nature portfolio

Peer Review File

C/EBPβ-dependent autophagy inhibition hinders NK cell function in cancer

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Autophagy, a highly conserved network of degradative pathways, plays a pivotal role in maintaining cellular and organismal homeostasis. Recent experimental evidence has underscored its critical function not only in regulating NK cell development and survival but also in orchestrating NK cell responses against infected cells (PMID: 27010363; PMID: 27210760). Autophagy-deficient NK cells typically exhibit the accumulation of damaged mitochondria, an excess of reactive oxygen species (ROS), and impaired IFNG secretion and cytotoxic activity. Intriguingly, inhibiting autophagy in tumor cells heightens their susceptibility to NK-mediated tumor cell killing. Despite these insights, the modulation of autophagy for improving NK cell-based immunotherapy remains an unresolved question. In their study, Portale et al focused on elucidating the role of autophagy in shaping NK cell anti-tumor responses. While supporting the essential role of autophagy in NK cell-mediated tumor killing, the authors identify CXCR4-C/EBPb as a crucial upstream signaling pathway for autophagy in NK cells. Notably, they demonstrate that activating autophagy enhances CAR NK cell anti-tumor activity in vivo. Overall, the study, rich in data, presents an interesting story. Nevertheless, there is modest novelty and direct mechanistic evidence supporting the CXCR4-C/EBPb-autophagy axis in NK cells may decrease the work's impact.

Major concerns:

1. If NK cells are considered insignificant in PCa development, it implies their dispensability despite acknowledged dysregulation in the tumor microenvironment (TME) of PCa. To enhance the study's robustness and relevance, I recommend the authors explore alternative PCa models for further validation or challenge of NK cell involvement and their reported findings.

2. Fig. 1F, the authors examine the splenic NK cell anti-tumor activity in tumor-bearing mice, which is not accurate, evaluating the function of tumor-infiltrating NK cells should be better.

3. Although tumor cells can suppress NK cell function via secreting some soluble factor, such as TGF-b, the impaired function of tumor-infiltrating NK cells is not solely due to tumor cells, but also due to other immune suppressive cells in the TME. Further exploration and clarification regarding the specific immune suppressive mechanisms would enhance our understanding of NK cell dysfunction in this context.

4. The GSEA results in Fig. 2B indicate that autophagy signaling is not ranked at the top. Considering the authors' mention of unfolded protein response and metabolic processes, which appear more profoundly altered in tumor-infiltrating NK cells, it raises a question about why autophagy was selected for further studies. To strengthen the rationale for focusing on autophagy, I recommend the authors conduct additional analyses to provide more comprehensive evidence, ensuring a convincing basis for selecting this pathway over others.

5. Fig. 2E, which sub-cluster of NK cells showed reduced autophagy? What’s the correlation of autophagy-related genes with NK effector, activating, and exhaustion molecules within these NK cell clusters?
6. Why did the authors use tumor supernatant to treat NK cells? The NK cells were preactivated? How is the autophagy status in NK cells that were co-cultured with tumor cells?

7. Fig. 4, the author used NK cell adaptive transfer to treat cancer. Can the author treat tumor-bearing mice with Metformin

and then evaluate its effect on NK cell activity and tumor growth in vivo?

8. Which factor from tumor cells induces CXCR4 expression in NK cells?

9. How CXCL12 upregulates C/EBPb in NK cells? Can KO C/EBPb abolish the inhibitory role of CXCL12 in NK cells?

10. Why CAR-NK92 cells did not show an anti-tumor effect in vivo?

11. Finally, the mechanism by which CXCL12- C/EBPb -autophagy axis regulates NK cell effector function remains uncovered. How does autophagy regulate mitochondrial fitness?

Minor:
1. Can the author show the representative flow dot plots data for Fig. 5 B-D and Fig. S4A? why do some places show MFI (Fig. S4A) and some show the percentage of CXCR4 in NK cells?

Reviewer #2

(Remarks to the Author)
Portale ed al. report on the role of autophagy in modulating NK cells' anticancer properties. They observe that NK cells from the tumor microenvironment are decreased in numbers, less mature, less cytotoxic, and present – among others – decreased autophagy as compared to NK cells in control healthy tissue. Next, the authors nicely demonstrate the relevance of autophagy for maintaining anticancer NK cell effector functions and wonder about the mechanism inhibiting autophagy in the cancer microenvironment. They find out that the CXCL12-CXCR4 axis is responsible for autophagy inhibition, providing insights into potential targets to improve NK cell antitumoral response.

The manuscript is nicely written, a lot of work has been performed and the experiments are well thought of.

Major concerns
- Figure 1C/D and 2G/H confirm that NK cells have a less differentiated profile in the tumor tissue. Figures 1E, 2B, and multiple others show a key analyses on cytotoxic, autophagic, and CXCR4-related parameters on total NK cells from tumor or control tissue. A major concern is that differences in cytotoxicity and possibly other readouts reflect different maturation stages; NK cell subsets shall be independently assessed.
Related to the first concern: Figure 1K (and experiments based on similar settings); the authors observed changes in the maturation markers after 24h incubation with tumor-conditioned medium. In how far are these changes induced or the result of selective survival?
- Axes are often cut. This shall be avoided and data shall provide a fair illustration of the mean and % difference achieved (2D, I, 3C, D, I, L, P, Q, 5B, C, D, G, H, I, J, K, L, 6F, G, 7D, and supplementary). When this is considered, many differences are smaller than they appear and require to re-tune some claims.
For instance, ex vivo CXCR4% positivity in NK cells from tumor or control is only mildly affected. Accordingly, specific lysis of target cells is reduced when acting on CXCR4 (5H, 5J vs. 1N, 2I).
- Figure legends are not sufficiently detailed; replicates, statistics and reproducibility information is missing. For instance, Figure 1F just says n=3. Are these technical replicates, if yes how many times was the result reproduced independently? In the Mat and Met section is written that t test was used, which seems inappropriate for selected comparisons. Also, it is written that paired or unpaired t test was applied "as specified", but in the checked legends this reviewer could not find the information. It is stated that SEM was used, but for some datasets this seems inappropriate. This lack of information renders difficult the evaluation of the presented data.
- Are mitochondria restored with metformin, CXCR4 signaling blockade, C-EBP inhibition is applied?
- Potential limitations of HA shall be mentioned.

Minor concerns
- From the reactome (2B), seemingly also performed on total NK cells from tumor and control, the authors chose to work on autophagy. If autophagy is a master regulator of immune cell differentiation, also other pathways are (UPR). Did the authors try to assess relevance of other pathways, or can they better justify their choice?
- Is there the possibility of providing data from (prostate) cancer patients taking metformin?
- Figure 3K; the authors might comment on how the overexpression of Beclin is expected to enhanced transcriptional regulation of many other autophagy players.
- Some descriptive parts of the text (e.g. description of all immune subsets not relevant to the scope of this manuscript in Fig 1 and 5) are distracting and could be limited.

Reviewer #3

(Remarks to the Author)
The team focuses on identifying the crucial role of autophagy in the anti-tumor function of Natural Killer (NK) cells. Researchers observed a disruption in the autophagic pathway in NK cells infiltrating tumors, resulting in a decrease in their anti-tumor functionality. This finding was confirmed both in patients with advanced prostate cancer and in various cancer

models. Exposure of NK cells to cancer reduces the autophagic process, impairs mitochondrial polarization, and decreases their effector functions. Mechanistically, the study identified the CCAAT enhancer binding protein beta (C/EBPb) as a regulator of NK cell metabolism downstream of the CXCL12-CXCR4 interaction. Inhibiting CXCR4 and C/EBPb restored NK cell fitness. Finally, genetic and pharmacological activation of autophagy improved NK cell effector and cytotoxic functions, enhancing the ability of NK and CAR-NK cells to control tumor growth. The study identifies autophagy as a novel intracellular checkpoint in NK cells and proposes a non-invasive approach to enhance NK-based immunotherapies in the clinic.

