nature portfolio

Peer Review File

IL-12 drives the expression of the inhibitory receptor NKG2A on human tumor-reactive CD8 T cells

Corresponding Author: Dr Thomas Duhen

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)
The manuscript by Fesneau and colleagues reports that the dominant population of tumor-infiltrating NKG2A+ CD8 T cells coexpress CD39 and CD103 (called DP), a phenotype shown by this same group to be associated with T cells displaying anti-tumor reactivities in various solid human cancers (Nature Com., 2018). The authors further demonstrate that NKG2A- CD8 T cells differentiate into NKG2A+ CD8 T cells in the tumor microenvironment and highlight that it is IL-12 secretion by APCs, potentiated by the CD40/CD40L pathway, that is responsible for inducing NKG2A expression on these cells. Nevertheless, the induction of NKG2A expression by IL-12 on CD8 T cells has already been documented in the literature, which is not mentioned in the article, as well as the ability of NKG2A+ CD8 TILs to recognize tumor cells. Although in this regard, this work presents no major novelty, the experiments, including advanced and varied technologies (CITE-Seq, TCR sequencing, whole exome and RNA sequencing, RNAscope) are very well conducted and the results obtained are very convincing, deciphering the origin of NKG2A+ CD8 TIL cells in the tumor microenvironment and the major role of IL-12, derived from myeloid cells, in increasing NKG2A expression.
This paper would benefit greatly from additional functional data on the real involvement of NKG2A in the anti-tumor immune response in the two models used (HNSCC and CRC). Here are a few recommendations, point by point, to improve the analysis and reinforce the main message. It should be noted that this paper is very well written and the figures have been designed with the greatest care.

Major revisions
1/ One of the critical points of this paper is the lack of references on the already well-known induction of NKG2A by IL-12, which weakens the innovative side of the authors' main message. Indeed, IL-12 has previously been shown to be a key cytokine in inducing NKG2A expression not only on NK cells (Saez Borderias et al, J Immunol, 2009; Cany et al., OncoImmunol., 2015) but also on CD8 T lymphocytes (Derré et al, J Immunol, 2002). More recently, it has been shown that CD94/NKG2A was strongly upregulated in IL-12-CAR T cells compared with conventional CAR T cells (Hombach et al., Mol Ther, 2022). This information should be introduced, discussed and the relevant references cited, especially as it reinforces the authors' message that future studies should carefully examine the effects of IL-12 on the phenotype and function of effectors of the antitumor immune response with a view to immunotherapies.

2/ A second point of criticism concerns the assessment of T-cell reactivity to different antigenic targets (viral proteins, peptides) by increasing the expression of 4-1BB (Figure 5). It would be more appropriate to carry out real functional tests than to assess cell reactivity solely by the expression of this activation marker. These experiments should enable the authors to support their CITE-seq results, notably the highly cytotoxic nature of the DP CD8 NKG2A+ subset, which is more cytotoxic than other CD8 subsets. Moreover, as CITE-seq results also showed that IFN-γ gene expression is associated with NKG2A gene expression, the authors could perform IFN-γ ELISPOT assay to evaluate the reactivity of tumor-specific NKG2A T cells. It should be noted that the authors mention in the "Methods" section the protocol of the IFN-γ ELISPOT assay but have not shown these results.
Since it is known that the anti-tumoral action of CD8 T cells is mediated by IFN-γ secretion and cytotoxic activity, it is essential that authors perform these functional tests, especially as they have the necessary equipment required to do so.

3/ Finally, in order to improve understanding of the results, can the authors modify their manuscript on the following points:
- Can the authors provide the gating strategy used for phenotype analysis of blood and tumor samples by flow cytometry. Do the graphs in Fig. 1b (and extended Fig. 1b) show the frequency of NKG2A+CD8+ cells and if so among the CD3+

population (or lymphoid cells based on FSC/SSC dot plot) or the percentage of NKG2A+ cells among CD8+ cells as implied in Fig. 1a (and extended Fig. 1a)? Can the authors clarify this point? Does the gating strategy include a gating step on αβ T cells to be sure not to include γδ T cells?
- Concerning the results of CiTE-seq analysis, the authors report on the expression of numerous genes (KLRG1, EOMES, GZMK, CCR7, SELL, XCL2, ANXA1, CTLA4) not shown in the associated figures (Fig. 2b and c and Extended Data Fig. 2b and c). These data should be shown in the figures, along with KLRC1 gene expression, the focus of this study.

Minor revisions
line 66: Please specify CD8 αβ T cells and add γδ T cells (notably Vδ2) which may also express NKG2A (Cazetta V. et al., Cell Rep., 2022; Cazetta V. et al., Cancers, 2023).
line 72: The authors write “its expression on human tumor-reactive CD8 T cells remains to be demonstrated”. Some studies have already shown this (Ducoin et al., OncoImmunol., 2022, article referenced at N°14 in the paper).
line 96: The authors identified two subsets of NKG2A+ CD8 T cells in the blood of CRC and HNSCC patients. Nevertheless, another subset of NKG2A+ cells (CD103+CD39-) appears on the UMAP representations (Fig. 1d-e and Extended Data Fig. 1d-e). Can the authors comment on this point? Can the authors specify their method of subgroup identification (manual or use of an algorithm)?
lines 98 to 110: Fig. 1f and Extended Data Fig. 1f are commented but not cited. Can the authors add them to the text?
lines 105 to 107: The authors report that cells in T2 subset express CD28 and CD127 but Figure 1F shows that these expressions are heterogeneous with approximately 40% and 80% of positive cells, respectively. Can the authors adapt their comment?
line 112: For figure 1g, a representation of the results on the same graph would enable statistical analysis to be added, in particular to validate the difference in frequency of DP (CD39+CD103+) cells in the NKG2A- and NKG2A+ populations.
line 117: Can the authors comment on the results shown in Fig. 1i? Are there any clinical differences between patients that might explain the increase of NKG2A expression in some of them (treatments, sample location, etc.)?
lines 144-145: The authors conclude this section by saying that the CD8 TIL cell clustering of CRC patients is similar to that of HNSCC. Nevertheless, the number of subgroups identified in each of the CD8 T cell groups is different, with in CRC, 4 subgroups DP, 2 SP and a single DN. Can the authors revise their conclusions?
line 162: There is a discrepancy between the trajectory indicated in the text and that of the associated figure (extended Fig. 2h) Can the authors correct this error?
line 167: The authors report that NKG2A+ DP CD8 TILs are more cytotoxic than other CD8 subsets. This conclusion is based solely on the increased gene expression of GZMB and GNLY, and therefore only reflects the cytotoxic capabilities of these cells. Functional cytotoxicity assays would be more appropriate to validate this function.
line 175: In the ISH/IF multiplex experiment, why examine NKG2A and CD39 expression in RNA and not in protein, which would seem more appropriate?
line 177: The authors state that "most tumor-infiltrating CD8 T cells express CD39", but the data presented in figure 3d essentially show an increased frequency of ENTPD1-expressing CD8+ cells within the tumor epithelium compared to the stroma. Can the authors adapt the commentary on this figure? In addition, can the authors comment on the absence of a CRC tumor in Fig. 3d and an HNSCC tumor in Fig. 3e given that in the legend it is stated that 3 samples of each tumor model were analyzed?
Lines 218-226: If the results presented in Figure 5e illustrate the recognition of common neoepitopes by the CD8 DP NKG2A- and NKG2A+ TILs, the results of Figure 5d do not go in this direction and undermine the message. Figure 5d could be removed.
line 239: Can the authors explain their choice to perform NKG2A upregulation experimentations on naive blood-derived CD8 T cells and not on DP CD8 NKG2A- TILs? This would have been more convincing.
line 251: Figure 6g, to better appreciate the individual roles of CD4 cells and monocytes and the contribution of their co-presence, it would be important to show results obtained with naive CD8 T cells stimulated by SEB (or not) in the presence or absence of CD4 T cells or monocytes.
line 286: Figures 7h and i, in multiplex analysis, can the authors justify why they didn't look at NKG2A instead of CD8?
line 316: The authors observed a decrease frequency of NKG2A+ cells in HPV-driven HNSCC. This result is surprising, given that TILs in these tumors include T cells reactive towards the E6, E2 and E7 viral proteins and demonstrated in this article. Can the authors comment on this result?
line 985: Can authors match the terms used between figures and their legends (macrostate vs microstates) (Fig.2g and extended data fig. 2g)?
lines 1029 and 1073: Can the authors perform statistical tests for the results shown in Fig. 6 and 7?
line 1073: Can the authors replace “HNSCC” by CRC and adapt the number samples (n=17)?

