nature portfolio

Peer Review File

NKG2A inhibition promotes NK cell-CD8+ T cell interactions to improve anticancer immunity in ovarian carcinoma

Corresponding Author: Dr Jitka Fucikova

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)
This manuscript presents a comprehensive and well-executed characterization of NK cell populations in NSCLC and HGSOC, offering valuable insight into their distinct immune landscapes. The comparative approach between 'hot' and 'cold' tumors is compelling, and the findings regarding NKG2A+ NK cells and their potential role in immune suppression are particularly noteworthy. While the narrative could benefit from improved flow and clarification in some sections, the study contributes meaningfully to our understanding of NK-T cell interactions in solid tumors. Revisions are required, and the reviewer provides constructive comments to support the authors in strengthening the manuscript:

Major Comments
1. Line 458: The manuscript implies differences in NK1 vs NK2 profiles are most evident in benign omentum vs metastasis, but it appears that the most distinct changes may be observed between region 2 (tumor-distant) and region 3 (peritumoral) versus region 4 (metastatic site). Please clarify which comparisons are being emphasized.
2. Lines 405–406: Please specify which additional signaling lymphocyte activation molecule (SLAM) family receptors were observed to be involved beyond SLAMF6 and SLAMF7.
3. Figure 4I: Consider changing the orange overlay to green to enhance contrast with the yellow background. Clarify what P1–P4 represent. Additionally, it is difficult to distinguish eTLSs from mTLSs in this panel—are extra markers being used compared to 4G?
4. Line 558: The data appear to suggest a correlation between both eTLSs and mTLSs with CD57+ NK cells in NSCLC. Why is the text emphasizing only mTLSs?
5. The manuscript mentions interactions between NKG2A+ NK cells and CD8+ T cells and survival outcomes. Please include supporting plots or correlation coefficients in the main or supplementary figures to substantiate these claims.

Minor Comments
6. Lines 404–408: The sentence describing gene expression in NK1 cells is difficult to follow. Consider splitting it and clarifying the contrasting expression patterns between NSCLC and HGSOC.
7. Line 410: The statement that KLRC1 is elevated in NK1 cells in HGSOC appears inconsistent with the figure. Please verify this.
8. Lines 423–424: Strengthen the logic linking NK cell subtype differences with CD8+ T cell differentiation—was this derived from transcriptomic data or predicted interactions?
9. Line 455: Add a figure reference to support the mention of previous observations in primary HGSOC.
10. Line 469: Grammatical correction: “with instead display” → “instead displaying”.
11. Line 523: Grammatical correction: “suggesting to potential negative…” → “suggesting a potential negative…”
12. Line 537: “bright” is misspelled—please correct.
13. Lines 561–572: Clarify the rationale for comparing both SO1 and BR5 models. State explicitly what each adds to the study.
14. Line 561 (Section Title): Suggest rephrasing the section title to reflect the bidirectional impact of CD8+ and NK cell depletion.
15. Line 578: Clarify how the observations in this paragraph recapitulate Figure 3 findings. Reference specific panels.
16. Lines 603, 607: Use “blockade” rather than “blockage” in the context of immune inhibition.
17. Figure Legends: Improve clarity in UMAP and flow cytometry figures, especially Supplemental Figures 3–5. Complex plots would benefit from more descriptive legends and clearer labeling.

18. Quantification of Immunofluorescence: Figures such as Supplemental Figures 2 and 5 should include quantitative data (e.g., TLS density, cell distances) to support image-based conclusions.

Additional observations
• In the SO1 model, early combination therapy didn't seem to suppress tumor progression effectively. Have the authors assessed metastatic spread or quantified total tumor burden?
• The translational relevance of these findings could be emphasized more clearly. For instance, how do the results inform clinical strategies involving NKG2A blockade (e.g., monalizumab) and PD-1 inhibitors? Could this inform future patient stratification?
• Consider briefly touching on how these observations may (or may not) differ in early-stage disease or post-treatment settings.
• Including a visual timeline or schematic of the in vivo experimental protocols would help readers navigate the mouse data more easily.
• Lastly, the Methods section should specify how spatial proximity thresholds (e.g., 10 μm) were chosen and whether they were empirically validated.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)
N/A

Reviewer #3

(Remarks to the Author)
\This work represents a multi-omic approach in several patient cohorts and mice to understanding the role of NK cells in HGSOC tumours. While I am impressed with the extent and breadth of the paper, inclusion of so much data perhaps interferes with a mechanistic or thorough interpretation of it and the story might better be told in separate parts.
The story moves through comparisons of HGSOC to NSCLC as putative checkpoint resistant and sensitive tumours, respectively, to assessing TLS formation, NK cell localization and finally co-blocking. While I agree that there are many associations present, they are each incompletely explored before moving to the next section. The observations are interesting and aligned with current understandings in the field, but they often fall short of providing or even speculating at the mechanistic reasons behind their presence. The focus on NSCLC is lost after the first couple of figures, and the overarching logical progression through the paper is missing. In general, results are described, but the hypotheses leading through the paper are not.
1. Line 82: The statement that HGSC has a low TMB is somewhat misleading. The statement would be more accurate if TMB were described as moderate or variable, as it tends to change substantially between patients (and even within them). Since the tumours are variable (some HGSC patients do respond to ICI), the authors should take caution in referring to HGSC as ICI resistant, laying the groundwork for how this is a model system and a generalized conclusion (likewise for NSCLC). This is noteworthy throughout, and especially for the text associated with figure 4. These are two different tumours (with subsets of each known to exist). It is an oversimplification to bucket them based on ICI sensitivity when there are many other reasons why these tumours are expected and known to differ.
2. Lines 83ff: NK cell recognition is not irrespective of antigen presentation on MHC I molecules. Interactions between NK Cells and MHC drive NK cell licensing, and also the peptide loaded can be involved in triggering receptors. Please rephrase. I also recommend using a more updated review article (present reference is from 2015; much modern research has unveiled the ways in which MHC I and loaded peptide impact NK:target/NK:self interactions). Likewise, refs 16 and 17 (line 93) refer to functional variation; both of those (albeit modern) papers focus more on transcriptomics of NK cells.
3. Study cohort descriptions: the specific sites of these samples need to be disclosed, as do the purposes of each study (this might be more efficiently achieved in a table). Presumably, RNAseq data and "tissue samples" refer to tumours, but this is unclear. How are samples normalized? Are they comparable, given that they were not expressly assessed for the purposes of comparison? How data were processed (including pre-processed), assessed and reported (i.e. fold change? Z-scores etc) is important information for the methods section.
a. Were the patients assessed for genomic alterations? In both cancers (but especially NSCLC), somatic mutations can impact disease progression and treatment approaches (and thus might impact the reported results).
b. The authors describe several cohorts studied; some of which were from public databases; others from their own sequencing. Ethics numbers are missing from at least Study cohort 6, and should be reconfirmed for all.
c. The specific interventions for each cohort are not given. For example, what was done with the patient materials in cohorts 5 and 6?
d. Were the pathology specimens ever overlapping with those assessed genomically?
e. In the results section, "study cohorts" are jumped between often; this makes the story difficult to follow. It would be better to be clear about what the comparisons are.

4. Recheck nomenclature throughout. For example, it's a Fortessa, not a FACSFortessa; IFNG is a gene name, but it's referred to as a protein that is being stained for (i.e. IFN-γ). Figures and display items should be presented in the order that they are introduced.
5. 15% as a higher threshold for mitochondrial gene content in single cell analysis is unconventionally high. The authors should explain the logic for this.
6. Single cell (lines 381 and following): the Rebuffet paper cited (ref 16) clusters NK cells into three groups with subclusters within them. As described, these clusters do not fully align: for example, the NK3 population is fully absent, and some of the features ascribed to the NK1 population might belong there. This work also understates the differences between the NK1 subpopulations, as they were described in the referenced paper (i.e. ABC, and also the NKint population).
a. How did the total number of NK cells compare between the HGSC and NSCLC patient groups? Were they represented similarly among patients within each disease cohort?
b. Likewise, how did the total numbers of NK cells compare between omental/metastatic sites?
7. In any expression analysis, there are always correlations that are not necessarily causations. The conclusions on line 431 and following state ER stress as being associated (it is) but were other pathways/GO terms explored? What about other features of NK cell biology in general, including, but not limited to those discussed throughout the paper (i.e. migration, cytokine production, regulation)? Considering the later inferences of the paper and spatial biology/interaction with T cells, as they are described, the authors could use the single cell data for more than NK cell analysis. Additional assessments of the immune architecture and interactions within these tumours would be informative.
8. Figure 2 E and following: The comparisons should be better described with respect to the logic. While I can infer why particular markers are being chosen, it might not be clear to the reader why NKG2A and NKp46 are being used. Have the authors considered the potential confounding contributions of NKp46+ ILCs? What is the purpose in comparing inhibitory marker expression to CD8+ T cells, where there will be some overlap, but not a reasonable expectation of the same expression levels?
a. Figure 2G's colour scheme is confusing since all of the dots are black and the legend is in the opposite order to the bars. This same problem persists in other figures (i.e. 3C-F). Likewise, the colour scheme in K is somewhat difficult to distinguish without zooming in extensively.
9. Have survival probabilities been adjusted for additional factors (stage, grade, treatment response, sex, comorbidities, somatic mutations?)
10. TLS contain many cells, but NK cells are not typically part of the considerations around these "neighbourhoods". What is the logic for comparing NK cells in this way? Why not associate neighbourhoods that have included considerations of NK cells, such as those described by Nersesian et al (Front Immunol 2024) or use an unsupervised approach to defining neighbourhoods? There are likely to be more associations that directly associate with NK cells with these approaches. As the paper goes on to conclude a positive cooperation between NK Cells and T cells (figure 5), this becomes an even more important consideration. Conversely, associations between other TLS components and NK cells is never explored further. What is the reasoning or expected logic linking NK cells and TLS? Is a TLS simply a mark of an immunologically "productive" tumour that also includes NK cell function?
11. In general, the authors have presented a lot of data, but fallen short of its immunologic interpretations. What is the mechanism(s) by which the combined NKG2A and PD1 are expected to work, for instance? While it is possible that they restore the function of NK cells and T cells, respectively (which is supported by Figure 6A), each population has been reported to express both markers (to varying extents).

(Remarks on code availability)
The code is provided and mostly from public sources.

Reviewer #4

(Remarks to the Author)
The paper by Tereza Lanickova et al. recapitulate a very interesting, sounded, well sounded and well-written study thta will be certainly of great interest for the community and reader of Nat Com. it certainly advances our knowledge in regard of the clinical translation and potential therapeutic uses of the checkpoint inhibitor NKG2A.

I have a few comments and queries that I would like authors could better address:

Major points:
1. The authors adopt NK1 and NK2 signatures to better identify NK cells once subclustered but never mention NK3. Although less common in tissues, there are genes (IL32, GZMH) that are upregulated in NK1 in NSCLC samples that could be index of NK3/adaptive NK cell signature, has this aspect been investigated?
2. Although the expression levels of CD57 and NKG2A correlates with CD56dim/NK1 and CD56bright/NK2 respectively, the expression of high levels of CD57 are mostly associated with terminally differentiated CD56dim only, while it is also true that clusters of CD56dim NK cells retain the expression of NKG2A, which hampers their validity as surrogate markers. Wouldn't it be better to combine a few more markers to better discriminate CD56dim and CD56bright? Additionally, in this setting the authors also point out the absent expression of GZMB in CD56bright, although coherent, several studies showed GZMB to be expressed by CD56dim only, while CD56bright mostly elicit cytotoxic features via other mediators.
3. The authors reported a functional exhaustion of CD56bright NK cells in primary HGSOC by comparing the transcriptional profile of these NK cells with CD56dim from benign omentum. However, the described transcriptional differences are already known features of CD56bright and CD56dim. To define a functional exhaustion of CD56bright NK cells in HGSOC, a comparison with CD56bright NK cells from a healthy tissue could be more informative.
4. The functional exhaustion of CD56bright NK cells described at the transcriptional level is not supported by functional

experiments. The authors reported a relative enrichment of NKG2A+ NK cells in HGSOC microenvironment compared to the NSCLC microenvironment. Do these cells show a lower ability to produce cytokines in vitro too?
5. As described by the authors in the discussion section, targeting NKG2A in a clinical setting is related to the expression of its ligand HLA-E. What about HLA-E expression in their cohorts? Is the expression of NKG2A related to the expression of HLA-E?
6. For what concerns the NSCLC cohort 4, the authors indicated that the patients had a stage disease III+IV. However, in the Suppl. Table 1B the stage diseases reported are mainly I and II. How do the authors explain this discrepancy?
The same for NSCLC cohort 9, in the text it has been specified that the samples were obtained from stage III+IV patients, but in the table are reported early stages too.
7. For Figure 3E the authors stated that NKG2A+ NK cells are equally represented in tumor cores and stromal areas in both HGSOC and NSCLC. Even if not statistically significant, the frequency of NKG2A+ NK cells is higher in the tumor core of HGSOC compared with the stromal area.
Moreover, in the HGSOC tumor core, there are different groups of patients with different frequencies of NKG2A+ NK cells. Is this related to any feature of the patients? Such as HLA-E expression?
8. In the tables with the information of the patients, the vital status is reported, after how much time is it calculated?
9. For the Suppl. Table 1B how is the percentage of NK cells, CD56dim, and CD56bright obtained?
10. In the Methods section, the authors state that “final cell suspensions were washed, counted, and resuspended in FACS buffer for further analysis”. Does this imply that all samples were processed as fresh material and not frozen? Have you accounted for batch effects related to sample processing and FACS acquisition timing? Regarding scRNA-seq library preparation, were libraries generated fresh from each individual sample, or were they prepared in batches? If the first option, have you accounted this while processing the scRNA-seq data?
11. In the scRNA-seq quality control steps, there is no mention of tools or strategies used for doublet removal. Have you assessed the presence of doublets in your dataset and excluded them accordingly?
12. Could you please specify which covariates were used for data integration in the scRNA-seq analysis?
13. In Figure 1I, are the counts comparable across samples? It would be more appropriate to represent the data as normalized frequencies (e.g., percentage of total cells per sample) rather than as absolute cell counts.

Minor points:
1. Some typing errors are present.
2. In the methods section “scRNA-seq sample preparation” the phrase regarding the study cohort 7 is not complete.
3. For the results of Figure 1A the authors used genes with documented NK-cell modulating effects, however the reference for these genes is missing.
4. In the legends of Suppl. Figures 3 and 4, the gating strategy used is not described.

(Remarks on code availability)

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
Overall, the authors have addressed previous reviewer comments comprehensively. The revised manuscript and rebuttal are clear, and the additional figures substantially strengthen the mechanistic and translational aspects of the study.

Only one minor clarification is suggested:

In the Introduction (lines 111–113), the sentence describing the SO1 model as having “high TMB” could be interpreted as implying that anti-NKG2A monotherapy confers a survival benefit. However, as shown in Figure 5 and Supplementary Figure 16 of the rebuttal, survival benefit was only observed when anti-NKG2A was combined with anti-PD-1. Consider rewording this sentence to reflect that therapeutic benefit occurred only in the combination setting.

Reviewer #2

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #3

(Remarks to the Author)
Lanickova et al have submitted an updated version of their manuscript and lengthy rebuttal in response to the reviewers’ comments. The paper continues to explore the immunologic landscapes of HGSC, in particular with respect to NK cells. By comparing transcriptomic approaches and HGSOC with NSCLC, the authors uncover an enrichment for NK2 NK cells in

HGSOC and the other populations – 1 and 3 in NSCLC. They conclude that this reflects an NK cell disregulation/exhaustion in HGSOC which might reflect differences in responsiveness to checkpoint inhibition and therapeutic intervention. The reader is lost in the details (which might more effectively be conveyed in table/figure format then many lines of text with gene symbols).

1. While I appreciate the thoroughness of the authors' responses, the paper, in general, needs to be edited for brevity and clarity. There is too much data presented in figures and supplementary material; all points to inferred relationships, but brevity would dramatically improve the impact of this paper. Seven Figures and 17 supplementary, all with >10 panels is just too much.

In some instances/newly added text, the writing is unclear and should be edited throughout. There is a lot of data and run-on sentences, which make interpretation difficult for the reader. As a reviewer, I do not see my role in editing, but provide just a few examples below:

Lines 887-893 (one sentence; 79 words):
"Harnessing multiple cohorts of patients with advanced HGSOC and an immunocompetent mouse model of the disease, here we demonstrate that (at odds with the immunologically "hot" NSCLC microenvironment) both primary and metastatic HGSOC microenvironment (including HGSOC-associated TLSs) is often enriched in CD56bright NK cells expressing the co-inhibitory receptor NKG2A, which (at least in preclinical settings) can be harnessed to unleash a therapeutically relevant interaction between intratumoral NK cells and CD8+ 893 CTLs in the context of concomitant PD-1 blockade. "

Or lines 850-856):
"Moreover, SO1 tumors responding to NKG2A blockade contained an increased frequency of CTLs co-expressing PD-1 and transcription factor 7 (TCF7, best known as
TCF1), which are known to respond to ICIs (57-60) and a decreased frequency of CD8+ CTLs
853 expressing NKG2A+ or co-expressing PD-1 and TIM3, both of which have previously been
854 associated with dysfuncton(42,61-63) (Fig. 6K).

Finally, the discussion lacks consideration of the limitations of the study – mainly that it is nearly all transcriptomic-based, and would benefit from an exploration of what would be necessary for protein-level and functional validation (beyond the one mouse model shown) to approach the phenotypic and challenging diversity of HGSOC.

2. The manuscript, in general, still suffers from inferring functionality from transcriptomic data. "Functional validation" is almost always conducted with more omics data, which, while supportive, cannot confirm function. For instance, lines 747-753 describe "functional" validation of T-NK interactions using spatial transcriptomics. Indeed, there is co-localization that associates with poorer outcomes, but whether this is causative is unknown. The authors, throughout, should be careful to dissociate actual from inferred function.
3. The mouse model supports a blockade strategy to promote anti-tumour activity against HGSOC. Is there evidence that this model recreates the complexity and immune structures described in the earlier figures of the paper? This would help to fill in some of the missing functional validations if their work in situ could be supported.
4. Spatial analysis: 10 neighbourhoods with respect to NK cells were defined – what is the logic for selecting such a large number of neighbourhoods. Are they, in fact, distinct? What were the criteria for inclusion/exclusion or definition of a neighbourhood, and are they expected to be functionally distinct?
5. I caution against the term "exhausted" for NK cells (i.e. line 651-658): they upregulate immune checkpoint transcripts, but functional conclusions cannot be made. In the context of T cells, such upregulation is often seen on activated T cells (and might simply reflect the same on NK cells).
6. The authors contend that their NK cells are defined by a "robust gating strategy" but have not described it. The possibility remains that NKp46+ cells from tumours are ILCs, such as those described by Crome et al. Further, (662-676) the authors should avoid making conclusions about function without performing functional studies – expression of checkpoints or GZB does not necessarily equate to functional changes unless they are experimentally tested. The following paragraph (T cells) similarly draws functional conclusions from phenotypic data.
7. The discussion should include some description about the limitations of transcriptomic/phenotypic data.

Reviewer #4

(Remarks to the Author)
In this revised form of the manuscript, authors properly addressed all my queries. Hence, I do confirm that this sound and well-written work by Tereza Lanickova et al. will be certainly of great interest for the community and reader of Nat Com. and remarkably advances our knowledge in regard of the clinical translation and potential therapeutic uses of the checkpoint inhibitor NKG2A.
Even the review process and rebuttal looks very meticulous by addressing every single issues and raised point.
Excellent work ready to be published

Version 2:

Reviewer comments:

Reviewer #4

(Remarks to the Author)
No additional comments. The paper is suitable too be published on Nature Communications in this presente re-revised form.

Ref: ***NCOMMS-25-31476***
Manuscript: NKG2A inhibition promotes NK cell-CD8+ T cell interactions in support of improved anticancer immunity in ovarian carcinoma
Authors: **Tereza Lanickova *et al.***
Corresponding author: **Jitka Fucikova**

POINT-BY-POINT REPLY TO REVIEWER N° 1

Reviewer n° 1 commented:

This manuscript presents a comprehensive and well-executed characterization of NK cell populations in NSCLC and HGSOC, offering valuable insight into their distinct immune landscapes. The comparative approach between 'hot' and 'cold' tumors is compelling, and the findings regarding NKG2A+ NK cells and their potential role in immune suppression are particularly noteworthy. While the narrative could benefit from improved flow and clarification in some sections, the study contributes meaningfully to our understanding of NK-T cell interactions in solid tumors. Revisions are required, and the reviewer provides constructive comments to support the authors in strengthening the manuscript.

Our response: We thank Reviewer #1 for the revision of our work and enthusiastic evaluation of our manuscript. Inspired by their constructive comments, we have performed various additional experiments and re-analyzed available data, overall providing additional mechanistic details into our findings and hence further strengthening our conclusions, as detailed below.

And raised these major comments:

1. Line 458: The manuscript implies differences in NK1 vs NK2 profiles are most evident in benign omentum vs metastasis, but it appears that the most distinct changes may be observed between region 2 (tumor-distant) and region 3 (peritumoral) versus region 4 (metastatic site). Please clarify which comparisons are being emphasized.

Our response: Inspired by the constructive comment of Reviewer #1, we now provide a separate analysis of NK cells residing in the omentum with metastatic lesions (tumor-distant), in the peritumoral region adjacent to tumor nodules (peritumoral), and within metastatic lesions (metastatic site), with clustering patterns consistent with those previously described by Rebuffet et al. (Nature, 2024) (Fig. 1A of this rebuttal letter; Study Cohort 8).

