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Transcriptional and chromatin profiling of human blood innate lymphoid cell subsets sheds light on HIV-1 pathogenesis

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Review #1

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Wang and colleagues present a very detailed, high quality paper describing phenotyping, transcriptional, epigenetic and functional differences between NK cells and ILCs in human peripheral blood. They overlay these studies which descriptions of differences in these populations in healthy and HIV infected (viremia, ART-treated, controllers), extending a their previous 2020 study.

Overall, this paper serves as an important resource for the field. There are areas in which the manuscript could be modified to improve clarity and detail regarding rigor.

My comments below are all addressable and therefore I class them all as 'major'.

1. The authors describe ILCs as being 'permanently deleted' in HIV infection. As written, this can be misinterpreted to suggest complete ablation of this cell subset. This is clearly not the case. Moreover, there looks to be some restoration of ILCs in ART-treated participants. I suggest revising text to quantify the level of cell loss or replace the word deleted with reduced.
2. The HIV EC are not clearly discussed in the paper and are not distinguished from viremic controllers. If ILCs are permanently reduced in this group, what does this suggest for the role of this cell subset in HIV control? Plasticity between ILC and NK cells is described. Is this plasticity relevant at all for HIV control, elite or otherwise?
3. The authors write that ILCs have a primary role in cytokine secretion and then distinguish the ILCs from NKs....but NKs also secrete cytokines. Can the authors please clarify their text?
4. Discussion (an also Results) - The authors use pseudotime analysis to show that CD56+NK cells sit between the ILCs and CD56-NKs. When initially described in the Discussion they don't really interpret this in terms of their (and others' observations) that CD56-NKs are increased in disease. Other groups have suggested CD56-NKs are dysfunctional. Here you show different granzyme profiles between CD56+ and CD56-NKs. Elsewhere you distinguish CD56dim and CD56- NKs in terms of functionality in HIV+ donors, though these cells cluster on the pseudotime analysis. The IL-2 and CD4+ T cell data add another layer of complexity.
- a. Altogether, I am unclear on the authors' interpretation of their collective data.
- b. Can you please clarify whether you are suggesting that CD56dimNKs are a distinct from CD56+ NKs but functional as opposed to CD56-NKs that are

dysfunctional?
- c. Are CD56-NKs end-stage or can they be rescued?
- d. CD8 T cell number remain high post ART and very much drive the ongoing inverted CD4:CD8 ratio. Have the authors consider how and if the need of these cells for IL-2 impact recovery of NK cell populations?
5. Discussion: It would be very helpful if the authors can pull this large volume of data together in a summary paragraph and possibly also a graphic.
6. "Thus, GZMK+CD8+T cells appear to be the adaptive counterpart of CD56hiNK cells, representing an intermediate state between cytokine producing CD4+T cells and cytotoxic CD8+T cells" For me, the 'cytokine-producing CD4+ T cells' comes from in from left field here. Can the authors please clarify or was the intent to write cytokine-producing CD8+ T cells?
7. Methods: Were Pearson correlations applied because of the large 'n' or were data first tested for normality?
8. Methods: Please clearly justify the use of t-tests throughout the manuscript rather than non-parametric based tests.
9. Methods: How many cells were acquired in flow cytometry? How many ILCs were acquired in healthy/HIV+ donors for these studies? Did this create limitations on interpretation of phenotypic data?
10. Methods: How was the cytokine concentrations used for in vitro assays determined?
11. Figure: In Figure 4A, should it be '+ILC- ' or '+ILC' (no negative symbol)?
12. It's unclear from you single cell analysis how many cells were acquired for each of the 4 subsets. I assume less ILCs were analyzed. If so, can you please clarify for the non-bioinformatician how your bioinformatic analysis took these differences into account?

2. Significance:

Significance (Required)

Overall, this paper serves as an important resource for the field.

There are areas in which the manuscript could be modified to improve clarity and detail regarding rigor (see above). The overall message of this work for understanding HIV pathogenesis is unclear but can be addressed.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Less than 1 month

4. *Review Commons* values the work of reviewers and encourages them to get credit for their work. Select 'Yes' below to register your reviewing activity at Web of Science Reviewer Recognition Service (formerly Publons); note that the content of your review will not be visible on Web of Science.

Web of Science Reviewer Recognition

Yes

Review #2

1. Evidence, reproducibility and clarity:

Evidence, reproducibility and clarity (Required)

Summary:

This manuscript makes use of RNA sequencing, ATAC-Seq and single cell sequencing to compare transcriptomes and identify relationships between various ILC/NK cell subsets in blood from uninfected controls. The authors describe differences in genes and pathways between subsets and determine that CD56high NK cells are an intermediate-connecting cell type between ILC progenitors and CD56dim NK cells. The authors show how HIV infection (viremic, ART+ or controllers) effect ILC/NK cell gene expression and pathways. One of the genes enhanced in NK cells is AREG which the authors demonstrate to be upregulated in IL/NK subsets in response to PMAI/I stimulation or stimulation with IL-2 or IL-15. The authors demonstrate that TGFB1 or knockout of RUNX3 modulates the frequency of AREG+ NK cells, implicating the Wnt signaling pathway in AREG regulation. Authors show that in

PLWH, the frequency of AREG+ NK cells is altered. Authors also report that with HIV infection there is an increase in CD56neg NK cells with corresponding loss of CD56dim cells. Using healthy control PBMCs, the authors suggest differentiation of CD56dim into CD56neg is prevented by CD4 T cell production of IL-2 but promoted by TGFB1. Also in healthy controls, the authors run metabolomics to describe how CD56dim are more metabolically functional than CD56neg and link this to MTOR activity.