Reviewer #2

(Remarks to the Author)
The study overall is very interesting. But I found a few parts to be not very solid and need revisions/clarifications:
(1) “Of note, TCR 195 clonotypes expressed by NKG2A and NKG2A+ DP CD8 TILs could be divided in three groups 196 whether they were shared at similar frequency between both subsets, shared but enriched in one 197 subset or not shared between subsets (Fig. 4c).” I don’t think this analysis tells us anything. There is no interpretation of the biological meanings of these three groups. We observe such “grouping” very commonly in TCR clonotype data when comparing two conditions
(2) “Furthermore, using overlapping single peptides for HPV16 E6, we showed for one 213 patient that NKG2A and NKG2A+ DP CD8 TILs recognized the same peptide (Fig. 5 c).” This is just one patient and one peptide. Not sure the authors can draw the conclusion “NKG2A and NKG2A+ DP CD8 TILs recognize the same tumor antigens” on a global scale.

(3) “For each patient, we detected TMG-reactive 219 T cells and their frequencies varied between NKG2A and NKG2A+ DP CD8 TILs (Fig. 5d).” Similarly, I do not see any pattern in this figure, and not sure how it supports the authors’ conclusion: “NKG2A and NKG2A+ DP CD8 TILs recognize the same tumor antigens”?
(4) “For the HNSCC patient, 223 DP CD8 TILs recognized a mutation in NOTCH1 while in the CRC patient a mutation in 224 HEATR5A was recognized (Fig. 5e).” Here again, only two peptides and no global analyses to show that “NKG2A and NKG2A+ DP CD8 TILs recognize the same tumor antigens” in an unbiased manner. There are also no proper control groups in these analyses. “memory CD8 T cells, DN CD8, SP CD8” would serve as good controls?
(5) “Interestingly, our analysis showed that CD8 T cells could be found close to IL-289 12+ cells in a TGF- -rich environment, with more than 50% of IL-12+ cells being within less than 290 30 m of a CD8 T cell (Fig. 7i).” There is no proper control in this experiment, and it is hard to draw the conclusion of whether the observed proportions should be considered high or low. For example, if the same analyses can be done for IL-12+ cells vs CD4 T cells or IL-12+ cells vs macrophages, and if the proportions for IL-12+ cells vs CD8 T cells are indeed higher, I think it would mean something. Maybe the authors can find a better control, but some kind of control is needed
(6) “NKG2A+ DP CD8 TILs are enriched in the tumor epithelium” and “IL12B mRNA was detected in the TME, 287 although in small numbers, and those cells were localized in the tumor stroma near the invasive 288 margin (Fig. 7h).” Are these two observations contradicting each other?

Reviewer #3

(Remarks to the Author)
Duhen et al report that CD8 TILs exhibit a phenotype suggestive of tumor reactivity express the inhibitory receptor NKG2A in response to IL-12, thus attributing a regulatory role to this cytokine.

The role of NKG2A as an immune checkpoint inhibitor has been defined in viral infections (e.g., flu), but its role in anti-tumor immunity, and what regulates the pathway, remains elusive. The study provides some insight into an unexpected regulatory activity for a cytokine traditionally considered as a driver of T cell activation. However, it could be argued that the upregulation of NKG2A in T cells in response to IL-12 is an inhibitory immune checkpoint to prevent overt activation (e.g., similar to PD-1); and not necessarily a regulatory function of IL-12. In fact, TGF-b and IL-12 synergize to drive NKG2A upregulation, which is not sufficiently emphasized in the abstract or the text.

The interesting, novel and relevant information is shown in Figure 7.

MAJOR COMMENTS:

-The expression of CD39 and CD103 is suggestive of tumor reactivity but not at all definitory. Figure 5 shows upregulation of 4-1BB among CD8 TILs in response to HPV, which is also NOT definitory of tumor reactivity. To define these TILs as truly tumor-reactive, IFNG and/or GZMB ELISPOTs against tumor lysates, predicted neoantigens or HPV antigens (as properly done for Figure 5) need to be conducted. It is puzzling that the experiment was basically done but the readouts were limited to 4-1BB expression, which is turned on in response to NF-kB.

-A factor that could influence the reported phenotypes is the presence of tertiary lymphoid structures in the tumors. This should be clarified. Whether T cells in TLS are NKG2A+CD8+ or not would be informative.

-There is no demonstration that NKG2A+ TILs have decreased effector activity. In fact, NKG2A+ TILs recognize a NOTCH mutated antigen more effectively than their NKG2A-CD8+ counterparts, if we assume that 4-1BB is a readout of tumor reactivity (Fig5). NKG2A may be, similar to PD-1, a mere marker of activation in cells that are otherwise superior effectors.

Reviewer #4

(Remarks to the Author)
the present paper is certainly of great interest can potentially contribute to a deeper understanding of the CD8 T cell response to tumors. Additionally, the investigation of the immuncheckpoint NKG2A is now a rising topic, broadening our perspective on immuncheckpoint-mediated regulation of CD8 T cell response
However, several issues need to be better addressed

Specific Comments
1. Line 65: ” In contrast to other checkpoint molecules, NKG2A is expressed exclusively on NK cells
and CD8 T cells,…”
There are no references added to this statement that, however, in some way are not correct. Besides NK cells and CD8 T cells, other immune cell populations expressing NKG2A include human γδ T cells, NKT cells, and MAIT cells (Cancers 2023, 15, p1264; Nat. Immunol., 2019, 20, p1110; Nat. Rev. Immunol., 2013, 13, p.101). Moreover, it has been observed that NKG2A-mediated signaling negatively regulates the activation of NKT cells and a specific subset of human γδ T cells, Vδ2

T cells (J Immunol. 2009, 182(1), p.250; Cell Reports, 2021, 37(3), 109871).
2. What is the rationale behind choosing HNSCC and CRC tumors for analysis? Were these tumors specifically selected due to tissue accessibility? Do the authors believe the results are consistent across different tumor types? Alternatively, do these tumors exhibit common characteristics that reveal a higher NKG2A+ CD8 T cell pattern? Please elaborate on this aspect.
3. Comments to Figure 1:
a) The whole gating strategy of CD8 NKG2A+ T cells in the tumor should be included.
b) Line 92) The statement of the NKG2A increases in tumors is inaccurate since the authors did not compare healthy tissue. Instead, it would be more accurate to discuss higher expression compared to blood.
c) Are there any differences in expression of NKG2A in the five CD8 T identified subsets? The expression of NKG2A should be added to Fig. 1f.
d) In Fig. 1h, provide rational for the HPV-related analysis. How the author explains higher expression of NKG2A+ CD8 T cells in HPV-negative individuals? Extend abbreviation for HPV. Abbreviations for HPV should be expanded.
e) f) Line 117: Fig. 1i. What does the author mean by sample collection at different time points? This results are poorly explained.
f) Line 119) This summary is poorly formulated.
4. Comments to Fig. 2:
a) The gating strategy here is confusing (should be rather included in Fig. 1) since the UMAP is the projection of all CD8 T cells in an unbiased way without any gating strategy performed.
b) KLRG1, EOMES and GZMK are rather markers of TEMRA CD8 cells and not effector CD8 T cells
c) In Fig. 2d, what specific genes were used to calculate the naïve/TCM and activation/exhaustion scores?
d) What is the difference between DP CD8 T cells in cluster 7 and cluster 2?
e) Line 166) The statement, "Overall, our analysis revealed that in the tumor, the DP CD8 NKG2A+ cells are more terminally differentiated and cytotoxic than other CD8 subsets," is overstated. No cytotoxicity has been performed; instead, the authors associated it with a higher cytotoxic potential.
5. Comments to Fig. 3:
a) The low resolution of the pictures in Fig. 3a does not allow for the appreciation of NKG2A and CD39 expression.
b) In Fig. 3c-e, statistics are lacking. Additionally, the authors should specify in the legend how many patients were analyzed for the study.
6. Comments to Fig. 4:
a) In Fig. 4a, the use of NK+ is confounding; please use NKG2A+.
b) The use of the terms "clonotypes" or "TCR repertoire" is not appropriate since the author only analyzes the beta chain and not the paired alpha-beta chains that accurately define a clone. This aspect is incorrectly described in the text. Additionally, the statement regarding shared TCR clonotypes between DP NKG2A+ and NKG2A- subsets is overstated.
c) Is there any publicly available single-cell TCR-seq data from CRC and/or HNSCC tumors that could be analyzed to define paired alpha-beta clonotypes in CD8+ T cells concerning NKG2A expression? In this scenario, it would be appropriate to explore whether such analysis could reveal whether shared clones undergo clonal expansion with heightened expression of NKG2A.
7. Comments to Fig. 5:
a) a) In Fig. 5a, the author shows similar activation of NKG2A+ and NKG2A- cells in response to HPV antigens. How does the author explain the significantly lower frequency of NKG2A+ CD8 T cells in HPV-negative subjects compared to HPV-positive subjects shown in Fig. 1h?
b) Considering the previous question regarding the low expression of NKG2A in HPV-negative patients, what is the rationale behind studying the response of NKG2A+ CD8 T cells in these patients?
c) In Fig. 5d, it is not clear what TMG 1, 2, 6, 7, etc. means.
d) Does the expression of NKG2A and CD39 increase upon HPV- and tumor-related antigens stimulations in vitro?
8. Comments to Fig. 6 and 7:
a) Abbreviations for SEB should be expanded.
b) There is a lack of statistical comparison with control examples, such as in Fig. 6h and 6f.
c) Regarding Fig. 7h, the resolution is insufficient, and the association of IL12 RNA expression with specific cells is not clearly delineated.
d) Viral infections typically induce IL12 secretion by innate cells. How do the authors explain the lower expression of NKG2A in HPV+ patients?