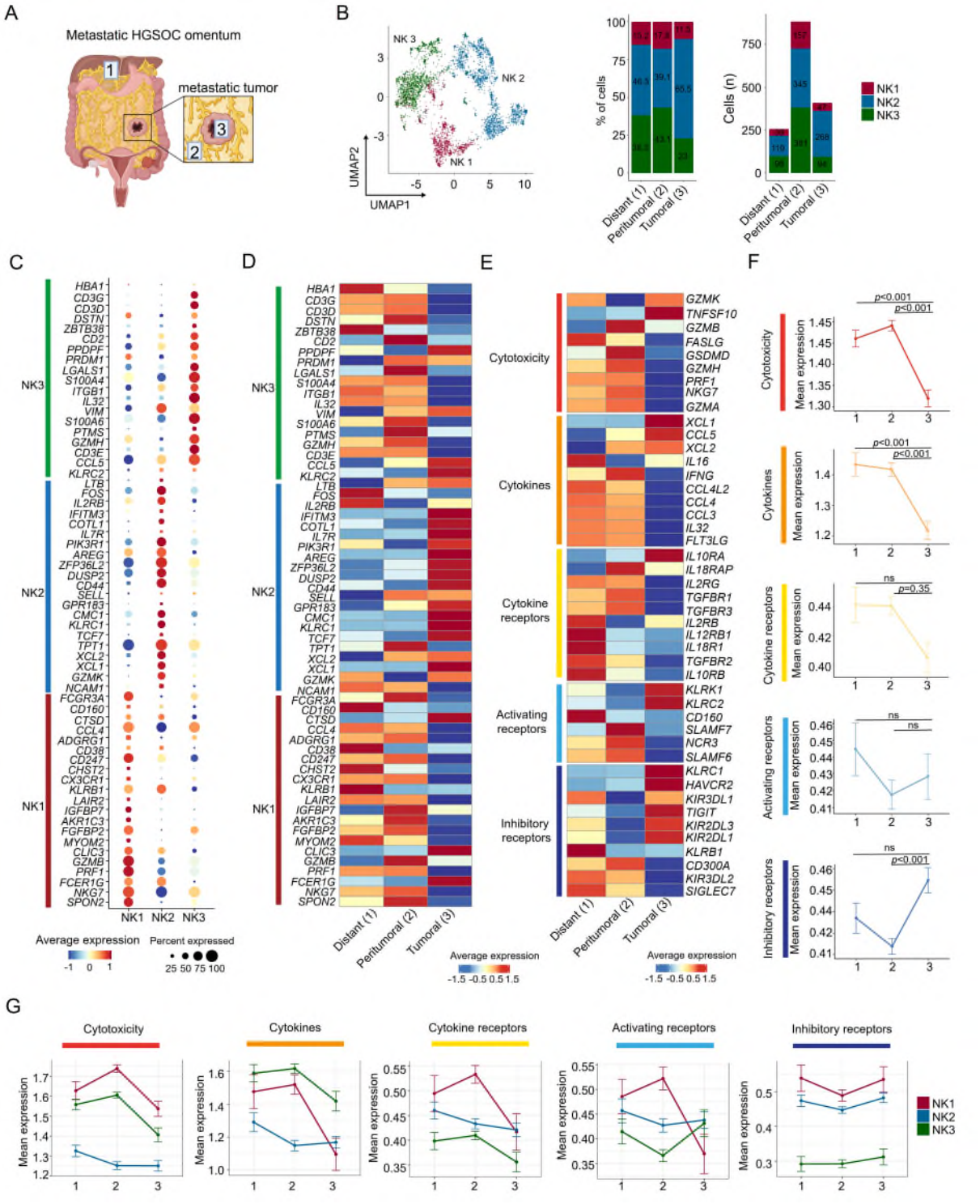

Figure 1. **Metastatic gradient in HGSOC reveals NK1 and NK3 polarization toward functionally exhausted NK2 cells. (A)** Overview of anatomical sampling sites across omentum with metastases (n=7), comprised of three different omental regions: macroscopically tumor-free sites distant to the omentum metastasis (1), and peritumoral region adjacent to the tumor (2), as well as metastatic tumors within the greater omentum (3) (Study Cohort 8). Created with BioRender.com. **(B)** UMAP and stacked plot of NK cells passing the quality control, colored by NK subtype within 3 histological sites (described in panel A) as determined by scRNAseq. **(C, D)** Dot plot and heatmap

of the 20 most distinguishing genes expressed for the NK1, NK2 and NK3 subsets of human NK cells within 3 previously defined histological compartments (as shown in panel A). The color indicates the z-score scaled gene expression levels. **(E)** Heatmap showing the differential expression of markers of interest among NK cell subsets within 3 previously defined histological compartments (as shown in panel A). The color scale is based on z-score-scaled gene expression. **(F, G)** Line plots of gene signatures associated with cytotoxicity, cytokines, cytokine receptors, activating receptors and inhibitory receptors expressed for entire NK cluster (F) and for the NK1, NK2 and NK3 clusters of human NK cells (G) within 3 previously defined histological compartments (as shown in panel A) as determined by scRNAseq. Gene expression was analyzed using the two-sided Wilcoxon rank-sum test with Benjamini-Hochberg adjustment.

Based on previously defined NK1, NK2 and NK3 signatures in HGSOC and NSCLC primary tumors (Fig. 1 and 2 of revised manuscript), we observed a significant shift in the polarization of NK1 and NK3 cells towards exhausted NK2 cells within metastatic gradients towards the tumor tissue as compared to distant and peritumoral regions adjacent to tumor nodules (Fig. 1B of this rebuttal letter). More specifically, we found NK cells in metastatic tumor regions to be characterized by high expression of *IFITM3*, *COTL1*, *IL7R*, *PIK3R1*, *AREG, ZFP36L2*, *CD44*, *GPR183*, *CMC1*, *KLRC1* and *TCF7*, a genetic signature pointing to a predominance of the NK2 ($CD56^{bright}$) population as compared to distant and peritumoral metastasized compartments (Fig. 1C, D of this rebuttal letter). In line with our previous findings, omental metastases were associated with an enrichment of NK cells showing transcriptional signs of functional exhaustion (*KLRC1*, *HAVCR2*, *TIGIT*, *KIR2DL3* and *KIR2DL1*), low expression of cytotoxic molecules (*GZMA*, *GZMB*, *GZMH*, *PRF1*, *GSDMD* and *NKG7*) and specific surface receptors (*CD160*, *NCR3*, *SLAMF6 and SLAMF7*) as compared to NK cells from both distant and peritumoral metastasized compartments (Fig. 1E, F of this rebuttal letter), which predominantly associate with NK2 ($CD56^{bright}$) cluster as indicated in separate analyses (Fig. 1G of this rebuttal letter). These findings extend our previous observations documenting that metastatic gradients toward the tumor tissue exhibit a significant shift in polarization of NK1 ($CD56^{dim}$) and NK3 cells towards NK2 ($CD56^{bright}$) phenotype with prominent signs of phenotypic and functional exhaustion as compared to distant and peritumoral locations, suggesting a detrimental impact of HGSOC cells on NK cell effector functions. These additional analyses were included in Fig. 1, 2 and Suppl. Fig.8 of revised version of the manuscript.

2. Lines 405–406: Please specify which additional signaling lymphocyte activation molecule (SLAM) family receptors were observed to be involved beyond SLAMF6 and SLAMF7.

Our response: As suggested by Reviewer #1, we analyzed the expression of SLAM genes, including *SLAMF1*, *CD48* (also known as SLAMF2), *LY9* (also known as SLAMF3), *CD244* (known as SLAMF4), *CD84* (known as SLAMF5), *SLAMF6*, *SLAMF7*, *SLAMF8* and *SLAMF9* within individual NK subsets of primary and metastatic HGSOC and primary NSCLC (Study Cohorts 6-8). In line with our previous findings, NK2 and NK3 cells expressed high levels of SLAM molecules in primary HGSOC as compared to similar NK subtypes in NSCLC (Fig. 2A of this rebuttal letter). Similarly, we observed high expression of most of SLAM molecules in NK2 cells in primary HGSOC as compared to metastatic HGSOC tumor and omental compartments (Fig. 2B, C of this rebuttal letter). These findings are in line with recent data suggesting the role of SLAM family receptors in late-stage activation of T and NK cells, cancer progression and being considered as potential immune checkpoints on T cells and NK cells, which have prompted the investigation of anti-SLAMF7 antibodies as therapeutic agents for multiple myeloma (*Lonial et al, NEJM, 2015; Dimopoulos et al, NEJM, 2018*).

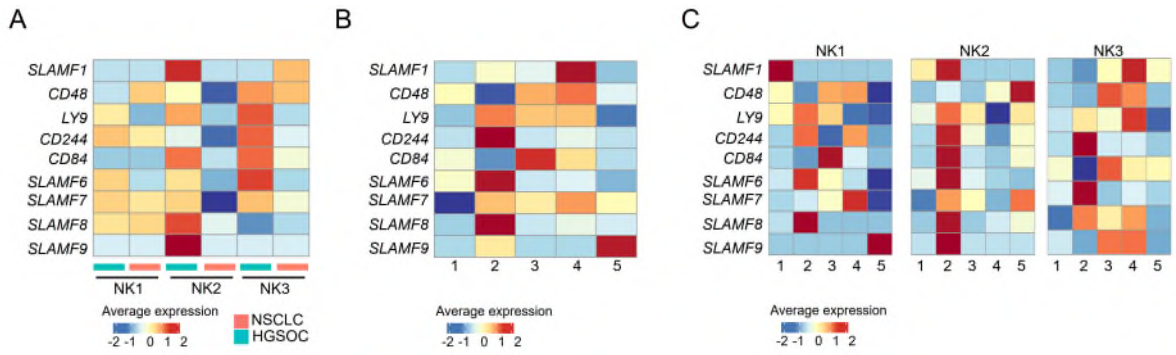

Figure 2. **SLAM molecules expression among NK cells clusters of advanced HGSOC and NSCLC tumor samples.** (**A-C**) Heatmap showing the differential expression of SLAM genes among NK1, NK2 and NK3 cell subsets in primary HGSOC and NSCLC tumor samples (Study Cohorts 6 and 7) (**A**) and across (1) benign omentum, (2) primary tumor and omentum with metastases, comprised of three different omental regions: (3) macroscopically tumor-free sites distant to the omentum metastasis, and (4) peritumoral region adjacent to the tumor, as well as (5) metastatic tumors within the greater omentum (Study Cohort 6+8) (**B, C**), as determined by scRNAseq. The color scale is based on z-score-scaled gene expression.

3. Figure 4I: Consider changing the orange overlay to green to enhance contrast with the yellow background. Clarify what P1–P4 represent. Additionally, it is difficult to distinguish eTLSs from mTLSs in this panel—are extra markers being used compared to 4G?

Our response: As pointed out by Reviewer #1, modifications were made in Figure 4 of revised version of manuscript with respect to immunofluorescence images, legends indicating patient samples and information indicating that all analyses targeting eTLS and mTLS within the figure refer to the panel presented in Figure 4G, using CD4, CD8, CD20, DCLAMP, GZMB, CD21 and CD23 multiplex immunofluorescence analysis.

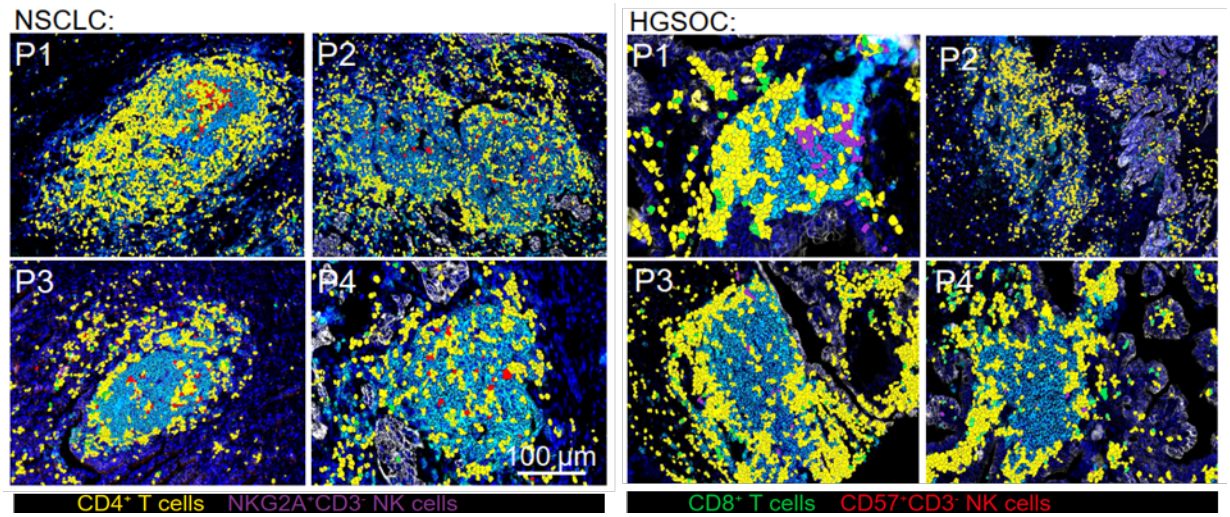

Figure 3. **Representative images of CD57^{+}CD3^{-} and NKG2A^{+}CD3^{-} NK cells within early (eTLS) and mature tertiary lymphoid structures (mTLS) of HGSOC (n=24) and NSCLC (n=24) as determined by immunofluorescence (Study Cohorts 9 and 10).**

4. Line 558: The data appear to suggest a correlation between both eTLSs and mTLSs with CD57+ NK cells in NSCLC. Why is the text emphasizing only mTLSs?

Our response: As suggested by Reviewer #1, our findings reflecting CD57^{+} NK cells in both eTLS and mTLS were presented and discussed in the revised version of manuscript.

5. The manuscript mentions interactions between NKG2A+ NK cells and CD8+ T cells and survival outcomes. Please include supporting plots or correlation coefficients in the main or supplementary figures to substantiate these claims.

Our response: Inspired by the constructive critique from Reviewer #1, we examined the clinical relevance of the spatial distribution of intratumoral NK cells—specifically CD57^{+}CD3^{-} and NKG2A^{+}CD3^{-} subsets—in relation to CD8^{+} T cells in advanced HGSOC and NSCLC (Fig. 4A–C of rebuttal letter). In HGSOC, a close proximity

between CD57$^+$ NK cells and CD8$^+$ T cells did not influence disease outcome, as shown by Kaplan–Meier survival analysis (Fig. 4D of rebuttal letter), likely due to the limited density of activated CD57$^+$ NK cells in this setting (Fig. 4B). Importantly, consistent with our previous findings on the detrimental impact of NKG2A$^+$ NK cells, we observed that a higher proportion of CD8$^+$ T cells located close to NKG2A$^+$ NK cells was associated with poor prognosis in primary HGSOC (p=0.012; Fig. 4E of rebuttal letter). By contrast, in NSCLC, increased frequency of CD8$^+$ T cell–CD57$^+$ NK cell contacts correlated with significantly improved clinical outcome (Stroma: p=0.045; Tumor: p=0.006), while interactions between CD8$^+$ T cells and NKG2A$^+$ NK cells had no significant effect (Fig. 4F, G of rebuttal letter). Together, these additional analyses extend our previous observations highlighting the clinical relevance of NK–T cell spatial intratumoral interactions and, in particular, the dominant negative prognostic role of NKG2A$^+$ NK cells—including their proximity to CD8$^+$ T cells—in primary HGSOC. These results are included in Fig. 3, Suppl. Figure 13 and Table 1 of the revised manuscript.

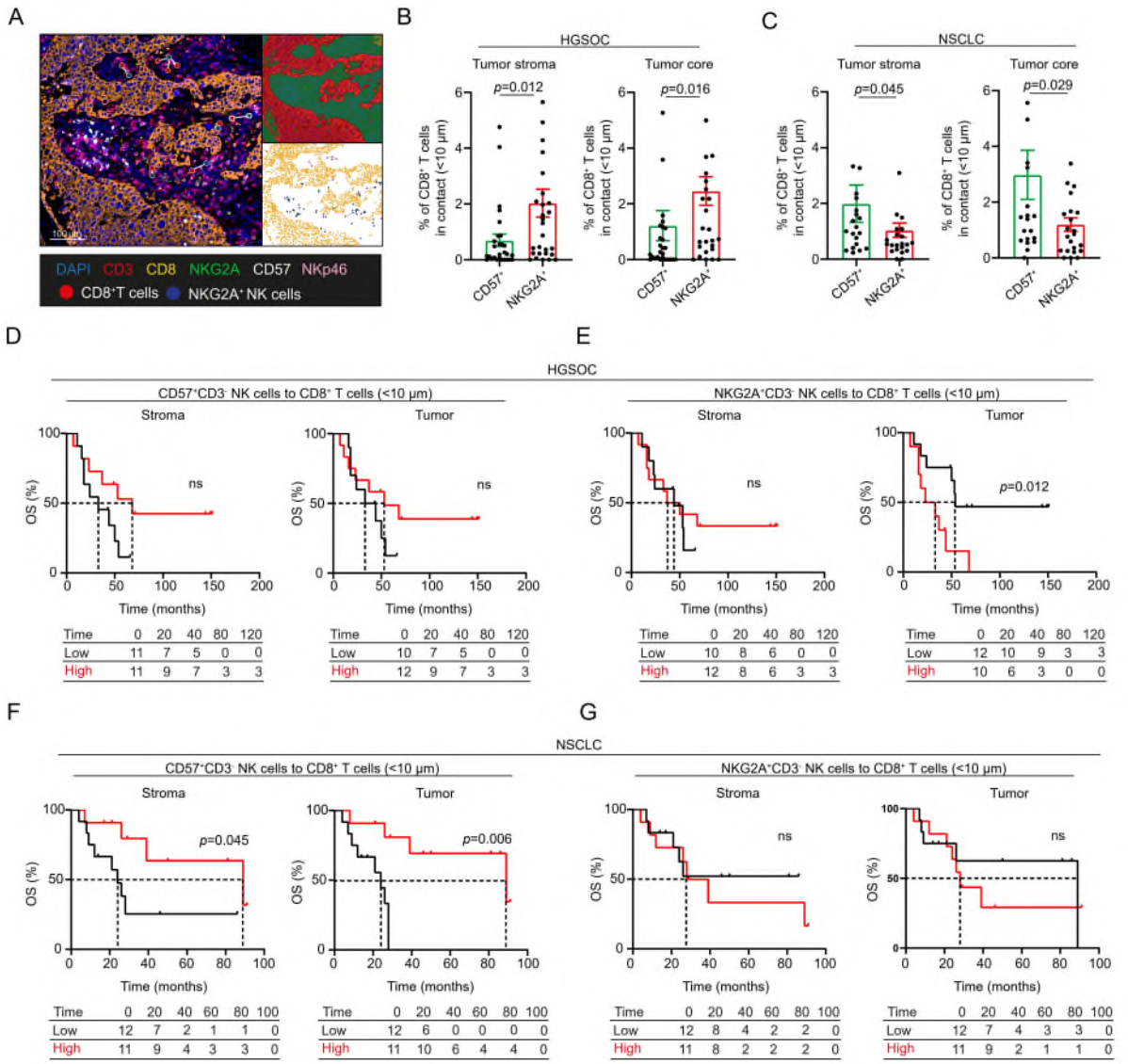

Figure 4. **The clinical relevance of spatial distribution of intratumoral NK cell subsets to CD8$^+$ T cells in advanced HGSOC and NSCLC tumor samples. (A-C)** Representative image of spatial distribution analysis on a digital pathology software (A) and bar plot showing the percentage of CD8$^+$ T cells in close proximity (<10 µm) to CD57$^+$CD3$^-$ and NKG2A$^+$CD3$^-$ NK cells within advanced HGSOC (n=22; Study Cohort 9)(B) and NSCLC (n=23; Study Cohort 10) chemo naïve tumor samples (C). Statistical significance was calculated by two-sided Mann–Whitney test. *p* values are indicated. **(D-G)** Overall survival (OS) of chemo-naïve HGSOC (n=22; Study Cohort 9)(D, E) and NSCLC (n=23, Study Cohort 10) (F, G), upon stratification based on median frequency of close proximity contacts of CD8$^+$ T cells to CD57$^+$CD3$^-$ and NKG2A$^+$CD3$^-$ NK cells. Survival curves were estimated by the Kaplan–Meier method, and differences between groups were evaluated using the log-rank test. The number of patients at risk is reported.

And raised these minor comments:

6. Lines 404–408: The sentence describing gene expression in NK1 cells is difficult to follow. Consider splitting it and clarifying the contrasting expression patterns between NSCLC and HGSOC.

7. Line 410: The statement that KLRC1 is elevated in NK1 cells in HGSOC appears inconsistent with the figure. Please verify this.

8. Lines 423–424: Strengthen the logic linking NK cell subtype differences with CD8+ T cell differentiation—was this derived from transcriptomic data or predicted interactions?

9. Line 455: Add a figure reference to support the mention of previous observations in primary HGSOC.

10. Line 469: Grammatical correction: "with instead display" → "instead displaying".

11. Line 523: Grammatical correction: "suggesting to potential negative…" → "suggesting a potential negative…"

12. Line 537: "bright" is misspelled—please correct.

13. Lines 561–572: Clarify the rationale for comparing both SO1 and BR5 models. State explicitly what each adds to the study.

14. Line 561 (Section Title): Suggest rephrasing the section title to reflect the bidirectional impact of CD8+ and NK cell depletion.

15. Line 578: Clarify how the observations in this paragraph recapitulate Figure 3 findings. Reference specific panels.

16. Lines 603, 607: Use "blockade" rather than "blockage" in the context of immune inhibition.

17. Figure Legends: Improve clarity in UMAP and flow cytometry figures, especially Supplemental

Figures 3–5. Complex plots would benefit from more descriptive legends and clearer labeling.

