Phosphodiesterase 4A confers resistance to PGE2-mediated suppression in CD25+/CD54+ NK cells

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(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)
Dear Dr. Lundqvist,

Thank you for the submission of your research manuscript to our journal. Since my colleague Achim Breiling is currently on vacation, I have taken over the handling of your manuscript for the time being. 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 point out several concerns and have a number of suggestions for how the study should be strengthened. The role of AMPK should be explored and explained, the different effect of IL-2 and IL-15 be further supported and the mechanism of cytotoxicity of CD25+CD45+ NK cells be discussed.

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 invite you to submit your manuscript within three months of a request for revision. This would be November 30th in your case. However, we are aware of the fact that many laboratories are not fully functional due to COVID-19 related shutdowns and we have therefore extended the revision time for all research manuscripts under our scooping protection to allow for the extra time required to address essential experimental issues. Please contact us to discuss the time needed and the revisions further.

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 is missing.
2) Your manuscript contains error bars based on n=2. Please use scatter blots showing the individual datapoints in these cases. The use of statistical tests needs to be justified.

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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.

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   - Please note that a maximum of 5 EV Figures can be typeset.
   - Additional figures that you do NOT wish to display as Expanded View figures should be bundled together with their legends in a single PDF file called *Appendix*, which should start with a short Table of Content (incl. page numbers). 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:

   - Movies: The nomenclature for these is Movie EV1 etc. Please provide the legend as a separate text file (README) and zip it together with the movie 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) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available.

9) 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.

10) Regarding data quantification:
- Please specify 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 and the test used to calculate p-values in each figure legend. Discussion of statistical methodology can be reported in the materials and methods section, but figure legends must contain a basic description of n, P and the test applied.
- Graphs must include a description of the bars and the error bars (s.d., s.e.m.).
- Please also include scale bars in all microscopy images.

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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 version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

Yours sincerely

Martina Rembold, PhD
Editor
EMBO reports

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Referee #1:

In this report, the Lundqvist lab defines how a link between IL-15-mTORC1-phosphodiesterase4 (PDE4) provides resistance to PGE2-mediated suppression of anti-tumor functions of human NK cells. Determining the differential resistance of NK cells cultured with IL-2 or IL-15 to PGE2 adds clinical significance to their study. Also, the extension of their observations to the zebrafish model and patient samples validates their in vitro findings. The novelty of this study as follows, a) IL-15 upregulates PDE4, which is essential to hydrolyze the cAMP into 5`AMP; b) IL-15 treatment resulted in the enrichment of CD25+CD54+ NK cells that upregulated PDE4A compared to that of CD25-CD54- NK cells; c) TCGA data correlates the survival of cancer patients with the levels of PGE2 synthase expression and the gene signature of NK cells; and d) authors identify CD25+CD54+ NK cells can mediate superior anti-tumor responses. Data is well-presented in the figures, and the overall writing is clear and appropriately described. The following concerns should be addressed before further consideration of this manuscript.
Major:
1) Authors should discuss why IL-15 but not IL-2 has this effect on the NK cells, in particular on PDE4A. Both IL-2 and IL-15 can initiate protein translation and gene transcriptions via mTORC1, albeit with significant differences.
2) IL-15 is known to activate AMPK in an Akt-independent manner. The cAMP is a major signal to activate AMPK. Can the authors describe the potential role played by AMPK in their model? PDE4A also has a negative role in AMPK function. Moreover, Compound C, an AMPK inhibitor, augments the expression of CD54 in endothelial cells. Addition of PGE2 to NK cells decreases the expression of CD54 in the current study. We also know that AMPK and mTORC1 reciprocally regulate each other's functions. Therefore, it is important to either determine the role of AMPK in CD25+/CD54+ NK cells.
3) Does the reciprocal regulation (lines 328-329) between mTORC1 and PDE4A reveals a role for AMPK?
4) Were there any significant differences in the NK gene signature within the different stages of lung adenocarcinomas in Figure E? Or all four stages were contained significantly reduced NK gene signature compared to the normal tissue?
5) If CD54-dependent clustering is not involved in the resistance mediated by PGE2, what are the other mechanisms that operate to suppress the effector functions? Why CD25+/CD54+ are able to mediate superior cytotoxicity? Did the authors check the levels of granzymes and perforin?
6) Figure 4B-D is confusing and not much explanation is given in the text on why the PGE2 is not having any effect.
7) Some parts of the abstract are confusing. Line 28-29:sentence does not make sense.

Minor points:
1) Figure 4H. What are the open bars?
2) 'CD25+/CD54+' and 'CD25 positive' or 'CD54 positive'. Authors should make them consistent.
3) Line 134: STAT3-spelling.
4) Line 165: 'IL2RA', change to IL-2RA.
5) Line 174: Please fix the sentence.
6) Line 221: 'K62', change to K562.