9 . Comment to Discussion:
a) The authors should address the implications of anti-NKG2A treatments
In the context of the expression of HLA-E.
b) Several statements in the discussion are overstated, as mentioned above. For instance, in Line 299, it is stated: "Our analysis revealed that NKG2A was induced in the TME as the cells differentiate into DP CD8 and its expression correlated with the acquisition of cytotoxic properties and effector functions." However, the authors did not demonstrate induction of NKG2A; instead, they observed higher expression compared to blood. No functional experiments were performed; rather, the cytotoxic potential was observed at the transcriptional level.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
We (reviewer 1 and early career researcher co-reviewer) would like to thank the authors for responding to all our comments and for all the changes made (figures, discussion, references...) to the manuscript which enhance the quality of this study. Nevertheless, we strongly recommend that the authors take into account the following points:
- Regarding the correlation between 4-1BB expression and IFN-g production by activated T cells, we thank the authors for providing ELISPOT and flow cytometry results that validate this correlation. Nevertheless, can the authors justify this read-out in the manuscript (line 207) with the addition of the references provided (or even the (particularly convincing) data provided in the appendix)?
- Although the preliminary results on the evolution of NKG2A expression by CD8 TILs (Figure 1i) are interesting, we recommend deleting this figure in view of the small number of samples analyzed, the different tumor models and the highly variable duration between the two surgeries which do not allow these data to be reliably exploited.
- We also ask the authors to comment in the discussion section (as they did in the reply to the review) on our remark about the surprising finding of the low frequency of CD8 NKG2A+ TILs within HPV+ HNSCC tumors (Figure 1h) despite the existence in this population of T cells reactive towards several viral peptides (Figures 5a-c).

Reviewer #2

(Remarks to the Author)
The authors have addressed all my concerns. Thank you!

Tao Wang

Reviewer #3

(Remarks to the Author)
The inclusion of new ELISPOT data clearly strengthens the manuscript. Similarly, the emphasis on presenting NKG2A as an immune checkpoint inhibitory mechanism, rather than a regulatory effect of IL-12, is also an improvement.
Everything can get better but I believe that the authors have proven the main conclusions.

Reviewer #4

(Remarks to the Author)
In this revised version of the manuscript, authors properly and exhaustively addressed all the queries I raised in the original version of the manuscript. Hence, I do find this revised from suitable to be published on Nature Communications

Manuscript NCOMMS-24-12327-T
Point-by-point responses to reviewer's comments

Reviewer #1 (Remarks to the Author):

The manuscript by Fesneau and colleagues reports that the dominant population of tumor-infiltrating NKG2A+ CD8 T cells coexpress CD39 and CD103 (called DP), a phenotype shown by this same group to be associated with T cells displaying anti-tumor reactivities in various solid human cancers (Nature Com., 2018). The authors further demonstrate that NKG2A- CD8 T cells differentiate into NKG2A+ CD8 T cells in the tumor microenvironment and highlight that it is IL-12 secretion by APCs, potentiated by the CD40/CD40L pathway, that is responsible for inducing NKG2A expression on these cells.
Nevertheless, the induction of NKG2A expression by IL-12 on CD8 T cells has already been documented in the literature, which is not mentioned in the article, as well as the ability of NKG2A+ CD8 TILs to recognize tumor cells. Although in this regard, this work presents no major novelty, the experiments, including advanced and varied technologies (CITE-Seq, TCR sequencing, whole exome and RNA sequencing, RNAscope) are very well conducted and the results obtained are very convincing, deciphering the origin of NKG2A+ CD8 TIL cells in the tumor microenvironment and the major role of IL-12, derived from myeloid cells, in increasing NKG2A expression.
This paper would benefit greatly from additional functional data on the real involvement of NKG2A in the anti-tumor immune response in the two models used (HNSCC and CRC).
Here are a few recommendations, point by point, to improve the analysis and reinforce the main message. It should be noted that this paper is very well written and the figures have been designed with the greatest care.

We thank the reviewer for the positive notes regarding our manuscript.

Major revisions
1/ One of the critical points of this paper is the lack of references on the already well-known induction of NKG2A by IL-12, which weakens the innovative side of the authors' main message. Indeed, IL-12 has previously been shown to be a key cytokine in inducing NKG2A expression not only on NK cells (Saez Borderias et al, J Immunol, 2009; Cany et al., OncoImmunol., 2015) but also on CD8 T lymphocytes (Derré et al, J Immunol, 2002). More recently, it has been shown that CD94/NKG2A was strongly upregulated in IL-12-CAR T cells compared with conventional CAR T cells (Hombach et al., Mol Ther, 2022). This information should be introduced, discussed and the relevant references cited, especially as it reinforces the authors' message that future studies should carefully examine the effects of IL-12 on the phenotype and function of effectors of the antitumor immune response with a view to immunotherapies.

We thank the reviewer for bringing those references to our attention and apologize for not acknowledging them in our manuscript. We have now included those references and are discussing them in the "Discussion" section of the manuscript. Of note, those above-cited

papers reported a role for IL-12 in increasing NKG2A expression. Our results demonstrate that IL-12 not only increases NKG2A expression on memory cells, but also induces its *de-novo* expression on human naïve CD8 T cells which is complementary. In the paper from Hombach et al, the authors do not show any clear evidence of NKG2A expression on IL-12-CAR. They indicate that IL-12-CAR express higher levels of CD94 than CAR without IL-12. However, CD94 can also pair with NKG2C which the authors did not address. For that reason, we would prefer not to cite that article.

2/ A second point of criticism concerns the assessment of T-cell reactivity to different antigenic targets (viral proteins, peptides) by increasing the expression of 4-1BB (Figure 5). It would be more appropriate to carry out real functional tests than to assess cell reactivity solely by the expression of this activation marker. These experiments should enable the authors to support their CITE-seq results, notably the highly cytotoxic nature of the DP CD8 NKG2A+ subset, which is more cytotoxic than other CD8 subsets. Moreover, as CITE-seq results also showed that IFN-γ gene expression is associated with NKG2A gene expression, the authors could perform IFN-γ ELISPOT assay to evaluate the reactivity of tumor-specific NKG2A T cells. It should be noted that the authors mention in the "Methods" section the protocol of the IFN-γ ELISPOT assay but have not shown these results.
Since it is known that the anti-tumoral action of CD8 T cells is mediated by IFN-γ secretion and cytotoxic activity, it is essential that authors perform these functional tests, especially as they have the necessary equipment required to do so.

We agree with the reviewer that one read-out of TCR-induced CD8 T cell activation is cytokine secretion, and most specifically IFN-γ. However, it is well established in the field that 4-1BB up-regulation can be used as a surrogate of TCR-mediated CD8 T cell activation. This was demonstrated in a seminal paper from Phil Greenberg's lab (Wolfl M et al., Blood 2007). It is also the main approach used in the Rosenberg lab at the National Cancer Institute to identify and isolate neoantigen-reactive TCRs (Tran E et al., Science 2015; Tran E et al., N Engl J Med 2016). Those three studies demonstrate a strong correlation between 4-1BB up-regulation and IFN-γ secretion. We have also shown similar results in our previous work published in *Nature Communications* (Duhen T et al., Nat Comm 2018). To illustrate this correlation, we have measured IFN-γ secretion by ELISPOT on 3 HPV+ HNSCC patients as requested by the reviewer and obtained results similar to the ones using 4-1BB upregulation as a readout (see figure below). We have in addition performed intracellular cytokine staining for IFN-γ following peptide stimulation for the reactivities shown in Figure 5 c and d and our results correlate with 4-1BB upregulation (see figure below).

