EMBO reports

Engagement of TRAIL Triggers Degranulation and IFNγ Production in Human Natural Killer Cells

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

Dear Dr. Körner

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge that the findings are potentially interesting. However, all three referees raise similar criticisms of the data and indicate that a major revision will be required to substantiate and extend the conclusions. Genetic evidence will be required to substantiate that TRAIL reverse signalling takes place and contributes to NK cytotoxicity. Moreover, assays to monitor the cytotoxicity of NK cells beyond CD107a expression/degranulation are required. I think that all of the concerns raised are important and should be addressed in a revision.

The comments are constructive and overall positive and we would therefore like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Since it is clear from the referee comments, that a major revision will be required we ask you to contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further.

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1) A data availability section is missing.
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6) We replaced Supplementary Information with Expanded View (EV) Figures and Tables that are collapsible/expandable online. A maximum of 5 EV Figures can be typeset. EV Figures should be cited as 'Figure EV1, Figure EV2" etc... in the text and their respective legends should be included in the main text after the legends of regular figures.

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- Additional Tables/Datasets should be labeled and referred to as Table EV1, Dataset EV1, etc. Legends have to be provided in a separate tab in case of .xls files. Alternatively, the legend can be supplied as a separate text file (README) and zipped together with the Table/Dataset file.

7) Please note that a Data Availability section at the end of Materials and Methods is now mandatory. In case you have no data that requires deposition in a public database, please state so instead of refereeing to the database.
See also < https://www.embopress.org/page/journal/14693178/authorguide#dataavailability>). Please note that the Data Availability Section is restricted to new primary data that are part of this study.

8) Figure legends and data quantification:
The following points must be specified in each figure legend:
- the name of the statistical test used to generate error bars and P values,
- the number (n) of independent experiments (please specify technical or biological replicates) underlying each data point,
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- If the data are obtained from n {less than or equal to} 2, use scatter blots showing the individual data points.
Discussion of statistical methodology can be reported in the materials and methods section, but figure legends should contain a basic description of n, P and the test applied.
See also the guidelines for figure legend preparation:
https://www.embopress.org/page/journal/14693178/authorguide#figureformat
- Please also include scale bars in all microscopy images.

9) We would also encourage you to include the source data for figure panels that show essential data. Numerical data should be provided as individual .xls or .csv files (including a tab describing the data). For blots or microscopy, uncropped images should be submitted (using a zip archive if multiple images need to be supplied for one panel). Additional information on source data and instruction on how to label the files are available .

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I look forward to seeing a revised form of your manuscript when it is ready. Please use this link to submit your revision:
https://embor.msubmit.net/cgi-bin/main.plex

Please let me know if you have questions or comments regarding the revision.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

Review of manuscript EMBOR-2021-54133-T by Höfle et al.

This manuscript investigates the function of TRAIL as an activating receptor for NK cells. The experiments are based on the interesting finding that NK cells which degranulate upon contact with autologous HIV-1 infected CD4 T cells express higher levels of TRAIL. The authors then go on to show that TRAIL is not simply up-regulated in response to the degranulation and provide some evidence that TRAIL engagement by up-regulated DR4 on HIV-1-infected T cells may contribute to NK cell degranulation and IFNg production.

The subject of reverse signaling of TRAIL is not new and the mechanism and relevance are being controversially discussed. Here, the authors use blocking antibodies to interfere with TRAIL reverse signaling or plate-bound TRAIL receptors or antibodies to induce it. The effects they observe are mostly modest. Therefore, it would be good to have a clearly defined system using genetically modified target or NK cells to definitively show an impact of TRAIL reverse signaling on NK cell function. This would also provide the opportunity to determine if TRAIL can actually induce NK cell cytotoxicity in addition to degranulation.

Specific points:

1. Figure 4: The effect of blocking degranulation using anti-TRAIL or anti-DR4/5 antibodies appears to be only significant once the data are normalized and expressed as percent inhibition. However, this normalization reduces variability in the data and amplifies the effect of the blocking antibodies. The same applies to Figure 6B. This may result in overestimating the effect of the blocking antibodies. To support their conclusions the authors should establish a cellular system where they can clearly interrogate the effect of TRAIL. They could knockout DR4/5 in 721.221 cells or overexpress OPG or DR4 on target cells to have a pair of target cells that only differ in their ability to trigger TRAIL on NK cells. Alternatively, they could use CRISPR/Cas9 to knockout TRAIL in NK cells to compare TRAIL+ and TRAIL- NK cells. As the observed effects are small (especially in light of the fact that the authors use cytokine stimulated NK cells to induce TRAIL expression in 70-90% of all NK cells for these experiments) and the mechanism of TRAIL reverse signaling are unclear, such a clean cellular system is essential to support the authors' conclusions.
2. The authors only determine CD107a expression as a marker for degranulation. They conclude from this that TRAIL can induce NK cell cytotoxicity e.g., against HIV-1 infected targets. However, they never show target cell death in their experiments. Interestingly, they observe most TRAIL-induced degranulation in CD56-bright NK cells (Fig. 2A), which are known to be not very cytotoxic. Therefore, a cytotoxicity assay actually determining the death of target cells that is induced upon TRAIL engagement is important.

Minor points:

3. Figure 1: The authors mention that TRAIL is just one of 48 markers that was enriched on degranulating NK cells upon contact with HIV-1 infected T cells. Were there also other known activating receptors among the 47 surface molecules? How important is the contribution of TRAIL to the killing of HIV-1 infected cells?
4. Figure 2 and Page 5 end of 1st paragraph: I do not understand the argument made by the authors that their data show that TRAIL is not just a surrogate marker for NK cells that possess inherently increased anti-HIV-1 activity. To me there is a clear correlation between NK subsets that express high TRAIL levels and that also degranulate more. Based on the data presented in this figure there does not need to be a causal relationship.
5. Figure 5: When stimulating NK cells with plate bound DR4 or OPG do only the TRAIL positive NK cells degranulate? This would be another proof of specificity.
6. Figure 5: Why is the degranulation reduced when using higher concentrations of DcR1?

Referee #2:

In this manuscript, Höfle et al establish that the killer ligand TRAIL is tightly associated with CD107a expression on NK cells, which was identified in a screen for NK cell receptors implicated in the recognition of HIV-infected CD4+ T cells. The authors then describe a pathway through which TRAIL activation or inhibition on NK cells either promote or diminish their functional activation via the regulation of CD107a expression / degranulation or the production of effector cytokines.
In general, the manuscript is well written, and the data are clear and well presented. Note that a previous study by others already reported that TRAIL regulates the degranulation / CD107a expression of (IL-18/poly I:C-) activated blood and liver human NK cells, which promoted the killing of target cells (PMID: 30748035), which takes away some of the novelty of the current study.
I have several comments to the authors, which are detailed below.

Major comments
• Line 97: the authors should be more explicit on why they conclude that TRAIL is not a "surrogate marker for subsets with inherently increased anti-HIV-1 activity". Indeed, they define "anti-HIV-1 activity" or "HIV-specific response" as frequency of CD107a+ on NK cells, and TRAIL expression is positively correlated with CD107a expression. Or do they mean there that

TRAIL is not specific to (CD107 positive) NKG2A+ versus KIR NK cells or to (CD107a positive) bright versus dim NK cells?
• Does the improved degranulation / increased expression of CD107a on NK cells results in increase target cell elimination or apoptosis (which is a more relevant readout than degranulation per se)?
• Along the previous comment: CD56dim (CD16+) NK cells are commonly referred as being more cytotoxic than CD56bright (CD16dim/–) NK cells (PMID: 19278419). However, their findings indicate increased general levels of degranulation in the latter population. How do they reconcile these contradicting facts?
• To determine TRAIL receptor expression by flow cytometry, a combination of antibodies against DR4 and DR5 is used (Fig. 3A&B, Fig. 4E). Yet they report in Fig. 5B that DR4 and DR5 have a differential effect on NK cells degranulation / CD107a expression. Therefore, it would be pertinent to distinguish DR4 from DR5 expression on NK target cells.
• Along these lines: are DR4/5 expressed on NK cells in their system? If yes, they should test the effect of TRAIL binding to TRAIL receptor(s) on their NK cells, regarding induction of apoptosis and/or degranulation. The latest would validate that the findings shown in this study represent bona fide reverse signaling of TRAIL.
• How do they mechanistically explain that soluble anti-TRAIL antibody blocks (Fig 4A) while plate-bound anti-TRAIL antibody activates (Fig 5A) NK cell degranulation in their system? A further comment: the anti-TRAIL antibody clone RIK-2.1 was used for flow cytometry studies while clone RIK-2 was used both for TRAIL blockade and TRAIL crosslinking. Are those the same clones?
• The same question applies to the experiments using anti-DR4 and anti-DR5 antibodies to block (while in soluble form) versus activate (after plate coating) NK cell degranulation. Were the same antibody clones used for these experiments (clone HS101 and clone HS201, respectively)?
• How do they explain the contrasting effects on NK cell degranulation of the different TRAIL receptors they tested? These TRAIL receptors apparently have different affinities for TRAIL (ligand) (PMID: 10770955), yet it does not appear that their relative ligand binding affinities may explain these differential effects on degranulation. Have they tested the ability of these (purchased / commercially available) receptors to bind TRAIL (and e.g. prevent TRAIL-induced cell death) after plate coating?
• Why is the inhibition of degranulation markedly smaller with anti-DR4/5 on 721.221 B cell targets compared to anti-TRAIL on NK effector cells? Fig 4B versus Fig 4G.
• Fig 5B: since not all NK cells express TRAIL in their system, they should demonstrate, as shown for the positive correlation between TRAIL and CD107a expression, that IFNg is preferentially produced in TRAIL-expressing NK cells.
• They refer to a previous publication (PMID: 31742873) indicating that TRAIL-deficient murine NK cells do not show difference in CD107a expression compared to wild-type controls (presented in the discussion). It this same study, it was reported that these TRAIL-deficient murine NK cells produce more IFNg upon activation (not discussed in the current manuscript). This is in contrast with their findings indicating that TRAIL signaling promotes CD107a expression and IFNg production. The authors suggest that there may be differences in TRAIL signaling between mice and humans. This is possible, yet reverse TRAIL signaling apparently activates cytokine production both in murine (PMID: 11466352) and in human T cells (PMID: 14872508). These different aspects need to be more discussed.
• The authors should better discuss how their findings are different from or extend the ones of Li et al., which they cite in the discussion (PMID: 30748035).