18. Quantification of Immunofluorescence: Figures such as Supplemental Figures 2 and 5 should include quantitative data (e.g., TLS density, cell distances) to support image-based conclusions.

Our response: All minor comments have been carefully addressed in the revised version of the manuscript.

And raised these additional comments:

19. In the SO1 model, early combination therapy didn't seem to suppress tumor progression effectively. Have the authors assessed metastatic spread or quantified total tumor burden?

Our response: We thank Reviewer #1 for this thoughtful comment. In our preclinical models of ovarian cancer, we primarily quantify overall survival (OS) to evaluate the impact of monotherapy versus combination treatment strategies, as we consider OS the most reliable and reproducible endpoint. To further strengthen our data, we conducted an additional experiment directly comparing αNKG2A and αPD1 monotherapies with their combination (Fig. 5A, rebuttal letter). Consistent with our previous findings, NKG2A blockade alone did not extend the survival of SO1 HGSOC-bearing mice. In contrast, the combination of NKG2A blockade with PD1 inhibition significantly enhanced the therapeutic efficacy of PD1 blockade ($p < 0.0001$). To complement the survival analysis, we also documented metastatic spread by photographing lesions observed at necropsy across individual mouse groups (Fig. 5B, rebuttal letter). These images provide qualitative evidence of reduced tumor dissemination in the αPD1+αNKG2A group compared with both control and monotherapy arms. However, because this preclinical model generates numerous small nodules throughout the omentum and peritoneal cavity—often embedded within fat deposits or dispersed across sites—not all lesions can be reliably detected or collected. Objective quantification across animals would therefore risk underestimation and potentially misleading conclusions. For this reason, we continue to regard OS as the principal endpoint in this model, with photographic documentation serving as supportive evidence of reduced metastatic burden. These images will be provided as Supplementary Fig. 16 in the revised manuscript.

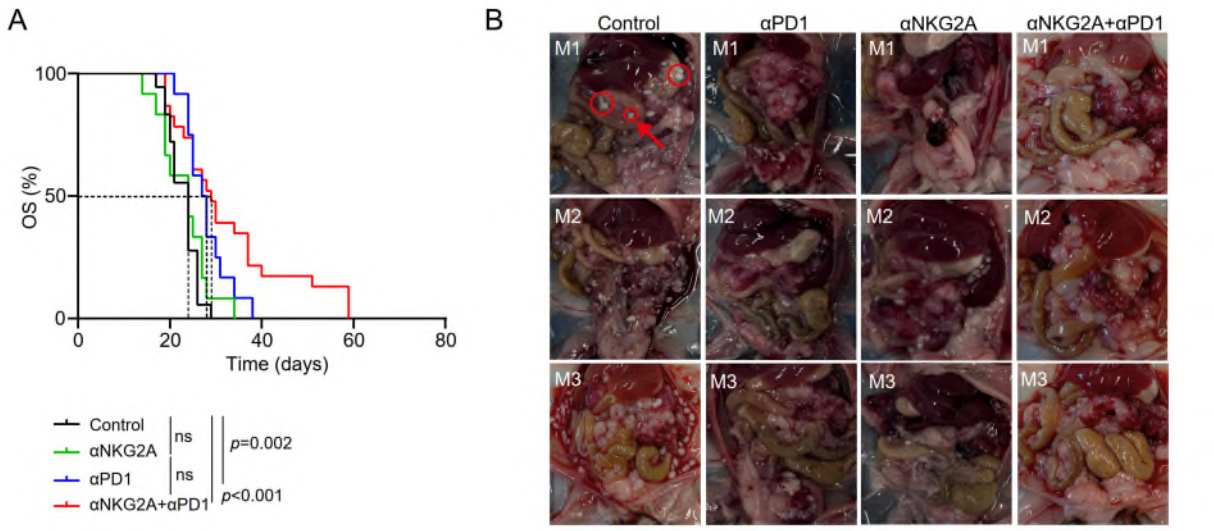

Figure 5. **Combined αPD1 and αNKG2A therapy improves survival and reduces metastatic tumor spread in the SO1 preclinical model of HGSOC. (A)** Overall survival (OS) of SO1-bearing mice treated with αNKG2A, αPD1, or the combination therapy (control: n=18; αPD1: n=12; αNKG2A: n=12; αPD1+ αNKG2A: n=23). Survival curves were estimated by the Kaplan–Meier method and differences between groups were evaluated using log-rank test. *p* values are indicated. **(B)** Representative necropsy images showing macroscopic peritoneal metastatic lesions from three individual mice (M1–M3) per treatment group. Red arrows indicate metastatic tumor sites in one representative image.

20. The translational relevance of these findings could be emphasized more clearly. For instance, how do the results inform clinical strategies involving NKG2A blockade (e.g., monalizumab) and PD-1 inhibitors? Could this inform future patient stratification?

21. Consider briefly touching on how these observations may (or may not) differ in early-stage disease or post-treatment settings.

Our response to #20 and #21: Inspired by the constructive comments from Reviewer #1, we performed additional analyses of *PDCD1* and *KLRC1* expression in $CD8^+$ T cell and NK cell clusters using scRNA-seq data from Study Cohort 6 (*Vazquez-Garcia et al, Nature, 2022*). A total of 548.965 cells (321.798 from primary HGSOC and 227.167 from metastatic HGSOC), including 244.626 malignant/stromal cells and 278.008 immune cells, were processed, leading to the identification of epithelial, stromal, and immune compartments using graph-based clustering and established lineage markers (Fig. 6A of rebuttal letter). After quality control and unsupervised hierarchical clustering, we identified 35.895 and 34.546 $CD8^+$ T cells and 14.304 and 12.267 bona fide NK cells

in both primary and metastatic HGSOC, respectively (Fig. 6B of this rebuttal letter). Consistent with our previous findings (Fig. 2O of revised version of manuscript), *PDCD1* expression was predominantly associated with $CD8^+$ T cells, while *KLRC1* was dominantly expressed by NK cells in both primary (pHGSOC) and metastatic (mHGSOC) HGSOC (Fig. 6B-D of rebuttal letter). No major differences were observed in *PDCD1* or *KLRC1* expression between primary and metastatic tumors in either cell type (Fig. 6C, D of this rebuttal letter). To validate these transcriptomic observations, we quantified the percentage of $PD1^+$ and/or $NKG2A^+$ $CD8^+$ T cells and $PD1^+$ and/or $NKG2A^+$ $CD56^+$ NK cells in native tissue samples of pHGSOC and mHGSOC using flow cytometry (Study Cohort 3) (Fig. 6E of this rebuttal letter). Consistent with the transcriptomic data, the percentages of $PD1^+CD8^+$, $NKG2A^+CD8^+$, $PD1^+CD56^+$, and $NKG2A^+CD56^+$ NK cells were comparable between pHGSOC and mHGSOC. Notably, the percentages of $PD1^+$ and $NKG2A^+$ $CD8^+$ T cells decreased following neoadjuvant chemotherapy (NACT) in mHGSOC (Study cohort 5), whereas the percentages of $PD1^+$ and $NKG2A^+$ NK cells remained stable (Fig. 6F, G of this rebuttal letter). Overall, these findings indicate that PD1 is predominantly associated with the $CD8^+$ T cell compartment (>50% of intratumoral $CD8^+$ T cells), whereas NKG2A is mainly expressed by intratumoral NK cells. Expression profiles of *PDCD1* and *KLRC1* in these compartments are comparable between pHGSOC and mHGSOC. Importantly, while $PD1^+$ and $NKG2A^+CD8^+$ T cells decreased after NACT, NK cells maintained stable expression of both these markers, highlighting them as a promising target for combined immunotherapy in advanced HGSOC. These additional analyses are incorporated into the revised version of the manuscript as Suppl. Figure 10.

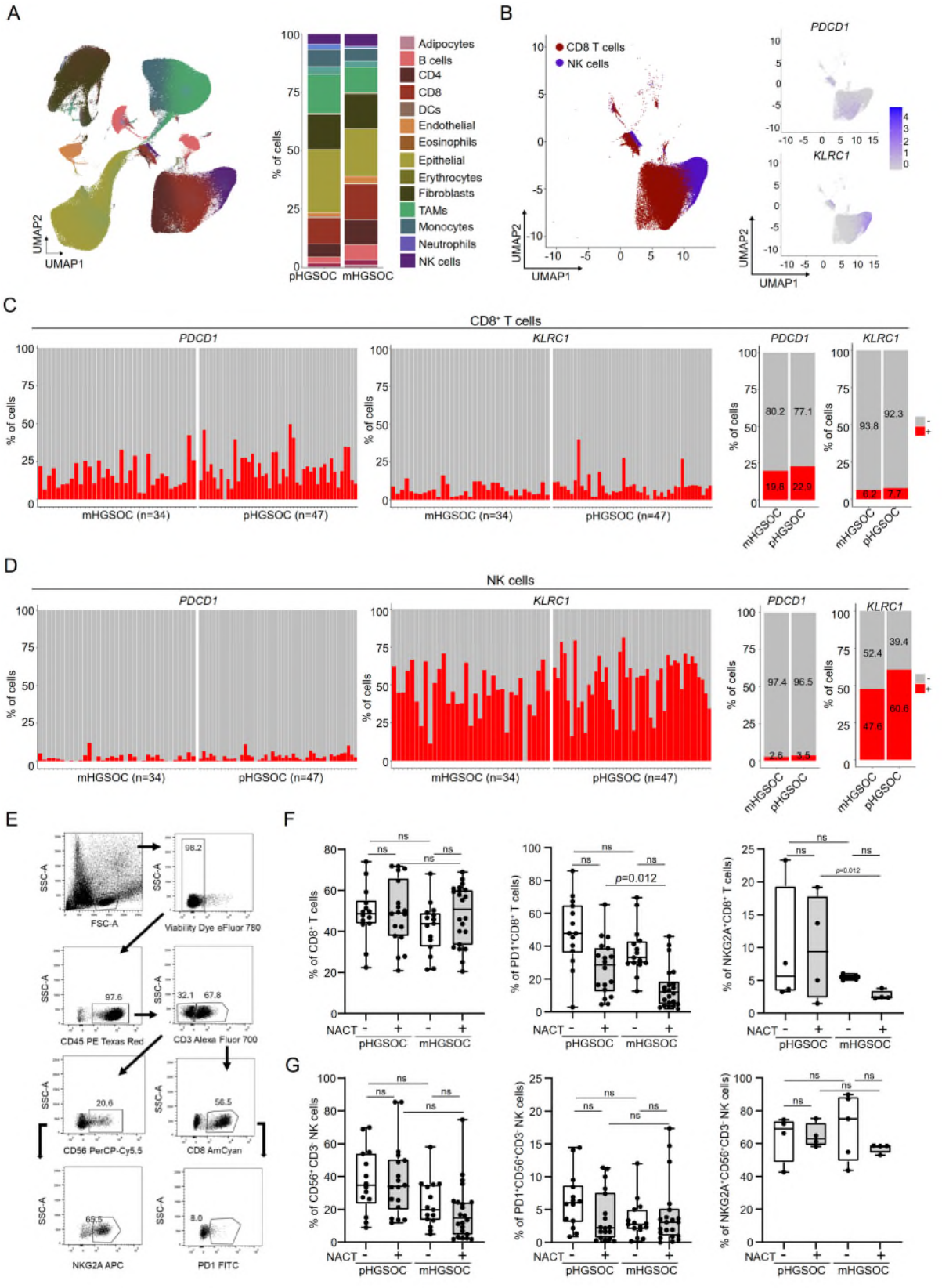

Figure 6. **Distribution of PD1⁺ and NKG2A⁺ CD8⁺ T cells and NK cells in chemo- naïve and neoadjuvant chemotherapy (NACT) treated primary and metastatic HGSOC samples. (A, B)** UMAP plot of all cells passing the quality control, colored by cell type. (B) UMAP and stacked plot showing expression of *PDCD1* and

KLRC1 expression in $CD8^+$ T cells and NK-cell clusters in primary and metastatic HGSOC samples as determined by scRNAseq (Study Cohort 6). **(C, D)** Proportion of $CD8^+$ T cells (C) and NK cells (D) expressing *PDCD1* and *KLRC1* in primary (pHGSOC) and metastatic (mHGSOC) HGSOC as determined by scRNAseq (Study Cohort 6). **(E-G)** Representative dot plot (E) and flow cytometry analyses quantifying intratumoral $CD8^+$ T cells, $PD1^+CD8^+$ and $NKG2A^+CD8^+$ T cells (F) and intratumoral $CD56^+CD3^-$ NK cells, $PD1^+CD56^+CD3^-$ and $NKG2A^+CD56^+CD3^-$ NK cells (G) within chemo-naive (Study Cohort 3) and NACT-treated primary and metastatic HGSOC (Study Cohort 5). Box plots: lower quartile, median, upper quartile; whiskers, minimum, maximum. Statistical significance was calculated by two-sided Mann–Whitney test. *p* values are indicated.

22. Including a visual timeline or schematic of the in vivo experimental protocols would help readers navigate the mouse data more easily.

Our response: We thank Reviewer #1 for this helpful suggestion. In line with the recommendation, all *in vivo* experiments presented in the study are now accompanied by visual timeline schematics to facilitate navigation and improve the clarity of the experimental design.

23. Lastly, the Methods section should specify how spatial proximity thresholds (e.g., 10 µm) were chosen and whether they were empirically validated.

Our response: We have decided to apply a spatial proximity threshold of 10µm, which was selected based on previously reported analyses describing biologically relevant interactions in the TME. Prior studies have shown that intercellular signaling and structural interactions typically occur within this spatial range (*Parra et al, Nat Commun, 2023; Monkman et al, J of Trans Med, 2024*). In addition, 10 µm approximates the average diameter of cells, thereby providing a biologically meaningful cutoff for identifying putative interactions.

Ref: ***NCOMMS-25-31476***
Manuscript: NKG2A inhibition promotes NK cell-CD8+ T cell interactions in support of improved anticancer immunity in ovarian carcinoma
Authors : **Tereza Lanickova *et al.***
Corresponding author: **Jitka Fucikova**

POINT-BY-POINT REPLY TO REVIEWER N° 2

Reviewer n° 2 commented:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Our response: We sincerely thank Reviewer #2 and Nature Communications for supporting this valuable initiative. We greatly appreciate the contribution of Early Career Researchers to the peer-review process and are grateful for the evaluation of our manuscript.

Ref: ***NCOMMS-25-31476***
Manuscript: NKG2A inhibition promotes NK cell-CD8+ T cell interactions in support of improved anticancer immunity in ovarian carcinoma
Authors : **Tereza Lanickova *et al.***
Corresponding author: **Jitka Fucikova**

POINT-BY-POINT REPLY TO REVIEWER N° 3

Reviewer n° 3 commented:

This work represents a multi-omic approach in several patient cohorts and mice to understanding the role of NK cells in HGSOC tumours. While I am impressed with the extent and breadth of the paper, inclusion of so much data perhaps interferes with a mechanistic or thorough interpretation of it and the story might better be told in separate parts. The story moves through comparisons of HGSOC to NSCLC as putative checkpoint resistant and sensitive tumours, respectively, to assessing TLS formation, NK cell localization and finally co-blocking. While I agree that there are many associations present, they are each incompletely explored before moving to the next section. The observations are interesting and aligned with current understandings in the field, but they often fall short of providing or even speculating at the mechanistic reasons behind their presence. The focus on NSCLC is

lost after the first couple of figures, and the overarching logical progression through the paper is missing. In general, results are described, but the hypotheses leading through the paper are not.

Our response: We thank Reviewer #3 for the thoughtful and constructive feedback, as well as for acknowledging the scope of our multi-omic analyses across patient cohorts and mouse models. We recognize the concerns regarding the balance between breadth of data and mechanistic depth, as well as the importance of presenting clear hypotheses within a cohesive narrative. In response, we have refined the articulation of hypotheses and rationale throughout the manuscript, expanded mechanistic interpretations whenever feasible (or provided literature-based context in the few instances where specific evidence was limited), and clarified the relevance of the NSCLC comparisons. In addition, we have reorganized sections and relocated selected data to the supplementary material to improve coherence and readability. We sincerely appreciate this guidance, which has substantially strengthened the clarity and mechanistic focus of the manuscript.

And raised these major comments:

1. Line 82: The statement that HGSC has a low TMB is somewhat misleading. The statement would be more accurate if TMB were described as moderate or variable, as it tends to change substantially between patients (and even within them). Since the tumours are variable (some HGSC patients do respond to ICI), the authors should take caution in referring to HGSC as ICI resistant, laying the groundwork for how this is a model system and a generalized conclusion (likewise for NSCLC). This is noteworthy throughout, and especially for the text associated with figure 4. These are two different tumours (with subsets of each known to exist). It is an oversimplification to bucket them based on ICI sensitivity when there are many other reasons why these tumours are expected and known to differ.

Our response: We thank Reviewer #3 for this important comment. We agree that introducing HGSOC as a solid malignancy with overall low TMB and labeling it universally ICI-resistant oversimplifies the heterogeneity of this neoplasm. Our statement was based on publicly available datasets, which demonstrate that level of somatic mutations and tumor mutational burden (TMB) is overall significantly higher in NSCLC compared with primary HGSOC (Fig. 7 of this rebuttal letter). Nevertheless, we fully acknowledge that TMB in HGSOC is moderate and variable across patients, and that subsets of HGSOC can still respond to ICI. Similarly, NSCLC represents a heterogeneous population with variable ICI sensitivity. We have revised our manuscript to clarify these points,

providing appropriate context for the use of these tumor types as model systems, and avoid overgeneralizing ICI responsiveness, particularly in the discussion associated with Figure 4.

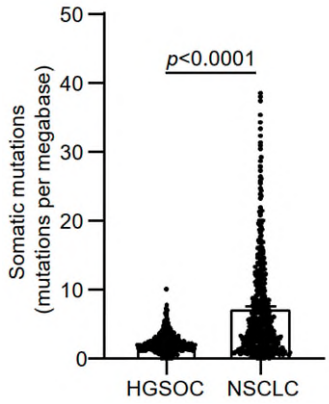

Figure 7. **Tumor mutational burden in primary high grade serous ovarian cancer (HGSOC) and non-small cell lung (NSCLC) carcinoma from TCGA public database (Study cohorts 1 and 2).**

2. Lines 83ff: NK cell recognition is not irrespective of antigen presentation on MHC I molecules. Interactions between NK Cells and MHC drive NK cell licensing, and also the peptide loaded can be involved in triggering receptors. Please rephrase. I also recommend using a more updated review article (present reference is from 2015; much modern research has unveiled the ways in which MHC I and loaded peptide impact NK:target/NK:self interactions). Likewise, refs 16 and 17 (line 93) refer to functional variation; both of those (albeit modern) papers focus more on transcriptomics of NK cells.

Our response: We thank Reviewer #3 for this insightful comment. We fully agree that the original statement oversimplified NK cell recognition and that interactions with MHC I molecules, including the presented peptide, are critical for NK cell licensing and target recognition. We have rephrased the text to more accurately reflect the current understanding of NK:MHC interactions. In addition, we have updated references to include more recent reviews and studies that discuss the impact of MHC I and associated peptides on NK cell function, moving away from transcriptomics-focused references to better support the mechanistic context.

3. Study cohort descriptions: the specific sites of these samples need to be disclosed, as do the purposes of each study (this might be more efficiently achieved in a table). Presumably, RNAseq data and "tissue samples" refer to tumours, but this is unclear. How are samples normalized? Are they comparable, given that they were not expressly assessed for the purposes of comparison? How data were processed (including

pre-processed), assessed and reported (i.e. fold change? Z-scores etc) is important information for the methods section. a. Were the patients assessed for genomic alterations? In both cancers (but especially NSCLC), somatic mutations can impact disease progression and treatment approaches (and thus might impact the reported results). b. The authors describe several cohorts studied; some of which were from public databases; others from their own sequencing. Ethics numbers are missing from at least Study cohort 6, and should be reconfirmed for all. c. The specific interventions for each cohort are not given. For example, what was done with the patient materials in cohorts 5 and 6? d. Were the pathology specimens ever overlapping with those assessed genomically? e. In the results section, "study cohorts" are jumped between often; this makes the story difficult to follow. It would be better to be clear about what the comparisons are.

Our response: We thank Reviewer #3 for these detailed and constructive comments. We agree that the description of study cohorts and sample handling could be clarified to improve transparency and reproducibility.

1. **Cohort details and sample sites**: We have added tables summarizing all cohorts (Suppl. Tables 1-3) and updated Suppl. Fig. 1 of revised version of manuscript (Fig. 8 of this rebuttal letter), including the tissue origin, number of samples, purpose of inclusion, and any interventions performed. This figure clarifies which samples correspond to RNA-seq, tissue analyses, or other assays.
2. **Sample normalization and comparability**: We have expanded the Methods section to describe how RNAseq and scRNAseq were pre-processed, normalized (e.g., z-scores), and analyzed to ensure comparability across cohorts, including publicly available and in-house datasets.
3. **Somatic mutations:** As correctly pointed by Reviewer #3, information on somatic mutations, particularly for NSCLC (ALK, EGFR), were used within univariate Cox proportional hazard analyses presented in Table 1 of revised version of manuscript.
4. **Ethics approvals**: We confirm that all cohorts for which we generated new data have appropriate ethics approvals (Study cohorts 3, 4, 5, 9 and 10), which are now included in the manuscript. For the publicly available datasets, we used previously published and de-identified data; as these datasets are already in the public domain, separate ethics approval for their use was not required.
5. **Cohort interventions and tissue handling**: We now better describe the interventions or procedures performed for each cohort, including cohorts 6-8 (Suppl. Fig. 1 or revised manuscript; methods section).
6. **Results presentation**: We have revised the Results section to better indicate which cohorts are being compared in each analysis, reducing confusion and improving logical flow.