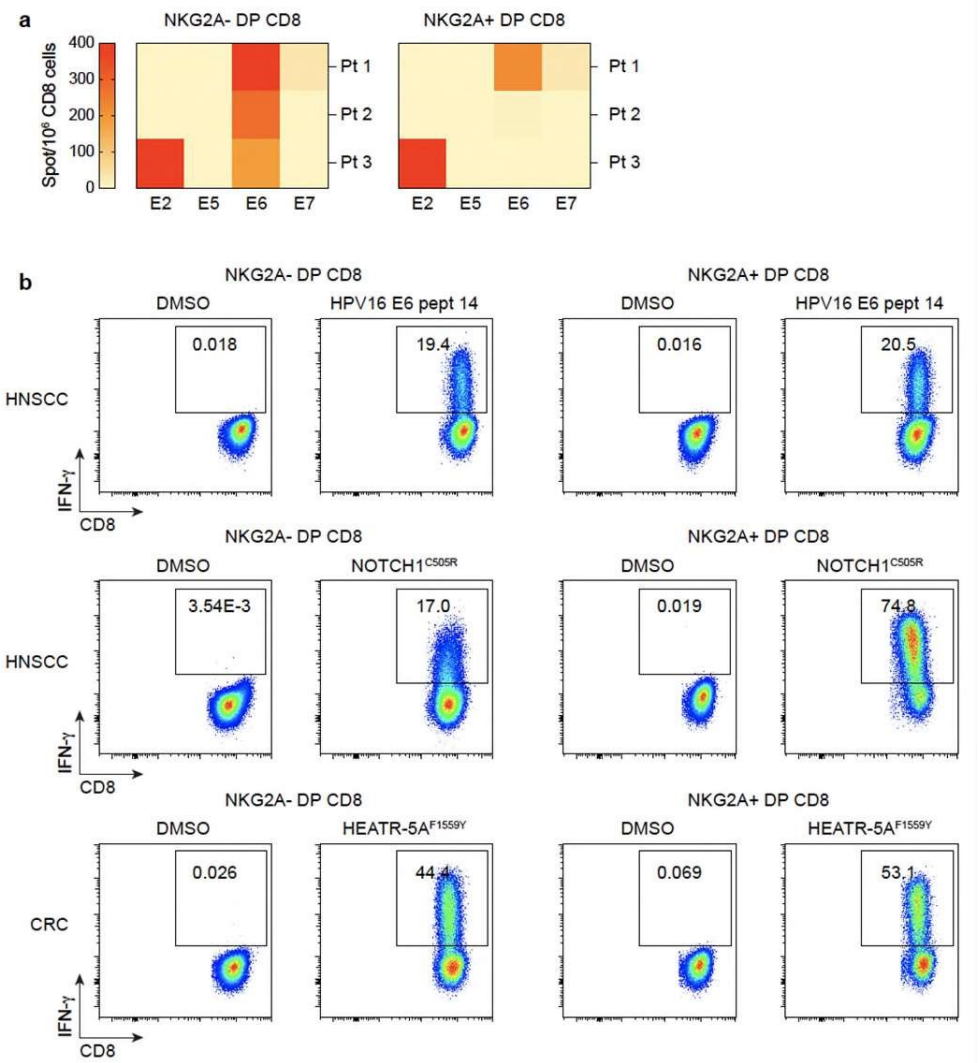

Figure: **(a)** Summary of the reactivity of NKG2A– and NKG2A+ DP CD8 TILs to HPV16 E proteins for 3 patients with HPV+ HNSCC, as measured by IFN-γ secretion by ELISPOT. The colors in the heatmap legend represent the number of spots per million cells. **(b)** NKG2A– and NKG2A+ DP CD8 TILs isolated from one HPV+ HNSCC patient (top), one HNSCC patient (middle) and one CRC patient (bottom) were cultured with autologous blood memory CD8 T cells pulsed with DMSO or the indicated peptides and reactivity was measured by IFN-γ secretion after 4 hours stimulation in the presence of brefeldin A. Those 3 patients correspond to the patients analyzed in Figure 5c and d of the manuscript. Of note, the experiments in **(b)** were performed on NKG2A- and NKG2A+ DP CD8 TILs enriched for their respective reactivities explaining the higher percentages of reactive cells as compared to the results presented in Figure 5c and d.

As for the cytotoxic activity of CD8 T cells, while this is a valid point raised by the reviewer, we are not sure it would be possible to demonstrate an advantage for NKG2A+ DP CD8 vs NKG2A- DP CD8 as both cells are already very efficient at inducing tumor cell killing in vitro. In vitro expansion but also the nature of the TCR between both subsets could hinder our

conclusions. Based on those fact, we would rather like to propose that NKG2A+ DP CD8 in the tumor have a higher cytotoxic potential, as indicated by higher expression of genes such as GZMB, GNLY and IFNG. We hope the reviewer will agree with our reasoning and our suggestion.

3/ Finally, in order to improve understanding of the results, can the authors modify their manuscript on the following points:
- Can the authors provide the gating strategy used for phenotype analysis of blood and tumor samples by flow cytometry.
The gating strategy used for the phenotypic analysis of blood and tumor samples is now included as Extended Figure 1.

Do the graphs in Fig. 1b (and extended Fig. 1b) show the frequency of NKG2A+CD8+ cells and if so among the CD3+ population (or lymphoid cells based on FSC/SSC dot plot) or the percentage of NKG2A+ cells among CD8+ cells as implied in Fig. 1a (and extended Fig. 1a)? Can the authors clarify this point?
The graphs in Fig. 1b and Extended Fig. 1b (now Extended Fig 2b) represent the frequency of NKG2A-expressing cells among CD8 T cells. To clarify this point, those two graphs have been modified.

Does the gating strategy include a gating step on αβ T cells to be sure not to include γδ T cells?
Our gating strategy does not include a step to exclude γδ T cells. However, Kalyan et al (Kalyan S, Cell Mol Immunol 2013) reported that the majority of γδ T cells lack CD8. Thus, we anticipate that the frequency of γδ T cells within the NKG2A+ CD8 T cells will be minimal and should not affect our conclusions.

- Concerning the results of CiTE-seq analysis, the authors report on the expression of numerous genes (KLRG1, EOMES, GZMK, CCR7, SELL, XCL2, ANXA1, CTLA4) not shown in the associated figures (Fig. 2b and c and Extended Data Fig. 2b and c). These data should be shown in the figures, along with KLRC1 gene expression, the focus of this study.
We apologize for this oversight. We have now modified Fig. 2c and Extended Fig. 2c (now Extended Fig. 3c). We have replaced the violin plots by feature plots as we believe those plots help with clarity. We have also reduced the number of gene per group for clarity purposes and added an “Effector memory genes” group displaying expression of KLRG1, EOMES and GZMK as requested by the reviewer. We have also included a feature plot for KLRC1 (NKG2A) in the “Activation/Exhaustion genes” group.

Minor revisions
line 66: Please specify CD8 αβ T cells and add γδ T cells (notably Vδ2) which may also express NKG2A (Cazetta V. et al., Cell Rep., 2022; Cazetta V. et al., Cancers, 2023).
Those references have been added to our manuscript.

line 72: The authors write “its expression on human tumor-reactive CD8 T cells remains to

be demonstrated". Some studies have already shown this (Ducoin et al., OncoImmunol., 2022, article referenced at N°14 in the paper).
We agree with the reviewer - even though the article from Ducoin et al only shows NKG2A expression on one T cell clone isolated from a CRC patient and recognizing a CRC tumor cell line. We decided to remove this sentence from the introduction.

line 96: The authors identified two subsets of NKG2A+ CD8 T cells in the blood of CRC and HNSCC patients. Nevertheless, another subset of NKG2A+ cells (CD103+CD39-) appears on the UMAP representations (Fig. 1d-e and Extended Data Fig. 1d-e). Can the authors comment on this point?
We agree with the reviewer that a small cell population of NKG2A+CD103+CD39- CD8 T cells exists in the blood. However, its frequency being very low (less than 100 cells for a total of 13 patients), we decided to not further analyze it. We hope the reviewer will agree with our decision.