Minor comments
• Fig 4A&C, Fig 5A&B: it would be helpful to see in these density plots were the cutoff was set to determine positivity versus negativity of CD107a expression.
• KIR-educated cells and NKG2A-educated cells should be defined in M&M in addition to the description in the legend of Fig. 2.
• They should indicate in the figure legends the number of independent experiments that were performed and produced data comparable to the ones presented. Alternatively, they should mention whether the data shown were pooled from several independent experiments.

Referee #3:

1. Does this manuscript report a single key finding? YES
If YES, please describe it in one sentence:
TRAIL triggers degranulation, granzyme B release and IFNgamma production in NK cells.

2. Is the reported work of significance (YES), or does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms etc (NO)?
YES. This is the first report of TRAIL reverse signaling in human NK cells. However, TRAIL reverse signaling has already been reported in murine NK cells and in T cells (as correctly discussed by the authors in lines 189-202 of this manuscript).

3. Is it of general interest to the molecular biology community? NO
If YES, please say why, in a single sentence. If NO, please state which more specialized community you feel it is aimed at (or none), in a single word or phrase.

The main conclusion of this study is certainly of interest for immunologists and virologists but I am afraid that its appeal beyond these disciplines remains limited.

4. Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longer¬format article (NO)?
NO
As outlined in my comments below, I feel that the conclusion of "reverse signaling" is not yet sufficiently backed up by the presented data.

In their study, Höfle et al. report that reverse signaling of TRAIL triggers degranulation and IFNgamma production in human NK cells. While performing a screen for NK cell receptors involved in the recognition of HIV-infected cells, the authors found TRAIL as a candidate. They show that NK cells which recognize HIV-infected CD4 T cells display an elevated TRAIL expression on their surface. They further show that TRAIL is neither a surrogate nor an activation marker in this setting and that surface expression of TRAIL-R1 and TRAIL-R2 is increased on HIV-infected CD4 T cells. Höfle and colleagues furthermore report that antibodies against TRAIL or TRAIL-R1/TRAIL-R2 diminish NK cell degranulation in co-culture experiments. Coating plates with TRAIL antibodies or with recombinant TRAIL-R1/TRAIL-R2, they report that this coating induces an enhanced degranulation and release of granzyme B in NK cells. Two other coated TRAIL-binding receptors, OPG and TRAIL-R3, likewise increase NK cell degranulation (TRAIL-R4 is not tested in this study). In supernatants from the coculture experiments (antibodies against TRAIL or TRAIL-R1/TRAIL-R2), the authors find reduced IFNgamma levels, whereas increased levels are reported in supernatants collected from the experiments using plates coated with TRAIL antibodies or with recombinant TRAIL-R1 (supernatants from the other ligands are not tested). Finally, the authors investigate whether the observed induction of degranulation and IFNgamma production are caused by an increased ability of the NK cells to adhere to their target cells, but do not find evidence for this. Based on these results, the authors suggest that TRAIL contributes to the anti-HIV-1 activity of NK cells beyond receptor-mediated cytotoxicity through reverse signaling.

The manuscript is very well and carefully written, and the experiments are at a high technical level. I have no concerns with the experimental design or the integrity and quality of the presented data. It is obvious that the authors have great experience in flow cytometry (almost all experiments are designed to use flow cytometry), and this is reflected by the way the data are presented. Yet, given that EMBO Reports is targeted at a broader audience, I feel that the authors may wish to enhance the accessibility and clarity of their presentation by adding more explanations, if the character limit permits. As one example: in line 48, the authors should explain that CD107a is a marker for NK cell degranulation for readers from non-related fields.

I feel that this study is certainly of interest, but needs to be strengthened to provide the novel substantial physiological insight that EMBO reports is seeking for. For me, it is not convincingly demonstrated that reverse signaling by TRAIL actually takes place. If yes, the question remains how important this pathway is for the cytotoxic response of NK cells.

Specific comments:

Major:

1. Currently, the authors demonstrate the presence of effects that are statistically significant, but nevertheless only of modest nature (e.g., in Figures 5AB when compared to the positive controls, but also throughout the study). For me, it remains open to which extent the engagement of TRAIL on NK cells contributes to the total cytotoxic response of NK cells against their targets. Is this a major pathway for the elimination of pathogens or just a minor additional mechanism, if at all? This could, e.g., be addressed by deleting TRAIL from primary human NK cells or NK cell lines via CRISPR/Cas (such gene deletion has been established, e.g., for primary T cells, PMID: 29436394).

2. The study is currently limited to measurements of degranulation, granzyme B release and IFNgamma production. Yet, it is not shown whether the manipulation of TRAIL reverse signaling has also an effect on the cytotoxic activity of NK cells as the ultimately relevant response. In my opinion, such a demonstration would significantly strengthen the relevance and impact of this study.

3. The existence of TRAIL reverse signaling is controversial due to the very short cytoplasmic moiety of TRAIL (PMID: 33215853). Therefore, the study would gain impact if the authors provided more conclusive evidence for intracellular signaling in response to TRAIL engagement (e.g., p38 activation, as discussed in line 231) beyond solely describing cellular responses after inhibition or engagement of TRAIL.

Minor:

1. The authors use OPG and TRAIL-R3 as engagers of TRAIL and should mention whether they are expressed on CD4 T cells, i.e., whether they have physiological relevance in this system.

2. In Figure. 4F, G, degranulation is reduced by antibodies to TRAIL-R1/TRAIL-R2. Have the authors excluded that this is due to agonistic killing of the target cells by the antibodies (i.e., less target cells, less engagement of NK cells, less degranulation)?

3. In Figure 6A, plate-coated anti-TRAIL antibodies induce IFNgamma, whereas in Figure 6B, soluble anti-TRAIL antibodies reduce its production in the coculture system. Please explain this discrepancy to the reader (no crosslinking of TRAIL by the soluble antibodies?).

Response to Referee #1

Referee #1:

Review of manuscript EMBOR-2021-54133-T by Höfle et al.
This manuscript investigates the function of TRAIL as an activating receptor for NK cells. The experiments are based on the interesting finding that NK cells which degranulate upon contact with autologous HIV-1 infected CD4 T cells express higher levels of TRAIL. The authors then go on to show that TRAIL is not simply up-regulated in response to the degranulation and provide some evidence that TRAIL engagement by up-regulated DR4 on HIV-1-infected T cells may contribute to NK cell degranulation and IFNg production. The subject of reverse signaling of TRAIL is not new and the mechanism and relevance are being controversially discussed. Here, the authors use blocking antibodies to interfere with TRAIL reverse signaling or plate-bound TRAIL receptors or antibodies to induce it. The effects they observe are mostly modest. Therefore, it would be good to have a clearly defined system using genetically modified target or NK cells to definitively show an impact of TRAIL reverse signaling on NK cell function. This would also provide the opportunity to determine if TRAIL can actually induce NK cell cytotoxicity in addition to degranulation.

Reply: We would like to thank the referee for their time and effort to critically review our manuscript. We are grateful for the constructive criticism and suggestions provided by the referee. These helped us improving the quality of the manuscript. In that regard, we conducted additional experiments, performed additional statistical analyses and revised the manuscript accordingly. Please see our detailed point-by-point response to the referee's comments below.

Specific points:
1. Figure 4: The effect of blocking degranulation using anti-TRAIL or anti-DR4/5 antibodies appears to be only significant once the data are normalized and expressed as percent inhibition. However, this normalization reduces variability in the data and amplifies the effect of the blocking antibodies. The same applies to Figure 6B. This may result in overestimating the effect of the blocking antibodies.
To support their conclusions the authors should establish a cellular system where they can clearly interrogate the effect of TRAIL. They could knockout DR4/5 in 721.221 cells or overexpress OPG or DR4 on target cells to have a pair of target cells that only differ in their ability to trigger TRAIL on NK cells. Alternatively, they could use CRISPR/Cas9 to knockout TRAIL in NK cells to compare TRAIL+ and TRAIL- NK cells. As the observed effects are small (especially in light of the fact that the authors use cytokine stimulated NK cells to induce TRAIL expression in 70-90% of all NK cells for these experiments) and the mechanism of TRAIL reverse signaling are unclear, such a clean cellular system is essential to support the authors' conclusions.