Major:

- Which population(s) of NK cells (high, dim or negative for CD56) are responding to stimulation with IL-2 and IL-15 by upregulating AREG in Figure 5? Does this NK population have higher expression of receptors for IL-2 and IL-15 (in single cell seq data or by flow) and if so how does receptor expression change in PLWH?
- Does signaling through IL-2 or IL-15 converge on the TCF7/Wnt signaling pathway? For example, is it possible to knockdown TCF7 then stimulate with IL-2 or IL-15 and measured AREG+ cells with the idea that loss of TCF7 would result in reduced cytokine-induced activation of AREG if mediated through the Wnt pathway?
- In Figure 8 the authors demonstrate that CD4 T cells promote the maintenance of CD56dim NK cells likely through production of IL-2. Was AREG expression examined in these studies and if so could you conclude that CD4 T cells also promote AREG expression in this population?
- In Figure 8D, although significant, the increase in percentage from 2-4% of the CD56neg is a minimal change. Could a higher dose of IL-2 be tested to see more substantial changes? Similar comment for Figure 10D, although significant, the increase in percentage from 2-4% of the NK population to CD56neg is not a large change with rapamycin. Could a higher dose be tested (that does not kill the cells) or a different inhibitor be used to see more substantial changes? More robust changes would strengthen the conclusions.
- In Figure 10, was the increase in pmTOR with IL-2/IL-15 stimulation specifically observed in CD56dim cells (rather than total NK cells or CD56neg cells)? This would strength the conclusion that CD56dim is more metabolically functional than CD56neg.

Minor

- Approximately how many ILC from PLWH were submitted for single cell sequencing: Since this cell population was depleted in HIV+, was there a sufficient number of cells to interpret/publish DEG results in Figure 4?
- On page 13 of text, it is a bit disconcerting to jump to Figure 7 then back to Figure 4. Would recommend reorganizing text or figure panels to flow better.

- Supplemental Figure 1 and 2: Dots for ILC population are purple in color but legend is mislabeled as pink color.
- Supplemental Figure 4: Dots for HIV+ controller are purple in color, but legend is mislabeled as pink in color.
- Would recommend adding quantification of Figure 5A for all donors tested.
- Figure 5B for IFNg it appears that part of the positive population is not gated on and thus percentages are likely higher if the gate is adjusted.
- For Figure 5F, need to add to text and panel that PMA/I was used to stimulate as described in figure legend. Current figure and text read that the Wnt agonist alone was responsible for levels depicted in NK subsets.
- On the x-axis of Figure 6F-J does "Lin-TBX21+" refer to Total NK cells? If so then would recommend sticking with nomenclature "Total NK" as in Figure 6A-B.
- Would recommend labeling gates for populations of interest in Supplemental Figure 5B-C, Supplement Figure 6A, Figure 8A.
- Other groups have shown that CD56neg are increased in HIV, functionally impaired and correlate with loss of CD4 T cells. Would recommend citing their studies.
- Would recommend adding quantification of Figure 8A for all donors tested. Also, for the 3rd flow plot does "NK+CD4" mean purified NK cells + autologous CD4 T cells? If so, then would clarify in figure legend. It may strengthen conclusion for IL-2/IL-15 to show differentiation of NK cells is not contact dependent with T cell via transwell assay.

2. Significance:

Significance (Required)

This study is valuable because it generates large datasets on the NK/ILC family from human blood that can be deposited in repositories and of special relevance to the HIV field because it examine show viral infection (controlled or not) effects these subsets.

The strengths of the study are the cohort of PBMC samples utilized (HIV-, HIV+ viremic, HIV+ ART+ and HIV+ controlled), the multi-omics approach for transcriptome and epigenetics and the in vitro mechanistic studies identifying key regulators of NK cell differentiation/function.

The study advances the field "mechanistically" by providing key targets that may be subject to therapeutic modulation in PLWH such as AREG, IL-2 signaling, IL-15 signaling, Wnt signaling, and mTOR activity (although more work will need to be done to examine these pathways using PBMCs from HIV+). This study advances the field "conceptually" by providing large datasets for others to mine if deposited.

The audience for this study "basic research" such as immunologists in the HIV field or immunologist interested in ILC/NK biology.

My field of expertise is infectious disease (HIV, SARS-CoV-2), basic immunology (ILC, NK, B cell) and autoimmune disease. Would recommend additional reviewers for assessment of metabolic genes/pathways in Figure 9-10.

3. How much time do you estimate the authors will need to complete the suggested revisions:

Estimated time to Complete Revisions (Required)

(Decision Recommendation)

Between 1 and 3 months

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Yes

Full Revision

Manuscript number: RC-2022-01810
Corresponding author(s): Jeremy Luban

[Please use this template only if the submitted manuscript should be considered by the affiliate journal as a full revision in response to the points raised by the reviewers.

*If you wish to submit a preliminary revision with a revision plan, please use our "Revision Plan" template. **It is important to use the appropriate template to clearly inform the editors of your intentions.**]*

1. General Statements [optional]

This section is optional. Insert here any general statements you wish to make about the goal of the study or about the reviews.

To the Editor,

We thank the reviewers for their generous efforts on behalf of our manuscript. We were very pleased to see that reviewer #1 considered our manuscript a "very detailed, high quality paper" that "serves as an important resource for the field." And that Reviewer #2 concurred, writing that our manuscript is "valuable because it generates large datasets on the NK/ILC family from human blood that can be deposited in repositories", and that it is of "special relevance to the HIV field because it examines viral infection effects on these subsets."

We believe the revisions made in response to the reviewers suggestions have improved the presentation of our data. Our responses to each reviewer comment are listed below in bold font. References mentioned here are listed in a bibliography at the end of this document.Changes to the text are also highlighted in the manuscript.

2. Point-by-point description of the revisions

This section is mandatory. Please insert a point-by-point reply describing the revisions that were already carried out and included in the transferred manuscript.

Reviewer #1 (Evidence, reproducibility and clarity (Required)):

Wang and colleagues present a very detailed, high quality paper describing phenotyping, transcriptional, epigenetic and functional differences between NK cells and ILCs in human peripheral blood. They overlay these studies which descriptions of differences in these populations in healthy and HIV infected (viremia, ART-treated, controllers), extending a their previous 2020 study.

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My comments below are all addressable and therefore I class them all as 'major'.

I. The authors describe ILCs as being 'permanently deleted' in HIV infection. As written, this can be misinterpreted to suggest complete ablation of this cell subset. This is clearly not the case. Moreover, there looks to be some restoration of ILCs in ART-treated participants. I suggest revising text to quantify the level of cell loss or replace the word deleted with reduced.