Can the authors specify their method of subgroup identification (manual or use of an algorithm)?
The identification of the subgroups of NKG2A-expressing cells was performed manually, which is now indicated in the text.

lines 98 to 110: Fig. 1f and Extended Data Fig. 1f are commented but not cited. Can the authors add them to the text?
We apologize for this oversight. It has now been corrected and those figures are cited in the text.

lines 105 to 107: The authors report that cells in T2 subset express CD28 and CD127 but Figure 1F shows that these expressions are heterogeneous with approximately 40% and 80% of positive cells, respectively. Can the authors adapt their comment?
We have adapted the description of the cell phenotype for the T2 subset. It now reads: "Cells in the T2 subset displayed intermediate levels of PD-1 and expressed heterogenous levels of CD39, CD28, and CD127, a phenotype consistent with tissue resident memory T cells".

line 112: For figure 1g, a representation of the results on the same graph would enable statistical analysis to be added, in particular to validate the difference in frequency of DP (CD39+CD103+) cells in the NKG2A- and NKG2A+ populations.
We thank the reviewer for this idea. We have now combined the CD8 subset composition among NKG2A- and NKG2A+ CD8 T cells and performed statistical analysis for both HNSCC and CRC patients. The resulting analysis indicates that the proportions of DN CD8 and DP CD8 are significantly different between NKG2A- and NKG2A+ CD8 TILs in HNSCC and CRC tumors.

line 117: Can the authors comment on the results shown in Fig. 1i? Are there any clinical

differences between patients that might explain the increase of NKG2A expression in some of them (treatments, sample location, etc.)?
The data in Fig. 1i include 3 HNSCC tumors, 2 ovarian tumors and 2 colorectal liver metastases (CRLM). It represents the fold change in NKG2A+ CD8 for each subset between the two time points. It is calculated by comparing the frequency of NKG2A+ DN, SP or DP among total CD8 T cells at each time point. A fold change >1 indicates an increase in the frequency of NKG2A+ CD8 at the second time point. We observed a fold change >2 for NKG2A+ DP CD8 in one HNSCC tumor, and two CRLM tumors. For the seven patients analyzed, the shortest amount of time between the two surgeries was 6 weeks and the longest 21 months. We did not see any association between the length of the interval between the surgeries and an increase in NKG2A+ DP CD8 cells among CD8 T cells. We do not have an explanation for this phenomenon, besides the fact that the more time tumor-reactive CD8 T cells spend in the TME, the more chance they have to further differentiate and potentially acquire NKG2A expression. If the reviewer believes that those results are too preliminary, we will consider removing them from our manuscript.

lines 144-145: The authors conclude this section by saying that the CD8 TIL cell clustering of CRC patients is similar to that of HNSCC. Nevertheless, the number of subgroups identified in each of the CD8 T cell groups is different, with in CRC, 4 subgroups DP, 2 SP and a single DN. Can the authors revise their conclusions?
We agree with the reviewer's comment, and we modified the text accordingly.

line 162: There is a discrepancy between the trajectory indicated in the text and that of the associated figure (extended Fig. 2h) Can the authors correct this error?
This error has been corrected. Thank you for noticing it.

line 167: The authors report that NKG2A+ DP CD8 TILs are more cytotoxic than other CD8 subsets. This conclusion is based solely on the increased gene expression of GZMB and GNLY, and therefore only reflects the cytotoxic capabilities of these cells. Functional cytotoxicity assays would be more appropriate to validate this function.
We agree with the reviewer's comment. We modified the text by saying that "DP CD8 NKG2A+ are more terminally differentiated than other CD8 TIL subsets and exhibit a high cytotoxic potential.

line 175: In the ISH/IF multiplex experiment, why examine NKG2A and CD39 expression in RNA and not in protein, which would seem more appropriate?
This is a valid comment. However, when those experiments were performed, we did not have a good antibody panel for multiplex IHC that we could use. We are still working on this panel – staining for CD39 and NKG2A is not straight forward and requires significant optimization.

line 177: The authors state that "most tumor-infiltrating CD8 T cells express CD39", but the data presented in figure 3d essentially show an increased frequency of ENTPD1-expressing

CD8+ cells within the tumor epithelium compared to the stroma. Can the authors adapt the commentary on this figure?
The commentary on this figure has been adapted to take into consideration the reviewer's comment. It now says "CD8 T cells present in the tumor epithelium were more prone to express CD39, and we observed an enrichment in NKG2A+ cells among CD8+CD39+ T cells in the tumor epithelium as compared to CD8+CD39+ in the stroma".

In addition, can the authors comment on the absence of a CRC tumor in Fig. 3d and an HNSCC tumor in Fig. 3e given that in the legend it is stated that 3 samples of each tumor model were analyzed?
We apologize for the error. It has now been corrected. Fig. 3d and Fig. 3e each display data from 3 HNSCC samples and 3 CRC samples.

Lines 218-226: If the results presented in Figure 5e illustrate the recognition of common neoepitopes by the CD8 DP NKG2A- and NKG2A+ TILs, the results of Figure 5d do not go in this direction and undermine the message. Figure 5d could be removed.
We agree with the reviewer. As a results, we decided to remove the data presented in Fig. 5d from our manuscript.

line 239: Can the authors explain their choice to perform NKG2A upregulation experimentations on naive blood-derived CD8 T cells and not on DP CD8 NKG2A- TILs? This would have been more convincing.
Our goal was to identify factors responsible for the de-novo induction of NKG2A by CD8 T cells. For that reason, we believe it is much more convincing to perform those experiments using sorted naïve CD8 T cells (which are negative for NKG2A) – in contrast to previous work performed on non-purified CD8 T cell populations. In the latter case, the upregulation of NKG2A could be due to preferential expansion of NKG2A-expressing cells instead of its induction. In a second time, we confirmed that TCR stimulation in the presence of IL-12 can also lead to NKG2A expression on NKG2A- DP CD8 TILs (Figure 7f).

line 251: Figure 6g, to better appreciate the individual roles of CD4 cells and monocytes and the contribution of their co-presence, it would be important to show results obtained with naive CD8 T cells stimulated by SEB (or not) in the presence or absence of CD4 T cells or monocytes.
We agree with the reviewer. We have done those experiments and those additional conditions have been added to Fig. 6g.

line 286: Figures 7h and i, in multiplex analysis, can the authors justify why they didn't look at NKG2A instead of CD8?
In our manuscript, we showed that IL-12 can drive the induction of NKG2A on CD8 T cells following TCR stimulation. To determine if those conditions exist in the TME, we analyzed the spatial location of CD8 T cells and IL-12-secreting cells. Our data show that IL-12-secreting cells can be found close to CD8 T cells which supports our hypothesis. In contrast, the expression of NKG2A by CD8 T cells is a consequence of this interaction, and

we cannot exclude that, once activated, CD8 T cells migrate in the tumor intraepithelial compartment and away from IL-12-secreting cells. This is of course our hypothesis and would need to be tested in the future.

line 316: The authors observed a decrease frequency of NKG2A+ cells in HPV-driven HNSCC. This result is surprising, given that TILs in these tumors include T cells reactive towards the E6, E2 and E7 viral proteins and demonstrated in this article. Can the authors comment on this result?
We agree with the reviewer that the lower frequency of NKG2A+ cells among DP CD8 TILs is surprising. So far, we do not have a conclusive explanation for this difference. It could be linked to differences in the immune cell composition in the TME between HPV- and HPV+ HNSCC tumors (e.g. B cells), resulting in secretome differences. However, we decided to include those results in our manuscript as it could help inform what patient groups might be more susceptible to respond to NKG2A blockade in the future.

line 985: Can authors match the terms used between figures and their legends (macrostate vs microstates) (Fig.2g and extended data fig. 2g)?
This has been corrected. Thank you for noticing it.

lines 1029 and 1073: Can the authors perform statistical tests for the results shown in Fig. 6 and 7?
Statistical analyses have been performed for those two figures.

line 1073: Can the authors replace “HNSCC” by CRC and adapt the number samples (n=17)?
This has been corrected. Thank you for noticing it.

Reviewer #2 (Remarks to the Author):

The study overall is very interesting. But I found a few parts to be not very solid and need revisions/clarifications:
(1) “Of note, TCR 195 clonotypes expressed by NKG2A– and NKG2A+ DP CD8 TILs could be divided in three groups 196 whether they were shared at similar frequency between both subsets, shared but enriched in one 197 subset or not shared between subsets (Fig. 4c).” I don’t think this analysis tells us anything. There is no interpretation of the biological meanings of these three groups. We observe such “grouping” very commonly in TCR clonotype data when comparing two conditions
We agree with the reviewer. We modified this sentence accordingly. It now reads “Of note, most of shared TCRb clonotypes were present at very similar frequencies between NKG2A- and NKG2A+ DP CD8, with a minority of shared TCRb clonotypes expanded in one subset and not the other”.

(2) “Furthermore, using overlapping single peptides for HPV16 E6, we showed for one 213

patient that NKG2A– and NKG2A+ DP CD8 TILs recognized the same peptide (Fig. 5 c)." This is just one patient and one peptide. Not sure the authors can draw the conclusion "NKG2A– and NKG2A+ DP CD8 TILs recognize the same tumor antigens" on a global scale.
We agree with the reviewer that we have demonstrated the recognition of the same peptide between NKG2A- and NKG2A+ DP CD8 TILs in one patient only. However, our TCR repertoire analysis performed in Figure 4 shows that a large proportion of TCRb clonotypes is shared between those two cell populations, implying the recognition of common antigens by NKG2A- and NKG2A+ DP CD8 TILs. We have modified our sentence to take the reviewer's comment into account.