MAJOR COMMENTS :

Figure 1
It would be interesting to study the cytokine functions of NK cells and to quantify the secretion of IFN-γ. In Figures N/O, FACS plots of CFSE should be included.

Figure 3
It is more important to see the protein validation of BECN1 expression in the main figure. The increase in expression of autophagy pathway genes should be shown at the protein level rather than at the RNA level. ROS and lactate, which are also markers of mitochondrial damage, should be investigated

Figure 4
It is important to show absolute cell counts and FACS analyses to check the differences between conditions.
The role of NKG2D and NKp46 when autophagy is blocked shown be analyzed.
It is important to study the cytokine function of NK cells.
In Figure O, there are more live NK cells; is it their lifespan or their proliferation that induces this difference?

Figure 5
In figures G, H, K, L, it would be interesting to have conditions where CXCL12 is added when there is Plerixafor and when CXCR4 is KO. That Plerixafor inhibits CXCR4 should be shown. CXCL12 should be quantified in the secretome of tumor cells to show that tumor cells secrete this chemokine, which will block the functions of NK cells.

Figure 6
The absolute counts of NK cells and the analysis of their cytokine function should be shown. Why is there no analysis on IFN-γ in Figure 6J?

Figure 7
It should be shown that Plerixafor inhibits CXCR4 on PD-L1 CAR NK-92 cells.
The absolute counts of NK cells and the analysis of their cytokine function should be shown.

MINOR COMMENTS :
• In figure 6J, there is a missing legend below the graph.
• In Figure 2G, there are white squares on the FACS plots.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have fully addressed all the comments from this reviewer. There are no additional comments. I now recommend publishing this work.

Mediating comments for R#3:

I have read the response to Reviewer #3. The authors have fully addressed the reviewer's comments. I suggest accepting this revised manuscript without further revision.

Reviewer #2

(Remarks to the Author)
The manuscript has been much improved and shall be accepted.

The only point for which I could not find an answer concerned whether differences in cytotoxicity were directly related to differences in maturation or not (first point). This does not affect relevance of the data, but shall be acknowledged as a possibility. E.g. "Tumor-infiltrating NK cells, isolated from Ptenpc-/- prostates (Fig. 1F), and splenic NK cells from Ptenpc-/- tumor-bearing mice (Extended Data 1H) presented a reduced ability to kill target cells when compared to splenic cells from WT mice, thus confirming their dysfunctional state."

shall be corrected to:
"Tumor-infiltrating NK cells, isolated from Ptenpc-/- prostates (Fig. 1F), and splenic NK cells from Ptenpc-/- tumor-bearing mice (Extended Data 1H) presented a reduced ability to kill target cells when compared to splenic cells from WT mice, thus confirming their dysfunctional state, which could directly relate to their altered maturation stages."

Title of the manuscript: C/EBPβ-dependent autophagy inhibition hinders NK cell function in cancer

Point by point response

To the attention of the Editor and reviewers

We appreciate the constructive feedback received and we now included additional data to address the concerns raised that we believe significantly strengthen our findings and enhanced the overall robustness and impact of our study. Changes to the text of the revised manuscript have been highlighted in yellow. We also included in the rebuttal a Table (Table 1) in which we listed all graphs that have been modified to respond to the reviewer's observations.
Please find below a point by point response to the reviewer's comments.

Reviewer #1 (Remarks to the Author):

Autophagy, a highly conserved network of degradative pathways, plays a pivotal role in maintaining cellular and organismal homeostasis. Recent experimental evidence has underscored its critical function not only in regulating NK cell development and survival but also in orchestrating NK cell responses against infected cells (PMID: 27010363; PMID: 27210760). Autophagy-deficient NK cells typically exhibit the accumulation of damaged mitochondria, an excess of reactive oxygen species (ROS), and impaired IFNG secretion and cytotoxic activity. Intriguingly, inhibiting autophagy in tumor cells heightens their susceptibility to NK-mediated tumor cell killing. Despite these insights, the modulation of autophagy for improving NK cell-based immunotherapy remains an unresolved question. In their study, Portale et al focused on elucidating the role of autophagy in shaping NK cell anti-tumor responses. While supporting the essential role of autophagy in NK cell-mediated tumor killing, the authors identify CXCR4-C/EBPb as a crucial upstream signaling pathway for autophagy in NK cells. Notably, they demonstrate that activating autophagy enhances CAR NK cell anti-tumor activity in vivo. Overall, the study, rich in data, presents an interesting story. Nevertheless, there is modest novelty and direct mechanistic evidence supporting the CXCR4-C/EBPb-autophagy axis in NK cells may decrease the work's impact.

Response:

We thank the reviewer for her/his comments, that helped to refine and improve the overall quality of our manuscript. We are glad that the referee appreciates the potential of targeting autophagy in NK cell therapy and finds our story interesting. Indeed, autophagy remains an underexploited pathway in NK cell therapy, and we believe our work offers significant insights into its therapeutic potential. Regarding the perceived limitation in novelty, we would like to underlie that, while autophagy has been investigated in the context of NK cell maturation and activation, its role within NK cells in the tumor microenvironment has not been thoroughly studied. Furthermore, we highlight the novel discovery of the CCAAT/enhancer-binding protein beta (C/EBPβ) as a critical transcription factor regulating

autophagy in NK cells. To our knowledge, this is the first report demonstrating the involvement of C/EBPβ in autophagy regulation within NK cells.
Please find below a point by point response to all comments:

Major Concerns:

1. If NK cells are considered insignificant in PCa development, it implies their dispensability despite acknowledged dysregulation in the tumor microenvironment (TME) of PCa. To enhance the study's robustness and relevance, I recommend the authors explore alternative PCa models for further validation or challenge of NK cell involvement and their reported findings.

Response:

We thank the reviewer for this comment, as it brought to information that in our opinion increased the overall impact of the paper. First of all, we now modified the text to add clarity. Indeed, we believe that NK cells are not insignificant in PCa, but that NK cells become dysfunctional at a certain stage after tumor establishment. Consequently, their depletion has little impact on tumor growth at later stages. To further investigate this concept and add strengthen to our data, we have addressed this concern by performing NK cell depletion experiments in an additional PCa model, specifically using the $Pten^{-/-}$; $Trp53^{-/-}$ transplantable mouse model. This model employs the $Pten^{-/-}$; $Trp53^{-/-}$ tumor cell line derived from the $Pten^{pc-/-}$; $Trp53^{pc-/-}$ conditional prostatic cancer model, which shares the same genotype. This PCa model accurately recapitulates the features of human aggressive prostate cancer, as previously described (PMID: 16079851). The advantage of using a transplantable model is the flexibility to experiment different timing for NK cell depletion. As noted in other studies, the timing of immune cell depletion can significantly impact the overall outcome. To explore this, we depleted NK cells at two different time points: an early time point (day 3 after tumor cell injection) and a later time point, when tumor is established. Our experiments demonstrate that early NK cell depletion results in tumor progression (Extended Data 2A-D), whereas depletion at later time points does not affect tumor growth (Extended Data 2E-H). These findings are consistent with the results presented in the original manuscript, when we depleted NK cells in established $Pten^{pc-/-}$ tumors. At the same time these data add a level of information on the dynamics of NK cell in PCa. They suggest that NK cells are effective in controlling tumor growth at early stages but become dysfunctional as the disease progresses. Consequently, NK cell depletion is impactful at early time points but not at later stages. We believe this enhances the understanding of NK cell involvement in prostate cancer progression and supports the significance of our findings.
We have prepared new panels showing the depletion strategy in the new model and the impact on tumor growth and on tumor microenvironment, which validate our findings in alternative PCa contexts (Extended Data 2A-H of the revised manuscript).

2. Fig. 1F, the authors examine the splenic NK cell anti-tumor activity in tumor-bearing mice, which is not accurate, evaluating the function of tumor-infiltrating NK cells should be better.