Referee #2:
In the manuscript by Z. Chen et al., the authors investigated if modulation of the tumor microenvironment in lung cancer with IL-2 or IL-15 would render NK cells resistant to suppression by prostaglandin E2 (PGE2). They found that in vitro, IL-15 but not IL-2 increased CD25+/CD54+ NK cells with superior mTOR activity that was linked to increased expression of the cAMP hydrolyzing enzyme phosphodiesterase 4A (PDE4A). This population of NK cells maintained their function in the presence of PGE2 and showed increased ability to infiltrate lung adenocarcinoma tumors in vitro and in vivo. The authors suggest the enrichment of CD25+/CD54+ NK cells could be used for adoptive cell therapy.

This is a very interesting and well-designed study. However, some minor points should be addressed:

1. Fig. 1: Which markers were used for the purification of normal NK cells.
2. Fig. 4: Could CD25+CD54+ produce cytokines or they exert only cytotoxicity. Was the cytotoxicity mediated by perforin or TRAIL?
3. The authors found that IL-15 induced CD54, however many NK cells are still CD54- after IL15 treatment followed testing their cytotoxicity (Fig. 4).

4. Fig. 6G: It would be interesting to characterize patients with high PTGES expression and NK cell gene expression signatures. Why do they show high NK signature despite high PTGES?

5. The references should be changed according to the journal requirements.

Referee #3:

This is a well-written study on the effect of PGE2 on the anti-tumor functions of NK cells. Though the authors show - through a set of various experiments - that PGE2 modulates certain NK functions, the argument that IL-2 and IL-15 would have different functionality in an in vivo setting is not fully convincing (the killing data seems similar).

Overall, the authors show that PGE2 and, particularly, CD25/CD54 NK cells have important anti-tumor function, the argument that IL-15 is somehow superior to IL-2 in the context of PGE2 is not fully convincing. It is true that certain properties on NK cells are restored with IL-15 instead of IL-2, but how this translates to practice needs perhaps more evidence.

There seem to be two stories here: one that focuses on the effect of PGE2 on IL-2 and IL-15-stimulated NK cells, and the other that focuses on showing that CD25/CD54 is a potent NK subset, and resistant to PGE2.

1. Figure 1A - 1B: Is there any statistical difference between IL-2 and IL-15 in the presence of PGE2? It seems that there is either no difference or no difference is shown.

2. Generally, the study shows that IL-15 protects NK cells from PGE2 immunosuppression to a greater extent than IL-2 does. However, the relevance of this finding based on the data provided is not fully realized. It is not clear what the baseline difference between IL-2 and IL-15 on NK functions is. In Figures 1A/B and 2E, for instance, cytotoxicity of NK cells with IL-2 or IL-15 looks to be the same. This is of course in vitro, and in vivo responses are different. However, the authors make no (or very few) direct comparisons between IL-2 and IL-15 with PGE2.

3. Is the level of PGE2 known (or measured) in the zebrafish LUAD model? This needs to be known.

4. Line 295: There is no Figure 6M. Generally, that entire section appears to mis-cite figures, text should be checked so that figure discussion follows the order in which they are shown in the text.

5. Figure 6I: Is seems as though the cluster of infiltrating NK cells is peripheral, and the A549 tumor in the DP- treatment is peripheral as well, whereas more central in the DP+ treated tumor. The authors should double check this result, or repeat it and discuss how tumor location might have affected NK infiltration.

Minor comments:
- Line 171: "the expression" is repeated
Dear Dr. Breiling,

We thank the editorial office for the opportunity to revise our manuscript entitled “Phosphodiesterase 4A confers resistance to prostaglandin E2-mediated suppression in CD25+/CD54+ NK cells” (EMBOR-2020-51329V2). We appreciate the editor and reviewers’ comments on the novelty and importance of our findings. Two additional authors, Lisa Liu and Le Tong has been added to the manuscript since they have provided experimental and intellectual input during the revision. We have added experimental data which has resulted in the inclusion of several more figures and reorganization of figures. As per journal requirements we have reduced the number of supplemental information.

Note the following changes to the figures.

Original figure 4B, C, and D has been changed.
Original figure 5C and D has been merged into new figure 5C
Original figure 61 has been changed.
Original figure EV1A and B = appendix 1A and figure EV1A
Original figure EV2A, 2B, and 2C = figures EV1B, 1C, 1D
Original figure EV3 = figure EV2
Original figure EV4 = figure EV3
Original figure EV5 = figure EV3F-G
Original figure EV6 = figure EV4
Original figure EV7A and B = figure EV4C and D
Original figure EV7C and D = appendix 1C and D

Added figures: 4E, 4F, 6J, 6K, EV1E, EV1F, appendix 1B, appendix 2, appendix 3.