1. Reply: We apologize for the missing p values for the cumulative data sets of figure 4 (A, right panel; C, right panel; F) and figure 6B and for any misleading assumptions that this may have created. Statistical comparisons of the cumulative data sets showed similar outcomes as shown for the transformed data sets in the panels B, D, and G (% inhibition of degranulation). After exposure to 721.221, levels of NK cell degranulation were significantly lower in the presence of αTRAIL as compared to the co-culture alone, while the addition of the respective isotype control had no effect on the relative frequency of $CD107a^+$ NK cells (αTRAIL: $p = 0.004$; Isotype: $p = 0.84$). A similar effect was observed when using autologous HIV-1-infected CD4 T cells as target cells (αTRAIL $p = 0.008$; Isotype: $p > 0.99$). Blocking DR4 and DR5 on 721.221 cells also led to significantly reduced levels of degranulating NK cells (αDR4/5: $p = 0.008$; Isotype: $p > 0.99$). The same applies to assessment of IFNγ production after TRAIL blocking in Figure 6B (αTRAIL: $p = 0.016$; Isotype: $p >$

0.99). Statistical analyses were performed using Wilcoxon signed-rank test adjusted for multiple comparisons (Bonferroni). Nevertheless, we do acknowledge the referee's concern and now report the respective p values in figure 4 and 6 and in the results section of the manuscript. We are aware that data transformation such as the conducted normalization is accompanied with loss of information, but can also provide additional information. Therefore, display of the relative inhibition of NK cell degranulation was not provided solely but rather in addition to representative and cumulative data. This allows the reader a clear and comprehensive assessment of all aspects of the results. In contrast to the cumulative data set, the reader will be able to see that inhibition of degranulation through blocking of TRAIL interactions was observed consistently in all conducted experiments.

We sincerely value the referee's ideas for further interrogation of the effects of TRAIL on degranulation and cytotoxicity. Therefore, we designed and conducted additional experiments based on the referee's suggestions. We generated a 721.221 double knockout cell line using CRISPR/Cas9 that featured the disruptions of the genes encoding DR4 and DR5 (.221-DR4/5KO). In addition, we transduced Raji cells to overexpress DR5 (Raji-DR5++). The desired changes in DR4 and DR5 expression of the transduced cell lines are displayed in **Figure R1-1.1**.

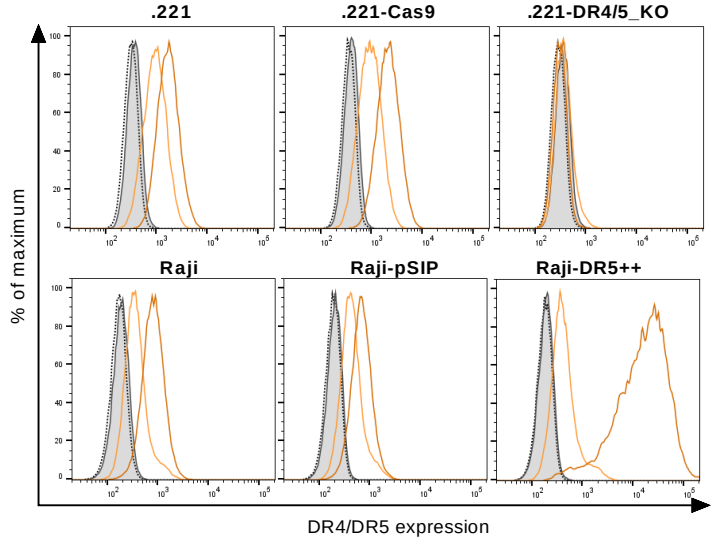

Figure R1-1.1 | Expression of TRAIL receptors on transduced 721.221 and Raji cells. The expression of DR4 and DR5 was assessed by flow cytometry. 721.221 and Raji cells were labelled with LIVE/DEAD Fixable Near-IR Stain, followed by incubation with biotin-conjugated mouse anti-human DR4 or DR5 and then labelled with Streptavidin-BV421. Expression was quantified as fluorescence intensity. Representative histogram of DR4 (light orange) and DR5 (dark orange) expression in comparison to the Streptavidin only control (grey) or the FMO control (dashed line). Upper panel (from left to right): untransduced 721.221 cells, Cas9-transduced .221s, DR4/5 double knockout .221s. Lower panel (from left to right): untransduced Raji cells, Raji cells transduced with an empty vector (pSIP), Raji cells overexpressing DR5.

Then we compared the ability of NK cells to lyse the respective cell lines in two competitive killing assays. In these assays, designated target cells and control cells were fluorescently labelled, mixed in a fixed ratio (app. 1:1) and then cultured in the presence or absence of NK cells (E:T ratio: 1:1) for five hours. Immediately after incubation, remaining cells were counted flow cytometrically and normalized

to counting beads. Specific lysis of target cells was calculated as previously described in Stary *et al.* (PMID: 33067380) with the following equation:

$[1 - (\#\text{control cells}/\#\text{target cells})_{\text{no NK cells}}/(\#\text{control cells}/\#\text{target cells})_{\text{with NK cells}}] \times 100$.

As shown in **Figure R1-1.2**, NK cells preferentially killed target cells (.221-Cas9: p = 0.002, Raji-DR5++: p = 0.0005) compared to the control cells which either lack DR4 and DR5 (.221-DR4/5KO) or express a lower amount of DR5 (Raji-pSIP). The results of these experiments clearly showed that target cells expressing death receptors or higher amounts of death receptors are increasingly sensitive to NK-cell-mediated cytotoxicity. We included and discussed these results in the revised manuscript.

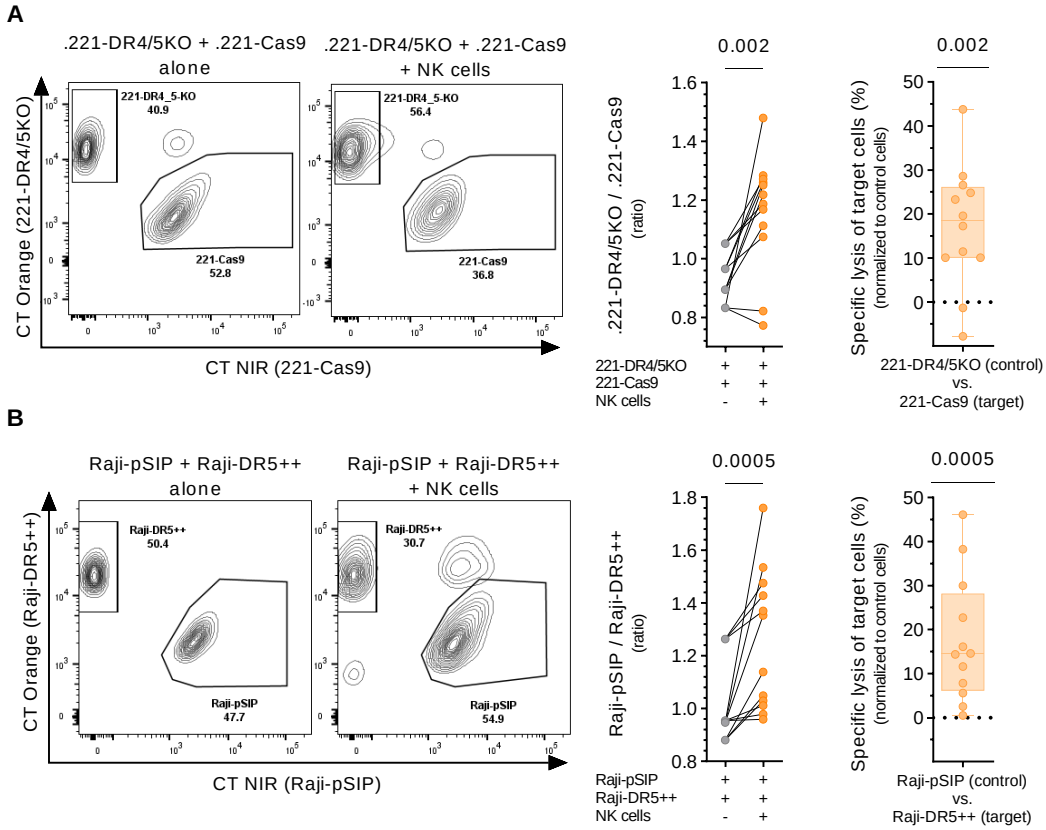

Figure R1-1.2 | Preferential killing of TRAIL receptor-positive target cells by NK cells. (A) Left panel: Representative contour plots displaying .221-DR4/5KO control cells (top left) and .221-Cas9 target cells (right). Numbers represent the relative frequency of the gated population. Middle panel: Ratio of .221-DR4/5KO and .221-Cas9 target cells after 5 h incubation in the absence or presence of NK cells (n = 12). Right panel: Specific lysis (in %) of .221-Cas9 cells (n = 12). **(B)** Left panel: Representative contour plots displaying Raji-DR5++ target cells (top left) and Raji-pSIP control cells (right). Numbers represent the relative frequency of the gated population. Middle panel: Ratio of Raji-pSIP and Raji-DR5++ target cells after 5 h incubation in the absence or presence of NK cells (n = 12). Right panel: Specific lysis (in %) of Raji-DR5++ cells (n = 12). **Data information:** Wilcoxon signed-rank test. Experiments were performed in four batches with three different donors each. No NK cell control served for all donors in each batch. Lines connect each data value of the NK cell condition with their designated No NK cell control. Each data point represents the mean of at least 3 technical replicates. Box plots represent the median and 25%/75% percentile. Whiskers indicate minimum and maximum data points.