These revisions provide full transparency regarding sample origin, processing, and analysis, and ensure that cohort comparisons are clearly defined and interpretable.

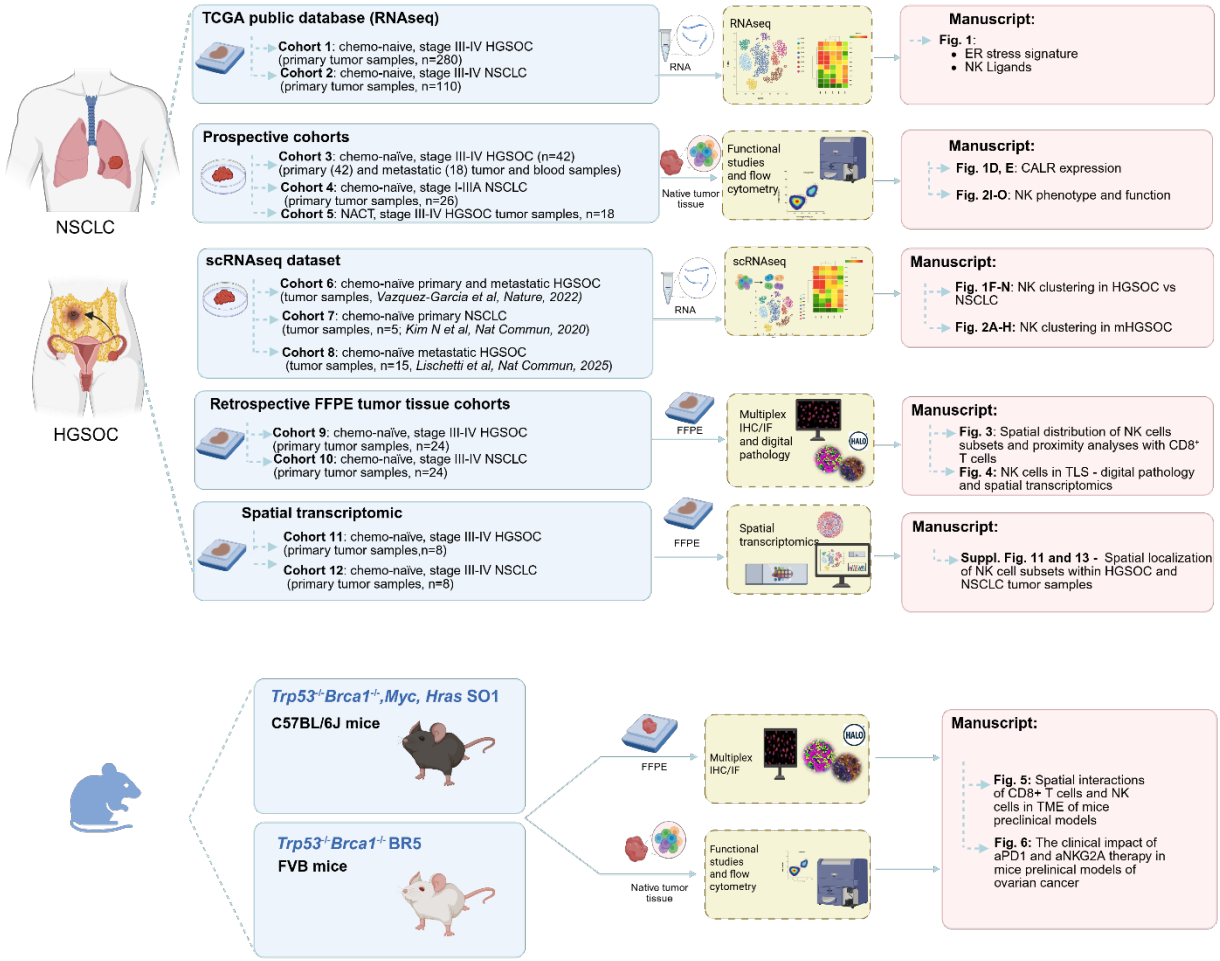

Figure 8. **Experimental design and study cohorts used within the study.**

4. Recheck nomenclature throughout. For example, it's a Fortessa, not a FACSFortessa; IFNG is a gene name, but it's referred to as a protein that is being stained for (i.e. IFN-γ). Figures and display items should be presented in the order that they are introduced.

Our response: We thank Reviewer #3 for bringing these points to our attention. In the revised version of the manuscript, we have specified the use of the BD LSRFortessa instrument. Regarding the gene/protein nomenclature, we hope Reviewer #3 will agree, that we have opted to retain *IFNG* in accordance with the official nomenclature listed in PubMed Gene/Protein, following the general recommendations of journals within the Nature Publishing Group (NPG).

5. 15% as a higher threshold for mitochondrial gene content in single cell analysis is unconventionally high. The authors should explain the logic for this.

Our response: We applied a 15% mitochondrial content threshold for cell filtering. While thresholds of 5–10% are commonly used, the optimal cutoff can vary depending on tissue type and experimental context. In our dataset, we observed that many high-quality cells from (tissue/source, e.g. omentum samples) exhibited elevated mitochondrial content, likely reflecting an underlying biological factor, e.g. higher metabolic activity, stress, or tissue dissociation effects. A stricter cutoff (e.g., 5–10%) would have excluded a substantial number of otherwise viable cells, thereby biasing against specific cell populations. To ensure robustness, we compared results across multiple thresholds and confirmed that key biological findings remained consistent. Based on this, we chose 15% as a balance between retaining biologically relevant cells and removing low-quality cells. We included this further information in material and method section of revised manuscript.

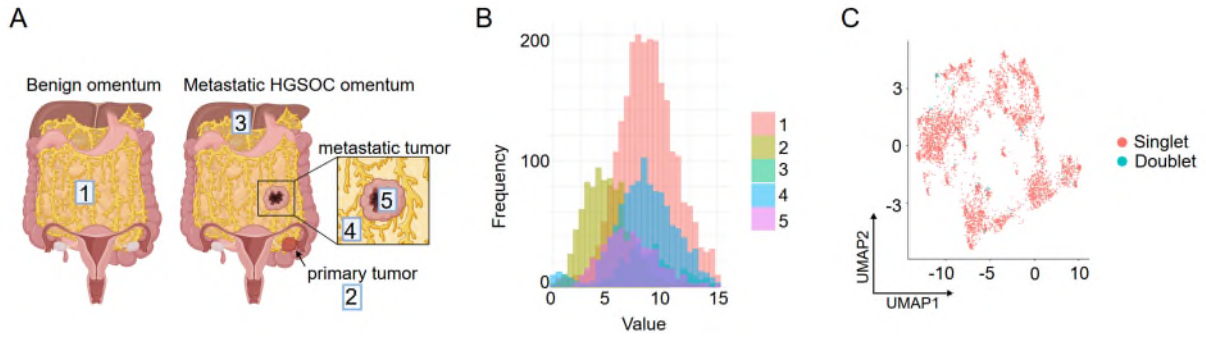

Figure 9. **Threshold or mitochondrial gene content in scRNAseq datasets in the study**. **(A)** Overview of anatomical sampling sites: (1) benign omentum; (2) primary tumor; omentum with metastases, comprising three distinct omental regions (3) macroscopically tumor-free sites distant from omental metastases, (4) peritumoral regions adjacent to tumor tissue, and (5) metastatic tumors within the greater omentum (Study Cohorts 6+8). Created with BioRender.com. **(B)** Histogram showing the distribution of mitochondrial gene content (*percent.mt*) across cells and within 5 histological sites (described in panel A), with frequency on the y-axis. **(C)** UMAP visualization of predicted singlets and doublets classified using DoubletFinder.

6. Single cell (lines 381 and following): the Rebuffet paper cited (ref 16) clusters NK cells into three groups with subclusters within them. As described, these clusters do not fully align: for example, the NK3 population is fully absent, and some of the features ascribed to the NK1 population might belong there. This work also understates the differences between the NK1 subpopulations, as they were described in the

referenced paper (i.e. ABC, and also the NKint population).

a. How did the total number of NK cells compare between the HGSC and NSCLC patient groups? Were they represented similarly among patients within each disease cohort?

b. Likewise, how did the total numbers of NK cells compare between omental/metastatic sites?

Our response: We thank Reviewer #3 for bringing up this important point. To provide a more detailed analysis of NK cell frequency and gene signatures, we have applied the NK1, NK2, and NK3 classification recently described by Rebuffet et al. (Nature, 2024) to our scRNA-seq datasets. The results are presented in Figures 1 and 2 of the revised manuscript (also shown in Figures 10 and 11 of this rebuttal letter). Consistent with our earlier findings, the NK1 cluster is characterized by high expression of *FCGR3A* (encoding CD16) and *CX3CR1*, along with elevated cytotoxic genes (*GZMB*, *PRF1*) and NK maturity markers (*NKG7*, *SPON2*). NK1 cells display lower *NCAM1* (CD56) expression relative to NK2 cells, consistent with a $CD56^{dim}$ phenotype. The NK2 cluster shows preferential expression of *NCAM1*, the inhibitory receptor *KLRC1* (encoding NKG2A), immune modulatory factors (*XCL1*, *XCL2*), migration/tissue-homing molecules (*CD44*), and *GZMK*, while *FCGR3A* expression is reduced, consistent with a $CD56^{bright}$ phenotype. The NK3 cluster is defined by expression of *CD2*, *KLRC2* (encoding NKG2C), *CD3* chain transcripts (*CD3D*, *CD3E*, *CD3G*), secreted cytokines/chemokines (*IL32*, *CCL5*), and *GZMH*. Thus, all three NK clusters described by Rebuffet et al. were readily identified in our scRNA-seq datasets from study cohorts 6-8.

These analyses reinforce our observation that the NK2 cluster is enriched in pHGSOC, whereas NK1 and NK3 clusters predominate in NSCLC tumor samples (Fig. 10C–D of this rebuttal letter). Similarly, when comparing NK cluster distributions across the benign omentum, primary tumors, and omental metastases, we observed a consistent enrichment of NK2 cells in both primary and metastatic HGSOC, while NK1 and NK3 clusters were more prevalent in the benign omentum, distant sites, and peritumoral regions (Fig. 11A–C of this rebuttal). These trends were corroborated by total NK cell counts across histological compartments (Supplementary Figs. 6 and 7 of the revised manuscript). Furthermore, pseudotime analysis revealed a clear shift toward functional exhaustion within the NK2 cluster, marked by upregulation of *LAG3*, *CTLA4*, *PDCD1*, and *KLRC1* genes. This exhausted NK2 phenotype was most pronounced in primary and metastatic HGSOC compared with the benign omentum and distant or peritumoral omental regions (Fig. 11F–H of rebuttal letter).

In summary, our findings demonstrate that NK cells in HGSOC preferentially polarize into the NK2 cluster, displaying features of functional exhaustion, whereas NK cells in NSCLC more commonly adopt effector NK1 and NK3 phenotypes. This polarization shift is consistently observed across distinct histological sites of omental

metastases, particularly in regions of direct NK–HGSOC cell interaction. The full analyses are included in Figures 1–2 and Supplementary Figures 6–7 of the revised manuscript.

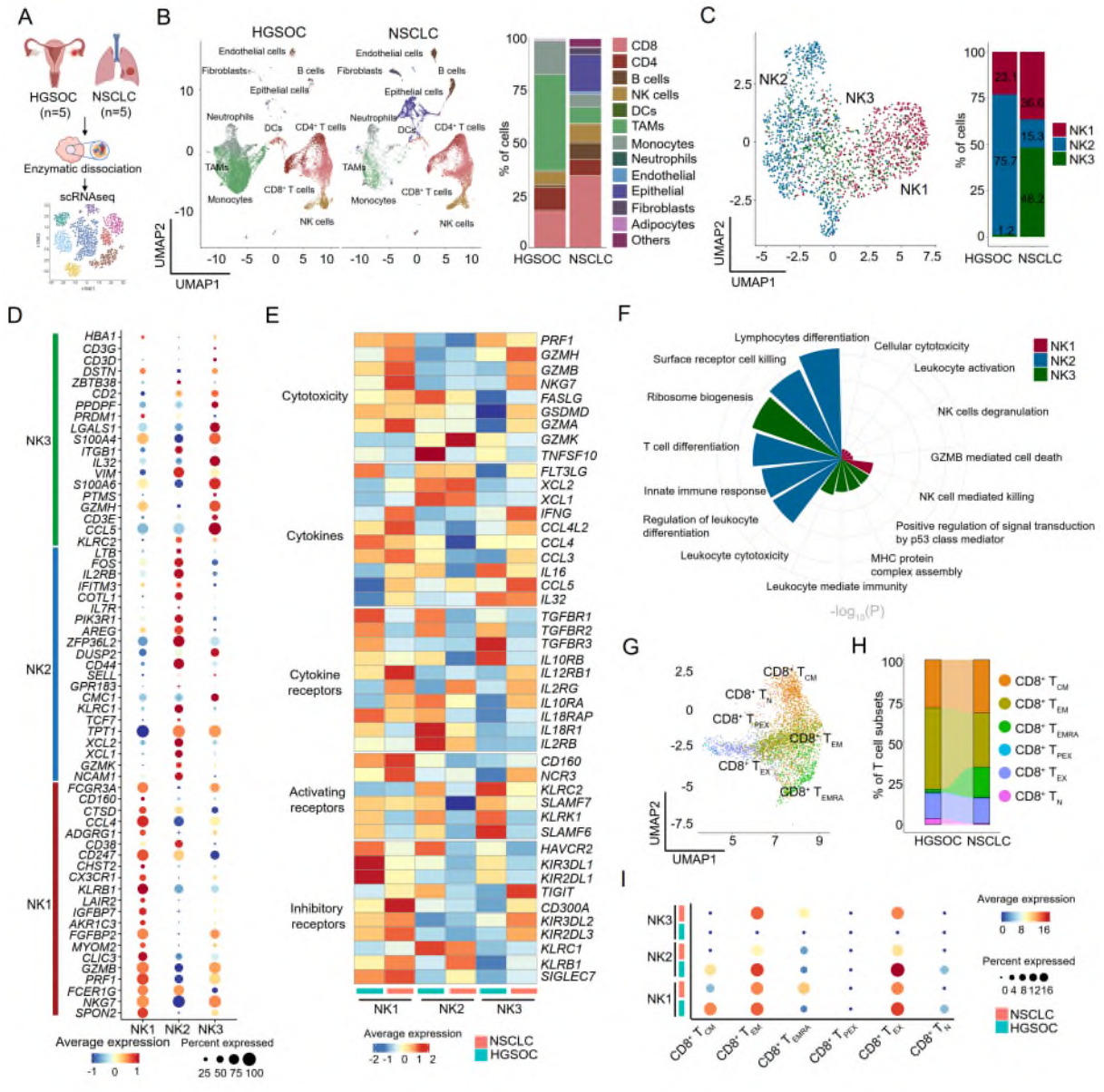

Figure 10. **Cellular stress and tumor adjuvanticity correlate with increased NK1 (CD56^{dim}) and NK3 cytotoxic NK cells in advanced NSCLC but not HGSOC. (A)** Design of experimental and sequencing workflow in 10 chemo-naïve patients with HGSOC (n=5) and NSCLC (n=5) (Study Cohorts 6 and 7), using scRNAseq. Created with BioRender.com. **(B, C)** UMAP and stacked plot showing all cells passing the quality control, colored by cell type (B) and NK1, NK2 and NK3 clusters (C) in chemo-naïve NSCLC and HGSOC samples as determined by scRNAseq. **(D)** Dot plot of the 20 most distinguishing genes expressed for the NK1, NK2 and NK3 subsets in NSCLC and HGSOC samples, as determined by scRNAseq. The color indicates the z-score scaled gene expression

levels. **(E)** Heatmap showing the differential expression of markers of interest among NK1, NK2 and NK3 cell subsets. The color scale is based on z-score-scaled gene expression. **(F)** Selected GO terms showing enrichment in the three major NK clusters. Benjamini–Hochberg-corrected $-\log_{10}(P)$ values were calculated by a hypergeometric test. The black dotted line indicates the significance threshold, which is $-\log_{10}(0.05)$. **(G, H)** UMAP and stacked plot showing expression of gene signatures related to intratumoral $CD8^+$ T cells states, including central memory (T_{CM}), effector memory (T_{EM}), terminally differentiated (T_{EMRA}), progenitor exhausted (T_{PEX}), exhausted (T_{EX}) and naïve (T_N) in chemo-naïve NSCLC and HGSOC samples as determined by scRNAseq (Study Cohorts 6 and 7). **(I)** Bubble plot showing cellular interactions between NK1, NK2 and NK3 with $CD8^+$ T cells subsets in HGSOC and NSCLC, as determined by scRNAseq.

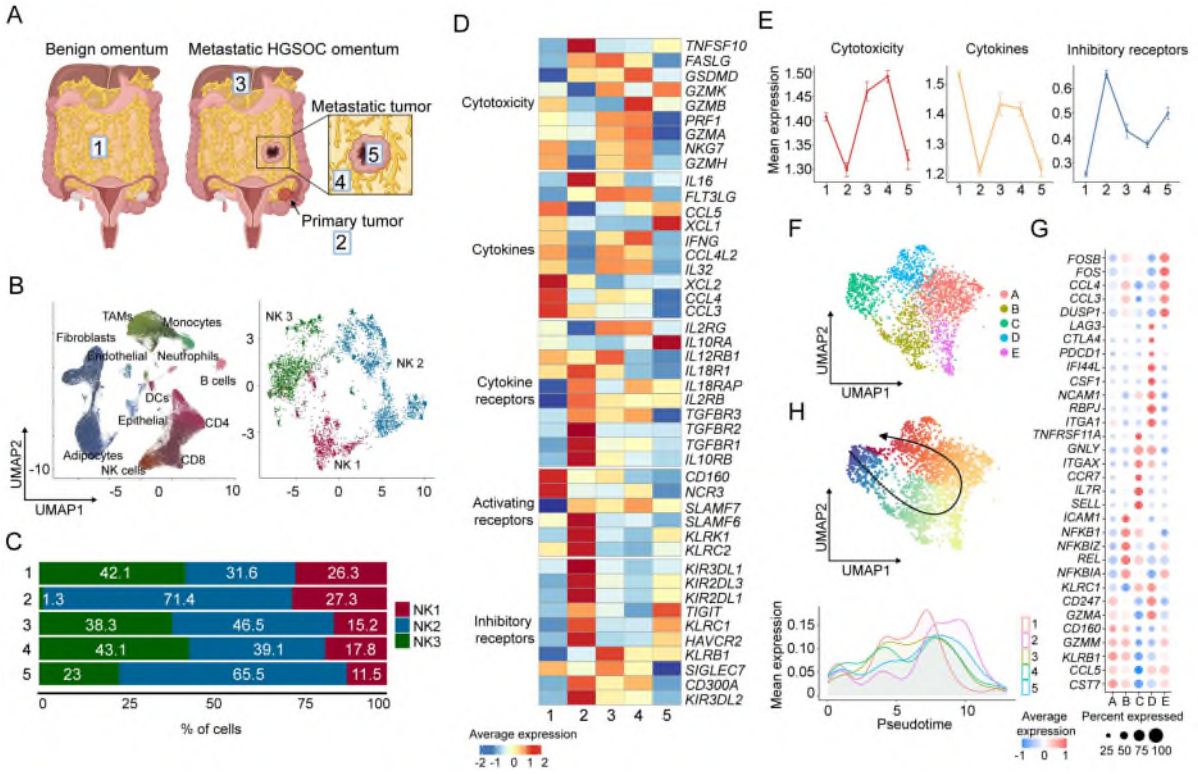

Figure 11. **NK cells from advanced HGSOC are phenotypically and functionally impaired. (A)** Overview of anatomical sampling sites across (1) benign omentum (n=8), (2) primary tumor (n=5) and omentum with metastases (n=7), comprised three different omental regions: (3) macroscopically tumor-free sites distant to the omentum metastasis, and (4) peritumoral region adjacent to the tumor, as well as (5) chemo-naïve metastatic tumors within the greater omentum (Study Cohorts 6 and 8). Created with BioRender.com. **(B, C)** UMAP (B) and stacked plot (C) of all cells passing the quality control, colored by cell type and NK subtypes across 5 previously defined histological compartments as shown in panel A) as determined by scRNAseq (Study Cohorts 6 and 8). **(D, E)** Heatmap (D) and line plots (E) showing the differential expression of markers and gene signatures of interest

among NK cell subsets within 5 previously defined histological compartments (as shown in panel A). The color scale is based on z-score-scaled gene expression. The z-score distribution ranges from −2 (blue) to 2 (red) (Study Cohorts 6 and 8) **(F-H)** Reclustering of NK2 cells (from the UMAP projection shown in panel B). Selected clusters were extracted and subjected to new normalization, scaling, integration, and dimensionality reduction. *n* = 2,241 cells. (F) Cells are colored by cluster. (G) Dot plot of selected marker genes distinguishing clusters presented in (F). Colors represent the average expression of a gene in a cluster, the size of dots represents the percentage of cells with non-zero expression. (H) UMAP projection colored by pseudotime (top) and density of NK2 cluster along the pseudotime trajectory. Pseudotime values calculated using the slingshot package. The arrow indicates the identified lineage trajectory, as determined by scRNAseq across 5 previously defined histological compartments as shown in panel A (Study Cohorts 6 and 8).

7. In any expression analysis, there are always correlations that are not necessarily causations. The conclusions on line 431 and following state ER stress as being associated (it is) but were other pathways/GO terms explored? What about other features of NK cell biology in general, including, but not limited to those discussed throughout the paper (i.e. migration, cytokine production, regulation)? Considering the later inferences of the paper and spatial biology/interaction with T cells, as they are described, the authors could use the single cell data for more than NK cell analysis. Additional assessments of the immune architecture and interactions within these tumours would be informative.