Referee #1:

In this report, the Lundqvist lab defines how a link between IL-15-mTORC1-phosphodiesterase4 (PDE4) provides resistance to PGE2-mediated suppression of anti-tumor functions of human NK cells. Determining the differential resistance of NK cells cultured with IL-2 or IL-15 to PGE2 adds clinical significance to their study. Also, the extension of their observations to the zebrafish model and patient samples validates their in vitro findings. The novelty of this study as follows, a) IL-15 upregulates PDE4, which is essential to hydrolyze the cAMP into 5’AMP; b) IL-15 treatment resulted in the enrichment of CD25+CD54+ NK cells that upregulated PDE4A compared to that of CD25-CD54- NK cells; c) TCGA data correlates the survival of cancer patients with the levels of PGE2 synthase expression and the gene signature of NK cells; and d) authors identify CD25+CD54+ NK cells can mediate superior anti-tumor responses. Data is well-presented in the figures, and the overall writing is clear and appropriately described. The following concerns should be addressed before further consideration of this manuscript.

Major:  
1) Authors should discuss why IL-15 but not IL-2 has this effect on the NK cells, in particular on
Both IL-2 and IL-15 can initiate protein translation and gene transcriptions via mTORC1, albeit with significant differences. This is indeed an important point in our present study. IL-15 is known to increase mTOR activity in human primary NK cells (Nat Immunol. 2014 Aug;15(8):749-757., Sci Signal. 2016 Feb 16;9(415):ra19., and Nat Commun. 2018 Nov 19;9(1):4874.). We previously reported that IL-15 is superior to IL-2 to increases mTOR and STAT5 activity and that priming with IL-15 increases differential translation compared with NK cells primed with IL-2 (Blood. 2016 Sep 15;128(11):1475-89.). To strengthen our findings on the association between IL-15 and PDE4A we performed additional experiments to first confirm that a two-day stimulation with IL-15 results in stronger STAT5 activation compared with IL-2 stimulation (Figure R1A). Figures for referees not shown. We next used the CistromeDB toolkit to analyze for transcription factors that bind to the PDE4A region (chr19:10,416,773-10,469,631/hg38). This analysis revealed several transcription factors including STAT5 might bind to the PDE4A region (Figure R1B). This result suggests that PDE4A expression can be regulated via IL-15/JAK/STAT5 signaling.
In light of this new information, the results and discussion, and methods sections has been changed on lines 154-161 and 357-364. We have also included the results from the CistromeDB analysis as figure EV1F and STAT5 analysis as figure EV1E.

2) IL-15 is known to activate AMPK in an Akt-independent manner. The cAMP is a major signal to activate AMPK. Can the authors describe the potential role played by AMPK in their model? PDE4A also has a negative role in AMPK function. Moreover, Compound C, an AMPK inhibitor, augments the expression of CD54 in endothelial cells. Addition of PGE2 to NK cells decreases the expression of CD54 in the current study. We also know that AMPK and mTORC1 reciprocally regulate each other's functions. Therefore, it is important to either determine the role of AMPK in CD25+CD54+ NK cells.

We thank the reviewer for this interesting and relevant comment. To address this question we performed additional experiments where we first analyzed the levels if pAMPK in IL-2 or IL-15 stimulated NK cells in the presence or absence of PGE2. In the absence of PGE2, we did not observe any difference in pAMPK levels between IL-2 and IL-15 stimulated NK cells. As expected, we observed a significant increase of pAMPK after PGE2 treatment in both IL-2 and IL-15 activated NK cells. In the presence of PGE2, a slight decrease in pAMPK was observed in IL-15 stimulated NK cells compared with IL-2 stimulated NK cells (Figure R2A).[Figures for referees not shown.]

We next performed experiments to ask whether inhibition of AMPK using Compound C would influence the frequency of CD25+/CD54+ NK cells. Following a two-day activation with IL-15, we observed that the frequency of CD25+/CD54+ NK cells was significantly reduced. In contrast to the issue raised by the reviewer on CD54 expression on endothelial cells, we found that the expression of CD54 was significantly reduced in both IL-2 and IL-15 activated NK cells following inhibition of AMPK (Figure R2B). We next purified CD25+/CD54+ and CD25+/CD54− NK cells and investigated if the levels of pAMPK differ in these two populations. Flow cytometry analysis revealed that the levels of pAMPK was significantly higher in CD25+/CD54+ compared with CD25+/CD54− NK cells (Figure R2C).
3) Does the reciprocal regulation (lines 328-329) between mTORC1 and PDE4A reveals a role for AMPK?