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2. The authors only determine CD107a expression as a marker for degranulation. They conclude from this that TRAIL can induce NK cell cytotoxicity e.g., against HIV-1 infected targets. However,

they never show target cell death in their experiments. Interestingly, they observe most TRAIL-induced degranulation in CD56-bright NK cells (Fig. 2A), which are known to be not very cytotoxic. Therefore, a cytotoxicity assay actually determining the death of target cells that is induced upon TRAIL engagement is important.

2. Reply: The referee is addressing the important aspect that degranulation is used as a well-established surrogate for NK cell cytotoxicity, however, cytotoxicity also depends on target cell susceptibility and granzyme release. To address this point, we quantified granzyme B concentration in the supernatant as an additional marker for degranulation in the plate-coating experiments (Figure 5C). Granzyme B concentration in the supernatant significantly correlated with levels of degranulation (Figure 5D), indicating that degranulation (release of Granzyme B) upon TRAIL engagement in fact took place.

Regarding the cytotoxicity of CD56Bright NK cells, the referee states the widely known paradigm that CD56Bright NK cells exhibit low natural cytotoxicity compared to their CD56Dim counterparts. Indeed, early findings indicated that CD56Dim NK cells might predominantly mediate NK cell cytotoxicity towards target cells whereas CD56Bright NK cells were considered to play an immune-regulatory role due to their ability to produce vast amounts of cytokines. While this is certainly true for freshly isolated peripheral blood NK cells (PMID: 2530273), the observed differences in cytotoxic abilities are leveled after treatment with cytokines. Several studies have shown that CD56Bright NK cells exert cytolytic activity to a remarkable degree, in particular after exposure to cytokines (PMID: 2530273, 1692080, 2139697, 28972539, 22384114). Hence, the fact that interleukin-activated CD56Bright NK cells displayed higher TRAIL-correlated degranulation rates compared to CD56Dim NK cells in our experimental set up is in line with other reports.

Evidence that TRAIL engagement is leading to target cell death has been previously shown for multiple types of effector and target cells (PMID: 22384114, 23719242, 11135622, 11745330, 20334827, 10523613, 10736093, 10209050, 9725211) but own experimental evidence was lacking in the initially submitted version of the manuscript. We therefore thank the referee for bringing this up and therefore performed a cytotoxicity assay using the 722.221 cell line. We cultured 721.221 cells alone or in co-culture with NK cells in the presence of either αTRAIL (10ug/ml) or the respective isotype control (10ug/ml). After 5-hour incubation, remaining cells were counted using a flow cytometer and then normalized to counting beads. Our results showed a generally strong ability of NK cells to lyse .221 cells (**Figure R1-2**). More importantly, presence of αTRAIL reduced NK-cell-mediated lysis compared to the isotype control ($p = 0.012$). Blockade of TRAIL interactions resulted in an increased number of remaining target cells (median 5.2 p.p. compared to isotype, $p = 0.012$). While the effect of the TRAIL blockade on target cell lysis seems modest in our experimental setup at first, it should be noted that .221 cells are HLA class I devoid and are therefore highly sensitive to NK-cell-mediated cytotoxicity. Given a certain redundancy of activating NK cell receptors that are triggered after exposure to .221 cells, the contribution of TRAIL to target cell lysis is therefore considerable.

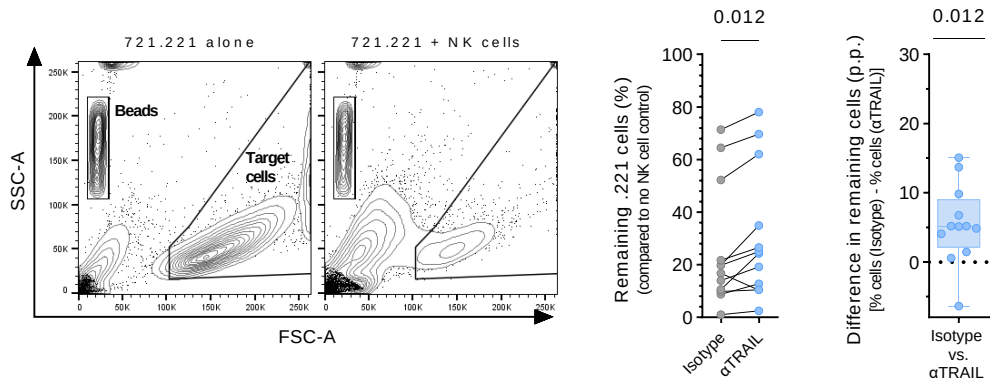

Figure R1-2 | Blocking of TRAIL interactions decreases NK-cell-mediated killing of .221 cells. Left panel: Representative contour plots displaying counting beads (top left), target cells (right) and NK cells (bottom left) based on size (FSC-A) and granularity (SSC-A). Middle panel: Percentage of remaining target cells in the presence of αTRAIL or isotype control compared to the culture condition without NK cells. Each data point represents one donor (n = 12). Right panel: Difference in remaining cells (percentage points, p.p.) between αTRAIL or isotype condition. **Data information:** Wilcoxon signed-rank test. Each data point represents the mean at least 2 technical replicates. Lines connect data points of the same donor. Box plots represent the median and 25%/75% percentile. Whiskers indicate minimum and maximum data points.

Minor points:

3. Figure 1: The authors mention that TRAIL is just one of 48 markers that was enriched on degranulating NK cells upon contact with HIV-1 infected T cells. Were there also other known activating receptors among the 47 surface molecules? How important is the contribution of TRAIL to the killing of HIV-1 infected cells?

3. Reply: The referee mentions an interesting aspect of our data set. Based on two selected cut-off criteria (five percentage points difference, p value < 0.05), 48 surface antigens were identified to be differentially expressed between CD107a^{+} and CD107a^{-} NK cells after exposure to autologous HIV-infected CD4 T cells. Of those, 43 antigens were enriched in degranulating NK cells. TRAIL ranked 8th (median difference: 22.4 p.p.). The identified group of antigens also comprised known activating receptors, such as NKG2D (10th, 18.9 p.p.) and NKp30 (32nd, 9.7 p.p.). Several studies have investigated the contribution of TRAIL-mediated elimination of HIV-1 infected cells. In 2001, Lum *et al.* observed an increased sensitivity against TRAIL-mediated apoptosis in peripheral blood lymphocytes (PBL) derived from HIV-infected individuals, in contrast to uninfected individuals (PMID: 11602752). CD4+ T cells from HIV-1+ donors showed increased expression of TRAIL-R1 (DR4), TRAIL-R2 (DR5) and TRAIL-4 (DcR2) compared to cells from HIV-1 negative donors. Similar findings have been reported by Herbeuval and colleagues (PMID: 16046522) with respect to increased levels of apoptosis of CD4 T cells from infected individuals and increased expression of DR5. Multiple other studies corroborated the hypothesis that TRAIL/TRAIL-R apoptotic pathway contributes to CD4 T cell depletion in HIV-1 pathogenesis (PMID: 16632604, PMID: 11602752, PMID: 32406872).

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4. Figure 2 and Page 5 end of 1st paragraph: I do not understand the argument made by the authors that their data show that TRAIL is not just a surrogate marker for NK cells that possess inherently increased anti-HIV-1 activity. To me there is a clear correlation between NK subsets that express high TRAIL levels and that also degranulate more. Based on the data presented in this figure there does not need to be a causal relationship.

4. Reply: We apologize for any ambiguity in the statement in question. Indeed, CD56Bright or NKG2A$^+$ NK cells not only display higher response rates after exposure to HIV-1-infected target cells but show overall higher expression of TRAIL compared to their pre-defined counterparts (CD56Dim or KIR-educated NK cells), indicating that TRAIL is involved in either improved target cell recognition or induction of degranulation. Given that we initially only looked at bulk NK cells we wanted to make sure that TRAIL was not just simply co-expressed in NK cell subsets that are superior in their anti-HIV-1 activity. For example, Davis *et al.* (PMID: 26828202) showed increased anti-HIV activity (degranulation) of CD56Bright as well as NKG2A$^+$ NK cells compared to their counterparts. Another study by our group (PMID: 28704647) observed inferior antiviral activity NK cells expressing self-inhibitory KIR (KIR-educated). These observations were confirmed by our experiments. By stratifying NK cells into these functionally divergent NK cell subsets, we demonstrated that within each of these subsets TRAIL was independently associated with increased degranulation.