Our response: We thank Reviewer #3 for bringing these points to our attention. To extend our analyses beyond the role of ER stress, previously identified as a driver of NK cell activation (*Dong et al, Nat Immunol, 2019; Wang et al, Nat Immunol, 2019; Ma et al, Sci Immunol, 2023; Sen Santara et al, Nature, 2023*), we evaluated additional aspects of NK cell biology related to chemotaxis and tumor migration across HGSOC and NSCLC samples, and further examined NK cells interactions with other immune and stromal populations within the scRNAseq datasets from study cohorts 6-8. Beyond the previously identified crosstalk with $CD8^+$ T cells (Figure 1 of the revised manuscript), NK cells displayed high levels of inferred interactions with tumor-associated macrophages (TAMs), B cells, and monocytes in HGSOC (Fig. 12A of this rebuttal letter). While similar interactions were observed in NSCLC samples, in this latter setting NK cells showed more frequent interplay with fibroblasts, endothelial, and epithelial cells, suggesting pronounced effector functions within the tumor microenvironment and tumor islets (Fig. 12B of rebuttal letter). Consistent with these findings, genes associated with NK cell chemotaxis and migration (*CXCR2, S1PR5, CX3CR1, S1PR1, S1PR4*) were enriched in NSCLC compared to HGSOC (Fig. 12C,

D of this rebuttal letter). Furthermore, when comparing the benign omentum to primary and metastatic HGSOC sites, we observed higher expression of chemotaxis-associated genes in the benign omentum, reflecting NK cell localization in distant and peritumoral areas relative to primary and metastatic tumors (Fig. 12E of this rebuttal letter). In summary, these analyses demonstrate that NK cell biology is shaped by multiple concurrent pathways and interactions within the tumor microenvironment, beyond ER stress. They also provide a broader view of immune architecture, supporting the spatial and functional inferences described in the manuscript. Notably, they highlight more pronounced NK cell effector crosstalk in NSCLC compared to HGSOC, consistent with the increased expression of chemotaxis-related genes in NSCLC. These results extend our previous findings presented in Fig. 1 and Suppl. Fig. 6 of revised version of manuscript, providing a detailed overview of NK cells crosstalk within TME.

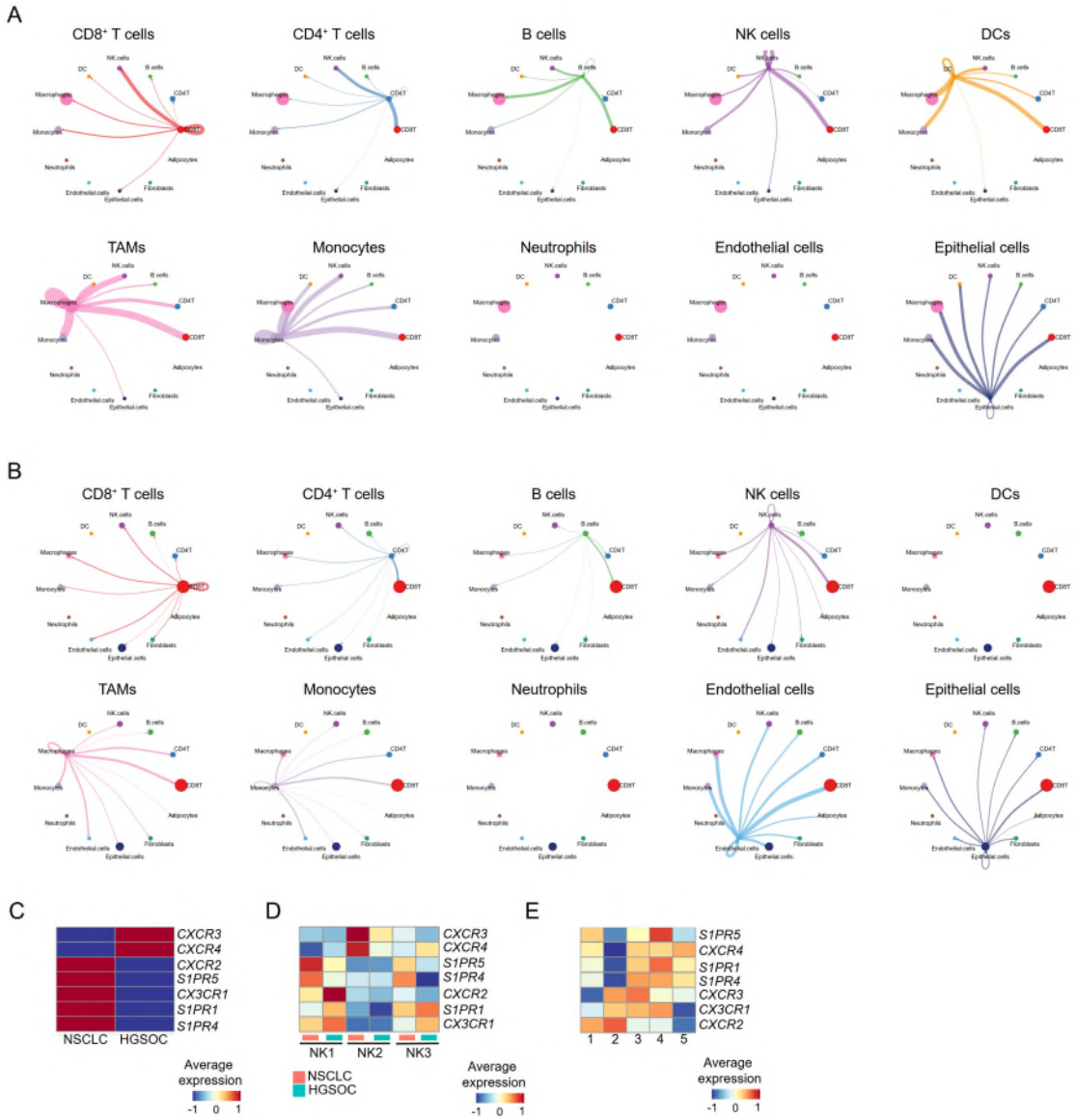

Figure 12. **The interplay between intratumoral cellular components and natural killer (NK) cells subsets within entire tumor microenvironment (TME) of advanced HGSOC and NSCLC tumor samples. (A, B)** Circle plots showing cellular interactions between NK cells and $CD8^+$ T, $CD4^+$ T cells, B cells, dendritic cells (DCs), tumor associated macrophages (TAMs), monocytes, neutrophils, endothelial cells and epithelial cells in HGSOC and NSCLC, as determined by scRNAseq (Study cohorts 6-8). Circle size and edge width are proportional to the number of cells in each cell cluster and the communication score between interacting cell clusters. **(C-E)** Heatmaps showing the differential expression of chemotaxis-associated genes among NK cells and NK1, NK2 and NK3 cell subsets in primary HGSOC and NSCLC tumor samples (**C, D**) and across (1) benign omentum, (2) primary tumor, and omentum with metastases, comprised three different omental regions: (3) macroscopically tumor-free sites distant to the omentum metastasis, and (4) peritumoral region adjacent to the tumor, as well as (5) metastatic tumors within the greater omentum (**E**), as determined by scRNAseq (Study cohorts 6-8).

8. Figure 2 E and following: The comparisons should be better described with respect to the logic. While I can infer why particular markers are being chosen, it might not be clear to the reader why NKG2A and NKp46 are being used. Have the authors considered the potential confounding contributions of NKp46+ ILCs? What is the purpose in comparing inhibitory marker expression to CD8+ T cells, where there will be some overlap, but not a reasonable expectation of the same expression levels?
a. Figure 2G's colour scheme is confusing since all of the dots are black and the legend is in the opposite order to the bars. This same problem persists in other figures (i.e. 3C-F). Likewise, the colour scheme in K is somewhat difficult to distinguish without zooming in extensively.

Our response: We thank the Reviewer #3 for these comments. We chose NKG2A and NKp46 based on their very established roles as inhibitory and activating NK cell receptors, respectively, and we have clearly mentioned this in the revised version of the manuscript. We recognize that NK cells possess many additional functional and phenotypic features that could also be assessed. However, due to limitations in the amount of available fresh tumor material and technological aspects of flow cytometry panels, we focused on these key markers to provide a clear and interpretable analysis. We are also aware that a minority of $NKp46^+$ ILCs may be present, but our analyses focus on NK cell–enriched clusters using a strict NK-specific gating strategy. Finally, we believe that comparing inhibitory marker expression of NK cells to $CD8^+$ T cells provide context for immune checkpoint regulation across cytotoxic populations in solid tumors, even if absolute expression levels differ. As discussed above in response to

comments 20 and 21 from Reviewer #1, our findings indicate that PD1 is predominantly associated with the $CD8^+$ T cell compartment (>50% of intratumoral $CD8^+$ T cells), whereas NKG2A is mainly expressed by intratumoral NK cells. Importantly, NK cells maintain stable expression of PD1 and NKG2A even after previous neo-adjuvant chemotherapy, thus highlighting them as a promising target for combined immunotherapy in advanced HGSOC. These additional analyses are now presented as Suppl. Figure 10 of revised version of manuscript.

Regarding figure clarity, we have revised the legend presented in Figures 2G, 3C–F, and K to use distinct, intuitive colors, match legend order to the bars/dots, and improve contrast for easier interpretation without zooming. These updates enhance readability while maintaining consistency across panels.

9. Have survival probabilities been adjusted for additional factors (stage, grade, treatment response, sex, comorbidities, somatic mutations?)

Our response: We thank the Reviewer #3 for bringing this point to our attention. Current Cox regression analyses presented within Table 1 of revised version of manuscript were adjusted for key clinical and molecular covariates, including stage, treatment response (not included, as these are chemo-naive samples), sex, and relevant somatic mutations. Comorbidities were not systematically available in our datasets and could not be included. The adjusted survival probabilities reported account for these factors, ensuring that the associations we observed reflect independent contributions of the features under study.

10. TLS contain many cells, but NK cells are not typically part of the considerations around these "neighbourhoods". What is the logic for comparing NK cells in this way? Why not associate neighbourhoods that have included considerations of NK cells, such as those described by Nersesian et al (Front Immunol 2024) or use an unsupervised approach to defining neighbourhoods? There are likely to be more associations that directly associate with NK cells with these approaches. As the paper goes on to conclude a positive cooperation between NK Cells and T cells (figure 5), this becomes an even more important consideration. Conversely, associations between other TLS components and NK cells is never explored further. What is the reasoning or expected logic linking NK cells and TLS? Is a TLS simply a mark of an immunologically "productive" tumour that also includes NK cell function?

Our response: Inspired by the constructive input from Reviewer #3, we applied an unsupervised niche analysis (Seurat BuildNicheAssay) that identifies spatial neighborhoods without prior assumptions about NK cell involvement. This approach revealed that NK cells consistently reside in the same niches as T cells (Fig. 13A, B

of this rebuttal letter), which was further supported by PCA and correlation analyses showing overlapping niche distributions between these two populations (Fig. 13C-D of this rebuttal letter). We also performed neighborhood analyses of NK cell subsets, which demonstrated distinct microenvironmental associations depending on tumor type, e.g., proximity to tumor cells and fibroblasts in NSCLC, versus T cells and myeloid cells in HGSOC (Fig.13E of this rebuttal letter). Together, these results support investigating NK cells in TLS-associated contexts, as their spatial distribution mirrors that of T cells and their local interactions vary across tumor types.

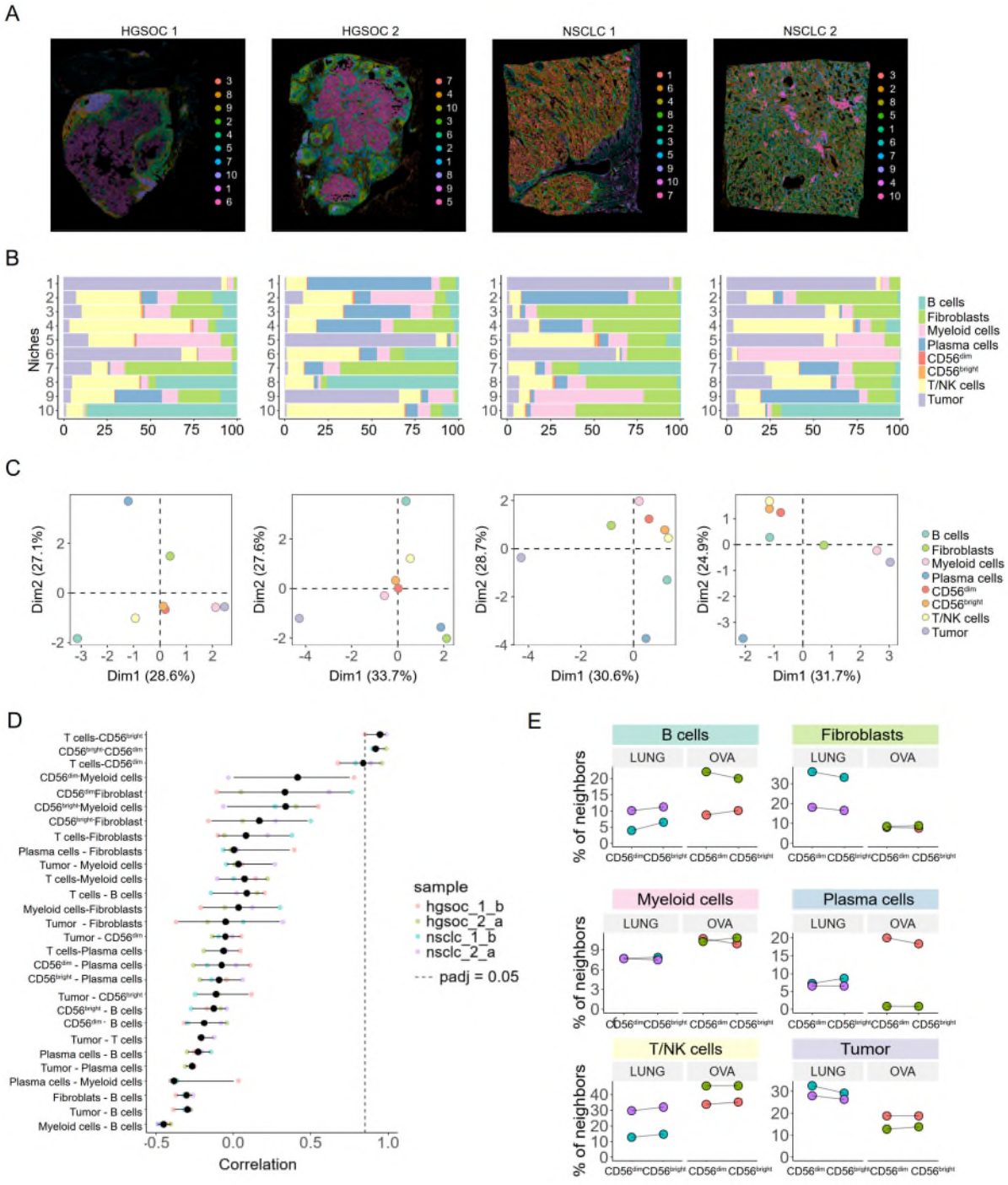

Figure 13. The spatial organisation of tumor niches and tertiary lymphoid structures (TLS) within high-grade serous ovarian cancer (HGSOC) and non-small cell lung carcinoma (NSCLC). (A) Spatial organization of cells in the HGSOC and NSCLC samples. Niches were defined based on the composition of of 30 nearest spatial neighbors using the *BuildNicheAssay* function in *Seurat*. **(B)** Stacked plots show of cell type compositions within the niches presented in A. **(C)** Principal component analysis (PCA) of niche distributions across cell types. **(D)** Pairwise correlations of niche distributions between cell types. Each colored dot represents one sample; large dots indicate mean values with 5–95% confidence intervals. Cell type pairs are ordered by mean correlation. Dashed line marks the significance threshold (adjusted $p = 0.05$). **(E)** Spatial neighbors of NK^{dim} and $NK2^{bright}$ cells. Each dot represents the frequency of a given cell type within 50 μm of an NK1 or NK2 cell centroid. NK1–NK2 pairs from the same sample are connected by lines.

11. In general, the authors have presented a lot of data, but fallen short of its immunologic interpretations. What is the mechanism(s) by which the combined NKG2A and PD1 are expected to work, for instance? While it is possible that they restore the function of NK cells and T cells, respectively (which is supported by Figure 6A), each population has been reported to express both markers (to varying extents).

Our response: We thank Reviewer #3 for raising this important point. To further investigate the mechanism underlying combined αPD1 and αNKG2A therapy, we performed additional experiments assessing the contribution of $CD8^+$ T cells and NK cells. These new findings confirm our previous observations showing that both $CD8^+$ T cell and NK cell subsets are required for therapeutic efficacy, as depletion of either population markedly reduced the clinical impact of the combined therapy (Fig. 14A, B of this rebuttal letter).

In addition, using multispectral flow cytometry, we found that $CD8^+$ T cell depletion did not alter the overall frequency of $PD1^+$ NK cells after therapy, but significantly increased the proportion of phenotypically exhausted $TIM3^+PD1^+$ NK cells (Fig. 14C,D of rebuttal letter). Conversely, depletion of $NK1.1^+$ NK cells did not affect the total pool of $PD1^+CD8^+$ T cells but resulted in a significant reduction of progenitor-like $TCF1^+PD1^+CD8^+$ T cells as compared to the combined treatment group (Fig. 14E of this rebuttal letter). These results demonstrate reciprocal regulation within the TME: depletion of NK cells diminishes progenitor-like $CD8^+$ T cells, while depletion of $CD8^+$ T cells skew NK cells toward exhaustion. We also observed a trend toward an increased frequency of $IFNG^+$ $NKG2A^+$ NK cells (relative to $NKG2A^-$ counterparts) following combined αPD1 and αNKG2A therapy, although this did not reach statistical significance ($p=0.061$), most likely due to the limited number of tumor-bearing mice available in this experimental group. Notably, this effect was not abrogated by $CD8^+$ T cell depletion (Fig. 14F of

this rebuttal letter). In summary, these analyses reinforce the importance of NK–CD8⁺ T cell cross-talk in shaping anti-tumor immunity and mediating response to αPD1 plus αNKG2A therapy, beyond the isolated effector functions of either subset. These findings are consistent with our previous observations demonstrating that the combined therapy promotes the formation of tertiary lymphoid structures (TLS) within the TME (Fig. 14G, H of this rebuttal letter), a key feature associated with ICI sensitivity and maintenance of effector-like CD8⁺ T cell phenotypes. The new results are presented in Fig. 6 and Supplementary Fig. 16 of the revised manuscript.

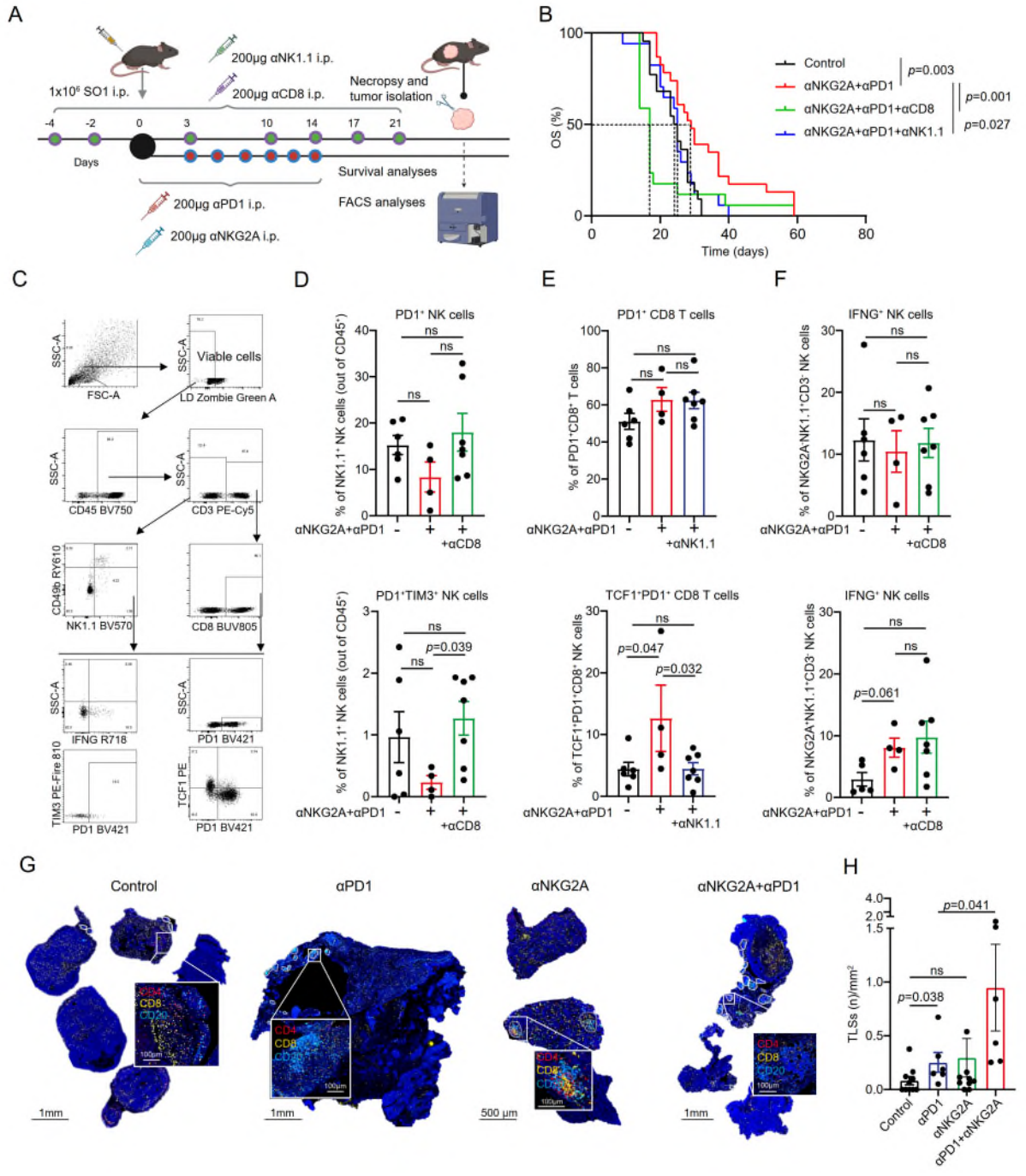

Figure 14. Combined αNKG2A and αPD1 therapy promotes NK cell- and CD8⁺ T cell-mediated anticancer immunity in preclinical models of HGSOC. (A) Experimental design for survival and flow cytometry analyses

of phenotypic and functional properties of $CD8^+$ T cells and $NK1.1^+$ NK cells after αPD1 and αNKG2A therapy, with and without $CD8^+$ T cells and NK1.1 cells depletion, in SO1 experimental syngeneic mouse model. Created with BioRender.com. **(B-F)** Overall survival (OS) of SO1 experimental model (B) and flow cytometry analyses (C) for percentage of $PD1^+$ and $PD1^+TIM3^+$ $CD45^+CD3^-NK1.1^+$ NK cells (D), percentage of $PD1^+$ and $TCF1^+PD1^+$ and $CD8^+$ T cells (E), and percentage of $IFNG^+NK1.1^+CD3^-$ $CD45^+$ NK cells within $NKG2A^+$ *vs* $NKG2A^-$ NK cells (F) after combined αPD1 and αNKG2A therapy following $CD8^+$ T cells (αCD8) and NK cells (αNK1.1) depletion. Survival curves were estimated by the Kaplan–Meier method and differences between groups were evaluated using log-rank test. Box plots: lower quartile, median, upper quartile; whiskers, minimum, maximum. Statistical significance was calculated by two-sided Mann–Whitney test. *p* values are indicated. **(G, H)** Representative immunostaining for CD4, CD8 and CD20 (G) and a box plot showing density of tertiary lymphoid structures (TLS) within control, αPD1, αNKG2A and combined αPD1+αNKG2A treated SO1 ovarian tumors (H). Scale bars 100, 500µm and 1mm. Box plots: lower quartile, median, upper quartile; whiskers, minimum, maximum. Statistical significance was calculated by two-sided Mann–Whitney test. *p* values are indicated.

