of OVA transgenic Listeria and then test how efficiently they can stimulate CD8+ T cells with titrated effector to target ratios.
2. DCs are often superior in their ability to induce potent CD8+ T cell responses. The authors only reported CD8+ T cell proliferation, activation marker up-regulation and cytotoxicity Can antigen cross-presenting B cells also induce Th1 associated cytokine production by primed CD8+ T cells (IFN-gamma and other cytokines)? Furthermore, acquisition of cytotoxic potential,

both expression of perforin and granzyme as well as degranulation in response to EL-4 cells should be measured in comparison to DC mediated priming.
3. The presented data seem to suggest that bacteria are transferred from infected DCs to B cells and then Atg16L1, listeriolysin and TAP are required for the observed cross-presentation. The authors should clarify which cellular pathways they envision. Do they suggest that Listeria are taken up then escape the endosome via listeriolysin and are then captured in autophagosomes. How would they then reach TAP for import into the ER and presentation on MHC class I molecules. Could Atg16L1 also redirect bacteria containing phagosomes via directly coupling LC3 to them, and Listeria or Listeria fragments possibly escape from these more efficiently for TAP import and presentation? Are bacteria taken up into LC3 positive endosomes or found in p62 positive autophagosomes? Do the authors have evidence that autophagy versus LC3 associated phagocytosis is involved in the observed cross-presentation? Is proteasomal or lysosomal antigen processing involved?

Referee #3:

The study deals with the role of B cells in crosspresentation to CD8+ T cells, the interaction with iDC and the relevance of this mechanism for clearance of intracellular bacterial infection. The manuscript is concise and clear, experiments are well described and technically sound and all controls well designed.

This is a very solid study containing many different experiments that altogether allow the conclusion that B cells are esssential for mounting bacterial antigen-specific CD8+ T cell responses. The finding is not new. Thus, despite the multitude of data I suggest that the authros revise the discussion to better clarify which of their findings are novel and not incremental advance towards known findings. It is further necessary to better connect the Tumor model to the infection model by better explaining why the authors believe that the experiment provides additional Information not seen in the infection model. Thus, the discussion should be expanded and more attention should be given to previous wor by other authors and models used (citations).

Referee #1:

This is an interesting report about B cells that are able to cross-prime CD8 T cells after taking up Listeria from DCs and presenting antigenic peptides on MHC I. The authors call the process they have found as "bacteria-trained B cells", a term which I find misleading and it should be avoided. They define this term as "bacterial encounter that trains B cells that acquire antigen presentation abilities". However, B cells always have antigen presentation abilities towards antigens which they take up by the BCR and present as peptides on MHC II. This is mixed up throughout the manuscript. The new thing here is the cross-priming of bacterial antigens and subsequent T cell activation. I would strongly suggest to avoid the term "bacteria-trained B cells", as it is completely unclear. Besides this, the study is interesting and mostly well done and contains many interesting data. Some other points should be addressed, as well:

We aimed to use the term “train” referring exclusively to the cross-presentation ability observed in B cells after *Listeria monocytogenes* encounter. It is true, as noticed by the reviewer, that it was not well explained in the text as we did not clearly exclude presentation by MHC-II. Note that we did not detect MCH-II presentation of bacterial antigens in BacB cells. We understand that the term “training”, in addition, may induce to confusion because it was coined for cells of the innate immunity that were able to develop a memory-like function (exclusive for adaptive immunity) after encounter with pathogens. So, following the suggestion of the reviewer 1, we decided to change the name of this phenomenon (B cells acquiring cross-presentation abilities after (some) bacteria encounter) and term it **bacteria instruction**. Therefore, BacB cells are from now on **bacteria-instructed B cells,** when only some bacteria are able to instruct B cells, not being a general mechanism occurring always (nor *Listeria* ΔLLO, neither *E. coli*, were able to trigger BacB-mediated cross-presentation)**.** We modified the text in order to make clear throughout the manuscript that bacteria-induced instruction of B cells refers to the acquired ability of cross-presentation. For example**,** the phrase in the abstract “bacterial encounter that trains B cells that acquire antigen presentation abilities" is now re-phrased as “Bacterial encounter ‘***instructs***’ the B cells that acquire antigen cross-presentation abilities, in a process that involves autophagy”.