In order to clarify the point brought up by the referee, we revised the manuscript accordingly. The passages in question now read:

"Based on this observation, we further investigated possible underlying causes and postulated the following three hypotheses: i) TRAIL acts as an activation marker, being upregulated during or after degranulation; ii) TRAIL is simply co-expressed on NK cell subsets with inherently higher anti-viral activity but not involved in the induction of degranulation; and iii) TRAIL is either directly or indirectly involved in degranulation." (Results, lines 63-68)

"Taken together, our data indicated that TRAIL is neither an activation marker in this experimental setting nor simply co-expressed in subsets with inherently higher anti-HIV activity. Instead, in all investigated subsets NK cell degranulation was associated with increased TRAIL expression, indicating a potential role for the induction of degranulation." (Results, lines 98 -102)

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5. Figure 5: When stimulating NK cells with plate bound DR4 or OPG do only the TRAIL positive NK cells degranulate? This would be another proof of specificity.

5. Reply: In our initial experimental setup, we did not distinguish between TRAIL$^+$ and TRAIL$^-$ NK cells. In response to the referee's question, we assessed degranulation and production of IFNγ in TRAIL$^+$ and TRAIL$^-$ NK cells. For this, we repeated plate-coating experiments using immobilized DR4 protein to engage TRAIL on NK cells. Following incubation, we labelled NK cells with anti-TRAIL-APC to identify TRAIL$^+$ and TRAIL$^-$ NK cells and additionally performed an intracellular cytokine

staining for IFNγ. When co-cultured with immobilized DR4 protein, TRAIL⁺ NK cells predominantly degranulated (p = 0.03) and produced IFNγ (p = 0.06) compared to TRAIL⁻ NK cells (**Figure R1-5**). Of note, measured TRAIL expression was lower in NK cells incubated with immobilized DR4 compared to the PBS and IgG controls, indicating that interaction of TRAIL⁺ NK cells with DR4 led to either internalization, shedding of TRAIL, or blocking of subsequent TRAIL labelling. Therefore, effector responses of TRAIL⁻ NK cells may be overestimated as the TRAIL⁻ subpopulation may in part contain initially TRAIL⁺ NK cells.

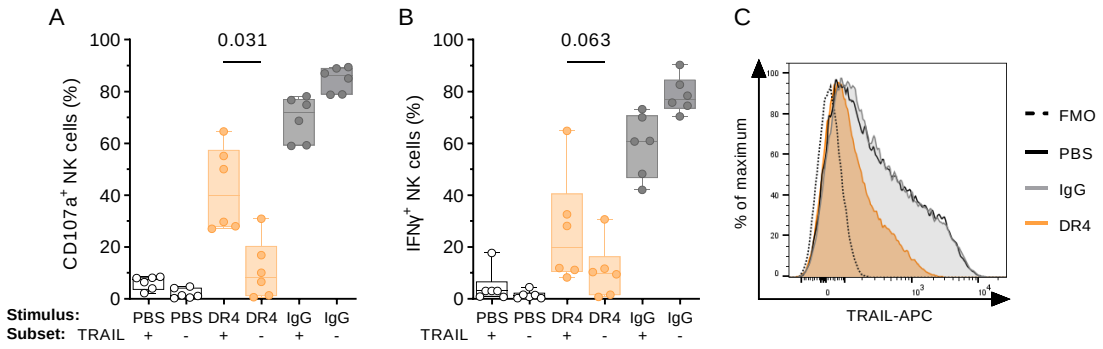

Figure R1-5 | TRAIL⁺ NK cells predominantly degranulate and produce IFNγ. NK cells from six different donors were incubated for 5 h in wells with immobilized DR4 protein, IgG or PBS in the presence of αCD107a-BV510 and Brefeldin A (added after 1 h). After incubation, TRAIL surface labeling and intracellular IFNγ staining was performed. **(A)** Relative frequency of CD107a⁺ NK cells, stratified by culture condition and subset. **(B)** Relative frequency of IFNγ⁺ NK cells, stratified by culture condition and subset. **(C)** Representative histogram of TRAIL expression for different culture conditions after 5 h incubation. **Data information:** Wilcoxon signed-rank test Circles represent individual data points, box plots show the median and 25%/75% percentile, whiskers define minimum and maximum data points.

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6. Figure 5: Why is the degranulation reduced when using higher concentrations of DcR1?

6. Reply: The referee raises an interesting question. The observed negative dose-dependent effect of the DcR1 protein on NK cell degranulation may have many underlying reasons, e.g. unspecific effects, increased formation of aggregates or negative intracellular feedback loops. An extended literature search has not yielded a satisfactory explanation. Therefore, we unfortunately cannot provide an answer to the referee's question that is beyond speculation. We have added a sentence in results section to address this phenomenon.

Referee #2:

In this manuscript, Höfle et al establish that the killer ligand TRAIL is tightly associated with CD107a expression on NK cells, which was identified in a screen for NK cell receptors implicated in the recognition of HIV-infected CD4+ T cells. The authors then describe a pathway through which TRAIL activation or inhibition on NK cells either promote or diminish their functional activation via the regulation of CD107a expression / degranulation or the production of effector cytokines.
In general, the manuscript is well written, and the data are clear and well presented. Note that a previous study by others already reported that TRAIL regulates the degranulation / CD107a expression of (IL-18/poly I:C-) activated blood and liver human NK cells, which promoted the killing of target cells (PMID: 30748035), which takes away some of the novelty of the current study.
I have several comments to the authors, which are detailed below.

0. Reply: We would like to thank the referee for their time and the positive view on the presented manuscript. To address the referee's questions and concerns, we conducted additional experiments and further revised the manuscript accordingly. Please see our detailed point-by-point response to the referee's comments below.

Major comments
• Line 97: the authors should be more explicit on why they conclude that TRAIL is not a "surrogate marker for subsets with inherently increased anti-HIV-1 activity". Indeed, they define "anti-HIV-1 activity" or "HIV-specific response" as frequency of CD107a+ on NK cells, and TRAIL expression is positively correlated with CD107a expression. Or do they mean there that TRAIL is not specific to (CD107 positive) NKG2A+ versus KIR NK cells or to (CD107a positive) bright versus dim NK cells?

1. Reply: We apologize for any ambiguity in the statement in question. Indeed, CD56Bright or NKG2A$^+$ NK cells not only display higher response rates after exposure to HIV-1-infected target cells but show overall higher expression of TRAIL compared to their pre-defined counterparts (CD56Dim or KIR-educated NK cells), indicating that TRAIL is involved in either improved target cell recognition or induction of degranulation. Given that we initially only looked at bulk NK cells we wanted to make sure that TRAIL was not just simply co-expressed in NK cell subsets that are superior in their anti-HIV-1 activity. For example, Davis *et al.* (PMID: 26828202) showed increased anti-HIV activity (degranulation) of CD56Bright as well as NKG2A$^+$ NK cells compared to their counterparts. Another study by our group (PMID: 28704647) observed inferior antiviral activity NK cells expressing self-inhibitory KIR (KIR-educated). These observations were confirmed by our experiments. By stratifying NK cells into these functionally divergent NK cell subsets, we demonstrated that within each of these subsets TRAIL was independently associated with increased degranulation.
In order to clarify the point brought up by the referee, we revised the manuscript accordingly. The passages in question now read:
"Based on this observation, we further investigated possible underlying causes and postulated the following three hypotheses: i) TRAIL acts as an activation marker, being upregulated during or after degranulation; ii) TRAIL is simply co-expressed on NK cell subsets with inherently higher anti-viral

activity but not involved in the induction of degranulation; and iii) TRAIL is either directly or indirectly involved in degranulation." (Results, lines 63-68)

"Taken together, our data indicated that TRAIL is neither an activation marker in this experimental setting nor simply co-expressed in subsets with inherently higher anti-HIV activity. Instead, in all investigated subsets NK cell degranulation was associated with increased TRAIL expression, indicating a potential role for the induction of degranulation." (Results, lines 98 -102)

• Does the improved degranulation / increased expression of CD107a on NK cells results in increase target cell elimination or apoptosis (which is a more relevant readout than degranulation per se)?

2. Reply: We thank the referee for this helpful comment. In order to answer this question we conducted multiple killing assays that demonstrated the impact of the TRAIL/TRAIL-R pathway for NK cell-mediated cytotoxicity. First, we generated a 721.221 double knockout cell line using CRISPR/Cas9 that featured the disruptions of the genes encoding DR4 and DR5 (.221-DR4/5KO) as well as a Raji cell line that overexpressed DR5 (Raji-DR5++). The altered expression levels of DR4 and DR5 in the transduced cell lines are shown in **Figure R2-2.1**.