Ref: ***NCOMMS-25-31476***
Manuscript: NKG2A inhibition promotes NK cell-CD8+ T cell interactions in support of improved anticancer immunity in ovarian carcinoma
Authors : **Tereza Lanickova *et al.***
Corresponding author: **Jitka Fucikova**

POINT-BY-POINT REPLY TO REVIEWER N° 4

Reviewer n° 4 commented:

The paper by Tereza Lanickova et al. recapitulate a very interesting, sounded, well sounded and well-written study that will be certainly of great interest for the community and reader of Nat Com. it certainly advances our knowledge in regard of the clinical translation and potential therapeutic uses of the checkpoint inhibitor NKG2A.

Our response: We thank Reviewer #4 for revision of our work and enthusiastic evaluation of our manuscript. Inspired by their constructive comments, we have performed various additional experiments and re-analyzed available data to provide additional mechanistic details into our findings, hence strengthening our conclusions, as detailed below.

And raised these major comments:

1. The authors adopt NK1 and NK2 signatures to better identify NK cells once subclustered but never mention NK3. Although less common in tissues, there are genes (IL32, GZMH) that are upregulated in NK1 in NSCLC samples that could be index of NK3/adaptive NK cell signature, has this aspect been investigated?

Our response: We thank Reviewer #4 for this insightful comment. The potential presence of NK3/adaptive NK cells and their associated gene signatures (e.g., *IL32, GZMH*) was carefully considered in the revised manuscript. This point is addressed in detail in our response to comment 6 from Reviewer #3 (see above). In summary, we incorporated NK cell gene signatures described by Rebuffet *et al.* (Nature, 2024) to capture these unique signatures across all scRNA-seq cohorts analyzed in this study. These findings are comprehensively discussed in Fig. 1, 2 and Suppl. Fig. 6 and 7 of revised version of manuscript.

2. Although the expression levels of CD57 and NKG2A correlates with CD56dim/NK1 and CD56bright/NK2 respectively, the expression of high levels of CD57 are mostly associated with terminally differentiated

CD56dim only, while it is also true that clusters of CD56dim NK cells retain the expression of NKG2A, which hampers their validity as surrogate markers. Wouldn't it be better to combine a few more markers to better discriminate CD56dim and CD56bright? Additionally, in this setting the authors also point out the absent expression of GZMB in CD56bright, although coherent, several studies showed GZMB to be expressed by CD56dim only, while CD56bright mostly elicit cytotoxic features via other mediators.

Our response: We fully agree with Reviewer #4 that, although CD57 and NKG2A are enriched in $CD56^{dim}$ and $CD56^{bright}$ NK cells, respectively, these markers cannot serve as surrogate identifiers of NK subsets. In this context, CD16 and CD56 (widely used in flow cytometry) would provide improved discrimination between $CD56^{dim}$ and $CD56^{bright}$ NK cells in digital pathology. However, as previously reported and confirmed by our own findings (*Bösmüller et al., Int J Gynecol Cancer, 2017; Völker et al., Diagn Pathol, 2008; Ohishi et al., Gynecol Oncol, 2007*), CD56 also stains tumor cells in ovarian cancer by immunohistochemistry, rendering it unsuitable as a specific NK cell marker in this setting. Similarly, CD16 alone is not reliable for NK cell identification due to its expression on other immune subsets, including monocytes, macrophages, neutrophils, dendritic cells, and NKT cells. Despite these limitations, we believe our 10-plex immunofluorescence panel (CD3, CD8, CD45, CD20, CD57, NKp46, DC-LAMP, GZMB, NKG2A, PanCK) provides a unique and comprehensive platform for digital pathology, enabling NK cell identification alongside spatial mapping and distance-based analyses in the HGSOC tumor microenvironment.

To extend our spatial digital pathology findings and integrate NK cell clusters identified by scRNAseq, we analyzed publicly available spatial transcriptomics (Visium) dataset on independent chemo-naïve primary HGSOC and NSCLC cohorts (Study cohorts 11 and 12) to assess the distribution of NK1, NK2, and NK3 subtypes across tumor and stromal compartments (Fig. 15A of this rebuttal letter). Using NK cell gene signatures derived from scRNA-seq, we confirmed that NK2 cells predominate in HGSOC, whereas NK1 and NK3 subsets are more abundant in NSCLC (Fig. 15B, C of this rebuttal letter). In NSCLC, NK1 and NK2 cells were more frequent in the tumor core than in the stroma, while in HGSOC the distribution between tumor and stroma was largely comparable, with the exception of NK2 cells, which were enriched in the tumor core (although not reaching statistical significance), consistent with our digital pathology observations (Fig. 15D, E of this rebuttal letter).

We further explored spatial relationships of NK cells with T cells subtypes, demonstrating that in NSCLC NK cells were preferentially located near $CD8^+$ central memory (T_{CM}) and effector memory (T_{EM}) T cells, whereas in HGSOC NK2 and NK3 cells were more often found in proximity to exhausted (T_{EX}) and terminally exhausted

(T_{EMRA}) $CD8^+$ T cells, respectively and, as well as $FoxP3^+$ regulatory $CD4^+$ T cells (Fig. 15G of this rebuttal letter). In summary, this independent spatial transcriptomic analysis supports and extends our digital pathology findings: NK cells polarize predominantly into NK1 and NK3 clusters in NSCLC, but into NK2 clusters in HGSOC. Moreover, NK cells are enriched in the tumor core of NSCLC but not in HGSOC, where NK2 cells dominate. These results further underscore the importance of NK–T cell crosstalk in shaping anti-tumor immunity in solid tumors. The new analyses are presented in Supplementary Figure 13 of the revised manuscript.

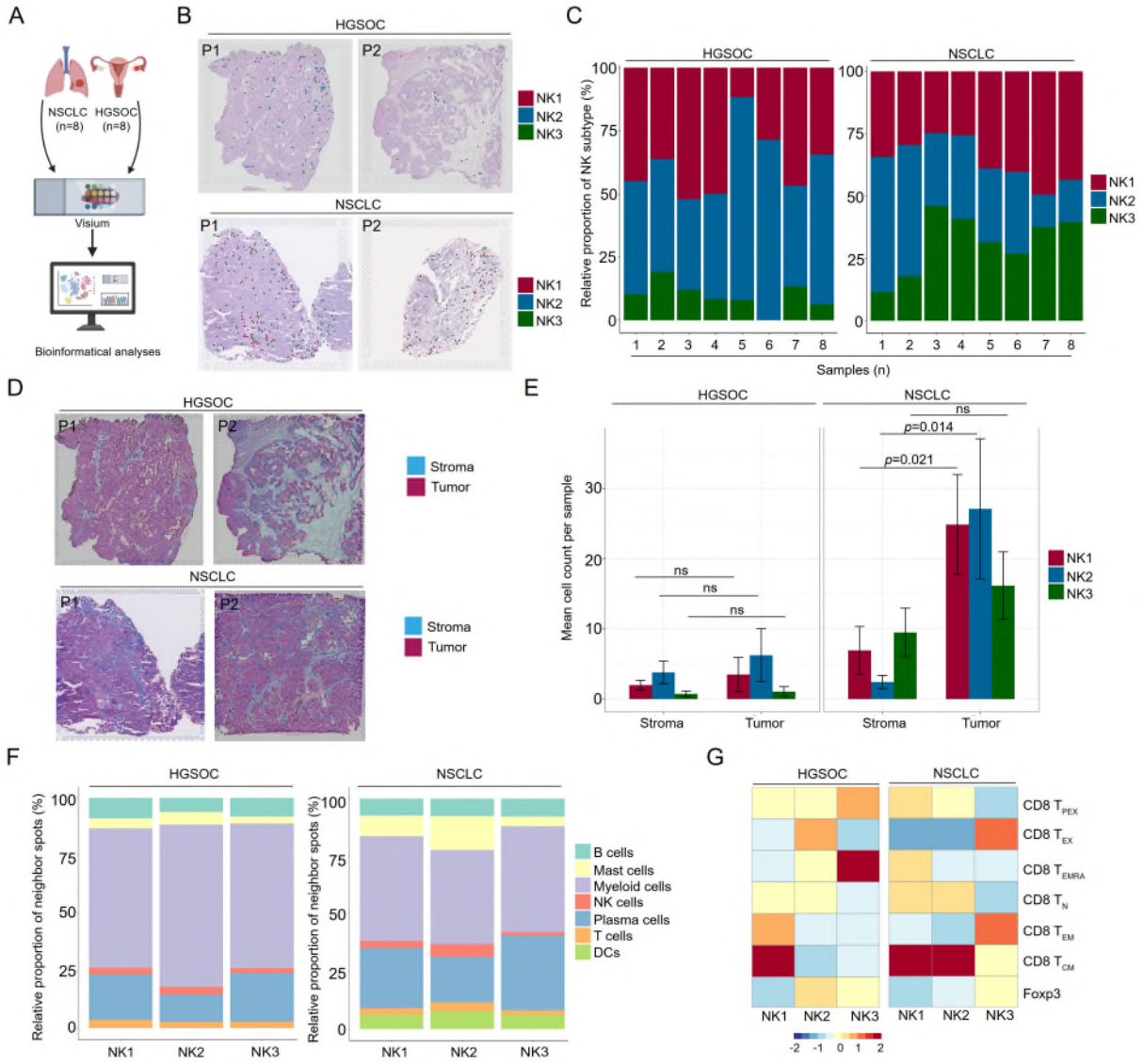

Figure 15. **Spatial mapping of NK cell subset localization and interactions with $CD8^+$ T cells across tumor and stromal regions in HGSOC and NSCLC. (A)** Experimental workflow in chemo-naïve HGSOC (n = 8; Study Cohort 11) and NSCLC (n = 8; Study Cohort 12) samples. Spatial transcriptomics was performed on FFPE tumor

specimens using the Visium in situ platform. Created with BioRender.com. **(B, C)** Representative spatial maps (B) and stacked plots (C) showing the distribution of NK1, NK2, and NK3 subsets in HGSOC and NSCLC samples, as determined by Visium transcriptomics. **(D, E)** Representative image of tumor core versus stroma annotation (D) and bar plots (E) showing NK1, NK2, and NK3 distribution within tumor core and stromal regions of HGSOC and NSCLC samples. **(F)** Stacked plots depicting the relative immune cell composition of spots adjacent to NK1, NK2, and NK3 subsets in HGSOC and NSCLC, as determined by Visium. **(G)** Spatial enrichment of $CD8^+$ T cell gene signatures, including central memory (T_{CM}), effector memory (T_{EM}), terminally differentiated (T_{EMRA}), progenitor exhausted (T_{PEX}), exhausted (T_{EX}), and naïve $CD8^+$ T cells (T_N), and $FoxP3^+$ regulatory T cells as determined by Visium spatial transcriptomics.

3. The authors reported a functional exhaustion of CD56bright NK cells in primary HGSOC by comparing the transcriptional profile of these NK cells with CD56dim from benign omentum. However, the described transcriptional differences are already known features of CD56bright and CD56dim. To define a functional exhaustion of CD56bright NK cells in HGSOC, a comparison with CD56bright NK cells from a healthy tissue could be more informative.

Our response: We thank Reviewer #4 for bringing this important point to our attention. To address the functional state of $CD56^{bright}$ NK cells, we directly compared the transcriptional profiles of the NK2 cluster across the benign omentum, primary HGSOC (pHGSOC), and metastatic HGSOC (mHGSOC) using scRNA-seq (Study Cohorts 6 and 8; Fig. 16A of this rebuttal letter). Consistent with our previous observations, NK2 cells displayed site-specific divergence in the expression of inhibitory receptors and cytokines. Specifically, pHGSOC-derived NK2 cells showed elevated expression of inhibitory molecules such as *KIR3DL2*, *KLRC1*, *KIR3DL1*, *CD300A*, *KIR2DL3*, *KIR2DL1*, *HAVCR2*, *IL16*, and *FLT3LG* compared to the benign omentum (Fig. 16B–D of this rebuttal letter). Similarly, mHGSOC-derived NK2 cells were enriched for *TIGIT*, *KIR2DL1*, and *HAVCR2* as compared to their counterparts in the benign omentum. In contrast, NK2 cells from the benign omentum expressed higher levels of cytokine and chemokine genes including *XCL2*, *CCL4*, *CCL3*, *CCL5*, *XCL1*, *IFNG*, and *CCL4L2*, highlighting an activated cytokine-secreting phenotype (Fig. 16C–D of this rebuttal letter). Together, these findings suggest that $CD56^{bright}$ NK cells (NK2 cluster) from both primary and metastatic HGSOC acquire features of functional exhaustion, in contrast to their counterparts in benign tissue.

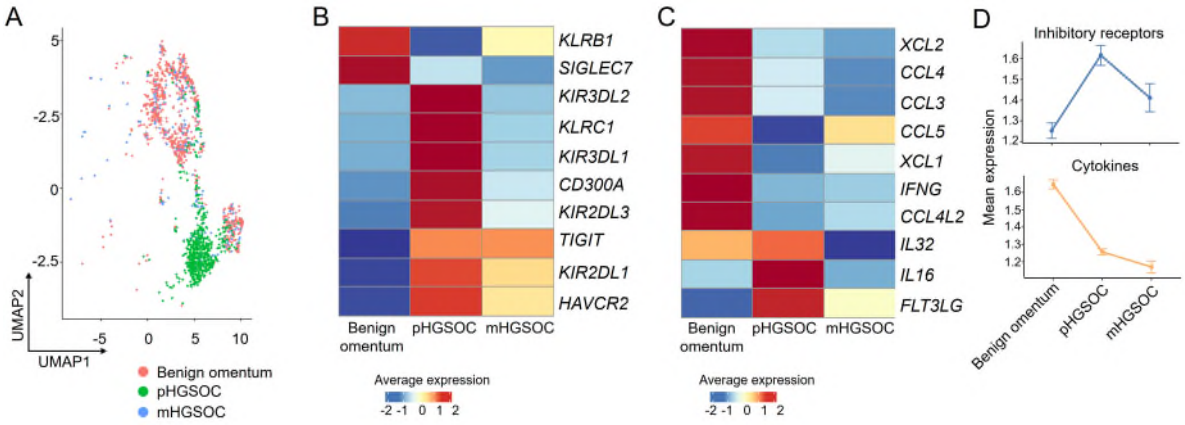

Figure 16. **NK^{bright} cells from primary and metastatic HGSOC are functionally impaired as compared to benign omentum. (A)** UMAP showing expression of NK2 cluster in benign omentum, primary (pHGSOC) and metastatic (mHGSOC) samples as determined by scRNAseq (Study cohorts 6 and 8). **(B-D)** Heatmap and lineplots showing the differential expression of gene signatures associated with inhibitory receptors (B) and cytokines (C, D) within 3 previously defined histological compartments (as shown in panel A) as determined by scRNAseq. The color scale is based on z-score-scaled gene expression.

4. The functional exhaustion of CD56bright NK cells described at the transcriptional level is not supported by functional experiments. The authors reported a relative enrichment of NKG2A+ NK cells in HGSOC microenvironment compared to the NSCLC microenvironment. Do these cells show a lower ability to produce cytokines in vitro too?

Our response: Inspired by the constructive comment from Reviewer #4, we performed additional analyses of the phenotypic and functional properties of NK cells in freshly resected tumor samples from primary (pHGSOC, n=7) and metastatic (mHGSOC, n=8) HGSOC and from primary NSCLC (n=4) using multispectral flow cytometry (Fig. 17A–B of this rebuttal letter). In line with our transcriptomic findings, these analyses demonstrated increased expression of inhibitory receptors such as PD1, TIM3, and NKG2A on total NK cells in pHGSOC as compared to primary NSCLC (Fig. 17C of this rebuttal letter). Notably, NK cells from pHGSOC displayed significantly reduced GZMB production compared with their counterparts from NSCLC patients (Fig. 17C of this rebuttal letter). Consistent with our previous findings, we also observed significantly higher expression of NKG2A on CD56^{bright} NK cells relative to CD56^{dim} NK cells in both pHGSOC and mHGSOC, a pattern that was not seen in NSCLC (Fig. 17D of this rebuttal letter). Finally, although CD56^{dim} NK cells generally expressed higher levels of GZMB than CD56^{bright} cells within NSCLC samples, we failed to observe similar findings in either pHGSOC or mHGSOC,

further supporting our earlier observation of functional impairment within the $CD56^{bright}$ NK cell compartment of HGSOC (Fig. 17D of this rebuttal letter).

Inspired by these observations, we assessed the functional properties of $NKG2A^+$ *versus* $NKG2A^-$ NK cells across pHGSOC, mHGSOC, and NSCLC (Fig. 17E, F of this rebuttal letter). $NKG2A^+$ NK cells consistently displayed a significantly lower frequency of GZMB expression compared with their $NKG2A^-$ counterparts within pHGSOC and mHGSOC, and a similar trend was observed in NSCLC although without statistical significance (Fig. 17F of this rebuttal letter). In addition, $NKG2A^+$ NK cells from NSCLC displayed significantly higher expression of $GZMB^+$ as compared to pHGSOC, further supporting our previous observation about functionally impaired NKG2A+ NK cells in HGSOC (Fig. 17F of this rebuttal letter). Similar trends were observed when analyzing IFNG expression in $NKG2A^+$ versus $NKG2A^-$ NK cells; however, the difference did not reach statistical significance, most likely due to the limited number of tumor samples analyzed (Fig. 17F of this rebuttal letter). Although the NSCLC cohort was limited to four patients, these findings lend extra support to our transcriptomic observations of functional NK cell impairment in HGSOC. Taken together, these functional experiments reinforce our conclusion that NK cells in HGSOC are functionally impaired, particularly in association with NKG2A expression. The new analyses are presented in Figure 2 and Supplementary Figure 9 of the revised manuscript.