(3) "For each patient, we detected TMG-reactive 219 T cells and their frequencies varied between NKG2A– and NKG2A+ DP CD8 TILs (Fig. 5d)." Similarly, I do not see any pattern in this figure, and not sure how it supports the authors' conclusion: "NKG2A– and NKG2A+ DP CD8 TILs recognize the same tumor antigens"?
We agree with the reviewer. As a result, we followed the suggestion from Reviewer 1 and decided to remove the data presented in Fig. 5d from our manuscript.

(4) "For the HNSCC patient, 223 DP CD8 TILs recognized a mutation in NOTCH1 while in the CRC patient a mutation in 224 HEATR5A was recognized (Fig. 5e)." Here again, only two peptides and no global analyses to show that "NKG2A– and NKG2A+ DP CD8 TILs recognize the same tumor antigens" in an unbiased manner. There are also no proper control groups in these analyses. "memory CD8 T cells, DN CD8, SP CD8" would serve as good controls?
For the first part of the comment, please see the response to point 2.
We believe that our TCR repertoire analysis showing strong TCR repertoire overlap between NKG2A- and NKG2A+ DP CD8 TILs support that those two T cell populations recognize shared antigens.
On the last point, we have screened memory CD8 T cells, DN CD8, and SP CD8 for tumor reactivity in those patients. Similarly to our previously published data (Duhen et al., Nat Comm 2018; van den Bulk J, JITC 2023), we did not detect tumor reactivity in any of those cell populations.

(5) "Interestingly, our analysis showed that CD8 T cells could be found close to IL-289 12+ cells in a TGF-β-rich environment, with more than 50% of IL-12+ cells being within less than 290 30μm of a CD8 T cell (Fig. 7i)." There is no proper control in this experiment, and it is hard to draw the conclusion of whether the observed proportions should be considered high or low. For example, if the same analyses can be done for IL-12+ cells vs CD4 T cells or IL-12+ cells vs macrophages, and if the proportions for IL-12+ cells vs CD8 T cells are indeed higher, I think it would mean something. Maybe the authors can find a better control, but some kind of control is needed.
We would like to disagree with the reviewer. The goal of Fig. 7 h and Fig. 7i was 1) to determine the presence of IL-12-secreting cells in the TME and 2) to evaluate whether CD8

T cells could be detected close to those IL-12-secreting cells. Our goal was not to claim a preferential enrichment of CD8 T cells next to IL-12-secreting cells. To clarify our message, we modified our sentence: “Interestingly, our results indicate that CD8 T cells can be found close to IL-12+ cells in a TGF-b-rich environment, consistent with the NKG2A-promoting culture conditions we identified”.

(6) “NKG2A+ DP CD8 TILs are enriched in the tumor epithelium” and “IL12B mRNA was detected in the TME, 287 although in small numbers, and those cells were localized in the tumor stroma near the invasive 288 margin (Fig. 7h).” Are these two observations contradicting each other?
We do not believe that those two observations are contradictory. Indeed, while CD8 T cells can interact with IL-12-secreting cells in the tumor stroma, the CD8 T cells can then infiltrate the tumor intraepithelial compartment to interact with their target cells, the tumor cells. As a result, NKG2A+ CD8 T cells might not necessarily colocalize with IL-12-secreting cells.

Reviewer #3 (Remarks to the Author):

Duhen et al report that CD8 TILs exhibit a phenotype suggestive of tumor reactivity express the inhibitory receptor NKG2A in response to IL-12, thus attributing a regulatory role to this cytokine.

The role of NKG2A as an immune checkpoint inhibitor has been defined in viral infections (e.g., flu), but its role in anti-tumor immunity, and what regulates the pathway, remains elusive. The study provides some insight into an unexpected regulatory activity for a cytokine traditionally considered as a driver of T cell activation. However, it could be argued that the upregulation of NKG2A in T cells in response to IL-12 is an inhibitory immune checkpoint to prevent overt activation (e.g., similar to PD-1); and not necessarily a regulatory function of IL-12. In fact, TGF-b and IL-12 synergize to drive NKG2A upregulation, which is not sufficiently emphasized in the abstract or the text.
This is correct. We do not believe that IL-12 has a regulatory function per se. Instead, we believe that the upregulation of NKG2A in response to IL-12 stimulation serves as a negative feedback loop to prevent overt activation similar to PD-1 or other immunoregulatory receptors. However, such mechanisms need to be uncovered and considered to fully benefit from the therapeutic properties of IL-12, as reagents exist to interfere with those immunoregulatory pathways. We also agree that TGF-b further increases NKG2A expression on CD8 T cells. Nonetheless, our results suggest that, in contrast to IL-12, TGF-b is not sufficient to induce NKG2A expression on those cells.
We have modified the abstract accordingly: “Our study revealed that NKG2A, an inhibitory receptor, can be induced on human tumor-reactive CD8 T cells by IL-12 in a TGF-b-rich environment, demonstrating a previously unappreciated immuno-regulatory feedback loop following IL-12 stimulation”.

The interesting, novel and relevant information is shown in Figure 7.

MAJOR COMMENTS:

-The expression of CD39 and CD103 is suggestive of tumor reactivity but not at all definitory. Figure 5 shows upregulation of 4-1BB among CD8 TILs in response to HPV, which is also NOT definitory of tumor reactivity. To define these TILs as truly tumor-reactive, IFNG and/or GZMB ELISPOTs against tumor lysates, predicted neoantigens or HPV antigens (as properly done for Figure 5) need to be conducted. It is puzzling that the experiment was basically done but the readouts were limited to 4-1BB expression, which is turned on in response to NF-kB.
We agree with the reviewer that CD39 and CD103 co-expression cannot be used alone to indicate tumor-reactivity. Instead, those two markers can be used to enrich in tumor-reactive CD8 T cells as we previously demonstrated in HNSCC and CRC tumors (Duhen T, Nat Comm 2018; Duhen R, Nat Comm 2021; van den Bulk J, JITC 2023).
As for 4-1BB up-regulation as a surrogate for TCR-mediated activation, please refer to our answer to Reviewer 1 Major comment 2, where we display both ELISPOT data and intracellular IFN-g staining.

-A factor that could influence the reported phenotypes is the presence of tertiary lymphoid structures in the tumors. This should be clarified. Whether T cells in TLS are NKG2A+CD8+ or not would be informative.
It is not clear what the reviewer is referring to here. While examining the expression of NKG2A on CD8 T cells in a TLS is a very interesting question, we believe it is outside of the scope of this manuscript.

-There is no demonstration that NKG2A+ TILs have decreased effector activity. In fact, NKG2A+ TILs recognize a NOTCH mutated antigen more effectively than their NKG2A-CD8+ counterparts, if we assume that 4-1BB is a readout of tumor reactivity (Fig5). NKG2A may be, similar to PD-1, a mere marker of activation in cells that are otherwise superior effectors.
We agree with the reviewer that we did not show decreased effector activity on NKG2A+ DP CD8 TILs. Our single cell RNA-seq analysis rather suggest that those DP CD8 are more differentiated and express higher levels of genes associated with CD8 effector function such as IFNG, GZMB, GNLY. We believe that like other “checkpoint” molecules, NKG2A is upregulated on CD8 T cells to control T cell activation and prevent tissue destruction. However, in the case of cancer, the expression of this receptor on T cells could impair their function if APCs and/or tumor cells express HLA-E.

Reviewer #4 (Remarks to the Author):

the present paper is certainly of great interest and can potentially contribute to a deeper understanding of the CD8 T cell response to tumors. Additionally, the investigation of the immunecheckpoint NKG2A is now a rising topic, broadening our perspective on immunecheckpoint-mediated regulation of CD8 T cell response.
However, several issues need to be better addressed

We thank the reviewer for recognizing the value of our manuscript.

Specific Comments

1. Line 65: ” In contrast to other checkpoint molecules, NKG2A is expressed exclusively on NK cells and CD8 T cells,…”

There are no references added to this statement that, however, in some way are not correct. Besides NK cells and CD8 T cells, other immune cell populations expressing NKG2A include human γδ T cells, NKT cells, and MAIT cells (Cancers 2023, 15, p1264; Nat. Immunol., 2019, 20, p1110; Nat. Rev. Immunol., 2013, 13, p.101). Moreover, it has been observed that NKG2A-mediated signaling negatively regulates the activation of NKT cells and a specific subset of human γδ T cells, Vδ2 T cells (J Immunol. 2009, 182(1), p.250; Cell Reports, 2021, 37(3), 109871).

We apologize for the lack of references. We have now added to references for NKG2A expression by NK cells, CD8 T cells and γδ T cells.