To address this question we performed additional experiments and analyzed the expression of PDE4A, pAMPK, and pS6 in the presence or absence of compound C or Torin-1 in IL-15 stimulated NK cells. As expected, Torin-1 strongly reduced pS6 and PDE4A expression, confirming our results provided in the original manuscript. However, the expression of pAMPK did not change upon addition of Torin-1. In contrast, and as expected, compound C strongly inhibited pAMPK levels. Furthermore, the expression of PDE4A and pS6 was significantly reduced in the presence of compound C (Figure R3). These results indicate that mTOR does not regulate AMPK whereas AMPK is associated with increased expression of PDE4A and pS6.
Taken points #2 and #3 into consideration these results suggest that AMPK acts to regulate the frequency of CD25+/CD54+ NK cells and that CD25+/CD54+ NK cells show higher levels of pAMPK. Furthermore, mTOR does not affect the expression of pAMPK whereas inhibition of AMPK reduces pS6 and PDE4A expression. In light of these new results we have amended the discussion on lines 345-356 and included figure R2 and R3 as Appendix 2.

4) Were there any significant differences in the NK gene signature within the different stages of lung adenocarcinomas in Figure 5E? Or all four stages were contained significantly reduced NK gene signature compared to the normal tissue?

To address this question we performed additional statistical analysis. Within the four stages, there were no statistically significant difference in NK gene expression signature between stage I and stage 2. However, stage I tumors showed a statistically higher NK gene expression signature compared with stage III tumors. We did not observe any significant difference between stage I and stage IV which could be explained by the low number of stage IV patients in this cohort. The original figures 5C and 5E has been combined and is now displayed as Figure 5C (figure R4) [Figures for referees not shown]. The results section on lines 258-260 have been amended to include this new information.
5) If CD54-dependent clustering is not involved in the resistance mediated by PGE2, what are the other mechanisms that operate to suppress the effector functions? Why CD25+/CD54+ are able to mediate superior cytotoxicity? Did the authors check the levels of granzymes and perforin? To investigate potential mechanisms of the superior cytotoxicity by CD25+/CD54+ NK cells we performed additional experiments to analyze the expression of granzyme B and TRAIL (Figure R5). We observed significantly higher expression of TRAIL in CD25+/CD54+ NK cells regardless of the NK cells had been stimulated with IL-2 or IL-15. We did not observe any difference in the expression of Granzyme B between CD25+/CD54+ and CD25-/CD54- NK cells. These new data have been added to figure 4E and F and the results section on lines 219-223.

6) Figure 4B-D is confusing and not much explanation is given in the text on why the PGE2 is not having any effect. The purpose of this figure is to highlight differences in phenotype between CD25+/CD54+ and CD25-/CD54- NK cells to potentially explain the difference in superior cytotoxicity between these two populations of NK cells, regardless of exposure to PGE2 or not (as also pointed out in comment #5). To avoid confusion, we have amended figure 4B-D and also included new data on the expression of TRAIL and granzyme B expression. Since no difference was observed between
IL-2 and IL-15 stimulated NK cells with regards to the activity by CD25+/CD54+ NK cell we have removed the data on IL-2 activated NK cells and only show the results from IL-15 stimulated NK cell populations. These data are also reflected in the figure legend to figure 4B-F and on lines 219-223.

7) Some parts of the abstract are confusing. Line 28-29: sentence does not make sense. This sentence of the abstract has been changed for improved clarity.

Minor points:

1) Figure 4H. What are the open bars? We apologize for this oversight. The aim of this experiment is to test if sorted CD25+/CD54+ NK cells could maintain their cytolytic ability under PGE2 suppression. These results were generated from NK cells pre-stimulated with IL-15 and tested in a 51Cr-release assay in the presence (filled bars) or absence (open bars) of PGE2. The figure legend of Figure 4J has been changed.

2) 'CD25+/CD54+' and 'CD25 positive' or 'CD54 positive'. Authors should make them consistent. We have amended the manuscript throughout for CD25+/CD54+.