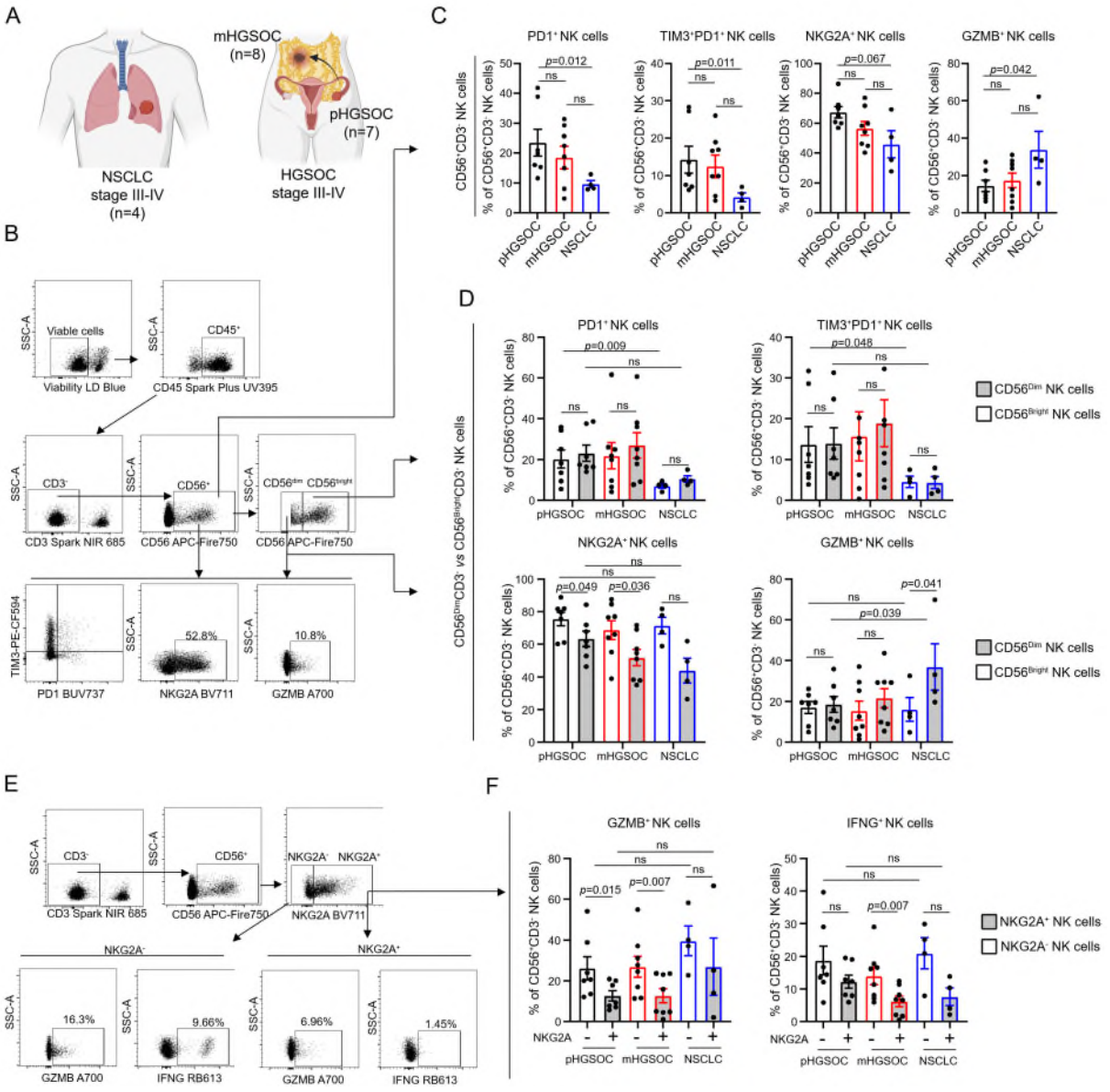

Figure 17. **NK cells from advanced HGSOC are phenotypically and functionally impaired as compared to NSCLC. (A)** Overview of anatomical sampling sites of collected samples for multispectral flow cytometry: non-small cell lung carcinoma (NSCLC) (n=4), primary HGSOC (pHGSOC) (n=7) and metastatic HGSOC (mHGSOC) (n=8) (reported as part of Study Cohort 3 and 4 in revised version of manuscript). Created with BioRender.com. **(B-D)** Representative dot plot (B) and bar plots (C-E) showing percentage of PD1$^+$, TIM3$^+$PD1$^+$, NKG2A$^+$ and GZMB$^+$ intratumoral CD56$^+$ NK cells (C) and CD56^{bright} vs CD56^{dim} (D) NK cells within pHGSOC, mHGSOC and NSCLC tumor samples as determined by flow cytometry. Mean and SEM are shown. Statistical significance was calculated by the Mann–Whitney test. *p* values are indicated. **(E, F)** Representative dot plot (E) and bar plots (F) showing the percentage of GZMB$^+$ and IFNG$^+$ among NKG2A$^+$ *vs* NKG2A$^-$ CD56$^+$ NK cells

among pHGSOC, mHGSOC and NSCLC. Mean and SEM are shown. Statistical significance was calculated by the Mann–Whitney and and the Wilcoxon test test. *p* values are indicated.

5. As described by the authors in the discussion section, targeting NKG2A in a clinical setting is related to the expression of its ligand HLA-E. What about HLA-E expression in their cohorts? Is the expression of NKG2A related to the expression of HLA-E?

Our response: Inspired by the constructive feedback from Reviewer #4, we performed additional analyses of NK cell ligands on epithelial cell clusters and their corresponding NK cell receptors across NK cell clusters in HGSOC and NSCLC, using publicly available scRNA-seq datasets (Study Cohorts 6 and 7; Fig. 18A–E of this rebuttal letter). Notably, HLA-E expression was markedly higher in HGSOC compared with NSCLC (Fig. 18D of this rebuttal letter). Consistent with our earlier findings, the HLA-E–associated receptor *KLRC1* (encoding NKG2A) was predominantly expressed in the NK2 cluster in both tumor types (Fig. 18E of this rebuttal letter). To validate these transcriptomic findings, we developed a multiplex immunostaining panel to assess HLA-E expression in HGSOC and NSCLC (Study Cohorts 9 and 10; Fig. 18F of this rebuttal letter). In contrast to scRNA-seq, HLA-E expression appeared comparable across tumor compartments, with significantly pronounced expression in stroma compartment of NSCLC as compared to HGSOC (Fig. 18G of this rebuttal letter). Supporting our prior observations, intratumoral HLA-E expression correlated significantly with the frequency of $NKG2A^+$ NK cells in HGSOC, with a similar but nonsignificant trend in NSCLC (Fig. 18H of this rebuttal letter).

To further investigate the functional role of the HLA-E–NKG2A axis, we performed *in vitro* assay using sorted $NK1.1^+$ cells from the SO1 preclinical mouse model (Fig. 18I of this rebuttal letter). Flow cytometry revealed that >70% of intratumoral $NK1.1^+$ cells expressed NKG2A, and this phenotype remained stable throughout the experiments (Fig. 18J of this rebuttal letter). To assess the effect of HLA-E on NK cell activity, we compared NK cytotoxicity against the SO1 cell line (low HLA-E) versus primary SO1 tumor cells freshly isolated from native tissue, which displayed significantly higher HLA-E expression induced by tumor progression (Fig. 18I of this rebuttal letter). Flow cytometry demonstrated that HLA-E expression on primary SO1 cells inhibited NK cells, resulting in reduced cytotoxicity. By contrast, NK cells co-cultured with the SO1 cell line (low HLA-E) displayed robust cytotoxicity, with increased frequencies of $annexin^+/DAPI^+$ target cells (Fig. 18K of this rebuttal letter). In summary, integrating transcriptomic, spatial pathology, and functional analyses, we highlight the NKG2A–HLA-E axis as a clinically relevant inhibitory checkpoint in HGSOC. These new results are incorporated in Figure 3 and Suppl. Fig. 12 of the revised manuscript.

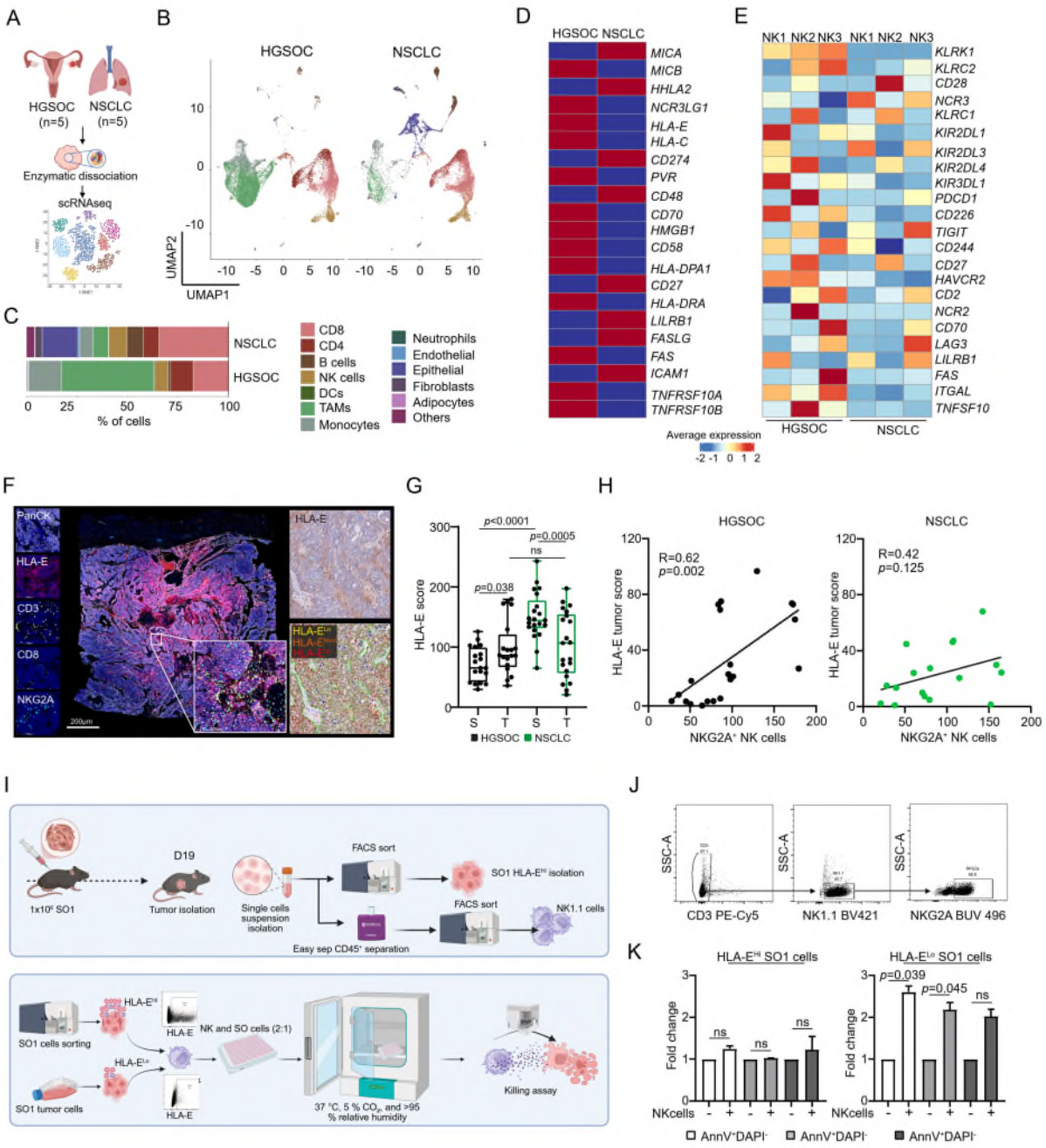

Figure 18. The NKG2A–HLA-E axis as a therapeutic checkpoint in HGSOC. (A) Experimental and scRNAseq workflow in chemo-naïve patients with HGSOC (n = 5) and NSCLC (n = 5) (Study Cohorts 6 and 7). Created with BioRender.com. **(B, C)** UMAP (B) and stack plot (C) of all cells passing quality control, colored by cell type. **(D, E)** Heatmaps showing differential expression of NK ligands in epithelial clusters (D) and corresponding NK receptors across NK cell subsets in HGSOC and NSCLC (E), as shown in panels A-C. **(F)** Representative image of HLA-E, CD45, CD8, CD3, and PanCK immunostaining (immunofluorescence panel 3). Scale bars: 100 µm and 200 µm, as indicated. **(G)** HLA-E score within tumor stroma and core of chemo-naïve HGSOC (n = 20; Study Cohort 9) and NSCLC (n = 21; Study Cohort 10) tumor samples, as determined by digital pathology. Box plots

display lower quartile, median, upper quartile; whiskers show minimum and maximum. Statistical significance was determined by Mann–Whitney test; *p* values indicated. **(H)** Correlation between intratumoral frequency of NKG2A^{+}CD3^{-}CD8^{-} NK cells and HLA-E score in HGSOC (Study Cohort 9) and NSCLC (Study Cohort 10), determined by multispectral immunofluorescence. Pearson correlation coefficient (R) and *p* values indicated. **(I)** Experimental workflow for NK killing assay using the SO1 preclinical ovarian cancer model. NK1.1^{+} cells were isolated from intraperitoneal SO1 tumors in syngeneic C57BL/6J mice (day 19) by FACS, showing stable NKG2A expression. SO1 cell line (HLA-E^{Lo}) and primary SO1 tumor cells (HLA-E^{Hi}) were used as targets cells. **(J)** Representative dot plot showing purity and stable NKG2A expression of FACS-sorted NK1.1^{+} cells from SO1 tumors. **(K)** Percentage of early apoptotic (AnnV^{+}/DAPI^{-}), late apoptotic (AnnV^{+}/DAPI^{+}), and necrotic (AnnV^{-}/DAPI^{+}) SO1 target cells with low versus high HLA-E expression after 4 h incubation with NK1.1^{+} cells (1:2 ratio), determined by flow cytometry. Statistical significance calculated by paired t-test; *p* values indicated.

6. For what concerns the NSCLC cohort 4, the authors indicated that the patients had a stage disease III+IV. However, in the Suppl. Table 1B the stage diseases reported are mainly I and II. How do the authors explain this discrepancy? The same for NSCLC cohort 9, in the text it has been specified that the samples were obtained from stage III+IV patients, but in the table are reported early stages too.

Our response: We thank Reviewer #4 for carefully reviewing the supplemental data reflecting the clinicopathological characteristics of patients in individual cohorts. We acknowledge that NSCLC Cohort 4 (but not Cohort 10) includes stage I (n=9) and II (n=6) patients and deeply apologize for the previous text indicating only stage III–IV. The inclusion of early-stage patients reflects the prospective nature of sample collection in the Study Cohort 4. We have carefully corrected Suppl. Figure 1 and Suppl. Table 2 and the corresponding text in the revised manuscript to accurately reflect the full stage distribution. Importantly, this correction does not affect any of the analyses or conclusions presented in our study.

7. For Figure 3E the authors stated that NKG2A+ NK cells are equally represented in tumor cores and stromal areas in both HGSOC and NSCLC. Even if not statistically significant, the frequency of NKG2A+ NK cells is higher in the tumor core of HGSOC compared with the stromal area.

Moreover, in the HGSOC tumor core, there are different groups of patients with different frequencies of NKG2A+ NK cells. Is this related to any feature of the patients? Such as HLA-E expression?

Our response: Please see our response to point 5 raised by Reviewer #4.

8. In the tables with the information of the patients, the vital status is reported, after how much time is it calculated?

Our response: We thank the Reviewer #4 for this important question. The vital status reported in the tables reflects the information available at the date of database lock for each cohort, which is the cutoff used to calculate survival outcomes. Each cohort has a distinct date lock, corresponding to the time when the clinical data were finalized for analysis. We have clarified this point in the table 3 legends of the revised manuscript.

9. For the Suppl. Table 1B how is the percentage of NK cells, CD56dim, and CD56bright obtained?

Our response: The full gating strategy on flow cytometry data from human tumor samples is displayed in detail in Suppl. figure 3 of revised version of manuscript.

10. In the Methods section, the authors state that "final cell suspensions were washed, counted, and resuspended in FACS buffer for further analysis". Does this imply that all samples were processed as fresh material and not frozen? Have you accounted for batch effects related to sample processing and FACS acquisition timing? Regarding scRNA-seq library preparation, were libraries generated fresh from each individual sample, or were they prepared in batches? If the first option, have you accounted this while processing the scRNA-seq data?

Our response: We thank the Reviewer#4 for raising this important point regarding sample processing and potential batch effects. Over FACS analysis, all human (Study Cohorts 3-5) and mice samples were processed as fresh material immediately following tissue dissociation. This approach was chosen to preserve minor cell populations, such as NK cells that can be lost or altered during freezing and thawing. We recognize that variability in sample handling and acquisition timing can introduce batch effects. To mitigate this, all FACS acquisitions were performed using standardized protocols, and instrument settings were calibrated daily. Additionally, we included internal controls and processed samples in a randomized order to minimize potential batch-related biases. For the single-cell analysis, we utilized three publicly available datasets (Study Cohorts 6-8). As reported in the original publications, these datasets were generated from fresh samples. To account for potential batch effects, we performed data integration using either Harmony or the RPCA-based approach, depending on the dataset.

11. In the scRNA-seq quality control steps, there is no mention of tools or strategies used for doublet removal. Have you assessed the presence of doublets in your dataset and excluded them accordingly?

Our response: We thank the reviewer for bringing this point to our attention. To assess doublets, we examined scatterplots of nCount_RNA versus nFeature_RNA, which allowed us to identify and exclude cells with abnormally high transcript and gene counts indicative of potential multiplets. While this approach efficiently removed obvious outliers, we acknowledge that dedicated algorithms such as DoubletFinder or Scrublet provide a more systematic method for doublet detection. We are confident that our scatterplot-guided thresholding effectively minimized the impact of doublets on downstream analyses, and we have now clarified this procedure in the Methods section of the revised manuscript.

12. Could you please specify which covariates were used for data integration in the scRNA-seq analysis?

Our response: For data integration, we used sample identity as the primary covariate to correct for batch effects across donors, ensuring that true biological differences between cells are preserved while minimizing technical variation.

13. In Figure 1I, are the counts comparable across samples? It would be more appropriate to represent the data as normalized frequencies (e.g., percentage of total cells per sample) rather than as absolute cell counts.

Our response: We have included the NK total numbers along with their corresponding percentage representation within individual scRNAseq datasets (Study Cohort 6-8) in the Fig. 1, 2 and Suppl. Fig. 7, 8 of revised version of the manuscript.

And raised these minor comments:

1. Some typing errors are present.

2. In the methods section “scRNA-seq sample preparation” the phrase regarding the study cohort 7 is not complete.

3. For the results of Figure 1A the authors used genes with documented NK-cell modulating effects, however the reference for these genes is missing.

4. In the legends of Suppl. Figures 3 and 4, the gating strategy used is not described.

Our response: All minor comments were addressed in the revised version of manuscript.

END

Ref: ***NCOMMS-25-31476A***
Manuscript: NKG2A inhibition promotes NK cell-CD8+ T cell interactions in support of improved anticancer immunity in ovarian carcinoma
Authors: **Tereza Lanickova *et al.***
Corresponding author: **Jitka Fucikova**

POINT-BY-POINT REPLY TO REVIEWER N° 1

Reviewer n° 1 commented:

Overall, the authors have addressed previous reviewer comments comprehensively. The revised manuscript and rebuttal are clear, and the additional figures substantially strengthen the mechanistic and translational aspects of the study.

Minor comment: The Introduction (lines 111–113), the sentence describing the SO1 model as having "high TMB" could be interpreted as implying that anti-NKG2A monotherapy confers a survival benefit. However, as shown in Figure 5 and Supplementary Figure 16 of the rebuttal, survival benefit was only observed when anti-NKG2A was combined with anti-PD-1. Consider rewording this sentence to reflect that therapeutic benefit occurred only in the combination setting.

Our response: We thank Reviewer #1 for positive evaluation of our work. We agree that the original phrasing in the Introduction section could be misinterpreted as suggesting that anti-NKG2A monotherapy provides a survival benefit. In the revised manuscript, we have clarified the sentence to indicate that significant therapeutic benefit was observed only when αNKG2A was combined with αPD-1, consistent with the data shown in Fig. 6L of the revised version of manuscript.

POINT-BY-POINT REPLY TO REVIEWER N° 2

Reviewer n° 2 commented:

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Our response: We acknowledge the contribution of the Early Career Researcher who co-reviewed this manuscript and provided constructive feedback as part of the Nature Communications initiative. Raised comments have been valuable in improving the clarity and quality of the manuscript.

POINT-BY-POINT REPLY TO REVIEWER N° 3

Reviewer n° 3 commented:

Lanickova et al have submitted an updated version of their manuscript and lengthy rebuttal in response to the reviewers' comments. The paper continues to explore the immunologic landscapes of HGSC, in particular with respect to NK cells. By comparing transcriptomic approaches and HGSOC with NSCLC, the authors uncover an enrichment for NK2 NK cells in HGSOC and the other populations – 1 and 3 in NSCLC. They conclude that this reflects an NK cell disregulation/exhaustion in HGSOC which might reflect differences in responsiveness to checkpoint inhibition and therapeutic intervention. The reader is lost in the details (which might more effectively be conveyed in table/figure format then many lines of text with gene symbols).

Our response: We thank Reviewer #3 for the constructive and thoughtful feedback, as well as for acknowledging the remaining issues that should be addressed to improve the focus and interpretability of our work. We appreciate the reviewer's careful reading of the revised manuscript and agree that some aspects—including overall brevity, writing quality, explicit discussion of study limitations, and a clearer distinction between inferred and experimentally validated function—require further refinement. In the revised manuscript, we also carefully addressed the comments from the other three reviewers, who expressed satisfaction with the revisions and confirmed that the central message of the study has been preserved. We sincerely appreciate this additional guidance, which has further enhanced the precision and mechanistic focus of the revised manuscript.

And raised these major comments:

1. While I appreciate the thoroughness of the authors' responses, the paper, in general, needs to be edited for brevity and clarity. There is too much data presented in figures and supplementary material; all points to inferred relationships, but brevity would dramatically improve the impact of this paper. Seven Figures and 17 supplementary, all with >10 panels is just too much.
In some instances/newly added text, the writing is unclear and should be edited throughout. There is a lot of data and run-on sentences, which make interpretation difficult for the reader. As a reviewer, I do not see my role in editing, but provide just a few examples below: Lines 887-893 (one sentence; 79 words): "Harnessing multiple cohorts of patients with advanced HGSOC and an immunocompetent mouse model of the disease, here we demonstrate that (at odds with the immunologically "hot" NSCLC

microenvironment) both primary and metastatic HGSOC microenvironment (including HGSOC-associated TLSs) is often enriched in CD56bright NK cells expressing the co-inhibitory receptor NKG2A, which (at least in preclinical settings) can be harnessed to unleash a therapeutically relevant interaction between intratumoral NK cells and CD8+ 893 CTLs in the context of concomitant PD-1 blockade.“ Or lines 850-856: “Moreover, SO1 tumors responding to NKG2A blockade contained an increased frequency of CTLs co-expressing PD-1 and transcription factor 7 (TCF7, best known asTCF1), which are known to respond to ICIs (57-60) and a decreased frequency of CD8+ CTLs 853 expressing NKG2A+ or co-expressing PD-1 and TIM3, both of which have previously been 854 associated with dysfuncton (42,61-63) (Fig. 6K). Finally, the discussion lacks consideration of the limitations of the study – mainly that it is nearly all transcriptomic-based, and would benefit from an exploration of what would be necessary for protein-level and functional validation (beyond the one mouse model shown) to approach the phenotypic and challenging diversity of HGSOC.