1) In Fig. 1 B, C and movie EV1 I do not see the intracellular location of Listeria in B cells very well. It looks as they would stick to the membrane, in actin-rich regions. If so, can the authors exclude that what they see in Fig.1G (labels missing) is just loss of Listeria from the membrane of B cells, in contrast to HELA cells? Killing of bacteria is not shown.

The new version includes new experiments of bacteria inside B cells detected by confocal imaging where B cell surface was staining using anti-IgM antibody. Bacteria can be detected clearly inside B cells (new **Figure EV 1B,C**) and new **Movies EV2** and **EV3**. Bacteria internalization was quantified by classical gentamicin survival assays, where all extracellular bacteria were killed by the antibiotic. Bacteria associated to B cells but not internalized, even if present in actin rich structures, would be killed.

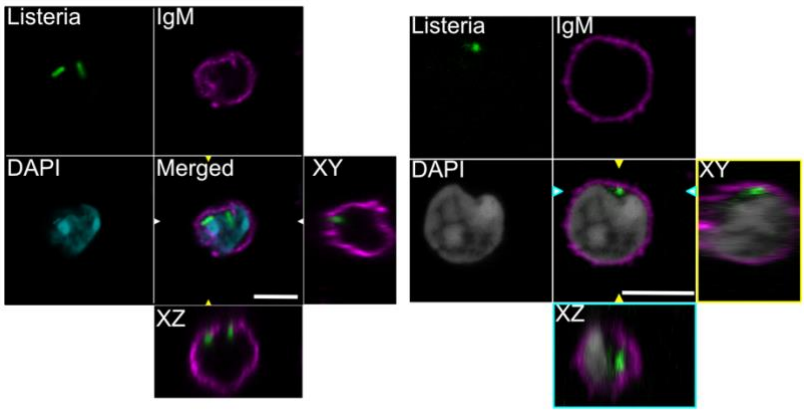

***Figure Legend**. **B-C** Confocal images of B cells from WT mice that have captured L. monocytogenes (green), after 4 hours of infected-DC/B cell contacts. B cell surface was detected using an anti IgM antibody (magenta) the B cell nucleus was stained with DAPI (cyan in B and grey in C). The orthogonal views show intracellular bacteria.*

To further analyze bacteria capture by B cells and in order to determine the cellular mechanisms involved in bacterial destruction, B cells capturing bacteria were treated with different inhibitors;
•Leupeptin + NH_4Cl. Leupeptin is an inhibitor of cysteine, serine and threonine peptidases in lysosomes and the ammonium chloride inhibit the acidic degradation in the lysosomes.
•Pepstatin A + E64D. Pepstatin A is an inhibitor of Cathepsin D and E64d inhibits cathepsin B
•Lactacystin is an inhibitor of the proteasome.

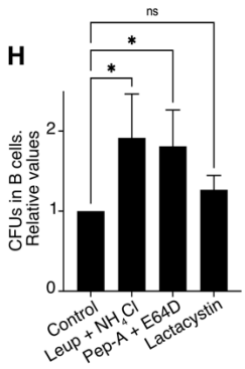

***Figure legend**: WT B cells were allowed to form conjugates with Listeria-infected DC, after 30 min samples were treated with the indicated inhibitors; Leupeptin + NH_4Cl, pepstatin-A + E64D, lactacystin, or PBS for controls. One hour later gentamicin was added to kill extracellular bacteria and two hours later B cells were reisolated by negative selection, lysed and plated in agar-containing plates. CFUs were counted; each colony represents one live intracellular bacterium inside B cells. Column bars represent the mean of at least three independent experiments showed intracellular bacteria respect to the control (non-treated samples). Error bars indicate the sd. Significant differences are represented by asterisk, non-significant differences are marked as (ns).*

As it is shown in new **Figure 1H,** both the combination of (Pepstatin A + NH_4Cl) and (Pepstatin A + E64D) partially inhibited intracellular bacterial degradation, suggesting that lysosome degradation plays an important role in intracellular bacterial clearance inside B cells. On the contrary, lactacystin did not impede bacterial degradation inside B cells.