Response:

We acknowledge that the evaluation of tumor infiltrating NK cell function is valuable. We have now included data reporting the lytic ability of tumor-infiltrating NK cells in the $Pten^{pc-/-}$ transgenic model. Specifically, we sorted NK cells from the tumor tissue and assessed their cytolytic activity against tumor cells *in vitro*. These data are included in Fig. 1F of the revised manuscript. Sorted tumoral NK cells were compared to splenic NK cells from healthy mice, isolated in the same manner. Unfortunately, it was not possible to sort NK cells from healthy prostatic tissue due to their paucity. Our results demonstrate that tumor-infiltrating NK cells exhibit cytolytic activity, although this activity is reduced compared to peripheral NK cells that have not been exposed to the tumor environment. To add further insights to the activity of NK cells in tumors, we included data about IFNγ expression in NK cells from $Pten^{pc-/-}$ tumors and $Pten^{pc+/+}$ normal prostatic tissues in Fig. 1E of the revised manuscript. Furthermore, we added a deeper characterization of the phenotype of prostatic $Pten^{pc-/-}$ and $Pten^{pc+/+}$ NK cells, including NK cells markers previously associated to NK cell effector functions and activation. These data are now included in the Extended Data Fig. 1I of the revised manuscript.

3. Although tumor cells can suppress NK cell function via secreting some soluble factor, such as TGF-β, the impaired function of tumor-infiltrating NK cells is not solely due to tumor cells, but also due to other immune suppressive cells in the TME.

Response:

We agree that the TME is characterized by a complex situation in which multiple cell subsets interact with each other. To gain further insights into NK cell interactions with other immune cells, we re-analyzed our single-cell RNA sequencing (scRNAseq) dataset from the $Pten^{pc-/-}$ model to identify specific immune-relevant mechanisms. We focused on interactions previously described by others, particularly targeting fibroblasts, dendritic cells, and lymphocytes. Our CellPhoneDB analysis revealed significant interactions between NK cells and other immune cells, such as macrophages, dendritic cells, and fibroblasts. These interactions are now illustrated in Fig. 1A-B of this rebuttal. We believe that these results provide valuable insights into cell-cell interactions within the prostate cancer environment, although a comprehensive exploration of these interactions is beyond the scope of the present study. If the reviewer finds these data of interest, we would be happy to include the graphs in the final version of the manuscript.

4. The GSEA results in Fig. 2B indicate that autophagy signaling is not ranked at the top. Considering the authors' mention of unfolded protein response and metabolic processes, which appear more profoundly altered in tumor-infiltrating NK cells, it raises a question about why autophagy was selected for further studies.

Response:

We have now modified the text to provide additional explanation on why we chose to investigate autophagy further. First, when biological processes are ranked for statistical significance (and not by normalized enrichment score as shown in the previous version of the manuscript), autophagy is among the three top deregulated pathways in tumoral NK cells. We have included this graph in the revised paper (Fig. 2B). Additionally, we applied a

second strategy by analyzing human scRNAseq data with Ingenuity Pathway Analysis (IPA). The IPA analysis confirmed that autophagy is critical in tumoral NK cells compared to non-tumoral cells, as shown in the graphical summary in Fig. 1C of this rebuttal. This approach based on different datasets of reference in GSEA and IPA demonstrates that autophagy is indeed highly modulated in tumoral NK cells. Furthermore, a literature search revealed that autophagy in tumoral NK cells was understudied, being explored primarily in the context of physiology and infection (PMID: 28103115, PMID: 27010363). We thus prioritized the investigation of autophagy in our project. We have now better explained this in the text that has been revised to provide a stronger rationale for focusing on autophagy.

5. Fig. 2E, which sub-cluster of NK cells showed reduced autophagy? What's the correlation of autophagy-related genes with NK effector, activating, and exhaustion molecules within these NK cell clusters?

Response:

To provide more information on autophagy in relation to NK cell maturation and activation states, we analyzed the autophagic flux in NK cell subsets *ex vivo*. As shown in Extended Data 3B, in human samples, autophagy is significantly deregulated in $CD56^{dim}CD16^{bright}$ effector NK cells. These results were partially confirmed in mouse models, where autophagy is significantly deregulated in $CD11b^{+}CD27^{-}$ subset (Extended Data 3B,C). However, we noticed a tendency for autophagy deregulation in most subsets, that may be indicative of a general modulation across NK cell subpopulations.

6. Why did the authors use tumor supernatant to treat NK cells? The NK cells were preactivated? How is the autophagy status in NK cells that were co-cultured with tumor cells?

Response:

We apologize for lack of clarity. In our *in vitro* assays, NK cells are activated with a cocktail of cytokines including IL-12, IL-15 and IL-18 (mouse) and IL-2 (human), we now added details in the text. We initially exposed NK cells to the supernatant of tumor cells to avoid the cell killing that would occur in direct co-culture. To complement these data, we have now included results from *in vitro* co-culture experiments. We conducted co-culture experiments with murine and human NK cells and tumor cells to assess autophagy status. Our new data, included in Fig. 1D-G of this rebuttal show the impact of direct cell-cell interactions on autophagy status and NK cell function. These results are consistent with those obtained from the supernatant exposure experiments.

7. Fig. 4, the author used NK cell adaptive transfer to treat cancer. Can the author treat tumor-bearing mice with Metformin and then evaluate its effect on NK cell activity and tumor growth in vivo?

Response:

We thank the reviewer for this comment. We have now implemented an additional *in vivo* preclinical trial by administering Metformin to tumor-bearing mice in the transplantable PCa

mouse model. After treatment, we evaluated its effect on NK cell activity and tumor growth. The new data, depicted in Extended Data 5G-K, indicate that Metformin systemic treatment reduces tumor growth. FACS analysis of tumor-infiltrating subsets shows a relative increase of $CD11b^{+}CD27^{-}$ cytotoxic NK cells and a higher autophagy levels in tumor-infiltrating NK cells. Splenic NK cells from treated mice show higher killing capability when compared to control (Extended Data 5K). These new results have been now included in the revised manuscript.

8. Which factor from tumor cells induces CXCR4 expression in NK cells?

Response:

We believe that this point is of strong interest. Therefore, to gain insights into the mechanisms by which tumor increases CXCR4 expression in NK cells, we re-analyzed our scRNAseq dataset from $Pten^{pc-/-}$ tumors. Employing NicheNet analysis, we identified factors predicted to regulate CXCR4 expression based on gene expression (Fig. 2A of this rebuttal). Specifically, we selected VEGFα, TGFβ, and CXCL12. A literature search confirmed that these three factors have been previously reported to modulate CXCR4 expression in other cell types. We thus tested the capability of these factors to upregulate CXCR4 on NK cells. Interestingly, in our setting TGFβ was able to induce CXCR4 upregulation in both murine and human NK cells (Fig. 6B of the revised manuscript and Figure 2A-F of this rebuttal). Consequently, we blocked TGFβ in the conditioned media of tumor cells by adding a selective TGFβ receptor inhibitor, LY2109761, and exposed NK cells to the media. In this situation, CXCR4 upregulation was inhibited (Fig. 2F of this rebuttal and Fig. 6B of the revised manuscript). These results suggest that TGFβ, in the tumor supernatant, is responsible for CXCR4 upregulation. Measurement by ELISA confirmed the release of TGFβ by the human PC3 cancer cell line and the murine $Pten^{-/-}$ and $Pten^{-/-}$;$Trp53^{-/-}$ cancer cell lines (Fig. 2G-H of this rebuttal and Fig. 6C-D of the revised manuscript). Findings are now also included and discussed in the manuscript.

9. How CXCL12 upregulates C/EBPβ in NK cells? Can KO C/EBPβ abolish the inhibitory role of CXCL12 in NK cells?

Response:

To explore this point, we conducted *in vitro* assays with NK cells exposed to Helenalin Acetate (C/EBPβ inhibitor) in the presence of CXCL12 and observed that inhibition of C/EBPβ diminishes the effects of CXCL12 on autophagy. These results are now presented in Fig. 6J of the revised manuscript.