The reviewer is correct that ILCs are not completely ablated. In response to this important point we have clarified the text, as follows:

- As indicated on page 5, we have changed the text to, "ILCs are decreased in the blood and intestinal lamina propria in people living with HIV-1, even after viremia has been suppressed by antiviral therapy".

- In the Results on page 13, we have changed the text to "ILCs were decreased in all subgroups of people living with HIV-1".

- The text on page 18 was changed to, "The percentage of ILCs in the $Lin^{-}CD56^{-}$ population decreased from 37.5% in $HIV\text{-}1^{-}$ controls to 7.34% in people living with HIV-1 who are viremic; the reduction in ILCs was not fully restored by ART (24.38%) or in spontaneous controllers (18.16%)"

- As indicated on page 26 in the Discussion, we have changed the text to "HIV-1 infection permanently reduces ILCs but not NK cells".

II. The HIV EC are not clearly discussed in the paper and are not distinguished from viremic controllers. If ILCs are permanently reduced in this group, what does this suggest for the role of this cell subset in HIV control? Plasticity between ILC and NK cells is described. Is this plasticity relevant at all for HIV control, elite or otherwise?

We thank the reviewer for asking us to clarify these important points in the manuscript:

- **Though elite controllers suppress HIV-1 viremia to undetectable levels, ILC numbers are still decreased in these individuals. Additionally, we do not detect an inverse correlation between ILC numbers and viremia. Further, our previous publication showed that ILC reductions in HIV-1 infection correlate inversely with markers of systemic inflammation, for example sCD14 (Wang *et al*, 2020b). Like other people living with HIV-1 infection, elite controllers have elevated microbial translocation, suggestive of disturbed gut homeostasis (Brenchley *et al*, 2006). Some studies have even reported higher rates of cardiovascular disease in elite controllers, presumably as a result of higher levels of systemic inflammation (Crowell et al. 2015; Caetano et al. 2022). Taken together, these observations suggest that ILCs play no direct role in control of HIV-1 replication. These points are discussed on page 26-27.**
- **The fascinating question of plasticity between ILCs and NK cells is one we have explored extensively, both in our previous work and in the current manuscript. Plasticity has been reported between intraepithelial ILC1s and NK cells from tumor tissue, and between ILC3s and NK cells in tonsil (Moreno-Nieves *et al*, 2021; Raykova *et al*, 2017; Cortez *et al*, 2017). When ILC2s are cultured in vitro with IL-12, IFN-γ and TBX21 expression are upregulated to the levels of $CD56^{hi}$NK cell (Lim *et al*, 2016), indicating possible plasticity between ILC2 and $CD56^{hi}$NK cells under certain inflammatory conditions. In HIV-1 infection, we do not detect correlations between the decrease in ILCs and increase in NK cells. In fact, the total NK cells did not change in HIV-1^{+} people who are viremic, on ART, or viremic controllers [(Figure 2A and 2B in (Wang *et al*, 2020b)]. Our transcriptome analysis indicates that, during HIV-1 infection, ILCs are likely depleted by inflammation-induced apoptosis (Figure 4I and Supplemental Table 6). However, whether blood ILCs (ILCPs or ILC2s) can upregulate TBX21 and EOMES to give rise to NK cells during HIV-1 infection is an interesting question worth further investigation. We now discuss the reviewer's question on pages 27.**

III. The authors write that ILCs have a primary role in cytokine secretion and then distinguish the ILCs from NKs....but NKs also secrete cytokines. Can the authors please clarify their text?

The reviewer is correct that, in this context, our statement is confusing. We have therefore deleted the sentence "Typical of cells with a primary role in secretion of cytokines…" on page 10. This change helps to focus the text on the specific genes that distinguish these subsets.

IV. Discussion (an also Results) - The authors use pseudotime analysis to show that CD56+NK cells sit between the ILCs and CD56-NKs. When initially described in the Discussion they don't really interpret this in terms of their (and others' observations) that CD56-NKs are increased in disease. Other groups have suggested CD56-NKs are dysfunctional. Here you show different granzyme profiles between CD56+ and CD56- NKs. Elsewhere you distinguish CD56dim and CD56- NKs in terms of functionality in HIV+ donors, though these cells cluster on the pseudotime analysis. The IL-2 and CD4+ T cell data add another layer of complexity.

a. Altogether, I am unclear on the authors' interpretation of their collective data.

b. Can you please clarify whether you are suggesting that CD56dimNKs are a distinct from CD56+ NKs but functional as opposed to CD56-NKs that are dysfunctional?

It would be best to respond to these two questions together. It is important to remember that our manuscript describes the characterization of these cells using several techniques.

- By flow cytometry, NK cells can be separated into $CD56^{hi}$, $CD56^{dim}$, and $CD56^{neg}$ populations (Figure 1F). Pseudotime analysis of our single cell RNA-Seq data showed that, at the transcriptional level, $CD56^{dim}$ and $CD56^{neg}$ NK cells are very similar to each other and cluster together (Figures 3A and 3F). Nonetheless, as compared with $CD56^{dim}$ NK cells, $CD56^{neg}$ NK cells express lower levels of GZMA and PRF1 (Supplemental Figure 4A), and produce less IFN-γ upon cytokine stimulation (Figures 8H and 8J). And when $CD56^{neg}$ NK cells are sorted and assessed by Bulk RNA-Seq, which gives deeper coverage than single cell RNA-Seq, metabolic gene expression is altered with respect to $CD56^{dim}$ NK cells (Figure 9).
- Our pseudotime analysis based on single cell RNA-Seq showed that $CD56^{hi}$NK cells form a distinct cluster, but that they share some transcriptional features with both the ILC cluster and the $CD56^{dim}$/$CD56^{-}$NK cell cluster (Figures 3A-3C and 3F).
- In people living with HIV-1, as assessed by flow cytometry, $CD56^{neg}$ NK cells increase in number at the expense of $CD56^{dim}$ NK cells (Figure 7C). And yes, we show here that $CD56^{neg}$ NK cells expand in tissue culture if $CD4^{+}$ T cells are depleted from the PBMCs, and addition of exogenous IL-2 prevents the switch from $CD56^{dim}$NK cells to $CD56^{-}$NK cells (Figures 8A-8C). These points are covered in the Results and Discussion on pages 11 and page 29-30.

c. Are CD56-NKs end-stage or can they be rescued?