2) Most other figures are well done and well controlled. In Fig. 2 A-D, what happens without bacteria ? Here a control is missing. The CD80 and CD86 up regulation could just be a consequence of DC-B cell interaction in the MD4-HEL system.

BCR activation (HEL system) is not necessary to trigger B cell instruction (cross-presentation of bacteria antigens). We found no differences in antigen cross-presentation abilities of BacB cells from WT or MD4 mice (HEL-activated). The differences (between B cells from WT vs MD4 mice) were detected only in bacteria capture and only during the first hours after B cell/infected-DC contacts. We decided therefore to perform most of the experimentation using WT B cells which is a more physiological setting that MD4 B cells/HEL decorated DC, as indicated in the manuscript.

The proposed experiments have already been performed in WT cells, not using the HEL system (Figure 4) using different bacteria (some of which do not stimulate neither the upregulation of MHC expression or CD80/86). The bacteria that did not induce cross-presentation show lower levels of CD80 CD86 and MHC-I.

Please find attached to this *responses-to-the-referees* letter a figure (not included in the manuscript) comparing the levels of CD80, CD86 and MHC-I in B cells instructed with *Listeria* or not instructed (B cells that have contacted non-infected, LPS-activated DC)

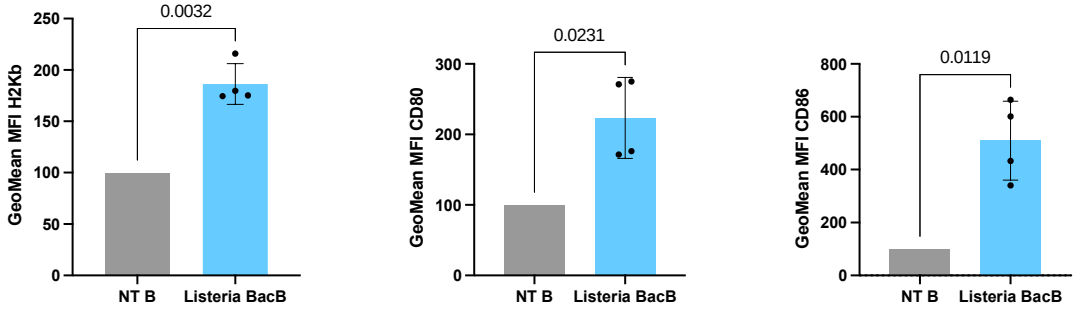

***Figure Legend**. Surface expression of H-2Kb, CD80, and CD86 in B cells that contacted non-infected, LPS-activated DC, (B cells not instructed; NT B, grey) or L. monocytogenes-infected, LPS-activated DC (Listeria BacB; blue). Panels show the relative data respect to NT B. The mean and the SD of four independent experiments* and the P values (always < 0.05) are shown.

3) Fig.5. Is there any significant difference in the survival curves detected? Please indicate clearly.

It is now indicated in the figure and the figure legend.

Referee #2:

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The authors demonstrate that Listeria bacteria can enter B cells through contact with infected bone-marrow derived dendritic cells (DCs). This transfer, however, was performed with hen egg lysozyme (HEL)

coated DCs and HEL specific B cell receptor (BCR) transgenic B cells. While bacteria were also transferred without decorating the infected DCs with HEL, BCR engagement via antigen on the DC surface increased bacterial transfer 3fold. B cells that had taken up transgenic bacteria expressing ovalbumin (OVA) were able to form mature immunological synapses with OT-1 CD8+ T cells. Accordingly, they were able to stimulate OT-1 proliferation and activation marker up-regulation (CD25). Listeria cross-presentation was unaltered when the DCs were H2 class I mismatched but reduced when the B cells were TAP deficient. Furthermore, listeriolysin deficient bacteria were less well cross-presented. Interestingly, Atg16L1 deficient B cells were also less able to perform this cross-presentation. Finally, the authors transferred different B cell populations with and without naïve CD8+ T cells into Rag1 deficient mice for challenge by Listeria infection or OVA expressing tumor cell transfer. They observed that only antigen presenting B cells in the presence of CD8+ T cells somewhat controlled Listeria infection and tumor growth. From these findings the authors conclude that bacteria cross-presenting B cells can efficiently prime protective CD8+ T cell responses.