3) Line 134: STAT3-spelling.
4) Line 165: 'IL2RA', change to IL-2RA.
5) Line 174: Please fix the sentence.
6) Line 221: 'K62', change to K562. We thank the reviewer for pointing these errors.
Referee #2:

In the manuscript by Z. Chen et al., the authors investigated if modulation of the tumor microenvironment in lung cancer with IL-2 or IL-15 would render NK cells resistant to suppression by prostaglandin E2 (PGE2). They found that in vitro, IL-15 but not IL-2 increased CD25+/CD54+ NK cells with superior mTOR activity that was linked to increased expression of the cAMP hydrolyzing enzyme phosphodiesterase 4A (PDE4A). This population of NK cells maintained their function in the presence of PGE2 and showed increased ability to infiltrate lung adenocarcinoma tumors in vitro and in vivo. The authors suggest the enrichment of CD25+/CD54+ NK cells could be used for adoptive cell therapy. This is a very interesting and well-designed study. However, some minor points should be addressed:

1. Fig. 1: Which markers were used for the purification of normal NK cells.
   *We used the NK Cell Isolation Kit from Miltenyi Biotech for isolation of the untouched NK cells. Non-NK cells are magnetically labeled by using a cocktail of biotin-conjugated antibodies and the NK Cell MicroBead Cocktail. For purity testing, isolated NK cells were stained with CD3 and CD56. The purity of NK cells was always greater than 95%. We have included the commercial name for the kit on line 464.*

2. Fig. 4: Could CD25+CD54+ produce cytokines or they exert only cytotoxicity. Was the cytotoxicity mediated by perforin or TRAIL?
   *Compared with CD25−CD54− NK cells, CD25+CD54+ produce higher levels of IFNγ and expressed higher perforin in the presence of K562 cells. To investigate further potential mechanisms of the superior cytotoxicity by CD25+CD54+ NK cells we performed additional experiments to analyze the expression of granzyme B and TRAIL (Figure R7)[Figures for referees not shown.]. We observed significantly higher expression of TRAIL in CD25+CD54+ NK cells regardless of stimulation with IL-2 or IL-15. We did not observe any difference in the expression of Granzyme B between CD25+CD54+ and CD25−CD54− NK cells. These new data have been added as figures 4E and F and the results section on lines 219-223.*
3. The authors found that IL-15 induced CD54, however many NK cells are still CD54- after IL15 treatment followed testing their cytotoxicity (Fig. 4).

*It is true that not all NK cells express CD54 following stimulation with IL-15. Still, they express significantly higher expression of CD54 than IL-2 stimulated NK cells. Throughout these experiments we activated NK cells during two days. While it may be of interest to investigate if the expression of CD54 would fluctuate during the time of activation, or if certain subpopulations upregulate CD54, but it is beyond the scope of the current study. The cytotoxicity assays displayed in figure 4 (4A and 4H) describe the ability of purified CD25+/CD54+ and CD25-/CD54- NK cells to kill K562 cells.*

4. Fig. 6G: It would be interesting to characterize patients with high PTGES expression and NK cell gene expression signatures. Why do they show high NK signature despite high PTGES?

*We thank the reviewer for this interesting and much relevant question. To investigate this, we performed GSEA analysis and compared PTGES\(^{hi}\)NK\(^{hi}\) and PTGES\(^{hi}\)NK\(^{low}\) TCGA-LUAD cohorts to identify putative gene expression programs. This analysis revealed that inflammatory-related pathways including T cell activation, T cell receptor signaling pathway, activation of innate immune response, and positive regulation of cytokine production (Figure R8)[Figures for referees not shown.]. These results indicate that tumors with PTGES\(^{hi}\)NK\(^{hi}\) are overall more inflamed than PTGES\(^{hi}\)NK\(^{low}\) and that such inflammatory mechanisms may contribute to the increased persistence of NK cell despite high levels of PTGES. The discussion has been amended to include these new data on lines 403-412.*
5. The references should be changed according to the journal requirements. *We have amended the reference style according to the journal style.*
Referee #3:

This is a well-written study on the effect of PGE2 on the anti-tumor functions of NK cells. Though the authors show - through a set of various experiments - that PGE2 modulates certain NK functions, the argument that IL-2 and IL-15 would have different functionality in an in vivo setting is not fully convincing (the killing data seems similar). Overall, the authors show that PGE2 and, particularly, CD25/CD54 NK cells have important anti-tumor function, the argument that IL-15 is somehow superior to IL-2 in the context of PGE2 is not fully convincing. It is true that certain properties on NK cells are restored with IL-15 instead of IL-2, but how this translates to practice needs perhaps more evidence. There seem to be two stories here: one that focuses on the effect of PGE2 on IL-2 and IL-15-stimulated NK cells, and the other that focuses on showing that CD25/CD54 is a potent NK subset, and resistant to PGE2.

1. Figure 1A - 1B: Is there any statistical difference between IL-2 and IL-15 in the presence of PGE2? It seems that there is either no difference or no difference is shown. 
   