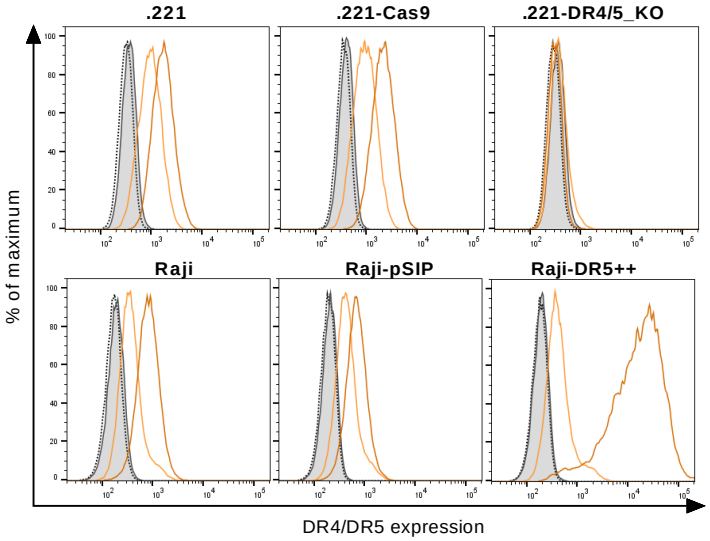

Figure R2-2.1 | Expression of TRAIL receptors on transduced 721.221 and Raji cells. The expression of DR4 and DR5 was assessed by flow cytometry. 721.221 and Raji cells were labelled with LIVE/DEAD Fixable Near-IR Stain, followed by incubation with biotin-conjugated mouse anti-human DR4 or DR5 and then labelled with Streptavidin-BV421. Expression was quantified as fluorescence intensity. Representative histogram of DR4 (light orange) and DR5 (dark orange) expression in comparison to the Streptavidin only control (grey) or the FMO control (dashed line). Upper panel (from left to right): untransduced 721.221 cells, Cas9-transduced .221s, DR4/5 double knockout .221s. Lower panel (from left to right): untransduced Raji cells, Raji cells transduced with an empty vector (pSIP), Raji cells overexpressing DR5.

Then we compared the ability of NK cells to lyse the respective cell lines in two competitive killing assays. In these assays, designated target cells and control cells were fluorescently labelled, mixed in a fixed ratio (app. 1:1) and then cultured in the presence or absence of NK cells (E:T ratio: 1:1) for five hours. Immediately after incubation, remaining cells were counted at a flow cytometer and

normalized to counting beads. Specific lysis of designated target cells was calculated as previously described in Stary *et al.* (PMID: 33067380) with the following equation:

$[1 - (\#\text{control cells}/\#\text{target cells})_{\text{no NK cells}} / (\#\text{control cells}/\#\text{target cells})_{\text{with NK cells}}] \times 100$.

As shown in **Figure R2-2.2**, NK cells preferentially killed the designated target cells (.221-Cas9: p = 0.002, Raji-DR5++: p = 0.0005) compared to the control cells which either lack DR4 and DR5 (.221-DR4/5KO) or express a lower amount of DR5 (Raji-pSIP). The results of these experiments clearly showed that target cells expressing death receptors or higher amounts of death receptors are increasingly sensitive to NK-cell-mediated cytotoxicity.

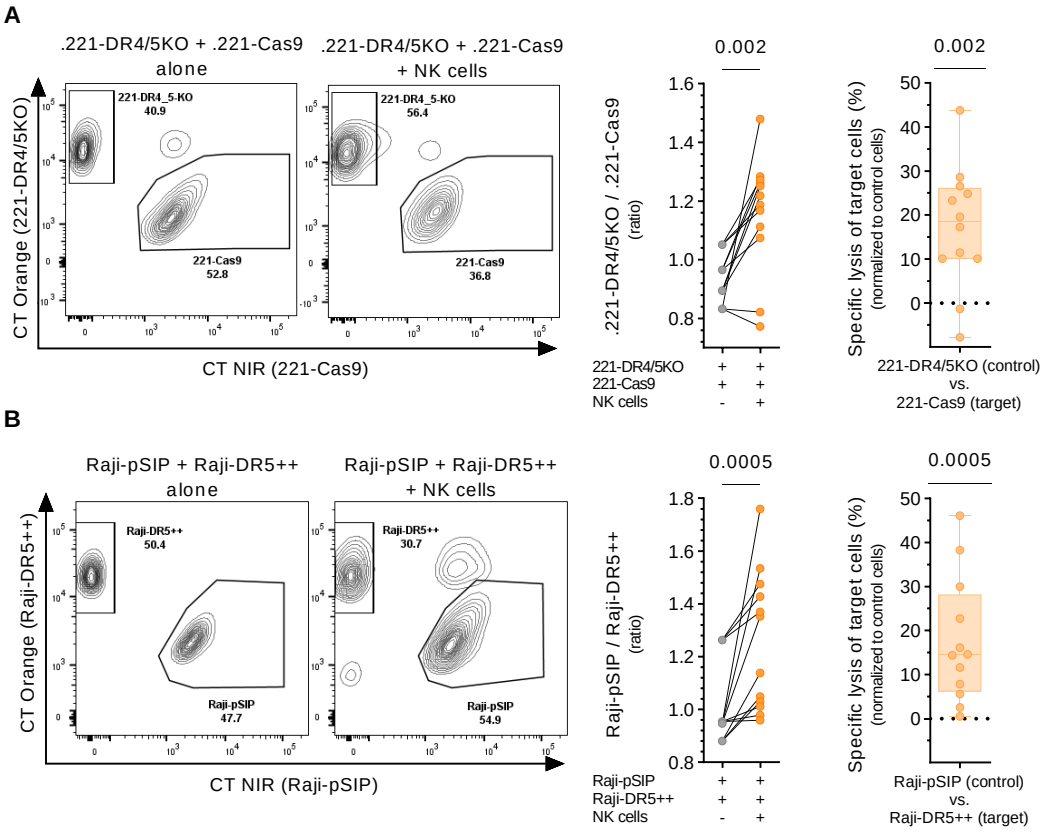

Figure R2-2.2 | Preferential killing of TRAIL receptor-positive target cells by NK cells. (A) Left panel: Representative contour plots displaying .221-DR4/5KO control cells (top left) and .221-Cas9 target cells (right). Numbers represent the relative frequency of the gated population. Middle panel: Ratio of .221-DR4/5KO and .221-Cas9 target cells after 5 h incubation in the absence or presence of NK cells (n = 12). Right panel: Specific lysis (in %) of .221-Cas9 cells (n = 12). **(B)** Left panel: Representative contour plots displaying Raji-DR5++ target cells (top left) and Raji-pSIP control cells (right). Numbers represent the relative frequency of the gated population. Middle panel: Ratio of Raji-pSIP and Raji-DR5++ target cells after 5 h incubation in the absence or presence of NK cells (n = 12). Right panel: Specific lysis (in %) of Raji-DR5++ cells (n = 12). **Data information:** Wilcoxon signed-rank test. Experiments were performed in four batches with three different donors each. No NK cell control served for all donors in each batch. Lines connect each data value of the NK cell condition with their designated No NK cell control. Each data point represents the mean of at least 3 technical replicates. Box plots represent the median and 25%/75% percentile. Whiskers indicate minimum and maximum data points.

Lastly, we cultured 721.221 cells alone or in co-culture with NK cells in the presence of either αTRAIL (10ug/ml) or the respective isotype control (10ug/ml). After 5-hour incubation, remaining cells were counted using a flow cytometer and then normalized to counting beads. Our results

showed a generally strong ability of NK cells to lyse .221 cells (**Figure R2-2.3**). More importantly, presence of αTRAIL reduced NK-cell-mediated lysis compared to the isotype control (p = 0.012). Blockade of TRAIL interactions resulted in an increased number of remaining target cells (median 5.1 p.p. compared to isotype, p = 0.012). While the effect of the TRAIL blockade on target cell lysis seems modest in our experimental setup at first, it should be noted that .221 cells are HLA class I devoid and are therefore highly sensitive to NK cell mediated cytotoxicity. Given a certain redundancy of activating NK cell receptors that are triggered after exposure to .221 cells, the contribution of TRAIL to target cell lysis is therefore considerable.

Collectively, our results showed that i) target cells with increased DR4 or DR5 expression are preferentially killed by NK cells and ii) that blockade of TRAIL interactions using soluble αTRAIL led to reduced killing by NK cells. We included and discussed these results in the revised manuscript.

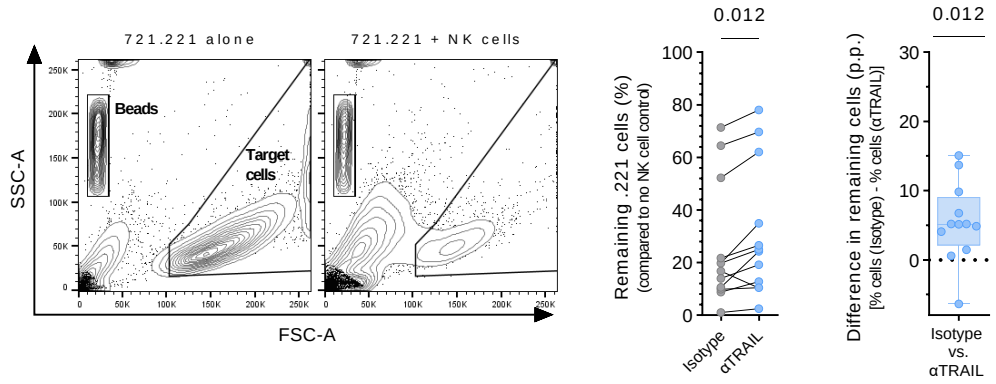

Figure R2-2.3 | Blocking of TRAIL interactions decreases NK-cell-mediated killing of .221 cells. Left panel: Representative contour plots displaying counting beads (top left), target cells (right) and NK cells (bottom left) based on size (FSC-A) and granularity (SSC-A). Middle panel: Percentage of remaining target cells in the presence of αTRAIL or isotype control compared to the culture condition without NK cells. Each data point represents one donor (n = 12). Right panel: Difference in remaining cells (percentage points, p.p.) between αTRAIL or isotype condition. **Data information:** Wilcoxon signed-rank test. Each data point represents the mean at least 2 technical replicates. Lines connect data points of the same donor. Box plots represent the median and 25%/75% percentile. Whiskers indicate minimum and maximum data points.