The reported findings are interesting but some of the underlying cell biology and the efficacy of cross-presentation in B cells compared to DCs need to be clarified.

To reply to the referee´s concerns about autophagy I contacted an expert in autophagy, Prof. Patricia Boya (Department of Neuroscience. University of Fribourg.1700 Fribourg, Switzerland). She helped us in experimental design and give us reagents.

Major comments:

1. The reported in vivo experiments suggest that B cell can significantly contribute to protective anti-Listeria and anti-melanoma CD8+ T cell responses in vivo. However, it remains unclear how their cross-presentation capacity compares to DCs or if they compensate a diminished cross-presentation capacity by higher numbers. Therefore, the authors should perform normalized experiments in which both DCs and B cells capture similar amounts of OVA transgenic Listeria and then test how efficiently they can stimulate CD8+ T cells with titrated effector to target ratios.

Bacterial instruction requires at least 24h and most of the DC died after 24 in the presence of *Listeria* (new Fig EV2B), surprisingly B cells are more resistant.

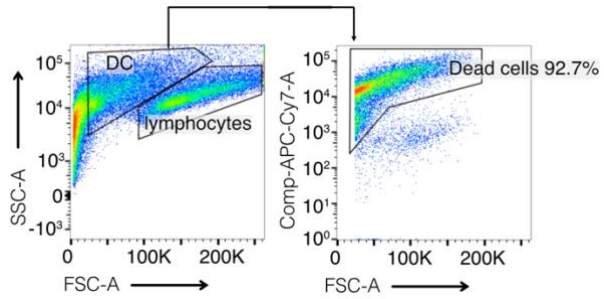

***Figure legend**: Analysis of DC viability during B cells instruction. DC population was taken by size and complexity after 24 infected-DC/B cells conjugates formation. Cell viability of DC population was further analyzed using Live/Dead Fixable Viability Near-IR staining. More that 90% of the DC died during the instruction protocol.*

As bacteria damage DC, we already performed another experiment using LPS-activated DC to present OVA antigen by decorating DC with OVAp. This maximizes the antigen presenting ability of DC. Even in this condition that favors DC-mediated antigen presentation, we observed higher CD8+ T cell proliferation when activated with BacB cells (Figure 3E).

2. DCs are often superior in their ability to induce potent CD8+ T cell responses. The authors only reported CD8+ T cell proliferation, activation marker up-regulation and cytotoxicity Can antigen cross-presenting B cells also induce Th1 associated cytokine production by primed CD8+ T cells (IFN-gamma and other cytokines)? Furthermore, acquisition of cytotoxic potential, both expression of perforin and granzyme as well as degranulation in response to EL-4 cells should be measured in comparison to DC mediated priming.

In the revised version we included the analysis of IFN-γ, granzyme B and perforin expression in: naïve CD8+ T cells, polyclonally activated (using antibodies anti CD3 + anti CD28) CD8+ T cells, CD8+ T cells activated with OVAp-loaded splenocytes and CD8+ T cells activated with BacB cells (capturing *Listeria* OVA). As shown in new Fig. 3 J-L similar, and even larger, levels of IFN-γ, granzyme B and perforin expression were observed in CD8+ T cells activated by BacB cells compared to the positive controls.