   We thank the reviewer for pointing this out. During the revision we performed additional experiments to investigate proliferation of NK cell in the presence or absence of PGE2. These additional data have been included in figure 1A. In the presence of PGE2, IL-15 activated NK cells showed significantly higher expression of ki-67 and displayed higher cytotoxicity against K562 target cells. We have amended figure 1A-B to include p-values comparing IL-2 vs. IL-15 activated NK cells (Figure R9)[Figures for referees not shown.], and amended the text on lines 108-112.

2. Generally, the study shows that IL-15 protects NK cells from PGE2 immunosuppression to a greater extent than IL-2 does. However, the relevance of this finding based on the data provided is not fully realized. It is not clear what the baseline difference between IL-2 and IL-15 on NK functions is. In Figures 1A/B and 2E, for instance, cytotoxicity of NK cells with IL-2 or IL-15 looks to be the same. This is of course in vitro, and in vivo responses are different. However, the authors make no (or very few) direct comparisons between IL-2 and IL-15 with PGE2.
   
   We have included p-values comparing IL-2 vs IL-15 activated NK cells in the presence or absence of PGE2 for figures 1A-B and figure 2E (figure R9 and R10). While both proliferation and cytotoxicity was significantly higher in IL-15 stimulated NK cell compared with IL-2 stimulated NK cells under PGE2 suppression, proliferation but not cytotoxicity was significantly
higher in IL-15 stimulated NK cells in the absence of PGE2. Yet, the difference between IL-2 and IL-15 stimulated NK cells in the presence of PGE2 is greater than in the absence of PGE2.

From earlier studies we know the E:T ratio plays a major role when comparing stimulation with either IL-2 and IL-15 NK cell activity. Throughout this manuscript we used an E:T ratio of 5:1 with the aim to minimize the difference in NK cell activity between IL-2 and IL-15 stimulated NK cells in the absence of PGE2. We used the same ratio in a previous study and found no difference in cytotoxicity between IL-2 and IL-15 stimulated NK cells (Blood. 2016 Sep 15;128(11):1475-89). However, at lower E:T ratios (e.g. 3:1) IL-2 activated NK cells display significantly lower cytotoxicity compared with IL-15 activated NK cells (Clin Invest. 2020 Oct 1;130(10):5508-5522). We have incorporated these results on lines 108-112.

3. Is the level of PGE2 known (or measured) in the zebrafish LUAD model? This needs to be known.

To test for this, we performed additional experiments and first cultured A549 cells as tumor spheroids and stained these with antibodies against PTGES and COX-2 (Figure R11A). Having confirmed that in vitro cultured tumor spheroids stain positive for PTGES and COX-2 we next inoculated A549 cells and explored immunohistochemistry staining in the zebrafish for PTGES and COX-2. However, since the zebrafish larva is too small to cut, we were unable to prepare slides for immunohistochemistry. We then made attempts to stain the zebra fish using whole-mounting method. Following 24 hours after tumor injection, the fish were fixed overnight by 4% PFA and stained for COX-2 and PTGES for two days and washing for two days. Although the fish showed positive staining for both COX-2 and PTGES, we did not detect COX2 or PTGES staining in the tumor area (Figure R11B)[Figures for referees not shown.]. Thus, we are unable to confirm the expression of COX-2 and PTGES in these tumors in vivo. It is possible that expression can be detected by inoculating I higher number of tumor cells or by using more sensitive detection methods (e.g. Fluorescence in situ hybridization and antibody staining, Nature Protocols volume 15, pages3361–3379(2020)). We are presently unable to establish such methods in our laboratory and agree that this is a limitation of our study.
4. Line 295: There is no Figure 6M. Generally, that entire section appears to mis-cite figures, text should be checked so that figure discussion follows the order in which they are shown in the text. Thank you for pointing this out. Please note that the figure has been changed.

5. Figure 6I: It seems as though the cluster of infiltrating NK cells is peripheral, and the A549 tumor in the DN treatment is peripheral as well, whereas more central in the DP treated tumor. The authors should double check this result, or repeat it and discuss how tumor location might have affected NK infiltration.

We appreciate this point by the reviewer. We realize that the original figure 6I did not accurately illustrate the tumor location. Indeed, the original figure 6I might be misleading in that the tumor location appears to be in the periphery in the DP treated group. The tumor was inoculated in the perivitelline space in all fish. We have amended Figure 6I and used a higher magnification of the tumor area (Figure R12). We have also visualized the tumor area with a dashed line in both figures 6H and I and amended the figure legend accordingly. While doing this analysis we also
added new parameters and investigated the frequency of NK cell infiltration in the tumors as well as the 3D distance from the NK cells to the tumor area. This analysis revealed that CD25+/CD54+ NK cells infiltrated A549 tumors to a significantly higher degree compared to CD25-/CD54- NK cells. Furthermore, the distance from CD25+/CD54+ NK cells to the tumor was significantly less than that of CD25-/CD54- NK cells. The figure legend and manuscript on lines 300-303 has been changed to incorporate these new findings.