10. Why CAR-NK92 cells did not show an anti-tumor effect in vivo?

Response:

We acknowledge that CAR NK-92 cells were expected to impact tumor growth even without treatment. We believe that this point can be explained by looking at the abundance of CAR NK-92 cells in the tumor. Importantly, we now calculated the absolute count of CAR NK-92

cells infiltrating cancer in all our preclinical trials. We thus noticed that both the relative abundance and the absolute count of infiltrating CAR NK-92 cells is augmented when cells are pre-exposed to Metformin, while infiltration is limited without the drug (Fig. 7G and Extended Data 7S of the revised manuscript). The low infiltration of CAR NK-92 cells might explain their scarce capability to kill tumor cells in our *in vivo* settings.

11. Finally, the mechanism by which CXCL12- C/EBPβ-autophagy axis regulates NK cell effector function remains uncovered.

Response:

Our hypothesis is that autophagy impairment leads to the accumulation of damaged mitochondria due to a lack of degradation. Previous findings have shown that NK cell functionality relies on mitochondrial fitness and that efficient clearance of damaged mitochondria is essential to maintain NK cell fitness. For example, Choi C et al. and Finlay DK et al. (PMID: 34090499; PMID: 30808985) demonstrated that cytotoxic NK cells maintain high levels of OXPHOS and glycolysis, essential for their cytotoxic functions, and that mitochondrial fitness is crucial for supporting NK cell functions. It has also been reported that during NK cell activation, mitochondria become damaged and fragmented, leading to ROS accumulation and metabolic impairment. Inefficient activation of autophagy and mitophagy results in the accumulation of dysfunctional mitochondria and subsequent NK cell death, underscoring the importance of mitochondrial dynamics and autophagy in NK cell function and memory cell development (PMID: 26858267). Accordingly, in liver cancer, tumor-infiltrating NK cells have been shown to possess fragmented mitochondria, which impairs their activation and the generation of memory cells.
To strengthen our evidence and increase mechanistic insights, we now conducted additional experiments aimed at elucidating the impact of autophagy inhibition on mitochondria. Our new findings indicate that levels of reactive oxygen species (ROS), indicative of mitochondrial dysfunction, are augmented when autophagy is deregulated upon exposure to the tumor media, and restored upon autophagy activation through Beclin 1 overexpression (now added in Fig. 3S and 3U of the revised manoscript). Furthermore, by combining TMRM and MitoTracker staining, we evaluated by FACS staining, the increase in cells containing dysfunctional mitochondria, defined as MitoTracker Green^{high} and TMRM^{low} upon exposure to tumor-conditioned medium (Extended Data 4I-K). We believe that, thanks to the reviewer's comment, we have now further substantiated the mechanism underlying autophagy alteration and NK cell dysfunction.

Minor Concerns:

1. Can the author show the representative flow dot plots data for Fig. 5 B-D and Fig. S4A? Why do some places show MFI (Fig. S4A) and some show the percentage of CXCR4 in NK cells?

Response:

We have included the representative flow cytometry dot plots for Fig. 5 B-D and Extended Data 6E-F in the revised manuscript (check Extended Data 6A-C and D). Additionally, we have standardized the presentation of data by showing percentage of CXCR4 expression in

all situations for consistency (we included percentage of CXCR4 on PC3 treated NK in Extended Data 6F).

Reviewer #2 (Remarks to the Author):

Portale ed al. report on the role of autophagy in modulating NK cells' anticancer properties. They observe that NK cells from the tumor microenvironment are decreased in numbers, less mature, less cytotoxic, and present – among others – decreased autophagy as compared to NK cells in control healthy tissue. Next, the authors nicely demonstrate the relevance of autophagy for maintaining anticancer NK cell effector functions and wonder about the mechanism inhibiting autophagy in the cancer microenvironment. They find out that the CXCL12-CXCR4 axis is responsible for autophagy inhibition, providing insights into potential targets to improve NK cell antitumoral response.

The manuscript is nicely written, a lot of work has been performed and the experiments are well thought of.

Response:

We are pleased to hear that the referee appreciates the extensive experimental efforts and the thoughtful design of our experiments. This positive feedback is highly encouraging and reinforces the significance of our findings. We believe that the reviewer's comments strongly helped to improve the quality of our manuscript. Please find below a point by point response to all comments.

Major concerns

- Figure 1C/D and 2G/H confirm that NK cells have a less differentiated profile in the tumor tissue. Figures 1E, 2B, and multiple others show a key analyses on cytotoxic, autophagic, and CXCR4-related parameters on total NK cells from tumor or control tissue. A major concern is that differences in cytotoxicity and possibly other readouts reflect different maturation stages; NK cell subsets shall be independently assessed.

Response:

We appreciate this insightful comment, that was also raised by Referee #1. We have now included FACS data on NK cell subsets, specifically assessing autophagy levels in different subpopulations in both the human tissues and murine model. These new data are now included in the revised manuscript. As shown in Extended Data 3B, in human samples, autophagy is significantly deregulated in $CD56^{dim}CD16^{bright}$ effector NK cells. These results were partially confirmed in mouse models, where autophagy is significantly deregulated in $CD11b^{+}CD27^{-}$ subset (Extended Data 3B,C). However, we noticed a tendency for autophagy deregulation in most subsets, that may be indicative of a general modulation across NK cell subpopulations.

Related to the first concern: Figure 1K (and experiments based on similar settings); the authors observed changes in the maturation markers after 24h incubation with tumor-conditioned medium. In how far are these changes induced or the result of selective survival?

Response:
We acknowledge the importance of this concern. Indeed, selective survival does play a role in the observed changes. Following the reviewer's suggestion, we have now checked murine NK cell vitality *in vitro*, upon exposure to tumor conditioned media. These new results are included in Extended Data 3E.
The comment from the referee unveiled an aspect that was not properly considered before, and addressing it has significantly improved the manuscript. By thoroughly examining selective survival, we now provided a more accurate interpretation of the changes in maturation markers.

- Axes are often cut. This shall be avoided and data shall provide a fair illustration of the mean and % difference achieved (2D, I, 3C, D, I, L, P, Q, 5B, C, D, G, H, I, J, K, L, 6F, G, 7D, and supplementary). When this is considered, many differences are smaller than they appear and require to re-tune some claims. For instance, ex vivo CXCR4% positivity in NK cells from tumor or control is only mildly affected. Accordingly, specific lysis of target cells is reduced when acting on CXCR4 (5H, 5J vs. 1N, 2I).

Response:

We have revised all relevant figures to avoid cutting axes. The revised graphs are included in the updated manuscript.

- Figure legends are not sufficiently detailed; replicates, statistics and reproducibility information is missing. For instance, Figure 1F just says n=3. Are these technical replicates, if yes how many times was the result reproduced independently? In the Mat and Met section is written that t test was used, which seems inappropriate for selected comparisons. Also, it is written that paired or unpaired t test was applied "as specified", but in the checked legends this reviewer could not find the information. It is stated that SEM was used, but for some datasets this seems inappropriate. This lack of information renders difficult the evaluation of the presented data.

Response:

We have revised the figure legends to include detailed information on replicates, statistics, and reproducibility. We have also reviewed and corrected the statistical analyses used, ensuring appropriate methods were applied. The revised figure legends and statistical details are now included in the updated manuscript. Please find in Table 1 the list of panels and graphs that were modified.

- Are mitochondria restored with metformin, CXCR4 signaling blockade, C-EBP inhibition is applied?

Response:

We thank the referee for this comment that helped to give strength to our conclusions. We have conducted FACS analyses to assess mitochondrial status under CXCR4 blockade by Plerixafor and C/EBPβ inhibition by Helenalin Acetate. Our new data indicate mitochondrial restoration under these conditions. These results are now included in Extended Data 7C and O.

- Potential limitations of HA shall be mentioned.