• Along the previous comment: CD56dim (CD16+) NK cells are commonly referred as being more cytotoxic than CD56bright (CD16dim/−) NK cells (PMID: 19278419). However, their findings indicate increased general levels of degranulation in the latter population. How do they reconcile these contradicting facts?

3. Reply: The referee refers to a common notion that CD56Bright NK cells exhibit low cytotoxicity compared to their CD56Dim counterparts. Indeed, early findings indicated that CD56Dim NK cells might predominantly mediate NK cell cytotoxicity towards target cells whereas CD56Bright NK cells were considered to play an immune-regulatory role due to their ability to produce vast amounts of cytokines. While this is certainly true for freshly isolated peripheral blood NK cells (PMID: 2530273),

the observed differences in cytotoxic abilities are leveled after treatment with cytokines. Several studies have shown that CD56Bright NK cells exert cytolytic activity to a remarkable degree, in particular after exposure to cytokines (PMID: 2530273, 1692080, 2139697, 28972539, 22384114). Hence, the fact that interleukin-activated CD56Bright NK cells displayed higher TRAIL-correlated degranulation rates compared to CD56Dim NK cells in our experimental set up is in line with other reports.

• To determine TRAIL receptor expression by flow cytometry, a combination of antibodies against DR4 and DR5 is used (Fig. 3A&B, Fig. 4E). Yet they report in Fig. 5B that DR4 and DR5 have a differential effect on NK cells degranulation / CD107a expression. Therefore, it would be pertinent to distinguish DR4 from DR5 expression on NK target cells.

4. Reply: Given the observation that TRAIL expression on NK cells was associated with increased degranulation against HIV-infected CD4 T cells, we first intended to investigate whether death receptors were present on *in vitro*-infected cells at all. Stratification of TRAIL receptor expression into the distinct receptors was deemed a lesser priority compared to the assessment of the global expression of relevant TRAIL interaction partners at that time. While degranulation levels did differ between the DR4 and DR5 proteins, both proteins induced degranulation on NK cells. In this context, it should be noted that the DR5 protein only contained the amino acids 52 to 183, in contrast to the full-length DR4 protein. This may have affected its biological activity to trigger degranulation in NK cells. To further dissect the expression patterns of TRAIL interaction partners on target cells given the observed differential effect of DR4 and DR5 protein on the induction of degranulation, we added experimental data on their surface expression on 721.221 and CD4 T cells. As displayed in **Figure R2-4**, DR4 and DR5 are both expressed on the surface of 721.221 cells and HIV-infected and uninfected primary CD4 T cells.

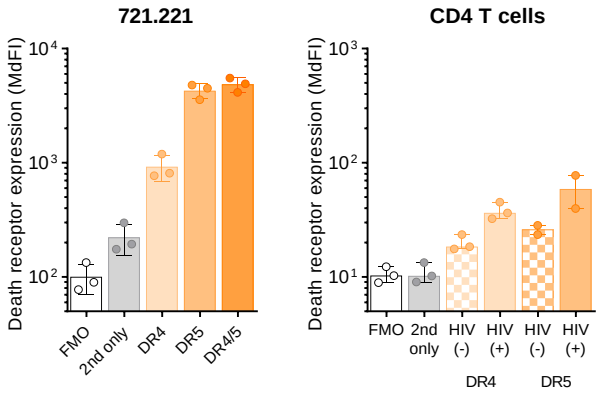

Figure R2-4 | Expression of death receptors 4 and 5 (DR4, DR5) on 721.221 cells, HIV-1-infected and uninfected CD4 T cells. 721.221s (.221s) were labelled with LIVE/DEAD Fixable Near-IR Stain, followed by incubation with biotin-conjugated mouse anti-human DR4 or DR5 and then labelled with Streptavidin-PE. CD4 T cells were stimulated for 3 days with αCD3/CD28 beads, and then infected with NL4-3 for 72 hours. Cells were labelled with LIVE/DEAD Fixable

Near-IR Stain, followed by incubation with biotin-conjugated mouse anti-human DR4 or DR5 and then labelled with Streptavidin-PE. Distinction between infected (CD4-/p24+) and uninfected (CD4+/p24-) cells was achieved by labelling cells with αCD4-APC and αp24-FITC. Expression was measured as median fluorescence intensity (MdFI) by flow cytometry. **(A)** Cumulative data of DR4 and DR5 expression of 721.221 cells displayed as MdFI. Data points represent 3 technical replicates of one independent experiment. Columns show the mean. Error bars show SD. **(B)** Cumulative data of DR4 and DR5 expression of infected (HIV+) and uninfected (HIV-) CD4 T cells displayed as MdFI. Data points represent different donors (n = 3 per condition, n = 2 for DR5). Columns show the median. Error bars show IQR.

• Along these lines: are DR4/5 expressed on NK cells in their system? If yes, they should test the effect of TRAIL binding to TRAIL receptor(s) on their NK cells, regarding induction of apoptosis and/or degranulation. The latest would validate that the findings shown in this study represent bona fide reverse signaling of TRAIL.

5. Reply: We tested the expression of TRAIL receptors on NK cells in our system by flow cytometry. In contrast to the target cells used in our experiments, we did not observe a ubiquitous expression of TRAIL receptors on NK cells (**Figure R2-5**). Those included death receptor 4 (DR4, TRAIL-R1), death receptor 5 (DR5, TRAIL-R2), decoy receptor 1 (DcR1, TRAIL-R3), and decoy receptor 2 (DcR2, TRAIL-R4). The lack of considerable expression of TRAIL receptors on NK cells further indicates that induction of degranulation and cytokine production was most likely mediated through reverse signaling of TRAIL itself and not through engagement of TRAIL receptors on NK cells in *cis* or *trans*.

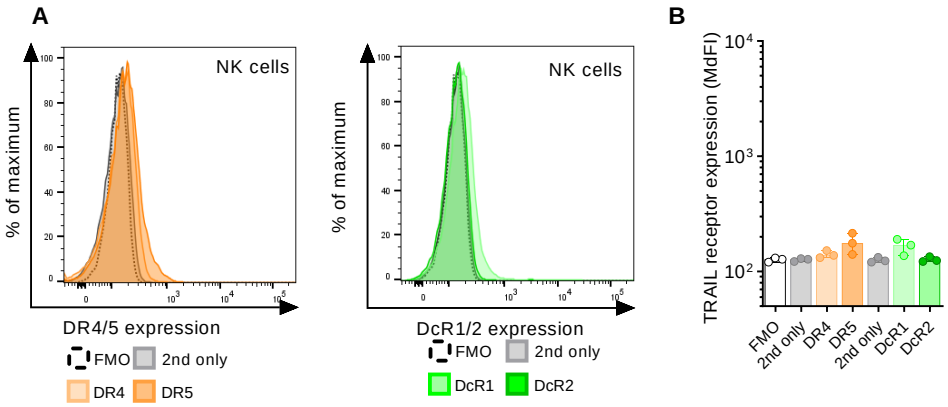

Figure R2-5 | Expression of TRAIL receptors on NK cells as determined by flow cytometry. Primary NK cells were cultured for three days in the presence of IL-2 and IL-15, analogue to previous experiments. Cells were labelled with biotin-conjugated mouse anti-human DR4 or DR5 or goat anti-human DcR1 or DcR2, and then labelled with the appropriate secondary dye (Streptavidin-PE or anti-goat-PE). Expression was measured as median fluorescence intensity (MdFI) by flow cytometry. **(A)** Left panel: Representative histogram of DR4 and DR5 expression on NK cells in comparison to the Streptavidin only control or the FMO control. Right panel: Representative histogram of DcR1 and DcR2 expression on NK cells in comparison to the anti-goat PE only control or the FMO control. **(B)** Cumulative data of TRAIL receptor expression on NK cells, displayed as MdFI. **Data information**: Data points represent individual donor NK cells (n = 3 per condition). Columns show the median. Error bars show IQR.

• How do they mechanistically explain that soluble anti-TRAIL antibody blocks (Fig 4A) while plate-bound anti-TRAIL antibody activates (Fig 5A) NK cell degranulation in their system? A further comment: the anti-TRAIL antibody clone RIK-2.1 was used for flow cytometry studies while clone RIK-2 was used both for TRAIL blockade and TRAIL crosslinking. Are those the same clones?