Minor comments:
- Line 171: "the expression" is repeated

Thank you.
Dear Dr. Lundqvist,

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you will see, all referees now support the publication of your study in EMBO reports.

Before we can proceed with formal acceptance, I have these editorial requests I ask you to address in a final revised manuscript:

- Please have your manuscript carefully proofread by a native speaker. See also the report of referee #3.

- Please order the sections like this: Title page - Abstract - Introduction - Results - Discussion - Materials and Methods - Ethics statement - DAS (data availability section) - Acknowledgements - Author contributions - Conflict of interest - References - Figure legends - Expanded View Figure legends.

- Please add the funding information to the acknowledgements and combine the patient consent part with the ethics statement. Please make sure that the funding information added in the online submission system is complete and similar to the one in the manuscript.

- Please remove the abbreviation list. Please define each abbreviation upon first mention in the text.

- Please shorten the title to not more than 100 characters (including spaces).

- Please provide the abstract written in present tense.

- Please add up to five keywords to the title page (or below the abstract).

- Please add the heading 'Expanded View Figure legends' before the EV legends, to separate them from the main figure legends.

- Please add author and manuscript information to the author checklist.

- Regarding data quantification and statistics, please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (also of the EV and Appendix figures). Please provide error bars and statistical testing where applicable.

- There is a callout to Figure EV7 on page 15. Please check.

- We need a proper Appendix file as single pdf. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables in the Appendix according to this nomenclature. Please add the three EV tables to the Appendix and call these Appendix Table Sx (and adjust their callouts in the manuscript text). Please move all legends regarding Appendix items directly to the
Appendix (below the respective figures or tables). Please remove their legends from the main manuscript text.

- The 2 movies need to be called Movie EV1 and Movie EV2. Please change this, also their callouts.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file with track changes, in order that we can see the modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript
- two to three bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Kind regards,

Achim Breiling
Editor
EMBO Reports

Referee #1:
Authors have addressed my concerns adequately

Referee #2:
The authors successfully addressed all concerns of this reviewer

Referee #3:
The authors have addressed all comments by carrying out additional experiments to demonstrate, firstly, the significance between the effect of IL-2 vs. IL-15 in the presence of PGE2 on NK cell activity and proliferation and, secondly, addressing PGE2 amounts in the zebrafish model used. These additional data strengthened the manuscript.

The manuscript still has a number of typos, and should be carefully checked before acceptance and publication. For instance:
Line 340: "inhibit" should be "inhibits"
Line 346: There should be a comma after "autophagy"
Line 348: "pAMPK levels was" should be "pAMPK levels were"
Line 362: correct "binding to the to"
These are just some examples. The manuscript should be carefully proof-read.
The authors have addressed all minor editorial requests.
Dear Dr. Lundqvist,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

At the end of this email I include important information about how to proceed. Please ensure that you take the time to read the information and complete and return the necessary forms to allow us to publish your manuscript as quickly as possible.

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If you do NOT want this File to be published, please inform the editorial office within 2 days, if you have not done so already, otherwise the File will be published by default [contact: emboreports@embo.org]. If you do opt out, the Review Process File link will point to the following statement: "No Review Process File is available with this article, as the authors have chosen not to make the review process public in this case."

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Thank you again for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

Yours sincerely,

Achim Breiling
Editor
EMBO Reports

*******************************************************************************

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Should you be planning a Press Release on your article, please get in contact with emboreports@wiley.com as early as possible, in order to coordinate publication and release dates.
Corresponding Author Name: Andreas Lundqvist
Journal Submitted to: EMBO Reports
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Reporting Checklist For Life Sciences Articles (Rev. June 2017)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal’s authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field’s best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n > 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified.
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (e.g. cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired or unpaired), Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of ‘center values’ as median or average;
  - definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

If the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

Please fill out these boxes! (Do not worry if you cannot see all your text once you press return.)

B- Statistics and general methods

1a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?

- Sample sizes were selected empirically from previous experimental experience with similar assays, and/or from sizes generally employed in the field.

1b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.

- No statistical methods were used to predetermine sample sizes.

2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analyses. Were the criteria pre-established?

- Larvae with tumor cells in the yolk or cellular debris were discarded.

3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.

- Yes.

3a. For animal studies, include a statement about randomization even if no randomization was used.

- Animals and samples from these animals were randomly selected and observed in this study.

3b. Are any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. binding of the investigator)? If yes please describe.