Response:

We acknowledge that while HA serves as a proper tool in dissecting C/EBPβ function, its potential off-target effects must be considered when interpreting the results. This limitation has been discussed in the revised manuscript to provide a balanced perspective on the use of HA in our study, also based on insights from our collaborator Thomas Schmidt, that contributed to the development of the drug. Briefly, Helenalin Acetate, a natural sesquiterpene lactone, has demonstrated potent anti-inflammatory and anti-cancer properties, partly through the inhibition of NF-κB and C/EBPβ. Recent studies, including those by our collaborator and colleagues (PMID: 27803164), have shown that HA is significantly more potent in inhibiting C/EBPβ than NF-κB, by binding to the N-terminal part of C/EBPβ and disrupting its interaction with the co-activator p300. However, despite its specificity towards C/EBPβ, HA may still exhibit off-target effects due to its ability to covalently interact with cysteine residues in other proteins. Given this, we now discussed in our manuscript that while HA effectively inhibits C/EBPβ, its off-target effects need to be considered when interpreting our results. Future studies could aim to utilize more specific inhibitors or genetic approaches to confirm our findings and minimize potential confounding effects.

Minor concerns

- From the reactome (2B), seemingly also performed on total NK cells from tumor and control, the authors chose to work on autophagy. If autophagy is a master regulator of immune cell differentiation, also other pathways are (UPR). Did the authors try to assess relevance of other pathways, or can they better justify their choice?

Response:

We have now modified the text to provide additional explanation on why we chose to investigate autophagy further. First, when biological processes are ranked for statistical significance, autophagy is among the top deregulated pathways in tumoral NK cells. We have included this graph in the revised paper (Fig. 2B). Additionally, we applied a second strategy not previously mentioned in the manuscript by analysing data with Ingenuity Pathway Analysis (IPA). The IPA analysis confirmed that autophagy is critical in tumoral NK cells compared to non-tumoral cells, as shown in the graphical summary in Fig. 1C of this rebuttal. This approach based on different datasets of reference in GSEA and IPA demonstrates that autophagy is indeed highly modulated in tumoral NK cells. Furthermore, a literature search revealed that autophagy in tumoral NK cells was understudied, being explored primarily in the context of physiology and infection (PMID: 28103115, PMID: 27010363). We have now better explained this in the text that has been revised to provide a stronger rationale for focusing on autophagy.

- Is there the possibility of providing data from (prostate) cancer patients taking metformin?

Response:

We consider this point of great interest. Unfortunately, we could not obtain relevant data from prostate cancer patients taking metformin for this study.

- Figure 3K; the authors might comment on how the overexpression of Beclin is expected to enhanced transcriptional regulation of many other autophagy players.

We appreciated this comment, as we realized that association between BECN1 overexpression and modulation of other players was not properly substantiated in the text. We have now added a sentence in the revised manuscript on why BECN1 overexpression was selected as a tool to enhance autophagy. We also clarified that BECN1 is not a direct transcriptional regulator of other ATG members. Briefly, we selected BECN1 for overexpression in NK cells to enhance autophagy based on its well-established role as a master regulator of this process. BECN1 plays a pivotal role in the initiation and regulation of autophagy by forming the core of the class III phosphatidylinositol 3-kinase (PI3K) complex, crucial for autophagosome formation. This selection is substantiated by multiple studies indicating that BECN1 is essential for maintaining cellular homeostasis and responding to metabolic stress (PMID: 20097051, PMID: 11306555). On light of these evidence, BECN1 was previously overexpressed by others to obtain autophagy hyperactivation (PMID: 28806762). However, Beclin 1 has not been reported to directly impact on the transcriptional levels of other ATG genes. Activation of autophagy by prolonged stimuli such as amino acid starvation, Torin1 or mitochondrial damage leads to upregulation of autophagy genes via transcription factors, such as TFEB/TFE3, ATF4 and FOXOs (PMID: 31312633; PMID: 37199330). Although Beclin 1 itself does not directly regulate the expression of ATG genes, its overexpression as well as the treatment with Tat-Beclin1 (PMID: 23364696) can induce autophagy. This induction may lead to an increase in autophagic gene expression, probably through positive feedback mechanisms that enhance the overall autophagic process, as demonstrated for mTOR inhibition by Torin1 (PMID: 22343943). This suggests that Beclin 1 overexpression may indirectly support autophagy through feedback loops involving key transcription factors. These concepts have now been commented in the Discussion of the revised manscript.

- Some descriptive parts of the text (e.g., description of all immune subsets not relevant to the scope of this manuscript in Figures 1 and 5) are distracting and could be limited.

Response:

We have rationalised the descriptive parts of the text, focusing on the most relevant data to the scope of this manuscript. The revised manuscript now presents a more concise and focused narrative.

Reviewer #3 (Remarks to the Author):

The team focuses on identifying the crucial role of autophagy in the anti-tumor function of Natural Killer (NK) cells. Researchers observed a disruption in the autophagic pathway in NK cells infiltrating tumors, resulting in a decrease in their anti-tumor functionality. This finding was confirmed both in patients with advanced prostate cancer and in various cancer models. Exposure of NK cells to cancer reduces the autophagic process, impairs mitochondrial polarization, and decreases their effector functions. Mechanistically, the study identified the CCAAT enhancer binding protein beta (C/EBPb) as a regulator of NK cell metabolism downstream of the CXCL12-CXCR4 interaction. Inhibiting CXCR4 and C/EBPb restored NK cell fitness. Finally, genetic and pharmacological activation of autophagy

improved NK cell effector and cytotoxic functions, enhancing the ability of NK and CAR-NK cells to control tumor growth. The study identifies autophagy as a novel intracellular checkpoint in NK cells and proposes a non-invasive approach to enhance NK-based immunotherapies in the clinic.

Response:

We thank this reviewer for the insightful comments and for highlighting the significance of our study in identifying autophagy as a novel intracellular checkpoint in NK cells. We now included absolute cell counts and protein analysis that strongly improved the quality of our manuscript. Please find below a point by point response to all comments.

MAJOR COMMENTS:

Figure 1
It would be interesting to study the cytokine functions of NK cells and to quantify the secretion of IFN-γ. In Figures N/O, FACS plots of CFSE should be included.

Response:

We agree with the referee that assessing the cytokine release by NK cells is critical to improve the quality of the data. We have now performed ELISA for IFNγ in murine NK cells exposed to tumor-conditioned medium from Pten$^{-/-}$ tumor cells. Our new data confirmed FACS data showing a reduction of IFNγ release in these settings. A new graph has been included in Fig. 1M of the revised manuscript. Also, the gating strategy for the cytotoxicity assay and FACS plots of CFSE are now provided in Extended Data 2I.

Figure 3
It is more important to see the protein validation of BECN1 expression in the main figure. The increase in expression of autophagy pathway genes should be shown at the protein level rather than at the RNA level. ROS and lactate, which are also markers of mitochondrial damage, should be investigated.

Response:

We acknowledge the importance of these points. We have now added Western Blot analyses for BECN1 (Figure 3L,M). We also explored the protein expression levels of main autophagy regulators (ATG7, ATG14, LC3 and p62) by flow cytometry and Western Blot analyses. New graphs show increased levels of ATG7, ATG14 and p62 in BECN1 OE (Extended Data 4B-E). On the contrary, decreased levels of total LC3 were observed in BECN1 OE (Extended Data 4F,G), that, however could be an index of induction of autophagic flux. Since the above-mentioned proteins are also autophagic substrates, it should be considered that their total amount is the result of the balance between transcriptional regulation and autophagy-mediated degradation.
Additionally, we investigated ROS levels and lactate production as markers of mitochondrial damage. These experiments have been included in the revised manuscript. Interestingly,

our new analysis showed that exposure to tumor conditioned media increases ROS and intracellular lactate levels in NK-92 cells, indicative of mitochondrial dysfunction and supporting our main hypothesis. Graphs have been now added in Fig. 3S and Extended Data 4L of the revised manuscript.