Our response : We thank Reviewer #3 for the constructive comments and fully agree that enhancing brevity and clarity strengthens the manuscript's overall impact. In response, we conducted a comprehensive revision to streamline the text, reduce redundancy, and improve readability, while balancing these changes with the modifications requested by the other reviewers during the revisions of this manuscript. Several long or run-on sentences—including those specifically highlighted by the reviewer—have been rewritten into clearer, more concise statements, and newly added sections were refined for coherence and precision. We also carefully re-evaluated all figures and supplementary materials, removing non-essential panels, consolidating overlapping datasets, and focusing the visual presentation on the most critical results. This allowed us to reduce overall volume without compromising scientific rigor. The revised manuscript now includes 6 main figures and 11 supplementary figures, balancing the previous requests of the reviewers and the editor.

Next, we agree that while our study leverages transcriptomic analyses across multiple independent human HGSOC and NSCLC cohorts, it inherently faces limitations related to the reliance on RNA-level data. Transcript abundance does not always directly reflect protein expression, post-translational modifications, or functional activity, and as such, our findings require further protein-level and functional validation. To partially address this, we incorporated flow cytometry (Fig. 2I-O), immunofluorescence (Fig. 3), and spatial transcriptomics (Fig. 4A-E) in multiple cohorts to validate NK cell subset composition, checkpoint expression, and spatial localization relative to $CD8^+$ T cells. Our SO1 mouse model further allowed functional interrogation of NK–T cell interactions and the impact of

NKG2A and PD1 blockade. Nevertheless, comprehensive protein-level validation across the full spectrum of NK cell heterogeneity in human HGSOC would require multiparametric flow or mass cytometry in larger cohorts, and functional validation remains challenging due to limited sample availability and variable NK cell frequencies. Future studies using ex vivo co-cultures, organoid systems, or live-cell imaging could capture dynamic NK–tumor and NK–T cell interactions. Additional preclinical models with diverse tumor mutational burdens and immune contexts will be necessary to generalize these findings. As per Reviewer comment, we have now added a paragraph in the Discussion section to explicitly acknowledge these points, emphasizing that although transcriptomic profiling provides comprehensive insights into NK cell heterogeneity, future work integrating protein-level and functional assays will be necessary for definitive validation.

We believe these revisions substantially strengthen the clarity, narrative focus, and contextual framing of the study's contributions and limitations.

2. The manuscript, in general, still suffers from inferring functionality from transcriptomic data. "Functional validation" is almost always conducted with more omics data, which, while supportive, cannot confirm function. For instance, lines 747-753 describe "functional" validation of T-NK interactions using spatial transcriptomics. Indeed, there is co-localization that associates with poorer outcomes, but whether this is causative is unknown. The authors, throughout, should be careful to dissociate actual from inferred function.

Our response: We thank the Reviewer #3 for raising this important point regarding the distinction between functional mechanisms and inferred associations. We agree that our multi-modal transcriptomic and spatial analyses identify correlative patterns rather than direct causal interactions. While our results derived from scRNA-seq, spatial transcriptomics, and mIF provide robust correlative evidence of T–NK spatial association and activation states, these analyses do not allow causal relationships to be inferred. Functional perturbation assays will be needed to strengthen these putative interactions with distinct technical limitations including viability and functionality of cells isolated from primary tumors. To address this, we have revised the text throughout the manuscript to avoid overstating functional implications and to ensure that all conclusions are framed as associations or potential mechanisms rather than established functions. Specifically, we removed the term "functional" in contexts referring to transcriptomic or spatial analyses, replaced phrasing such as "functional validation" with "supportive multimodal evidence," and clarified in the Results and Discussion that co-localization and pathway signatures indicate putative interactions rather than proven cause–effect relationships.

3. The mouse model supports a blockade strategy to promote anti-tumour activity against HGSOC. Is there evidence that this model recreates the complexity and immune structures described in the earlier figures of the paper? This would help to fill in some of the missing functional validations if their work in situ could be supported.

Our response: We thank Reviewer #3 for this insightful comment. The SO1 model was selected after evaluating several established ovarian cancer systems, including Trp53$^{-/-}$ Brca1$^{-/-}$ BR5, Trp53$^{-/-}$ Brca1$^{-/-}$ Hras/Myc SO1, and Trp53$^{-/-}$ Brca1$^{-/-}$ ID8 (*Walton et al., Cancer Res 2016; Kasikova et al., Nat Commun 2024; Lanickova et al., Clin Cancer Res 2024; Righi et al., Cancer Res 2011; Xing et al., Cancer Res 2006*). Although existing mouse model only partially reflect the immunological heterogeneity of human HGSOC (*Maniati et al., Cell Rep 2020; Cook et al., Commun Biol 2023; Rodriquez et al., Cancer Res Commun 2022*), we selected the SO1 model because it most closely reflects the immune architecture characteristic of advanced human HGSOC (*Kasikova et al., Nat Commun 2024*). First, SO1 tumors display a particularly robust intratumoral NK-cell compartment, confirmed by both immunostaining (NKp46^{+}CD3^{-} cells) (Fig. 1A, B of this rebuttal letter) and flow cytometry (NK1.1^{+}CD3^{-} cells) (Fig. 1C of the this rebuttal letter; Fig. 5–6 in the revised manuscript). Importantly, more than 50% of intratumoral NK1.1^{+} NK cells express NKG2A, closely resembling the phenotype and frequency observed in advanced human HGSOC samples (Fig. 1D of this rebuttal letter). Thus, with respect to NK-cell abundance and phenotypic features relevant to this study, SO1 is comparable to advanced HGSOC. Second, SO1 tumors exhibit a distribution of major immune cell components that closely parallels the cellular composition of human HGSOC, as determined by scRNA-seq (Fig. 1E of this rebuttal letter). Notably, intratumoral CD8^{+} T cells in SO1 tumor samples preferentially differentiate into T_{EF} and T_{EX} states, with only limited T_{PEX} populations—mirroring the dominant T-cell phenotypes observed in human HGSOC by both scRNA-seq (Fig. 1F of this rebuttal letter) and flow cytometry (Fig. 1G of this rebuttal letter). This enriched innate and adaptive immune contexture enables a systematic interrogation of NK–CTL interactions and facilitates evaluation of immunotherapy responses with greater resolution than many alternative models. Finally, the SO1 model uniquely develops TLS-like lymphoid aggregates, providing an opportunity to study lymphoid organization highly reminiscent of that observed in human metastatic HGSOC (Fig. 1H of this rebuttal letter) (MacFawn et al., Cancer Cell 2024; Lanickova et al., Clin Cancer Res 2024).

Together, we hope the Reviewer will agree that the characteristics presented support SO1 as an immunologically relevant model for studying NKG2A- and PD-1–directed immunotherapy in the context of HGSOC. As noted in

the revised Discussion, complementary in situ functional validation in human samples remains an important future step to fully translate these mechanistic insights.

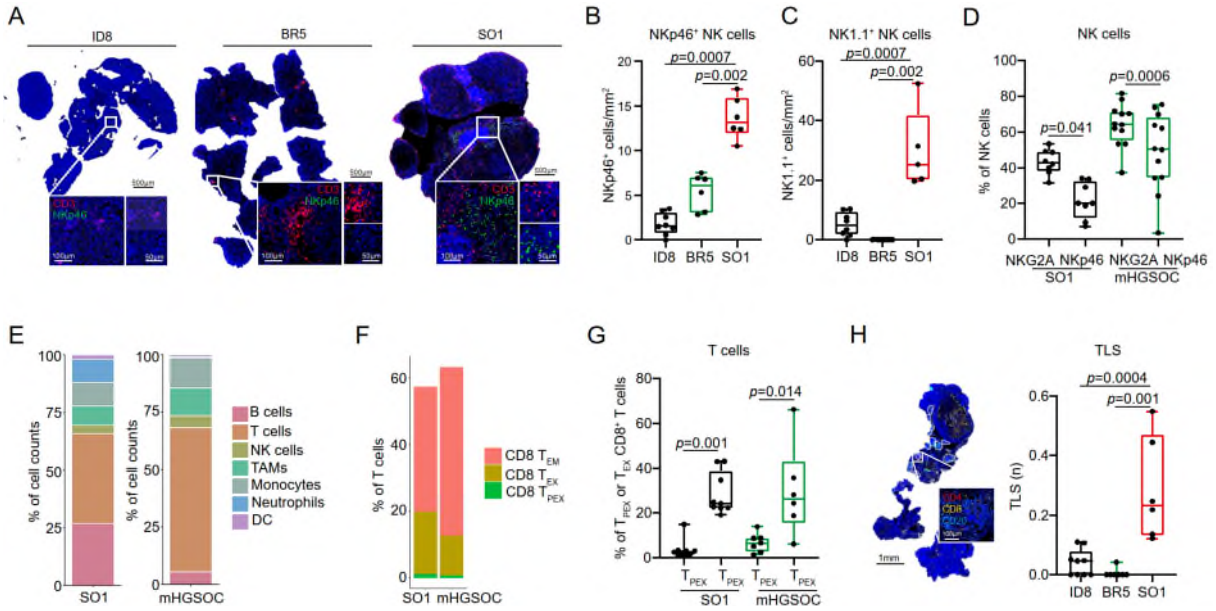

Figure 1. **SO1 mouse tumors recapitulate key immune features associated with advanced HGSOC. (A, B)** Representative immunostaining for CD3 and NKp46 (A) and a box plot showing density of NKp46$^+$ NK cells in *Trp53*$^{-/-}$ *Brca1*$^{-/-}$ ID8 (n=8), *Trp53*$^{-/-}$*Brca1*$^{-/-}$ BR5 (n=6) and *Trp53*$^{-/-}$ *Brca1*$^{-/-}$ *Hras*/*Myc* SO1 (n=6) ovarian tumors (B). **(C)** Box plot showing density of intratumoral NK1.1$^+$ NK cells in tumor samples of *Trp53*$^{-/-}$ *Brca1*$^{-/-}$ ID8 (n=8), *Trp53*$^{-/-}$*Brca1*$^{-/-}$ BR5 (n=6) and *Trp53*$^{-/-}$ *Brca1*$^{-/-}$ *Hras*/*Myc* SO1 (n=6) ovarian tumors, as determined by immunostaining. **(D)** Box plot showing percentage of NKG2A$^+$ and NKp46$^+$ NK cells in *Trp53*$^{-/-}$ *Brca1*$^{-/-}$ *Hras*/*Myc* SO1 (n=8) and advanced HGSOC tumor samples (n=12), as determined by flow cytometry. NK cells were determined as NK1.1$^+$CD3$^-$CD45$^+$ cells in murine model and CD56$^+$CD3$^-$CD45$^+$ in human tumor samples. **(E, F)** Stacked bar plots showing distribution of immune cellular components (E) and T cells states (F) across *Trp53*$^{-/-}$ *Brca1*$^{-/-}$ *Hras*/*Myc* SO1 (n=3) and advanced HGSOC tumor samples (n=30), as determined by scRNAseq. **(G)** Box plot showing percentage of progenitor exhausted (T_{PEX}) and exhausted (T_{EX}) CD8$^+$ T cells in *Trp53*$^{-/-}$ *Brca1*$^{-/-}$ *Hras*/*Myc* SO1 (n=9) and advanced HGSOC tumor samples (n=12), as determined by flow cytometry. **(H)** Representative immunostaining for CD4, CD8 and CD20 and a box plot showing density of tertiary lymphoid structures (TLS) in *Trp53*$^{-/-}$ *Brca1*$^{-/-}$ ID8 (n=9), *Trp53*$^{-/-}$*Brca1*$^{-/-}$ BR5 (n=6) and *Trp53*$^{-/-}$ *Brca1*$^{-/-}$ *Hras*/*Myc* SO1 (n=6) ovarian tumors. Scale bars 100µm and 1mm. Box plots: lower quartile, median, upper quartile; whiskers, minimum, maximum. Statistical significance was calculated by two-sided Mann–Whitney test. *p* values are indicated.

4. Spatial analysis: 10 neighbourhoods with respect to NK cells were defined – what is the logic for selecting such a large number of neighbourhoods. Are they, in fact, distinct? What were the criteria for inclusion/exclusion or definition of a neighbourhood, and are they expected to be functionally distinct?

Our response: We thank Reviewer #3 for this comment. The ten spatial neighborhoods ("niches") were identified in an unbiased, data-driven manner using the *BuildNicheAssay* function in Seurat and therefore do not correspond to prospectively defined biological or functional entities (see Fig. 2A–C of this rebuttal letter). While the method requires specifying the number of niches a priori, we assessed the robustness of our findings by repeating the analysis using 4, 6, and 8 niches. In all cases, we observed highly consistent spatial patterns and NK–T-cell associations (Fig. 2D–F of this rebuttal letter), indicating that our conclusions are not dependent on the specific number of neighborhoods selected. We have clarified the niche definition strategy and robustness analyses in the revised Methods and Results sections.

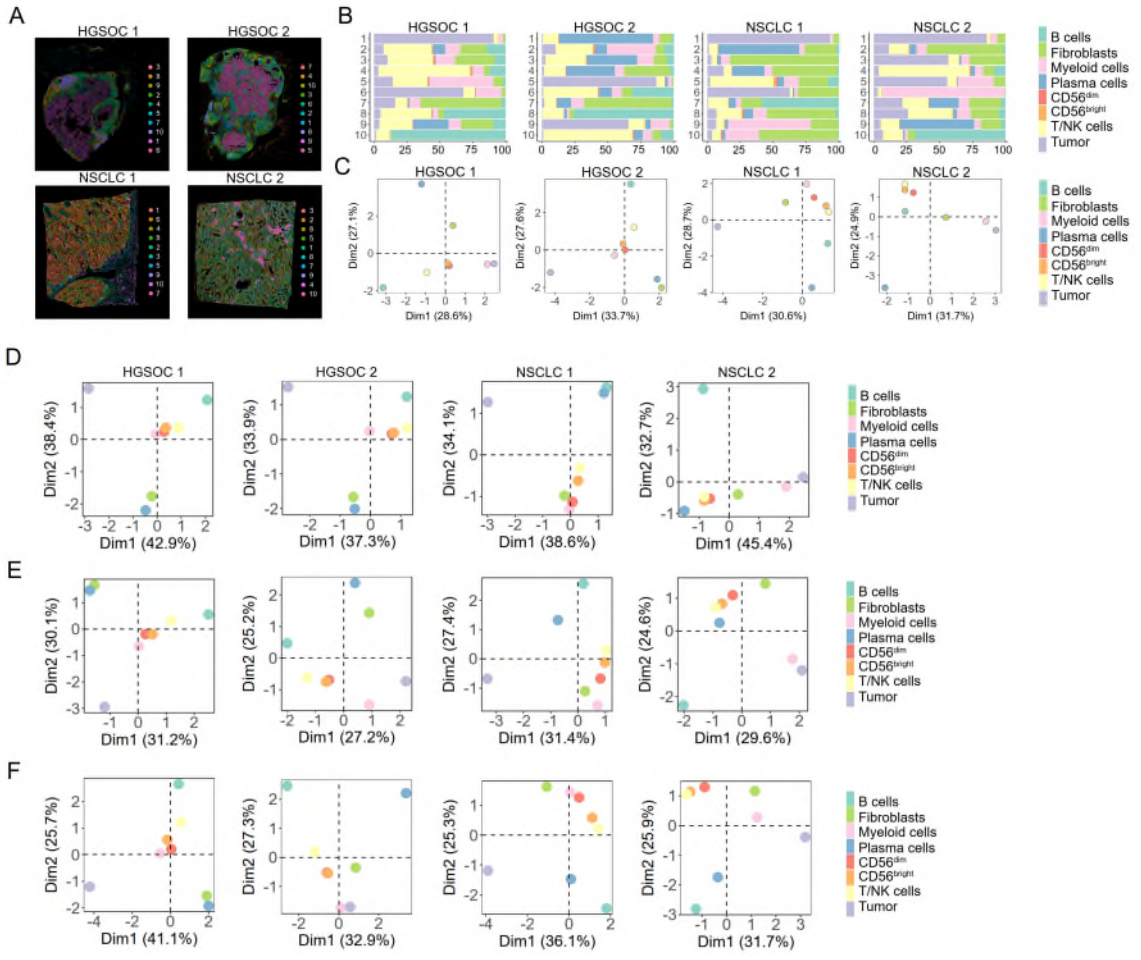

***Figure 2*. The interplay between CD8$^+$ T and NK cells subsets within HGSOC and NSCLC tumor samples.** **(A)** Spatial organization of cells in HGSOC and NSCLC samples. Niches were defined based on the composition

of 30 nearest spatial neighbors using the *BuildNicheAssay* function in *Seurat*. **(B)** Stacked bar plots show cell type compositions within the niches presented in A. **(C-F)** Principal component analysis (PCA) of niche distributions across cell types, while using 10 (C), 4 (D), 6 (E) and 8 (F) cancer niches, respectively.

5. I caution against the term "exhausted" for NK cells (i.e. line 651-658): they upregulate immune checkpoint transcripts, but functional conclusions cannot be made. In the context of T cells, such upregulation is often seen on activated T cells (and might simply reflect the same on NK cells).

Our response: We thank the Reviewer #3 for bringing this important point to our attention. We agree that the upregulation of immune checkpoint transcripts in NK cells cannot be interpreted as functional exhaustion. Similar to T cells, NK cells may upregulate these transcripts upon activation, and transcriptomic data alone do not allow conclusions about functional impairment. In response, we have revised the manuscript to avoid the term "exhausted" for NK cells, instead describing them as expressing immune checkpoint–associated transcripts. We have also clarified in both the Results and Discussion that functional interpretations rely on experimental validation in our mouse model rather than solely on transcriptomic observations.

6. The authors contend that their NK cells are defined by a "robust gating strategy" but have not described it. The possibility remains that NKp46+ cells from tumours are ILCs, such as those described by Crome et al. Further, (662-676) the authors should avoid making conclusions about function without performing functional studies – expression of checkpoints or GZB does not necessarily equate to functional changes unless they are experimentally tested. The following paragraph (T cells) similarly draws functional conclusions from phenotypic data.

Our response : We fully agree with Reviewer #3 that the commonly used gating strategy for identifying NK subsets—defining $CD56^{dim}$ cells as $CD56^{+}CD16^{+}$ NK cells and $CD56^{bright}$ cells as $CD56^{+}CD16^{-}$ NK cells (*Tang et al, Cell, 2023 ; Dean et al, Nat Commun, 2024 ; Mell et al, Front Immunol, 2022 ; Wagner et al, J Clin Invest, 2017*), may not completely exclude a minor contribution from $CD56^{+}$ innate lymphoid cells (ILCs) (*Crome et al, Nat Med, 2017 ; Crome et al, JITC, 2018 ; Jacquelot et al, Nat Immunol, 2022*). Additional markers such as CD127, CD117, EOMES, or KIRs would indeed provide a more definitive separation of NK cells from ILC subsets. However, because our study was based on prospectively analyzed, freshly dissociated tumor samples, it

was not technically feasible to repeat staining with an expanded phenotyping panel. In response to the reviewer's feedback, we have now: (i) expanded and clarified our gating strategy in the Methods and Supplementary Figures; (ii) explicitly acknowledged in the Discussion part that CD56/CD16 gating strongly enriches for NK cells but cannot fully exclude a minor contribution from $CD56^{+}$ ILCs; and (iii) softened statements that previously implied absolute lineage assignment.

Regarding functional conclusions, we agree that the expression of immune checkpoints or granzyme B does not, on its own, establish functional activation or impairment. We have therefore revised the relevant NK-cell and T-cell sections to focus on descriptive phenotypes, avoiding overinterpretation of transcriptional or surface marker data. Functional conclusions in the revised manuscript are now limited to those supported by experimental evidence in our mouse model, and we explicitly note that full functional validation—such as cytotoxicity assays, receptor-blockade assays, or in situ functional readouts—will require future work. These revisions ensure that our interpretation remains rigorous and transparent, and that phenotype-based observations are clearly distinguished from experimentally validated function.

7. The discussion should include some description about the limitations of transcriptomic/phenotypic data.

Our response : Inspired by Reviewer #3 comment, we have included in the revised version of manuscript a full paragraph describing the main limitations of our transcriptomic/phenotypic based data and conclusions.

POINT-BY-POINT REPLY TO REVIEWER N° 4

Reviewer n° 4 commented:

In this revised form of the manuscript, authors properly addressed all my queries. Hence, I do confirm that this sound and well-written work by Tereza Lanickova et al. will be certainly of great interest for the community and reader of Nat Com. and remarkably advances our knowledge in regard of the clinical translation and potential therapeutic uses of the checkpoint inhibitor NKG2A. Even the review process and rebuttal looks very meticulous by addressing every single issues and raised point. Excellent work ready to be published

Our response: We thank Reviewer #4 for the revision of our work and enthusiastic evaluation of our manuscript

- END -

Ref: ***NCOMMS-25-31476B***
Manuscript: NKG2A inhibition promotes NK cell-CD8+ T cell interactions to improve anticancer immunity in ovarian carcinoma
Authors: **Tereza Lanickova *et al.***
Corresponding author: **Jitka Fucikova**

POINT-BY-POINT REPLY TO REVIEWER N° 4

Reviewer n° 4 commented:

No additional comments. The paper is suitable to be published on Nature Communications in this presente re-revised form.

Our response: We thank Reviewer #4 for the revision of our work and enthusiastic evaluation of our manuscript.

- END -