2. What is the rationale behind choosing HNSCC and CRC tumors for analysis? Were these tumors specifically selected due to tissue accessibility? Do the authors believe the results are consistent across different tumor types? Alternatively, do these tumors exhibit common characteristics that reveal a higher NKG2A+ CD8 T cell pattern? Please elaborate on this aspect.

The rationale behind choosing HNSCC and CRC tumors for the analysis is multiple.
First, our lab is interested in deciphering the antitumor immune response in HNSCC and CRC tumors as those tumors do not respond well to the current checkpoint blockade agents targeting mostly PD-1/PD-L1 and CTLA-4/CD80, CD86. As such, other therapeutic options are necessary to induce tumor control for those patients and the NKG2A/HLA-E pathway could be a valid candidate.
Second, our lab has a fruitful collaboration with HNSCC and CRC surgeons at our institution that allowed us to collect enough patient samples to perform our analysis.
Our results on HNSCC and CRC are consistent and indicate that NKG2A is expressed on a subset of DP CD8 T cells which are more differentiated and with a higher cytotoxic potential for both cancers. Because of that, we believe our results might be able to be extrapolated to other solid tumors, but future work will need to be done to verify that point.

3. Comments to Figure 1:

a) The whole gating strategy of CD8 NKG2A+ T cells in the tumor should be included.

We have now included in the Extended Fig. 1 the gating strategy used for Figure 1.

b) Line 92) The statement of the NKG2A increases in tumors is inaccurate since the authors did not compare healthy tissue. Instead, it would be more accurate to discuss higher expression compared to blood.

We agree with the reviewer. We have modified the sentence accordingly: “We observed that, while NKG2A+ CD8 T cells were present in the periphery, there was a higher frequency of those cells in the tumor for both cancers”.

c) Are there any differences in expression of NKG2A in the five CD8 T identified subsets? The expression of NKG2A should be added to Fig. 1f.
Following the reviewer's recommendation, we added the histograms for NKG2A expression on the five CD8 T cell populations in Fig. 1f.
We compared the expression level of NKG2A between the five different populations and did not observe a significant difference (not shown).

d) In Fig. 1h, provide rational for the HPV-related analysis. How the author explains higher expression of NKG2A+ CD8 T cells in HPV-negative individuals? Extend abbreviation for HPV. Abbreviations for HPV should be expanded.
Besides tobacco and alcohol, human papilloma virus (HPV) is another main risk factor for HNSCC cancer, and the prevalence of HPV-driven HNSCC is increasing in high-income countries. While the response to standard of care is higher than for the HPV-negative subgroup, there is still a need to find novel therapeutic options for patients that do not respond to treatment. For this reason, we decided to evaluate NKG2A expression by DP CD8 TILs in HPV-negative and HPV-positive HNSCC tumors to help identify the patient population most likely to respond to NKG2A/HLA-E blockade.
The abbreviation for HPV has been added.

e) f) Line 117: Fig. 1i. What does the author mean by sample collection at different time points? These results are poorly explained.
We apologize for the confusion. The goal for Fig. 1i was to determine whether the proportion of NKG2A+ cells for each CD8 subset changed overtime. Such analysis is difficult to perform with human samples as surgical specimens are a snapshot of the CD8 T cell phenotype at a given time. However, some patients require more than one surgery to help control tumor growth. In that case, sequential tumor samples are available and can be used to address our question. In our patient cohort, we had 7 patients for whom we received tumor samples collected a two different timepoints that we could use for this analysis. Those samples include 3 HNSCC tumors, 2 ovarian tumors and 2 colorectal liver metastases (CRLM). As mentioned to Reviewer 1, if the reviewer believes that those results are too preliminary, we agree to removing them from our manuscript.

f) Line 119) This summary is poorly formulated.
We agree with the reviewers. We modified our summary accordingly: "Altogether, our results indicate that in HNSCC and CRC tumors NKG2A is mainly expressed by DP CD8 TILs".

4. Comments to Fig. 2:
a) The gating strategy here is confusing (should be rather included in Fig. 1) since the UMAP is the projection of all CD8 T cells in an unbiased way without any gating strategy performed.
We apologize for the confusion. We decided to include the density plot for CD4 and CD8 (protein, Total-seq antibodies) to indicate our approach for selecting CD8 T cells for the

downstream analysis, instead of using mRNA expression. On our scRNA-seq dataset, we also wanted to identify cells that express CD39 and CD103 at the protein level (Total-seq antibodies). By doing so, we were able to determine the cluster composition of the different CD8 T cell subsets (DN CD8, SP CD8, and DP CD8).

b) KLRG1, EOMES and GZMK are rather markers of TEMRA CD8 cells and not effector CD8 T cells
Based on the article from Willinger et al, J Immunol 2005, where the authors sorted naïve, central memory (TCM), effector memory (TEM) and terminally differentiated effector memory (TEMRA) CD8 T cells based on CD45RA and CCR7 expression and performed microarray analysis, KLRG1 and EOMES were expressed at higher levels in TEM and TEMRA, while GZMK was higher in TCM and TEM. To consider the reviewer's comment, we changed the text to "*cluster 0 and 5 expressed genes characteristic of CD8 T effector memory (TEM) and terminally differentiated effector memory (TEMRA) cells such as KLRG1, EOMES and GZMK*".

c) In Fig. 2d, what specific genes were used to calculate the naïve/TCM and activation/exhaustion scores?
Here are the genes used to calculate the naïve/TCM and activation/exhaustion scores:
Naïve/TCM score: SELL, TCF7, LEF1, CCR7, KLF2
Activation/exhaustion score: ACP5, CFS1, CTLA4, CXCL13, ENTPD1, GLNY, GZMB, HAVCR2, LAG3, PELI1, PHLDA1, PRF1, RBPJ, TNFRSF18

d) What is the difference between DP CD8 T cells in cluster 7 and cluster 2?
Many of the genes expressed by cells in cluster 7 are also detected on cells in cluster 2 but at higher expression level, such as ADGRG1, CSF1, KLRC1, TNFRSF18. Those genes display a gradual expression increase from cluster 4>2>7.

e) Line 166) The statement, "Overall, our analysis revealed that in the tumor, the DP CD8 NKG2A+ cells are more terminally differentiated and cytotoxic than other CD8 subsets," is overstated. No cytotoxicity has been performed; instead, the authors associated it with a higher cytotoxic potential.
We agree with the reviewer. We have modified the text accordingly: "*Overall, our analysis revealed that in the tumor, the DP CD8 NKG2A+ are terminally differentiated and exhibit a high cytotoxic potential*".

5. Comments to Fig. 3:
a) The low resolution of the pictures in Fig. 3a does not allow for the appreciation of NKG2A and CD39 expression.
We apologize for that. We have selected four areas for each image, and those area are displayed at a higher magnification. We hope that will facilitate the appreciation of NKG2A and CD39 expression.

b) In Fig. 3c-e, statistics are lacking. Additionally, the authors should specify in the legend how many patients were analyzed for the study.
Statistical analysis has now been performed on Fig. 3c-e. The number of patients analyzed is indicated in the figure legend (3 HNSCC tumors and 3 CRC tumors).

6. Comments to Fig. 4:
a) In Fig. 4a, the use of NK+ is confounding; please use NKG2A+.
“NK” has been replaced by “NKG2A”. in Fig. 4a and 4b.

b) The use of the terms "clonotypes" or "TCR repertoire" is not appropriate since the author only analyzes the beta chain and not the paired alpha-beta chains that accurately define a clone. This aspect is incorrectly described in the text. Additionally, the statement regarding shared TCR clonotypes between DP NKG2A+ and NKG2A- subsets is overstated.
As noted by the reviewer, we used the ImmunoSEQ kit from Adaptive Biotechnologies which sequences the variable region (CDR3) of the TCRb chain of the TCR. We and others have been referring to those unique TCRb chains as “clonotypes” (Becattini S, Science 2015; Duhen T, Nat Comm 2018). To clarify that our analysis is focused primarily on the TCRb chain of the TCR, we modified the text as indicated here “To address that, we performed a quantitative assessment of TCRb clonotypes by deep sequencing of blood memory CD8 T cells, DN CD8, SP CD8, NKG2A- DP CD8 and NKG2A+ DP CD8 TILs”. We hope the reviewer will accept this modification. As for shared clonotypes, we are referring to unique TCRb clonotypes that are detected in two T cell populations (their frequency can be different). Using this approach, our analysis revealed that approximately half of the TCRb clonotypes detected in the NKG2A+ DP CD8 were also detected in the NKG2A- DP CD8 for a specific patient.