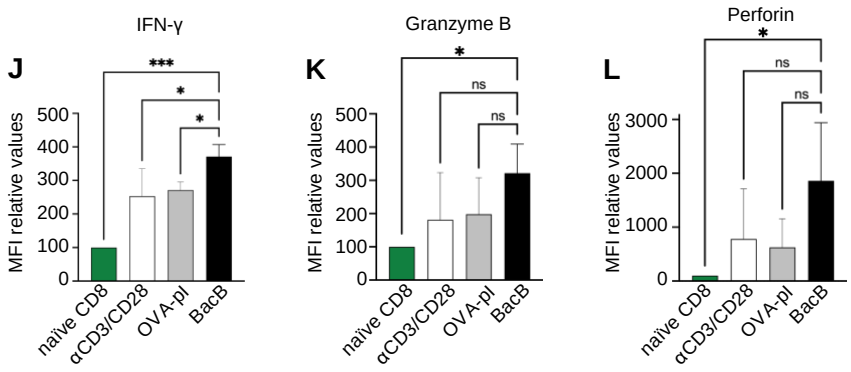

***Figure legend**: Intracellular expression, measured by flow cytometry, of the indicated proteins in non-activated naïve CD8+ t cells or in CD8+ T cells activated with either αCD3 + αCD28 antibodies, OVA-decorated splenocytes (OVA-pI), or Listeria-OVA BacB cells (BacB). The relative mean fluorescence intensity (MFI) and the sd corresponding to three independent experiments is shown. Statistical significant differences analyzed by ANOVA is represented by *.*

3. The presented data seem to suggest that bacteria are transferred from infected DCs to B cells and then Atg16L1, listeriolysin and TAP are required for the observed cross-presentation. The authors should clarify which cellular pathways they envision. Do they suggest that Listeria are taken up then escape the endosome via listeriolysin and are then captured in autophagosomes. How would they then reach TAP for import into the ER and presentation on MHC class I molecules. Could Atg16L1 also redirect bacteria containing phagosomes via directly coupling LC3 to them, and Listeria or Listeria fragments possibly escape from these more efficiently for TAP import and presentation? Are bacteria taken up into LC3 positive endosomes or found in p62 positive autophagosomes? Do the authors have evidence that autophagy versus LC3 associated phagocytosis is involved in the observed cross-presentation? Is proteasomal or lysosomal antigen processing involved?

In an effort to respond to the reviewer concern, we analyzed the autophagic flux in BacB cells to determine whether bacteria internalization triggers canonical or non-canonical autophagy (LC3 associated phagocytosis, or LAP, is considered as non-canonical autophagy). We followed the protocol as described in (Martinez-Martin *et al*, 2017). Briefly, we measured by flow cytometry the accumulation of vesicular LC3 using bafilomycin A1 (BafA1) or chloroquine, two commonly used autophagy inhibitors, which block LC3 degradation differently. The results from flow cytometry analyses were quantitated using an autophagy index (AI), wherein we defined total autophagy flux as the mean fluorescence intensity (MFI) after chloroquine treatment, canonical autophagy flux as the MFI after BafA1 treatment, and the noncanonical autophagy flux as the difference between the two normalized by the MFI of LC3 before inhibitor addition. *Listeria* exposure triggered a three-fold increase in non-canonical autophagy in BacB cells, compared to non-instructed B cells (Fig. EV4 A). This increase in non-canonical autophagy was not observed when using *Listeria* Δhly, which maintain levels similar to those observed in non-instructed B cells (Fig. EV4 A). No differences were detected in any condition regarding canonical autophagy. Therefore *Listeria*-mediated instruction promote both, the increase of non-canonical autophagy and antigen cross-presentation in BacB cells.