IL-2 treatment of CD56^{-}NK cells from people living with HIV-1 can be converted back to CD56^{dim}NK cells, though function is not fully restored to the level of CD56^{dim}NK cells (Mavilio *et al*, 2005). In our manuscript, we showed that IL-2 or IL-15 treatment can prevent CD56^{dim}NK cells from becoming dysfunctional CD56^{-}NK cells, and that these cytokines maintain mTOR activity (Figure 8A-D, 8G, 8I and Figure 10). Taking together our data with the published data, CD56^{-}NK cells appear to be an end-stage dysfunctional cell. Whether conditions can be found for full restorage of function is an important question that is worth pursuing. This point is now stated on page 30.

d. CD8 T cell number remain high post ART and very much drive the ongoing inverted CD4:CD8 ratio. Have the authors consider how and if the need of these cells for IL-2 impact recovery of NK cell populations?

Two orthogonal experiments indicate that IL-2 secreted by CD4^{+} T cells, but not from CD8^{+} T cells, is responsible for the recovery of CD56^{dim}NK cells after treatment with ART. In our *ex vivo* culture of PBMCs, depletion of CD4^{+} T cells , but not of CD8^{+} T cells, was associated with increased numbers of CD56^{-}NK cells (Supplemental Figure 6A). Additionally, the IL-2 concentration in plasma from people living with HIV-1 correlated with CD4^{+} T cell numbers, but not with CD8^{+} T cell numbers (Figures 8N and 8O).

V. Discussion: It would be very helpful if the authors can pull this large volume of data together in a summary paragraph and possibly also a graphic.

We thank the reviewer for the suggestion. We have now summarized our data in the discussion (page 31). Additionally, we have added bullet points with a 2 sentence summary to accompany our graphic abstract (page 3).

VI. "Thus, GZMK+CD8+T cells appear to be the adaptive counterpart of CD56hiNK cells, representing an intermediate state between cytokine producing CD4+T cells and cytotoxic CD8+T cells" For me, the 'cytokine-producing CD4+ T cells' comes from in from left field here. Can the authors please clarify or was the intent to write cytokine-producing CD8+ T cells?

To clarify what we meant we need to discuss two points:

First, NK cells can be considered as the innate immune counterparts of CD8^{+}T cells, in that both cell types are cytotoxic killer cells. In contrast, ILCs may be considered as the innate counterparts of non-cytotoxic CD4^{+} T helper cells. For example, like Th1 cells, ILC1 cells are TBX21^{+} producers of IFN-γ. And like Th2

cells, ILC2s are GATA3+ producers of IL-13. The relationship between ILCs and NK cells is similar to the relationship between $CD4^+$T help cells and $CD8^+$ cytotoxic T cells (Vivier *et al*, 2018).

Second, previous studies showed that $GZMK^+CD8^+$T cells are distinguished from other $CD8^+$T cells by higher expression of IL7R, TCF7, IFN-γ and TNF-α, and by decreased cytotoxic activity (Jonsson *et al*, 2022). Thus, the relationship between $GZMK^+CD8^+$T cells and $GZMK^-CD8^+$T cells may be similar to the relationship between $CD56^{hi}$NK cells ($GZMK^+$, $IL7R^+$, $TCF7^+$, higher IFN-γ production and lower cytotoxicity) and cells from the $CD56^{dim}$ and $CD56^-$NK cell cluster ($GZMK^-$, $IL7R^-$, $TCF7^-$, lower IFN-γ production and higher cytotoxicity). In response to this comment we have modified the text on page 25-26 to make these points more clear.

VII. Methods: Were Pearson correlations applied because of the large 'n' or were data first tested for normality?

After checking the normality, nonparametric spearman correlation was performed for panels that failed the normality test. For panels with smaller n, the normality test may be not applicable, and Pearson correlation was performed.

VIII. Methods: Please clearly justify the use of t-tests throughout the manuscript rather than non-parametric based tests.

We tested the normality before performing parametric or nonparametric test. The t-test, Wilcoxon test or Mann-Whitney test used in our analysis was now specified for each panel in the legend. For panels that calculate cell percentage or numbers with smaller n, normality test may be not applicable, t-test was performed as done previously in these papers, Figure 7C and 7D in (Wang *et al*, 2020a), and in Figure 3 and 4 in (Xue *et al*, 2022). We confirmed these analyses with two statisticians in our institute.

IX. Methods: How many cells were acquired in flow cytometry? How many ILCs were acquired in healthy/HIV+ donors for these studies? Did this create limitations on interpretation of phenotypic data?

ILCs constitute roughly 1,000 cells per million PBMCs. To assess ILCs, we acquired data from 500,000 PBMCs, or roughly 500 ILCs per sample. NK cells constitute roughly 100,000 cells per million PBMCs, we acquired data from 200,000 PBMCs, or roughly 20,000 NK cells per sample. For each patient group, whether HIV-1-negative, HIV-1 viremic, etc, we analyzed PBMCs from at least 19

blood donors. This information is now stated in the "Flow cytometry" section in "Methods", on page 35.

X. Methods: How was the cytokine concentrations used for in vitro assays determined?

The cytokine concentrations were determined according to previous publication and our previous studies (Romee *et al*, 2016, 2012; Wang *et al*, 2020b; Silverstein *et al*, 2022). We have added the related references to the "Stimulation conditions" section in "Method", on page 36.

XI. Figure: In Figure 4A, should it be '+ILC- ' or '+ILC' (no negative symbol)?

Yes, the reviewer is correct. We have deleted the typo "-".

XII. It's unclear from you single cell analysis how many cells were acquired for each of the 4 subsets. I assume less ILCs were analyzed. If so, can you please clarify for the non-bioinformatician how your bioinformatic analysis took these differences into account?