c) Is there any publicly available single-cell TCR-seq data from CRC and/or HNSCC tumors that could be analyzed to define paired alpha-beta clonotypes in CD8+ T cells concerning NKG2A expression? In this scenario, it would be appropriate to explore whether such analysis could reveal whether shared clones undergo clonal expansion with heightened expression of NKG2A.
It is a very good idea, and we have scTCR-seq data for our HNSCC and CRC single cell datasets. Unfortunately, limitations inherent to scRNA-seq might prevent such an analysis. One limitation of scRNA-seq is the high number of dropout events. While this limitation is overcome by using clustering algorithm and grouping cells that are the most similar, it can become an issue when trying to perform an analysis such as the one suggested by the reviewer. In that scenario, a KLRC1+ cells can appear negative due to dropouts. An alternative to that would be to generate new single cell data adding an anti-NKG2A Total-seq antibody. In that scenario, the antibody will identify all cells with detectable NKG2A at their cell surface.
With this limitation in mind, we selected KLRC1+ cells among DP CD8. Those cells were separated into four groups based on clonal expansion levels: not expanded (1 cell), small expansion (2-5 cells), medium expansion (6-), and large expansion, and we analyzed

KLRC1 expression level for each group. Results from the HNSCC dataset suggest an increased expression of KLRC1 as cells expand. The results are not as clear for the CRC dataset, where the medium expanded cells appear to have the highest expression of KLRC1.
While interesting, we would prefer not to add this analysis to the manuscript due to the limitations associated with scRNAseq as discussed above.

7. Comments to Fig. 5:
a) a) In Fig. 5a, the author shows similar activation of NKG2A+ and NKG2A- cells in response to HPV antigens. How does the author explain the significantly lower frequency of NKG2A+ CD8 T cells in HPV-negative subjects compared to HPV-positive subjects shown in Fig. 1h?
The lower frequency of NKG2A+ cells in DP CD8 from patients with HPV+ HNSCC shown in Fig. 1h is by itself interesting. However, we currently do not have an explanation to account for that difference. It might be associated with differences in the cellular composition of the microenvironment between those two groups of HNSCC tumors and this question will be addressed in future studies.

b) Considering the previous question regarding the low expression of NKG2A in HPV-negative patients, what is the rationale behind studying the response of NKG2A+ CD8 T cells in these patients?
While the frequency of NKG2A+ cells in DP CD8 TILs is lower in HPV+ HNSCC tumors, those cells are still present, and their reactivity can be analyzed. We chose to evaluate reactivity to HPV antigens because those antigens are known, and their sequences are conserved between patients. In contrast, tumor-specific somatic mutations are private to a patient and the identification of neoantigens requires performing whole exome sequencing, mutation calling and screening of a potentially large number of putative neoantigens. The amount of work and cost associated with the latter limits the number of patients that can be analyzed.

c) In Fig. 5d, it is not clear what TMG 1, 2, 6, 7, etc. means.
We apologize for the lack of clarity. TMG stands for tandem minigenes. Those are plasmid construct containing a succession of minigenes (up to 16 minigenes/construct). A minigene consists of the mutant amino acid flanked by 12 amino acids of the wild-type protein sequence. Those plasmid constructs are used to screen multiple putative neoantigens at once. In the case of a positive signal, short peptides for each of the mutations present in the TMG construct can then be tested to identify the mutation(s) recognized by the T cells. In response to a comment from reviewer 1, we decided to remove the data presented in Figure 5d from our manuscript.

d) Does the expression of NKG2A and CD39 increase upon HPV- and tumor-related antigens stimulations in vitro?
While CD39 expression decreases during in vitro expansion following the sort of DP CD8 TILs, its expression is upregulated after peptide stimulation (higher mean fluorescence

intensity). As for NKG2A, while we have shown that peptide stimulation is not sufficient to induce NKG2A expression on DP CD8 TILs (Fig. 7f), we did not analyze the effect of peptide stimulation on the level of expression of NKG2A by NKG2A+ DP CD8 TILs.

8. Comments to Fig. 6 and 7:
a) Abbreviations for SEB should be expanded.
We apologize for this oversight. We have now added the abbreviation for SEB to the manuscript.

b) There is a lack of statistical comparison with control examples, such as in Fig. 6h and 6f.
We have now included a statistical comparison with control examples for Fig 6.

c) Regarding Fig. 7h, the resolution is insufficient, and the association of IL12 RNA expression with specific cells is not clearly delineated.
We apologize for that. We have now selected four areas of image, and those area are displayed at a higher magnification. We hope that will facilitate the appreciation of IL-12, TGF-b and CD8 expression.

d) Viral infections typically induce IL12 secretion by innate cells. How do the authors explain the lower expression of NKG2A in HPV+ patients?
We agree with the reviewer that IL-12 is produced by innate cells in response to viral infection. However, in the case of HPV+ HNSCC tumors, the viral genome integrates into DNA regions of genomic instability of infected cells. This enables HPV to establish persistent infection and continue to replicate, but, although viral proteins are synthesized, no viral particles are produced by the virus. The infection leads to carcinogenic transformation of the cells by escaping cell-cycle checkpoints through E6- and E7-mediated degradation of p53 and Rb proteins, respectively. This is a situation distinct from a classical viral infection with active viral replication and viral particle production and might not lead to IL-12 secretion.
The lower expression of NKG2A in HPV+ patients might instead be linked to differences in the cellular composition of the tumor microenvironment. Additional work would need to be done in the future to address that point.

9. Comment to Discussion:
a) The authors should address the implications of anti-NKG2A treatments in the context of the expression of HLA-E.
We have now addressed the implications of anti-NKG2A treatments in the context of the expression of HLA-E in the Discussion.

b) Several statements in the discussion are overstated, as mentioned above. For instance, in Line 299, it is stated: "Our analysis revealed that NKG2A was induced in the TME as the cells differentiate into DP CD8 and its expression correlated with the acquisition of cytotoxic properties and effector functions." However, the authors did not demonstrate induction of NKG2A; instead, they observed higher expression compared to blood. No

functional experiments were performed; rather, the cytotoxic potential was observed at the transcriptional level.

We have modified the discussion and tempered down our statements. As mentioned by the reviewer, we did not demonstrate induction of NKG2A on CD8 T cells in our TIL analysis. Instead, our trajectory analysis indicates that NKG2A expression on CD8 T cells increases as cells differentiate and acquire a DP CD8 phenotype. This increase in NKG2A expression correlated with an increased expression of effector genes and cytotoxic genes implying a higher cytotoxic potential for those cells.

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Point-by-point responses to reviewer's comments

Reviewer #1 (Remarks to the Author):

We (reviewer 1 and early career researcher co-reviewer) would like to thank the authors for responding to all our comments and for all the changes made (figures, discussion, references…) to the manuscript which enhance the quality of this study.

We are glad that our responses and the changes we made to our manuscript enhanced the quality of our study.

Nevertheless, we strongly recommend that the authors take into account the following points:
- Regarding the correlation between 4-1BB expression and IFN-g production by activated T cells, we thank the authors for providing ELISPOT and flow cytometry results that validate this correlation. Nevertheless, can the authors justify this read-out in the manuscript (line 207) with the addition of the references provided (or even the (particularly convincing) data provided in the appendix)?

We have included the references to our manuscript. We have also added the IFN-g production data as a Suppl Figure (Suppl Fig. 6).

- Although the preliminary results on the evolution of NKG2A expression by CD8 TILs (Figure 1i) are interesting, we recommend deleting this figure in view of the small number of samples analyzed, the different tumor models and the highly variable duration between the two surgeries which do not allow these data to be reliably exploited.

We agree with the reviewer, and we decided to remove these results from the manuscript.

- We also ask the authors to comment in the discussion section (as they did in the reply to the review) on our remark about the surprising finding of the low frequency of CD8 NKG2A+ TILs within HPV+ HNSCC tumors (Figure 1h) despite the existence in this population of T cells reactive towards several viral peptides (Figures 5a-c).

We have added a paragraph in the Discussion section regarding the low frequency of CD8 NKG2A+ TILs within HPV+ HNSCC tumors.

Reviewer #2 (Remarks to the Author):

The authors have addressed all my concerns. Thank you!

Thank you for reviewing our manuscript.

Tao Wang

Reviewer #3 (Remarks to the Author):

The inclusion of new ELISPOT data clearly strengthens the manuscript. Similarly, the emphasis on presenting NKG2A as an immune checkpoint inhibitory mechanism, rather than a regulatory effect of IL-12, is also an improvement.
Everything can get better but I believe that the authors have proven the main conclusions.

We are glad that the modifications we introduced in our manuscript were accepted by the reviewer.

Reviewer #4 (Remarks to the Author):

In this revised version of the manuscript, authors properly and exhaustively addressed all the queries I raised in the original version of the manuscript. Hence, I do find this revised from suitable to be published on Nature Communications

Thank you for your exhaustive review of our manuscript.