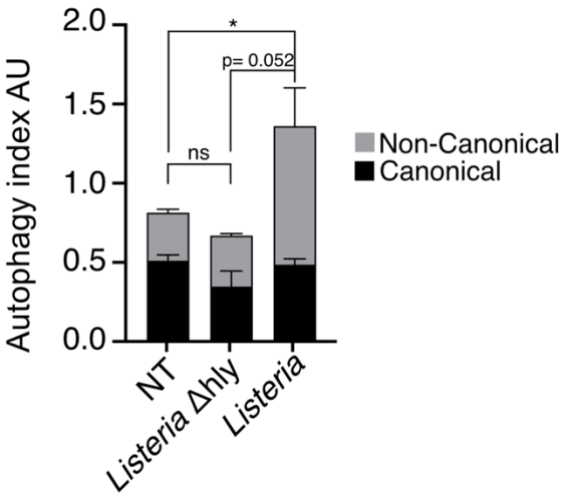

***Figure Legend.** Autophagy index (AI) measuring LC3 by flow cytometry. The total autophagy flux is defined as the mean fluorescence intensity (MFI) after chloroquine treatment, the canonical autophagy flux as the MFI after BafA1 treatment, and the noncanonical autophagy flux as the difference between the two normalized by the MFI of LC3 before inhibitor addition. The Bar charts show the mean of the Autophagic Index in non-instructed B cells (B cells contacting non-infected DC), and BacB cells instructed with either L. monocytogenes or L. monocytogenes Δhly, from three independent experiments. No statistical differences between the groups were noticed in canonical autophagy. The statistical differences between groups related to non-canonical autophagy are shown; * p <0.05. No statistical differences is marked as "ns". AU, arbitrary units. Error bars indicate SEM*

With the data we obtained, i.e. BacB cells capturing *Listeria*, but not *Listeria ⊿hly,* trigger non-canonical autophagy and develop cross-presentation abilities. Moreover, Atg16L1-depend autophagy is necessary for such cross-presentation, which is also dependent on TAP-1, we can speculate on the molecular mechanisms occurring in BacB cells. It is known that LLO protein is sufficient to trigger autophagy, even in the absence of bacteria (Meyer-Morse *et al*, 2010). So, the presence of LLO could prepare BacB cells for cross-presentation. In parallel bacteria or bacterial products could escape the phagosome by the pore forming activity of LLO and this could help antigens cargo in MCH-I in the ER via TAP-1.

This theory deserves further investigations. Several studies relate autophagy with the MHC class I cross-presentation. However, the importance of autophagy as a source of antigen for cross-presentation and the molecular mechanisms behind remains to be defined despite a global effort (Øynebråten, 2020).

Referee #3:

The study deals with the role of B cells in crosspresentation to CD8+ T cells, the interaction with iDC and the relevance of this mechanism for clearance of intracellular bacterial infection. The manuscript is concise and clear, experiments are well described and technically sound and all controls well designed.

This is a very solid study containing many different experiments that altogether allow the conclusion that B cells are esssential for mounting bacterial antigen-specific CD8+ T cell responses. The finding is not new. Thus, despite the multitude of data I suggest that the authros revise the discussion to better clarify which of their findings are novel and not incremental advance towards known findings.
It is further necessary to better connect the Tumor model to the infection model by better explaining why the authors believe that the experiment provides additional Information not seen in the infection model. Thus, the discussion should be expanded and more attention should be given to previous wor by other authors and models used (citations).

We revised and discuss what is known about antigen cross-presentation by B cells and clarify the advances of this work from what was already known. We included these paragraphs in the discussion:

"Antigen cross-presentation is important for the activation of naïve CD8⁺ T cells and the generation of a cytotoxic response. It is widely believed that only some DCs subsets have the ability to cross-present antigens. Contrary to this theory, several reports have shown that B cells can cross-present external antigens. Coupling the TLR9 ligand CpG to OVA enhanced non-cognate B cell endocytosis ability and promoted B cell activation. CpG-OVA complex-loaded B cells cross-primed CD8 T cells in vivo (Heit *et al*, 2004)*. Using plasmid-encode antigen as a DNA vaccination tool, Hon and colleagues found that B cells mediate cross-priming of antigen-specific CD8 T cells* (Hon *et al*, 2005)*. Furthermore, BCR-recognition of specific antigen drove an efficient antigen uptake and cross-presentation in MHC class-I, stimulating CD8 T cells responses in vitro comparable to those ones elicited by bone marrow-derived DCs* (Robson *et al*, 2008)*. The authors used immune-stimulating complexes where OVA and HEL proteins were incorporated to stimulate HEL-specific B cells and, thus, the uptake of immune complexes triggered by cognate BCR/antigen interactions. Immunization studies indicated that HEL-specific B cells have a major role in maintaining the clonal expansion of OVA-specific CD8 T cells* (Robson *et al*, 2008)*. This study set up the groundwork of targeting B cells for promoting CD8 T cell responses as a therapeutic tool. In agreement with these studies, mouse B cells have been shown to prime antigen specific cytotoxic response following DNA stimulation better than DCs in vivo* (Colluru & McNeel, 2016)*. Besides, Mariño and colleagues found in an autoimmune mouse model of diabetes that B cells cross-present autoantigens to self-reactive CD8⁺ T cells by MHC class-I and BCR-dependent mechanisms* (Mariño *et al*, 2012)*.*
Here, we have demonstrated that BacB cells cross-prime naïve CD8⁺ T cells triggering a potent cytotoxic response. The cytotoxic activity of CTLs activated by BacB cells cross-priming was higher than that observed in CTLs activated by OVAp-decorated splenocytes. A previous study found that cross-presentation of Salmonella antigens by B cells result in the activation of a CD8⁺ cytotoxic T cell response in vitro, requiring the help of CD4⁺ T cells. The mechanism of cross-presentation of Salmonella antigens by B cells is unknown (de Wit *et al*, 2010)*. In our model cross-presentation occurred when bacteria-instructed B cells were able to activate autophagy. Autophagy is related to many physiological functions, such as development, differentiation, homeostasis and renewal of the immune system cell. Moreover, autophagy has been suggested to participate in antigen presentation* (English *et al*, 2009; Mintern *et al*, 2015; Münz, 2016) *and the innate immune response to pathogens* (Germic *et al*, 2019; Tao & Drexler, 2020)*. Our results matched with previous observations about the requirement of endosomal acidification, proteasomal processing and classical MHC class-I/peptide transport for cross-presentation by B cells* (Robson *et al*, 2008)*. Here, we have described the non-canonical autophagy as a crucial pathway in BacB cell instruction, which is necessary to cross-prime naïve CD8⁺ T cells. The bacteria unable to activate non-canonical autophagy were not able to trigger the activation of CD8⁺ T cells."*

We also changed the linkage of the proof-of-concept experiment showing the antitumor potential of BacB as a novel immunotherapy to make the rationale behind it clearer.

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Dear Dr. Veiga

Thank you for the submission of your revised manuscript to EMBO reports. We have now received the full set of referee reports that is copied below.

As you will see, all referees are very positive about the study and request only minor changes to clarify text and figures.

From the editorial side, there are also a few things that we need before we can proceed with the official acceptance of your study.

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With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

all concerns addressed.

Referee #2:

Manuscript Nr.: EMBOR-2022-56131V2
Garcia-Ferreras et al., "Bacteria-instructed B cells (BacB) cross-prime naïve CD8+ T cells triggering effective cytotoxic responses"

The authors demonstrate that Listeria bacteria can enter B cells through contact with infected bone-marrow derived dendritic cells (DCs). This transfer, however, was performed with hen egg lysozyme (HEL) coated DCs and HEL specific B cell receptor (BCR) transgenic B cells. While bacteria were also transferred without decorating the infected DCs with HEL, BCR engagement via antigen on the DC surface increased bacterial transfer 3fold. B cells that had taken up transgenic bacteria expressing ovalbumin (OVA) were able to form mature immunological synapses with OT-1 CD8+ T cells. Accordingly, they were able to stimulate OT-1 proliferation and activation marker up-regulation (CD25). Listeria cross-presentation was unaltered when the DCs were H2 class I mismatched but reduced when the B cells were TAP deficient. Furthermore, listeriolysin deficient bacteria were less well cross-presented. Interestingly, Atg16L1 deficient B cells were also less able to perform this cross-presentation. Finally, the authors transferred different B cell populations with and without naïve CD8+ T cells into Rag1 deficient mice for challenge by Listeria infection or OVA expressing tumor cell transfer. They observed that only antigen presenting B cells in the presence of CD8+ T cells somewhat controlled Listeria infection and tumor growth. From these findings the authors conclude that bacteria cross-presenting B cells can efficiently prime protective CD8+ T cell responses.

In their revised manuscript version, the authors have addressed several of my concerns. They claim to cannot use OVA encoding Listeria for DC cross-presentation due to toxicity, but do not present a titration curve to investigate at which Listeria to DC ratio DCs would still survive, and if then cross-priming of OVA specific T cells would occur. They now demonstrate that T cells primed by OVA cross-presenting B cells also produce IFN-gamma. Finally, they suggest that Listeria primarily induced non-canonical autophagy in B cells, suggesting that phagosome modification by the autophagy machinery might allow for the listeriolysin dependent release of OVA from phagosomes, presumably proteasomal degradation and then import into the ER via TAP for presentation on MHC class I molecules to CD8+ T cells. Thus, the revised manuscript provides more insights into mechanism and T cell differentiation upon priming by OVA cross-presenting B cells.