Indeed, we were concerned that we might have too few ILCs and therefore set up conditions so that similar numbers of each cell type were assessed. To accumulate enough ILCs for single cell analysis, ILCs were sorted from 3 donors. ILCs, $CD56^{hi}$, $CD56^{dim}$ and $CD56^{-}$NK cells were sorted in parallel from the same 3 donors. The sorting strategy is shown in Supplemental Figure 1D. Equal numbers of ILCs, $CD56^{hi}$, $CD56^{dim}$ and $CD56^{-}$NK cells were sorted and then mixed together before library preparation. In total, libraries were generated from 5,210 single cells using 10 x genomics. 1,478 ILC2s, 897 ILCPs, 1,116 $CD56^{hi}$NK cells, and 1,486 $CD56^{dim}$ and $CD56^{-}$NK cells were shown in UMAP. We have added this information in Figure 3 legend.

Reviewer #1 (Significance (Required)):

Overall, this paper serves as an important resource for the field.

There are areas in which the manuscript could be modified to improve clarity and detail regarding rigor (see above). The overall message of this work for understanding HIV pathogenesis is unclear but can be addressed.

We appreciate the reviewer's efforts on behalf of our manuscript. By carefully addressing the reviewer's questions and comments, we believe that the rigor of our manuscript is improved and that the message regarding HIV-1-induced abnormalities of ILCs and NK cells are clarified.

Full Revision

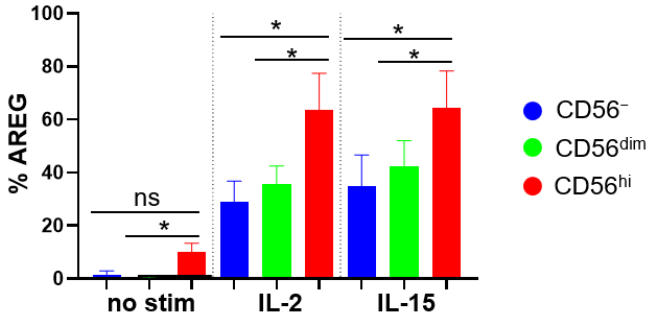
Reviewer #2 (Evidence, reproducibility and clarity (Required)):

Summary: This manuscript makes use of RNA sequencing, ATAC-Seq and single cell sequencing to compare transcriptomes and identify relationships between various ILC/NK cell subsets in blood from uninfected controls. The authors describe differences in genes and pathways between subsets and determine that CD56high NK cells are an intermediate-connecting cell type between ILC progenitors and CD56dim NK cells. The authors show how HIV infection (viremic, ART+ or controllers) effect ILC/NK cell gene expression and pathways. One of the genes enhanced in NK cells is AREG which the authors demonstrate to be upregulated in IL/NK subsets in response to PMAI/I stimulation or stimulation with IL-2 or IL-15. The authors demonstrate that TGFB1 or knockout of RUNX3 modulates the frequency of AREG+ NK cells, implicating the Wnt signaling pathway in AREG regulation. Authors show that in PLWH, the frequency of AREG+ NK cells is altered. Authors also report that with HIV infection there is an increase in CD56neg NK cells with corresponding loss of CD56dim cells. Using healthy control PBMCs, the authors suggest differentiation of CD56dim into CD56neg is prevented by CD4 T cell production of IL-2 but promoted by TGFB1. Also in healthy controls, the authors run metabolomics to describe how CD56dim are more metabolically functional than CD56neg and link this to MTOR activity.

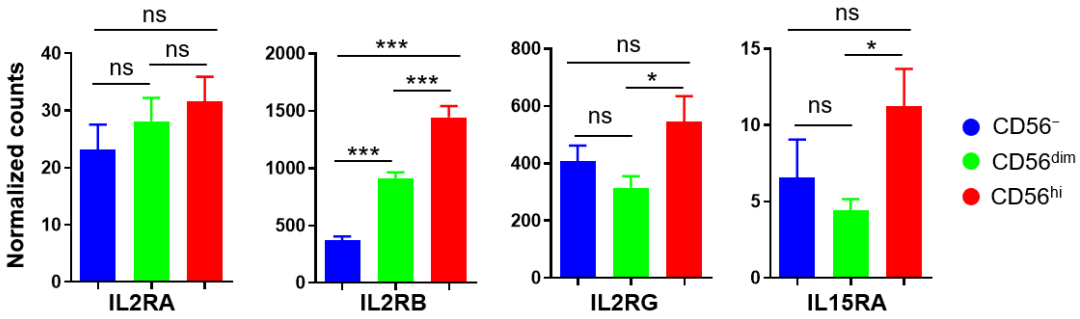
Major:

• Which population(s) of NK cells (high, dim or negative for CD56) are responding to stimulation with IL-2 and IL-15 by upregulating AREG in Figure 5? Does this NK population have higher expression of receptors for IL-2 and IL-15 (in single cell seq data or by flow) and if so how does receptor expression change in PLWH?

We thank the reviewer for these excellent questions. In response, we have added the new figure below to Supplemental Figure 4E. In general, CD56^{hi} NK cells have higher AREG RNA and protein than do the other NK cell subsets. After IL-2 or IL-15 stimulation, all three NK cell populations upregulate AREG, though CD56^{hi}NK cells produce significantly higher levels of AREG than do either CD56^{dim} or CD56^{neg}NK cells.



Consistent with the above results, the expression of IL2RB, IL2RG and IL15RA was higher in CD56^{hi}NK cells than in CD56^{dim}NK cells (new Supplemental Figure 4F).



We examined IL2RA, IL2RB, IL2RG, and IL15RA expression in the different NK cell subsets from the three groups of people living with HIV-1, ART, viremic, and controllers. As compared with cells from HIV-1-negative people, the only clearly significant difference for these four genes was a reduction in IL2RB expression in CD56^{-} and CD56^{dim} NK cells from viremic people. These points are now mentioned on page 15.