Figure 4
It is important to show absolute cell counts and FACS analyses to check the differences between conditions.

Response:

We acknowledge the importance of the absolute cell count of tumor-infiltrating NK cells, that may be different in behavior than relative abundance. Absolute cell counts have been now calculated and included for all preclinical trials to better illustrate the differences between conditions. Importantly, calculation of absolute cell count revealed key differences that further support the role of autophagy not only in NK cell activation, but also in the capability of NK cell to better migrate to the tumor bed. Results are now shown in Fig. 4J, Fig. 4N, Fig. 5O, Fig. 6N and Fig. 7G.

The role of NKG2D and NKp46 when autophagy is blocked shown be analyzed.

Response:

To explore the expression of these two markers on tumor-infiltrating NK cells, we performed *in vitro* assays and we also repeated two preclinical trials (with NK-92 cells pre-exposed to Metformin or to HA) and included antibodies against NKG2D and NKp46 in our panels. *In vitro* we analysed the expression of NKG2D and NKp46 when autophagy is blocked by chloroquine. Results show that autophagy inhibition reduces the expression of both NKG2D and NKp46 on murine NK cells isolated from the spleen of WT mice. On the contrary, autophagy inhibition in NK-92 cells does not impact the expression of neither marker (Fig. 3A-D of this rebuttal). In the new *in vivo* trials we analysed the expression of NKG2D and NKp46 on tumor-infiltrating NK-92 cells, after exposure to Metformin and administration to tumor bearing mice. In this *ex vivo* settings we could not detect any modulation of the two markers (Fig. 3E,F of this rebuttal). These data may indicate that NKG2D and NKp46 expression are not indicative of NK cell activation upon autophagy activation.

It is important to study the cytokine function of NK cells.

Response:

We now included the analysis of IFNγ by intracellular staining in all trials performed (Fig. 1E, Fig. 4F-K-P, Fig. 5P, Fig. 6P and Fig. 7I). Our analysis shows that IFNγ expression is not affected upon autophagy regulation, with the exception of tumor-infiltrating BECN1 overexpressing NK-92 cells. Additionally, we now performed ELISA assays to detect the release of IFNγ by NK-92 cells, upon exposure to Metformin (NK-92 in Fig. 3H and CAR NK-92 in Fig. 3J of this rebuttal) or HA (Fig. 3I of this rebuttal). We performed similar experiment by comparing IFNγ release by scramble or BECN1 overexpressing NK-92 (Fig. 3K of this

rebuttal). Interestingly, ELISA showed a strong increase of IFNγ release in BECN1 overexpressing NK-92 cells. IFNγ is not affected in the other conditions, and even decreased in NK-92 cells treated with HA.

In Figure O, there are more live NK cells; is it their lifespan or their proliferation that induces this difference?

Response:

Regarding NK cell persistence *in vivo*, we appreciate the reviewer's insightful comment highlighting this critical aspect. To address this, we performed additional analyses to evaluate NK cell viability and proliferation in tumor models. Specifically, we repeated the *in vivo* trial with metformin-treated NK-92 cells and assessed NK cell abundance and proliferation (via absolute cell count and Ki67 staining) in the tumor and peripheral blood of tumor-bearing mice. Our results demonstrate that both metformin and HA treatment, which activate autophagy, increase the absolute number of viable NK cells within the tumor (Fig. 4Jand Fig. 6N). However, absolute count and proliferation rate of NK-92 cells in the peripheral blood of mice were not affected (Fig. 3 L-O of this rebuttal).

Figure 5
In figures G, H, K, L, it would be interesting to have conditions where CXCL12 is added when there is Plerixafor and when CXCR4 is KO. That Plerixafor inhibits CXCR4 should be shown. CXCL12 should be quantified in the secretome of tumor cells to show that tumor cells secrete this chemokine, which will block the functions of NK cells.

Response:

We performed *in vitro* experiments to assess this point. We now show in Extended Data 7D,G that Plerixafor reverts the downregulation of autophagy and lytic activity of NK-92 cells upon exposure to CXCL12. Same results were obtained with CXCR4 KO NK-92 cells (Extended Data 7I,J). Additionally, ELISA assays detected CXCL12 in the supernatant of PC3 human cancer cells and $Pten^{-/-}$, $Pten^{-/-}$;$Trp53^{-/-}$ murine tumor cells (Extended Data Fig. 7M,N).

Figure 6
The absolute counts of NK cells and the analysis of their cytokine function should be shown. Why is there no analysis on IFN-γ in Figure 6J?

Response:

As mentioned above, absolute cell counts have now been included (Fig. 6N). IFNγ expression has also been included in Fig. 6P of the revised manuscript.

Figure 7
It should be shown that Plerixafor inhibits CXCR4 on PD-L1 CAR NK-92 cells.

Response:

We performed *in vitro* experiments to assess this point. We now show in that Plerixafor reverts the downregulation of autophagy and lytic activity of CAR NK-92 upon exposure to CXCL12 (Extended Data 7Q-R).

The absolute counts of NK cells and the analysis of their cytokine function should be shown

Response:

Absolute cell counts have now been included (Fig. 7G) and IFNγ expression has been shown in Fig. 7I.

MINOR COMMENTS :
• In figure 6J, there is a missing legend below the graph.

Response:

We apologize and we corrected the error.

• In Figure 2G, there are white squares on the FACS plots.

Response:

We apologize and we deleted the white squares.