Minor comments:
1. The authors should point out some limitations of their study, namely that the cross-presenting ability strongly depends on a pore forming protein of the used bacterium (Listeria). The ability of Listeria to release antigen from phagosomes is superior to most other bacteria. Therefore, the authors should call their B cells Listeria-instructed, because this most likely does not reflect generally bacteria-instructed B cells.
2. The manuscript would benefit from a schematic figure that explains the authors' mechanistic hypothesis how Atg16L1, listeriolysin and TAP work together for cross-presentation. With the new data in the revised manuscript version, non-canonical autophagy coupling LC3 to the phagosome membranes presumably allows listeriolysin to more efficiently damage this vesicular compartment for OVA release, possibly due to altered trafficking of LC3 labelled vesicles, followed by antigen release into the cytosol, proteasomal degradation and finally import into the ER via TAP. In the ER MHC class I might then be loaded and stimulate CD8+ T cells after transport to the cell surface.
3. Since the authors did not compare DCs directly with B cells (titrating OVA-Listeria and APC to T cell ratios) for their cross-presenting ability but rather use the argument that peptide pulsed and LPS matured DCs were less efficient than OVA-Listeria exposed B cells, they should include a sentence that DCs might still be more efficient in antigen cross-presentation, in particular when the antigenic cargo does not encode a pore forming protein that facilitates antigen release into the cytosol.

Referee #2:

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Minor comments:
1. The authors should point out some limitations of their study, namely that the cross-presenting ability strongly depends on a pore forming protein of the used bacterium (Listeria). The ability of Listeria to release antigen from phagosomes is superior to most other bacteria. Therefore, the authors should call their B cells Listeria-instructed, because this most likely does not reflect generally bacteria-instructed B cells.

In agreement with the reviewer, we made clear throughout the manuscript, including in the synopsis image, that instruction is a process that (among the bacteria tested) requires *Listeria*. We, however cannot exclude that other bacteria could induce similar instruction process. We are currently working on it

2. The manuscript would benefit from a schematic figure that explains the authors' mechanistic hypothesis how Atg16L1, listeriolysin and TAP work together for cross-presentation. With the new data in the revised manuscript version, non-canonical autophagy coupling LC3 to the phagosome membranes presumably allows listeriolysin to more efficiently damage this vesicular compartment for OVA release, possibly due to altered trafficking of LC3 labelled vesicles, followed by antigen release into the cytosol, proteasomal degradation and finally import into the ER via TAP. In the ER MHC class I might then be loaded and stimulate CD8+ T cells after transport to the cell surface.

We included now a synopsis image reflecting the relevant features of antigen cross-presentation by BacB cells. We agree with the reviewer ´s opinion, however, the role of LC3 and LLO regarding TAP-1-dependent antigen cross-presentation, beyond participating in autophagy, is at this moment, speculative.

3. Since the authors did not compare DCs directly with B cells (titrating OVA-Listeria and APC to T cell ratios) for their cross-presenting ability but rather use the argument that peptide pulsed and LPS matured DCs were less efficient than OVA-Listeria exposed B cells, they should include a sentence that DCs might still be more efficient in antigen cross-presentation, in particular when the antigenic cargo does not encode a pore forming protein that facilitates antigen release into the cytosol.

This point was specifically addressed (Fig EV2C) and discussed throughout the manuscript. The differential role of DC- vs bacB- mediated antigen cross-presentation and their physiological roles were, in addition, discussed in the *in vivo* experiments showing that cross-presenting B cells are required for a complete control of bacterial infections (Fig. 5E).

Dr. Esteban Veiga
CSIC
Molecular and Cellular Biology
darwin 3
CNB-CSIC
Madrid 28049
Spain

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The data shown in figures should satisfy the following conditions:

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Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
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Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
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Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
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