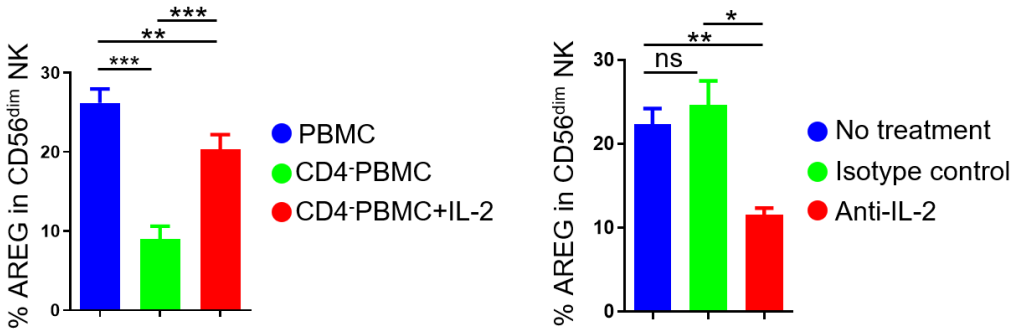
• Does signaling through IL-2 or IL-15 converge on the TCF7/Wnt signaling pathway? For example, is it possible to knockdown TCF7 then stimulate with IL-2 or IL-15 and measured AREG+ cells with the idea that loss of TCF7 would result in reduced cytokine-induced activation of AREG if mediated through the Wnt pathway?

Indeed, this question was very important to us and we attempted to answer it with lentiviral vector-mediated knockdown with shRNA and with electroporation of Cas9 ribonucleoprotein complexes (RNPs). Though we were successful in decreasing TCF7 protein, to render NK cells competent for lentiviral transduction or for electroporation with the Cas9 RNPs, NK cells must first be cultured for at least 7 days in high concentration IL-2. This treatment with IL-2 activates AREG production before TCF7 protein is decreased. These points are now discussed in the manuscript on page 29.

• In Figure 8 the authors demonstrate that CD4 T cells promote the maintenance of CD56dim NK cells likely through production of IL-2. Was AREG expression examined in these studies and if so could you conclude that CD4 T cells also promote AREG expression in this population?

Yes, indeed, CD4^{+}T cells, or IL-2, promote AREG production by CD56^{dim}NK cells. Depletion of CD4^{+}T cells from PBMCs decreased AREG production and addition of exogenous IL-2 was sufficient to restore AREG production by NK cells when CD4^{+} T cells were depleted (see figure below, left panel). Additionally, IL-2

neutralizing antibodies decreased AREG production by NK cells in total PBMC cultures (right panel). These results are now included as Supplemental Figure 6G and 6H in the revised manuscript.

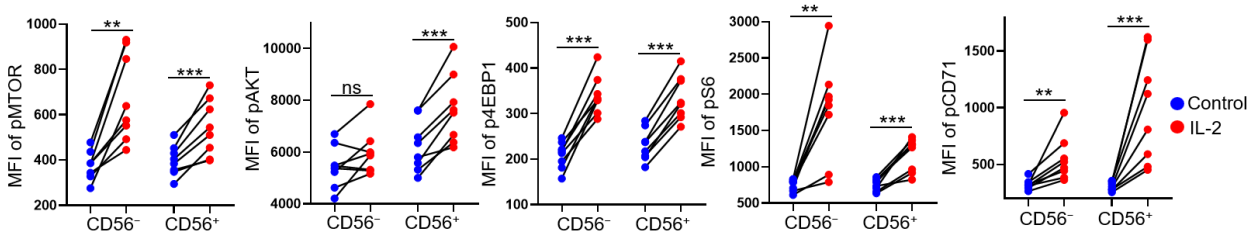


• In Figure 8D, although significant, the increase in percentage from 2-4% of the CD56neg is a minimal change. Could a higher dose of IL-2 be tested to see more substantial changes? Similar comment for Figure 10D, although significant, the increase in percentage from 2-4% of the NK population to CD56neg is not a large change with rapamycin. Could a higher dose be tested (that does not kill the cells) or a different inhibitor be used to see more substantial changes? More robust changes would strengthen the conclusions.

First to clarify, as concerns figure 8D, we believe the reviewer is referring to IL-2 neutralizing antibody, not IL-2. Regarding Figure 8D, which used IL-2 neutralizing antibody at 4 ug/ml, we tried a higher dose (10 ug/ml) but this caused cell death. We also tried longer culture time with 4 ug/ml IL-2 neutralizing antibody, and found the cell viability decreases after 7 days culture. Similarly, the limited *in vitro* culture time in the presence of anti-IL-2 antibody restricted experimental conditions in Figure 10D.

• In Figure 10, was the increase in pmTOR with IL-2/IL-15 stimulation specifically observed in CD56dim cells (rather than total NK cells or CD56neg cells)? This would strength the conclusion that CD56dim is more metabolically functional than CD56neg.

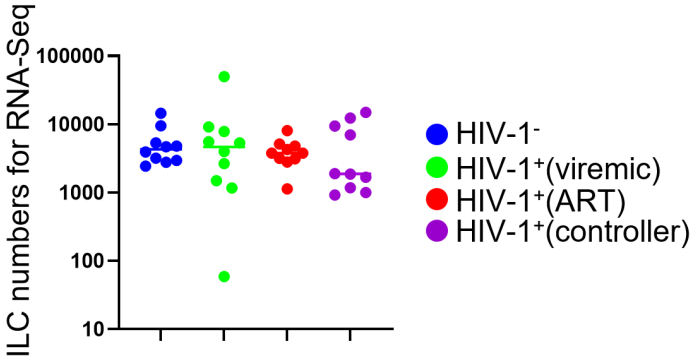
Our data indicate that the mTOR response to IL2 in $CD56^{-}$NK cells was similar to that in $CD56^{+}$NK cells (including $CD56^{dim}$ and $CD56^{hi}$) (see figure below). This result is consistent with a previous report showing that, when stimulated with exogenous IL-2, sorted $CD56^{-}$ NK cells become $CD56^{dim}$NK cells (Mavilio *et al*, 2005). Our data extend this observation by showing that upregulation of CD56 on CD56neg NK cells by IL-2 correlates with mTOR upregulation.



Minor

• Approximately how many ILC from PLWH were submitted for single cell sequencing: Since this cell population was depleted in HIV+, was there a sufficient number of cells to interpret/publish DEG results in Figure 4?

In Figure 4, ILCs were sorted from HIV-1 negative donors and different groups of PLWH, and then these cells were subjected to bulk-RNA seq using CEL-Seq2. In Figure 4, for most blood donors, whether HIV-1 negative or PLWH, well over 1,000 ILCs were sorted and used to construct high quality libraries (see figure below, was included as new Supplemental Figure 3C). PCA plots also showed that all ILC libraries clustered together as a distinct population (Figure 4A), indicating the quality of all ILC libraries was comparable.