- Yes. The investigators were blinded to animal samples collection.

4. For every figure, are statistical tests justified as appropriate?

- Yes.

5. Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.

- Yes. We tested for normality using the GraphPad.

6. In there an estimate of variation within each group of data?

- Yes.
| Section | Question/Requirement | Yes/No/NA/Certified | References/Comments |
|---------|----------------------|---------------------|--------------------|
| **C- Reagents** | 8. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | N/A | For all hyperlinks, please see the table on the top right of the document. |
| | 7. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodies (see link list at top right), 1DegreeBio (see link list at top right). | Yes | We provided information in our Materials and Methods “Human specimen” section. |
| | 6. Provide a “Data Availability” section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g., RNA-Seq data: Gene Expression Omnibus GSE19962, Proteomics data: PRIDE PXD000208 etc.) (see link list at top right). Please refer to our author guidelines for ‘Data Deposition’. | NA | We provided the “Data Availability” section at the end of the Materials and Methods in our manuscript, which included the information of how and where we deposited our RNA-seq data. |
| | 5. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals. | Yes | We provided information in our Materials and Methods “Human specimen” section. |
| | 4. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | N/A | NA |
| | 3. Identify the committee(s) approving the study protocol. | Yes | Yes, we followed the journal’s data policy. |
| | 2. Include a statement confirming that consent to publish was obtained. | NA | NA |
| | 1. For publication of patient photos, include a statement confirming that consent to publish was obtained. | NA | NA |
| **D- Animal Models** | 10. Report any restrictions on the availability (and/or on the use) of human data or samples. | N/A | NA |
| | 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | Yes | Yes, we reported confirmation in animal “Zebrafish tumor model” section. |
| | 8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals. | Yes | Yes, we reported these information of animal (zebrafish) used in this study. |
| | 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | N/A | N/A |
| | 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodies (see link list at top right), 1DegreeBio (see link list at top right). | Yes | Yes, we showed these detailed information of these used antibodies in this study. |
| | 5. Report any restrictions on the availability (and/or on the use) of human data or samples. | N/A | NA |
| | 4. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | N/A | NA |
| | 3. Identify the committee(s) approving the study protocol. | Yes | Yes, we followed the journal’s data policy. |
| | 2. Include a statement confirming that consent to publish was obtained. | NA | NA |
| | 1. For publication of patient photos, include a statement confirming that consent to publish was obtained. | NA | NA |
| **E- Human Subjects** | 17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). Please confirm you have followed these guidelines. | N/A | See author guidelines, under ‘Reporting Guidelines’. Please confirm you have followed these guidelines. |
| | 16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under ‘Reporting Guidelines’. Please confirm you have submitted this list. | N/A | See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance. |
| | 15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable. | NA | NA |
| | 14. Report any restrictions on the availability (and/or on the use) of human data or samples. | N/A | NA |
| | 13. For publication of patient photos, include a statement confirming that consent to publish was obtained. | NA | NA |
| | 12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | N/A | NA |
| | 11. Identify the committee(s) approving the study protocol. | Yes | Yes, we followed the journal’s data policy. |
| | 10. Report any restrictions on the availability (and/or on the use) of human data or samples. | N/A | NA |
| | 9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments. | Yes | Yes, we reported confirmation in animal “Zebrafish tumor model” section. |
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| | 7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination. | N/A | N/A |
| | 6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodies (see link list at top right), 1DegreeBio (see link list at top right). | Yes | Yes, we showed these detailed information of these used antibodies in this study. |
| | 5. Report any restrictions on the availability (and/or on the use) of human data or samples. | N/A | NA |
| | 4. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report. | N/A | NA |
| | 3. Identify the committee(s) approving the study protocol. | Yes | Yes, we followed the journal’s data policy. |
| | 2. Include a statement confirming that consent to publish was obtained. | NA | NA |
| | 1. For publication of patient photos, include a statement confirming that consent to publish was obtained. | NA | NA |
| **F- Data Accessibility** | 23. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM machine-readable form. | N/A | N/A |
| | 22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could. | N/A | N/A |
| | 21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM machine-readable form. | N/A | N/A |
| | 20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGaP (see link list at top right) or EGA (see link list at top right). | N/A | N/A |
| | 19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal’s data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a ‘Supplementary Document’ (see author guidelines under ‘Expanded view’ or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right)). | Yes | Yes, we showed these detail information of these used antibodies in this study. |
| | 18: Provide a “Data Availability” section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g., RNA-Seq data: Gene Expression Omnibus GSE19962, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for ‘Data Deposition’. | We provided the “Data Availability” section at the end of the Materials and Methods in our manuscript, which included the information of how and where we deposited our RNA-seq data. | |