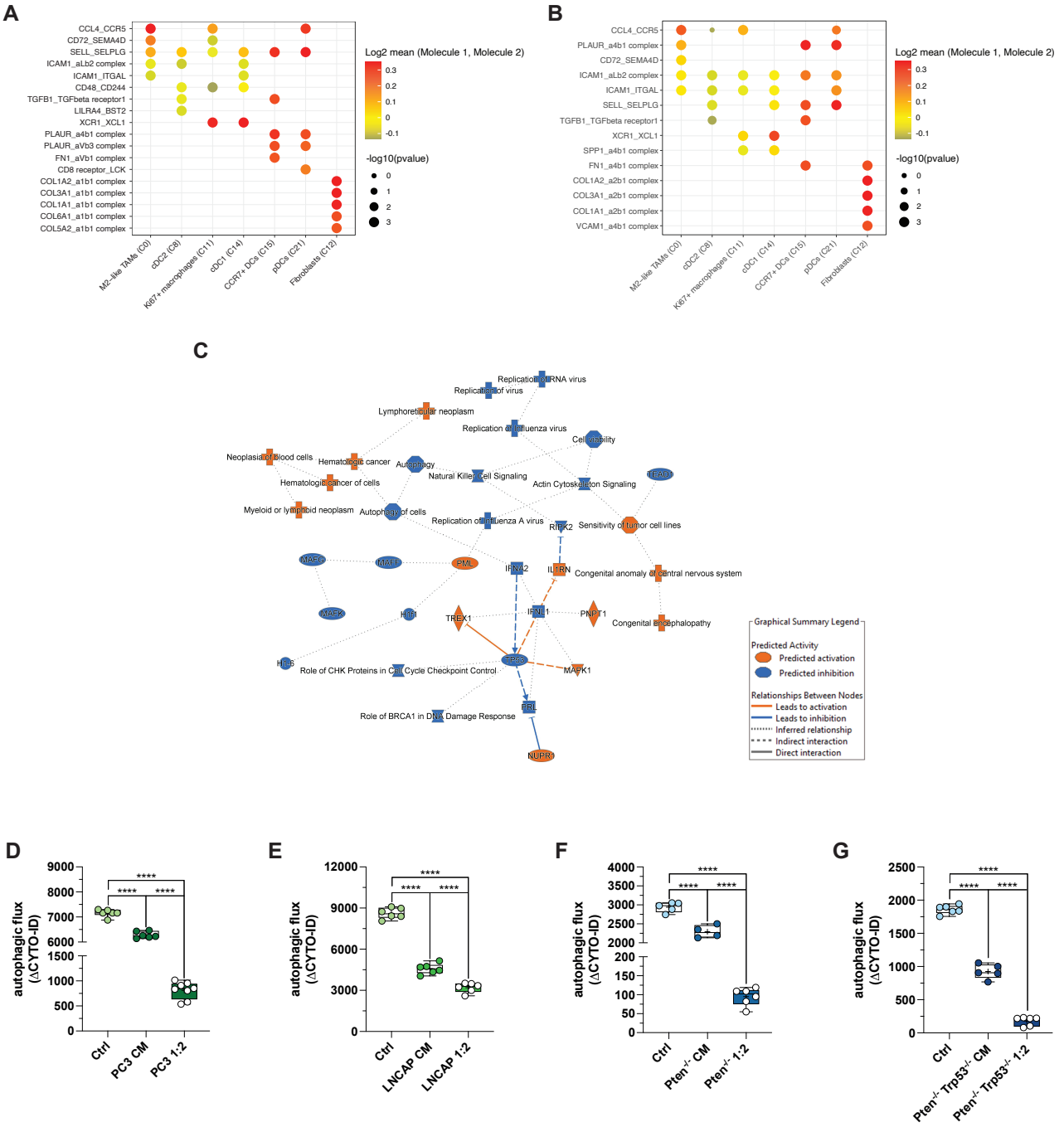

Figure 1

A,B) CellPhoneDB intercellular communication analysis between NK cell subsets, cluster 2 (A) and cluster 13 (B), and selected immune cell clusters identified by scRNA-seq. Bubble plot indicates the mean L:R expression (color scale) and the corresponding p value (size of the bubble). For each clusters, the top 5 interactions, according to mean L:R expression, are shown. C) Network of differentially expressed genes in tumor *vs* non-tumor NK cells, as computed with Ingenuity Pathway Analysis software. Continuous lines indicate direct functional relationships, dashed lines indicate indirect or inferred relationships. Network nodes are labeled with gene name and canonical pathway name. D-G) Graph showing the ΔMFI of CYTO-ID (autophagic flux) in murine NK cells (D,E) and in human NK-92 cells (F,G) upon exposure to tumor-conditioned medium (CM) or upon co-culture at 1:2 Effector-to-Target ratio with specified prostate cancer cell lines.

Data in (D-G) are presented as Min to Max box-and-whisker plot with median indicated at center line and mean value indicated as "+"; one-way ANOVA test. ****p < 0.0001.

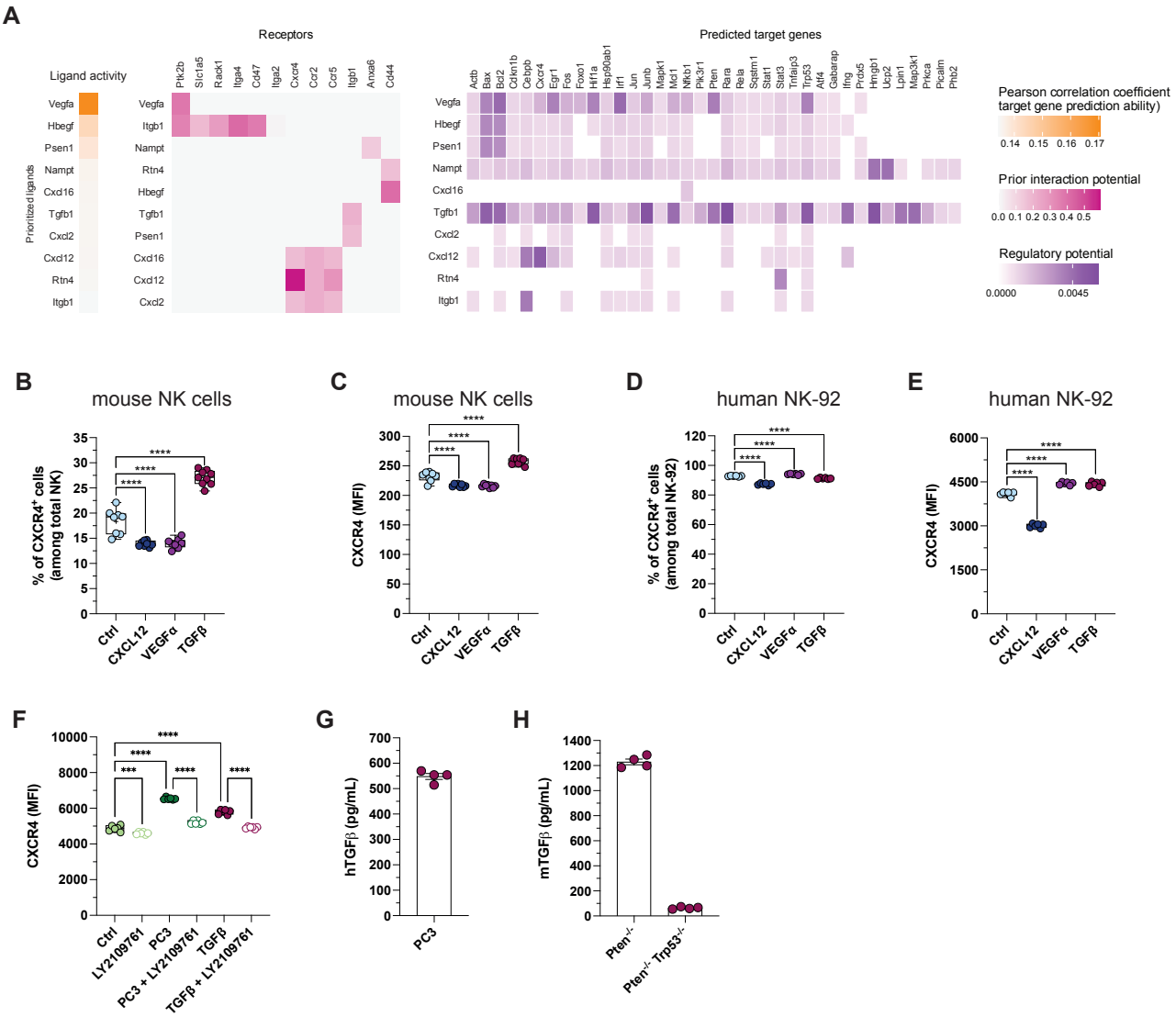

Figure 2

A) Results of the NicheNet analysis performed on scRNA sequencing, described in 4E. Ligands predicted in basal epithelial cells (cluster 17) were ranked by the likelihood that the ligand would affect gene expression changes in autophagic pathway (expressed as Pearson correlation coefficient). Receptors, expressed in NK cells (cluster 13), were selected based on their known potential to interact with the prioritized ligands. Target genes belonging to the autophagy pathway are shown, according to their regulatory potential to affect the L:R interaction. B,C) Graph showing the percentage of $CXCR4^+$ cells (B) and the MFI of CXCR4 (C) in splenic NK cells, treated with CXCL12, VEGFα or TGFβ (n=7 biological replicates). D,E) Graph showing the percentage of $CXCR4^+$ cells (D) and the MFI of CXCR4 (E) in NK-92 cells, treated with CXCL12, VEGFα or TGFβ (n=5 biological replicates). F) Graph showing the MFI of CXCR4 in NK-92 cells, treated with TGFβ or tumor-conditioned medium, in presence or absence of TGFβ receptor type I/II inhibitor LY2109761 (n=6 replicates, independently collected batches of tumor-conditioned medium). G,H) ELISA-based quantification of TGFβ release from human (G) and murine (H) prostate cancer cell lines (n=4 biological replicates).

Data in (B-F) are presented as Min to Max box-and-whisker plot with median indicated at center line and mean value indicated as "+"; one-way ANOVA test. Data in (G) and (H) are presented as scatter plot with mean ± SEM. ***p < 0.001, ****p < 0.0001.