• On page 13 of text, it is a bit disconcerting to jump to Figure 7 then back to Figure 4. Would recommend reorganizing text or figure panels to flow better.

We have deleted the premature mention of Figure 7A.

• Supplemental Figure 1 and 2: Dots for ILC population are purple in color but legend is mislabeled as pink color.

• Supplemental Figure 4: Dots for HIV+ controller are purple in color, but legend is mislabeled as pink in color.

Full Revision

Thank you, these mistakes in the labeling of the colors have been corrected.

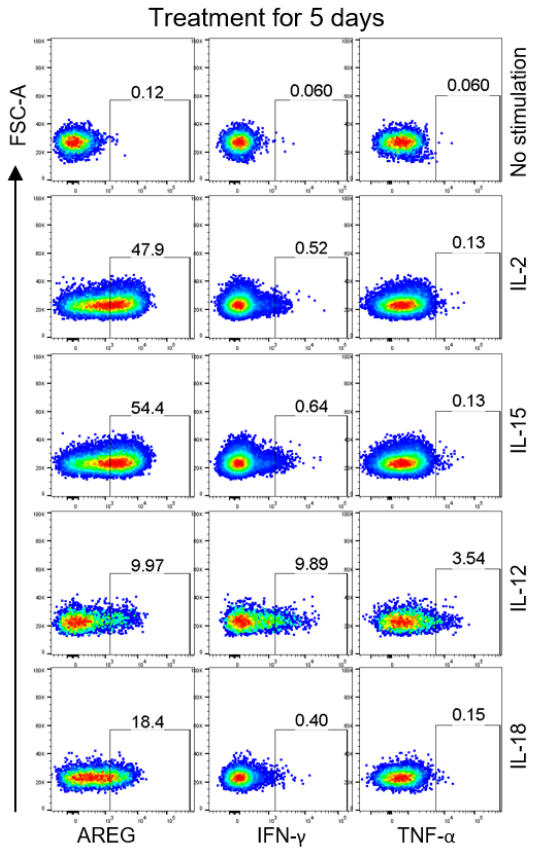
• Would recommend adding quantification of Figure 5A for all donors tested.

In fact, the AREG production by CD56^{hi}, CD56^{dim}, CD56^{-}NK cells and ILCs from HIV-1 negative donors were quantified in Figure 6A.

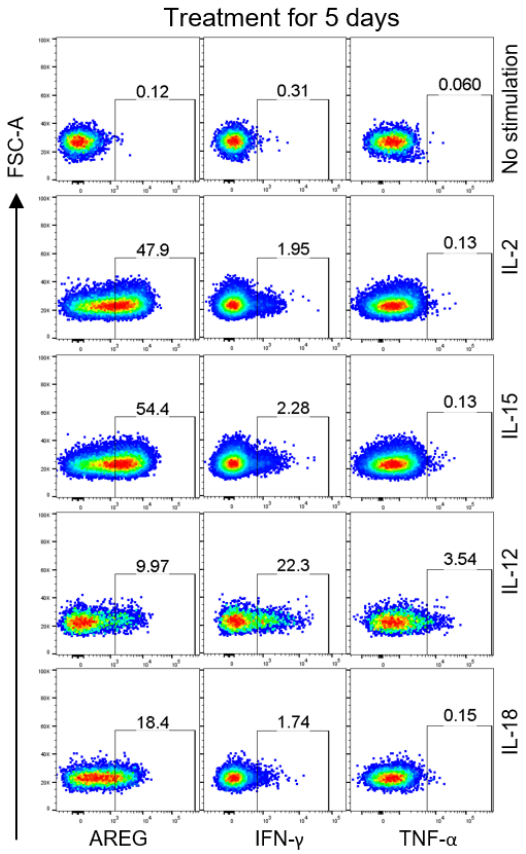
• Figure 5B for IFNg it appears that part of the positive population is not gated on and thus percentages are likely higher if the gate is adjusted.

We thank the reviewer for the suggestion. The gating for IFN-γ has been adjusted in the revised manuscript, as suggested. The original Figure 5B and associated histogram Figure 5C were replaced accordingly. Please see the newly gated figure below:

Original



Re-gating for IFN-γ



• For Figure 5F, need to add to text and panel that PMA/I was used to stimulate as described in figure legend. Current figure and text read that the Wnt agonist alone was responsible for levels depicted in NK subsets.

We now state that PMA+ionomycin was used to stimulate the cells in Figure 5F and 5G, and in the text (page 16 and 28).

• On the x-axis of Figure 6F-J does "Lin-TBX21+" refer to Total NK cells? If so then would recommend sticking with nomenclature "Total NK" as in Figure 6A-B.

The x-axis of Figure 6F-J was changed to "total NK (Lin^-TBX21^+)".

• Would recommend labeling gates for populations of interest in Supplemental Figure 5B-C, Supplement Figure 6A, Figure 8A.

The populations of interest ($CD56^{dim}$NK, $CD56^-$NK, and ILCs) were labeled as suggested.

• Other groups have shown that CD56neg are increased in HIV, functionally impaired and correlate with loss of CD4 T cells. Would recommend citing their studies.

The original manuscript had mentioned only Mavilio et al. The revised manuscript now has a more complete list of references (Cao *et al*, 2021; Mavilio *et al*, 2005; Cocker *et al*, 2022; Alter *et al*, 2005; Barker *et al*, 2007). These references are cited in the Introduction (page 5) and in the Results (page 20).

• Would recommend adding quantification of Figure 8A for all donors tested. Also, for the 3rd flow plot does "NK+CD4" mean purified NK cells + autologous CD4 T cells? If so, then would clarify in figure legend. It may strengthen conclusion for IL-2/IL-15 to show differentiation of NK cells is not contact dependent with T cell via transwell assay.