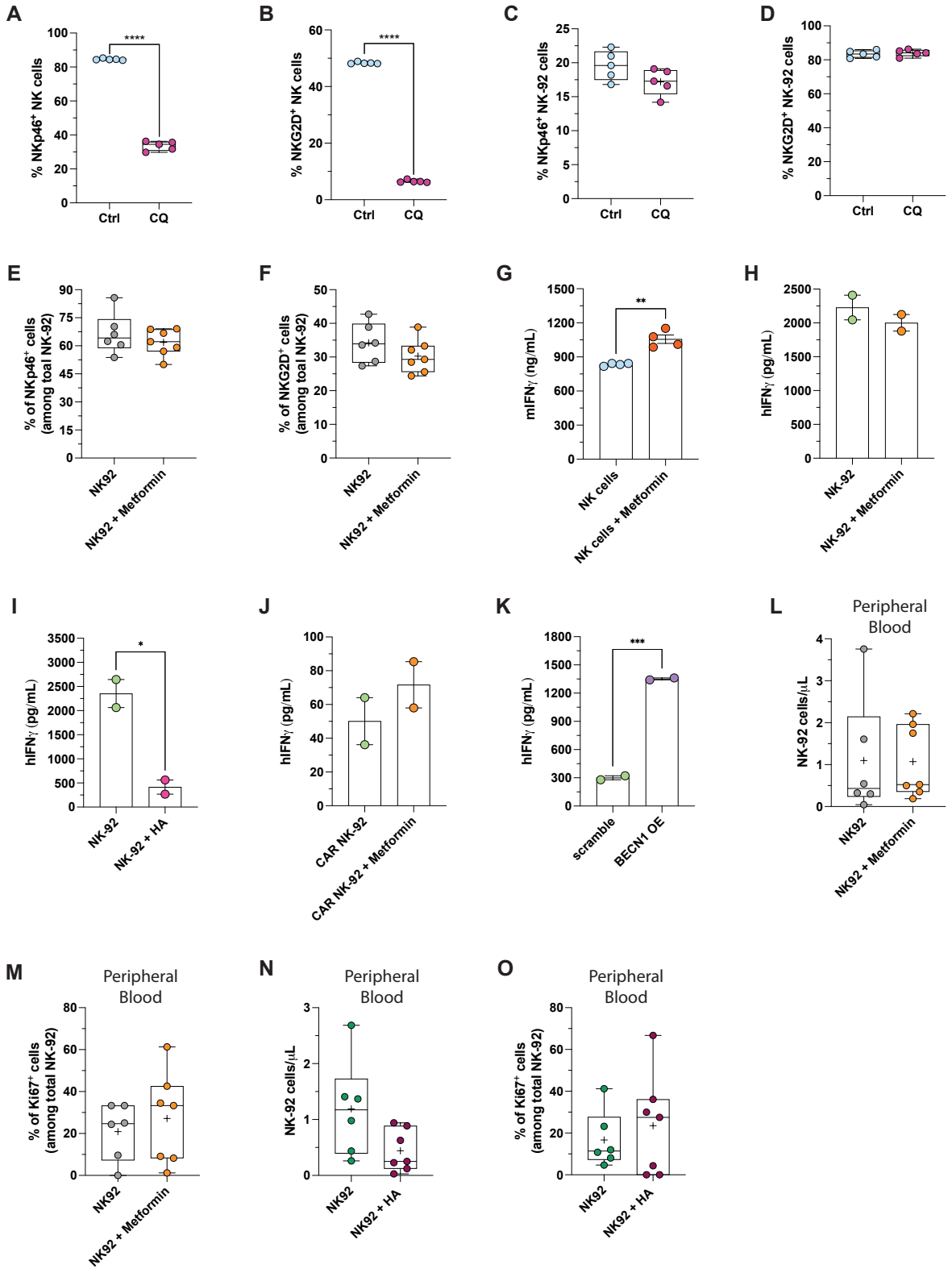

Figure 3

A,B) Graph showing the percentage of NKp46$^+$ (A) and NKG2D$^+$ (B) murine NK cells, in control condition or upon exposure to chloroquine (CQ) treatment, as evaluated by FACS analysis (n=5 biological replicates, NK cells from different mice). C,D) Graph showing the percentage of NKp46$^+$ (C) and NKG2D$^+$ (D) NK-92 cells, in control condition or upon exposure to chloroquine (CQ) treatment, as evaluated by FACS analysis (n=5 biological replicates). E,F) Graph showing the frequency of NKp46$^+$ (C) and NKG2D$^+$ cells (D) in tumor-infiltrating NK-92 cells in mice injected with untreated or Metformin-treated NK-92 cells, as determined by flow cytometry. G) ELISA-based quantification of IFNγ release

from murine NK cells, exposed or not for 24h to metformin treatment. H-K) ELISA-based quantification of IFNγ release from NK-92 cells; H) untreated *vs* metformin-treated NK-92 cells, I) untreated *vs* HA-treated NK-92 cells, J) untreated *vs* metformin-treated CAR NK-92 cells, K) scramble *vs* BECN1 overexpressing NK-92 cells. L) Graph showing the absolute cell count of circulating NK-92 cells in mice injected with untreated or Metformin-treated NK-92 cells, as determined by flow cytometry (n=6 mice injected with control NK-92, n=7 mice injected with Metformin-treated NK-92). M) Graph showing the frequency of $Ki67^+$ NK-92 cells in peripheral blood of mice injected with untreated or Metformin-treated NK-92 cells, as determined by flow cytometry (n=6 mice injected with control NK-92, n=7 mice injected with Metformin-treated NK-92). N) Graph showing the absolute cell count of circulating NK-92 cells in mice injected with untreated or HA-treated NK-92 cells, as determined by flow cytometry (n=6 mice injected with control NK-92, n=7 mice injected with HA-treated NK-92). O) Graph showing the frequency of $Ki67^+$ NK-92 cells in peripheral blood of mice injected with untreated or HA-treated NK-92 cells, as determined by flow cytometry (n=6 mice injected with control NK-92, n=7 mice injected with HA-treated NK-92).

Data in (A-F) and (L-O) are presented as Min to Max box-and-whisker plot with median indicated at center line and mean value indicated as "+"; unpaired *t* test. Data in (G-K) are presented as scatter plot with mean ± SEM; unpaired *t* test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Table 1

Old manuscript		Revised manuscript		New panels	
Figure	Panel	Figure	Panel	Figure	Panel
1	A	1	A	1	E
1	B	1	B	1	F
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Point by point response

Title of the manuscript: C/EBPβ-dependent autophagy inhibition hinders NK cell function in cancer

To the attention of the Editor and reviewers,

we appreciate the constructive feedback received and we believe that the final version of the manuscript, that we are now submitting, is much improved thanks to the referee's comments and constructive suggestions.

Please find below a point by point response to the last comments.

Reviewer #1 (Remarks to the Author):

The authors have fully addressed all the comments from this reviewer. There are no additional comments. I now recommend publishing this work.

Mediating comments for R#3:

I have read the response to Reviewer #3. The authors have fully addressed the reviewer's comments. I suggest accepting this revised manuscript without further revision.

Reviewer #2 (Remarks to the Author):

The manuscript has been much improved and shall be accepted.

The only point for which I could not find an answer concerned whether differences in cytotoxicity were directly related to differences in maturation or not (first point). This does not affect relevance of the data, but shall be acknowledged as a possibility. E.g. "Tumor-infiltrating NK cells, isolated from Ptenpc-/- prostates (Fig. 1F), and splenic NK cells from Ptenpc-/- tumor-bearing mice (Extended Data 1H) presented a reduced ability to kill target cells when compared to splenic cells from WT mice, thus confirming their dysfunctional state."
shall be corrected to:
"Tumor-infiltrating NK cells, isolated from Ptenpc-/- prostates (Fig. 1F), and splenic NK cells from Ptenpc-/- tumor-bearing mice (Extended Data 1H) presented a reduced ability to kill target cells when compared to splenic cells from WT mice, thus confirming their dysfunctional state, which could directly relate to their altered maturation stages."

We thank the referee for this comment, we now modified the text accordingly.