Quantification for all donors in Figure 8A has been added to the revised manuscript (Figure 8A, right panel). The figure legend has been modified accordingly. As far as the comment about contact-dependence, the fact that $CD56^{dim}$NK cells were maintained by IL-2 in the absence of $CD4^+$ T cells demonstrates that direct contact with $CD4^+$ T cells is not required.

Reviewer #2 (Significance (Required)):

Significance: This study is valuable because it generates large datasets on the NK/ILC family from human blood that can be deposited in repositories and of special relevance to the HIV field because it examine show viral infection (controlled or not) effects these subsets.

The strengths of the study are the cohort of PBMC samples utilized (HIV-, HIV+ viremic, HIV+ ART+ and HIV+ controlled), the multi-omics approach for transcriptome and epigenetics and the in vitro mechanistic studies identifying key regulators of NK cell differentiation/function.

The study advances the field "mechanistically" by providing key targets that may be subject to therapeutic modulation in PLWH such as AREG, IL-2 signaling, IL-15 signaling, Wnt signaling, and mTOR activity (although more work will need to be done to examine these pathways using PBMCs from HIV+). This study advances the field "conceptually" by providing large datasets for others to mine if deposited.

The audience for this study "basic research" such as immunologists in the HIV field or immunologist interested in ILC/NK biology.

My field of expertise is infectious disease (HIV, SARS-CoV-2), basic immunology (ILC, NK, B cell) and autoimmune disease. Would recommend additional reviewers for assessment of metabolic genes/pathways in Figure 9-10.

We were very pleased to see that the reviewer thought our manuscript makes a valuable contribution to HIV-1 immunology and ILC/NK biology, that it advances mechanistic understanding of pathogenesis in people living with HIV-1, and that it provides a valuable data resource.

- In Figure 9, the metabolism related genes were defined as canonical targets of, or regulated by, MTOR signaling, in previous publications (Saxton & Sabatini, 2017; Bayeva *et al*, 2012).

- In Figure 10, we used pMTOR, pAKT, p4EBP1, pS6 and CD71 to monitor MTOR pathway activation as reported previously by others (Marçais *et al*, 2014). We have cited this paper on pages 23 and 24.

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Dear Jeremy,

Thank you for submitting your manuscript to The EMBO Journal. The manuscript was transferred to The EMBO Journal from Review Commons where the review process was carried out. The manuscript was revised according to the referees' comments and transferred to The EMBO Journal.

Your revision has now been seen by the two original referees and their comments are provided below. As you can see the referees appreciate the introduced revisions and support publication here. I am therefore pleased to let you know that we will accept the manuscript for publication here.

Before, formally accepting the manuscript it needs to be formatted and fitted to EMBO Journal style - we also need source data. My colleagues Bojana and Hannah (CCed in) will get get back to you within the next week with editorial revision points and a source data list that need to be dealt with before resubmission.

Once we get the revised version in, we will do the figure checks on the revision.

Just a few comments from my side, I think we should consider having the manuscript marked as a resource paper rather than a regular article. Resource papers should include a structured Material and Methods section that includes a Reagents and Tools Table followed by a Methods and Protocols section - see also our Author guidelines.

Can you also take a careful look at the title and abstract, I almost find both too detailed.

Congratulations on a nice study!

Best Karin

Karin Dumstrei, PhD
Senior Editor
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Referee #1:

Summary: This manuscript details differences between NK cells and ILCs in HIV+ individuals (ART+/-, controllers) compared to healthy controls using a variety of techniques (flow phenotyping, RNAseq, single cell seq, ATAC seq, metabolomics, in vitro exp). The many differences reported between cells types make this a great asset to the NK/ILC/HIV field.

Opinion: In the revised manuscript the authors have thoroughly addressed the concerns of both reviewers by modifying the text and adding data to the figures as needed. The manuscript is improved in clarity and rigor. No major or minor concerns remain from this reviewer.

Referee #2:

The authors have addressed referee comments satisfactorily.

***
Rev_Com_number: RC-2022-01810
New_manu_number: EMBOJ-2023-114153
Corr_author: Luban
Title: Global profiling of human blood ILC subtypes reveals that NK cells produce homeostatic cytokine amphiregulin and sheds light on HIV-1 pathogenesis

Editorial Revision Points

Dear Prof. Luban,
I am following up with you regarding the decision below with a list of formatting points that need to be addressed prior to the resubmission of your manuscript.

Please make sure to resolve the following formatting issues:

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2) The main figures need to be removed from the manuscript and uploaded separately, as high-resolution figure files. Please
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manuscript vs. Pamela StLouis in eJP
4) The paragraph with "address correspondence to..." should be removed from p 32; alternatively can be placed on the title page
5) Make sure the funding information in the manuscript and in submission system is congruent - it appears that U54AI170856 is
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6) Please add up to five keywords, which may or may not appear in the title, in alphabetical order, below the abstract, with each
word separated by a slash.
7) To list the primary data generated in your study, please include a formal "Data Availability Section" (after Materials &
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8) We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and
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Thank you.
Kind regards,
Bojana
Bojana Perkucin
Editorial Assistant
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The authors have addressed all minor editorial requests.

Dear Jeremy,

Thank you for submitting your revised MS to the EMBO Journal. I have now had a chance to take a careful look at it and it all looks good.

I am therefore very pleased to accept the manuscript for publication here.

with best wishes

Karin

Karin Dumstrei, PhD
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The data shown in figures should satisfy the following conditions:

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- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
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- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
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- definitions of statistical methods and measures:

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Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

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Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Not Applicable	

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods
Animal observed in or captured from the field: Provide species, sex, and age where possible**.**	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Yes	Dataset EV6

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgements

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)

If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	**Yes**	Materials and Methods
Include a statement about **blinding** even if no blinding was done.	**Yes**	Materials and Methods
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	**Yes**	Materials and Methods
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	**Yes**	Materials and Methods

Sample definition and in-laboratory replication	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Not Applicable	
In the figure legends: define whether data describe **technical or biological replicates**.	**Yes**	Figure Legends

Ethics

Ethics	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	**Yes**	Materials and Methods
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	**Yes**	Materials and Methods
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	**Yes**	Materials and Methods
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	**Information included in the manuscript?**	**In which section is the information available?** (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	**Yes**	Data Availability Section
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	**Yes**	References and Data Availability Section