6. Reply: Here, the referee raises an interesting experimental and biological aspect. Antibodies in solution may bind to the targeted antigen and therefore block interaction with the respective binding partner. However, in this form the antibodies are usually not able to induce clustering of multiple receptor molecules and therefore fail to trigger receptor signaling. In contrast, immobilized (plate- or bead-coated) antibodies are thought to mimic the cell surface, providing a higher molecule density that allows clustering of the targeted receptor/antigen. Differential mode of actions of the same antibody in either solution (blocking) or immobilized (cross-linking) have been utilized in the past. Recent examples can be found in a study by Garcia Beltran *et al.* (Nat Immunol, 2016, PMID: 27455421) for the activating NK cell receptor KIR3DS1 and in a recent study by Niehrs *et al.* (Nat Immunol, 2019, PMID: 31358998) for the activating NK cell receptor NKp44.
With respect to the clones of the TRAIL antibodies used in this study, the clone marketed by Miltenyi under the name RIK-2.1 is the same clone as RIK-2.

• The same question applies to the experiments using anti-DR4 and anti-DR5 antibodies to block (while in soluble form) versus activate (after plate coating) NK cell degranulation. Were the same antibody clones used for these experiments (clone HS101 and clone HS201, respectively)?

7. Reply: In contrast to the TRAIL antibodies used in blocking and cross-linking experiments, different types of reagents were used in the two experimental setups mentioned by the referee. We incubated 721.221 target cells with anti-DR4 (clone HS101) and anti-DR5 (clone HS201), in order to block interactions between TRAIL on NK cells and its receptors on 721.221 cells. In the plate-coating experiments (TRAIL cross-linking), we did not use antibodies against DR4 and DR5, but rather used biologically active DR4 (Abcam, ab641) and DR5 (Abcam, ab243777) proteins to engage TRAIL on NK cells. This latter setup sought to serve as a more physiological approach, demonstrating that degranulation of NK cells was not only induced by antibody-mediated cross-linking of TRAIL but also through its actual receptors, in the form of plate-coated proteins.

• How do they explain the contrasting effects on NK cell degranulation of the different TRAIL receptors they tested? These TRAIL receptors apparently have different affinities for TRAIL (ligand) (PMID: 10770955), yet it does not appear that their relative ligand binding affinities may explain these differential effects on degranulation. Have they tested the ability of these (purchased / commercially available) receptors to bind TRAIL (and e.g. prevent TRAIL-induced cell death) after plate coating?

8. Reply: The referee raises an interesting question. The study referenced by the referee (Truneh *et al.*, 2000, PMID: 10770955) observed that the TRAIL receptor DR5 exhibited the highest affinity to TRAIL at 37 °C, followed by DR4 and lastly OPG, exhibiting the weakest affinity. Indeed, this ranking does not reflect the observed levels of degranulation induced in NK cells after incubation in TRAIL

receptor-coated wells. In our experimental setting, using 10 µg/ml as a protein concentration, OPG induced the strongest response in NK cells; then in declining order, DR4, DR5 and lastly DcR1. Multiple reasons may account for the observed discrepancy. Truneh and colleagues determined binding affinities, using isothermal titration calorimetry (ITC). ITC is a highly sensitive and accurate physical technique, intended to use thermodynamics for the calculation of binding affinity between molecules, among other things. In the present study, we assessed the cellular response of NK cells after TRAIL engagement. In contrast to ITC, our experimental setup contains a cellular system with all its complexity. Therefore, sole binding affinities between TRAIL and its interaction partner may not coincide with the functional readouts we conducted.

In terms of the referee's second question on that matter, we have not tested the ability of these receptors to bind TRAIL after plate coating but rather relied on the information about biological activity provided by the vendor. For all three proteins (DR4, DR5, OPG), biological activity was tested by the vendor. The underlying methods described in the respective data sheets of the products are stated below:

- Recombinant human DR4 protein (Abcam, Cat#ab641): The biological activity of this product was determined by its inhibitory effect of IL-8 production in human PBMC by Human sTRAIL/Apo2L. 95% inhibition (100ng/ml of sTRAIL/Apo2L) was reached using a concentration of 100ng/ml of sTRAIL Receptor-1.
- Recombinant human DR5 protein (Abcam, Cat#ab243777): Fully biologically active when compared to standard. rHusTRAIL-R2 reduced the production of LPS-induced TNF by its ability to neutralize endogenous TRAIL in fresh human PBMC. In this assay, endogenous TRAIL is induced during a 24 hour exposure to LPS (10 ng/mL) but in the presence of rHusTRAIL-R2, TRAIL-induced TNF is suppressed.
- Recombinant human Osteoprotegerin protein (OPG) (Abcam, Cat#ab182688): Immobilized ab182688 at 10 µg/ml (100 µl/well) can bind Human TNFSF11 Fc Chimera with a linear range of 4-256 ng/ml.

Aside from the statement above, it should be noted that DR5 does not contain the full protein (amino acids 52 - 183), in contrast to DR4. This may be an additional reason for the reduced ability of DR5 to induce NK cell degranulation, as compared to DR4.

• Why is the inhibition of degranulation markedly smaller with anti-DR4/5 on 721.221 B cell targets compared to anti-TRAIL on NK effector cells? Fig 4B versus Fig 4G.

9. Reply: Phenotypical assessment of 721.221 cells (.221s) showed a clear expression of DcR1 as well as a low, potentially negligible expression of DcR2 (**Figure R2-9**). Given the ability of DcR1 to induce degranulation on NK cells after engagement, blocking of DR4 and DR5 on .221s might have not been sufficient to block all interactions between TRAIL on NK cells and its respective receptors

on .221s. We report this finding in the revised manuscript and discuss its implication for the outcome of the anti-DR4/5 mediated blocking of NK cell degranulation. In a speculative manner, it is possible that binding of the TRAIL antibody to TRAIL is more effective in blocking the interaction through steric hindrance. Vice versa, binding of anti-DR4 and anti-DR5 to its respective receptors may occur in a manner that still allows residual binding of DR4 and DR5 to TRAIL and therefore enables remaining, but weaker, engagement of TRAIL.

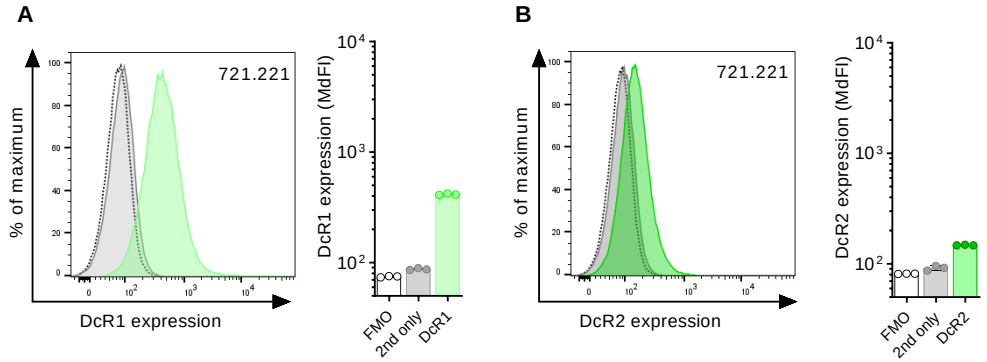

Figure R2-9 | Expression of Decoy Receptors 1 and 2 on 721.221 cells. .221s were labelled with LIVE/DEAD Fixable Near-IR Stain, followed by incubation with goat anti-human DcR1 or DcR2 and then labelled with anti-goat-PE. Expression was measured as median fluorescence intensity (MdFI) by flow cytometry. **(A)** Left panel: Representative flow cytometry histogram of DcR1 expression on .221s (light green) in comparison to the Streptavidin only control (grey) or the FMO control (dashed line). Right panel: Cumulative data of DcR1 expression displayed as MdFI. **(B)** Left panel: Representative histogram of DcR2 expression on .221s (green) in comparison to the Streptavidin only control (grey) or the FMO control (dashed line). Right panel: Cumulative data of DcR2 expression displayed as MdFI. **Data information:** Data points represent 3 technical replicates (n = 3) of one independent experiment. Columns show the mean. Error bars show SD.

• Fig 5B: since not all NK cells express TRAIL in their system, they should demonstrate, as shown for the positive correlation between TRAIL and CD107a expression, that IFNg is preferentially produced in TRAIL-expressing NK cells.

10. Reply: In response to the referee's suggestion, we assessed the production of IFNγ in TRAIL$^+$ and TRAIL$^-$ NK cells. For this, we repeated plate-coating experiments using immobilized DR4 protein to engage TRAIL on NK cells. Following incubation, we labelled NK cells with anti-TRAIL-APC to identify TRAIL$^+$ and TRAIL$^-$ NK cells and additionally performed an intracellular cytokine staining for IFNγ. Our results show that IFNγ is predominantly produced by TRAIL$^+$ NK cells when co-cultured with immobilized DR4 protein (**Figure R2-10**). Of note, measured TRAIL expression was lower in NK cells incubated with immobilized DR4 compared to the PBS and IgG controls, indicating that interaction of TRAIL+ NK cells with DR4 led to either internalization or shedding of TRAIL on NK cells, or blocking of subsequent TRAIL labelling. Therefore, effector responses of TRAIL- NK cells may be overestimated as the TRAIL- subpopulation may in part contain initially TRAIL$^+$ NK cells.

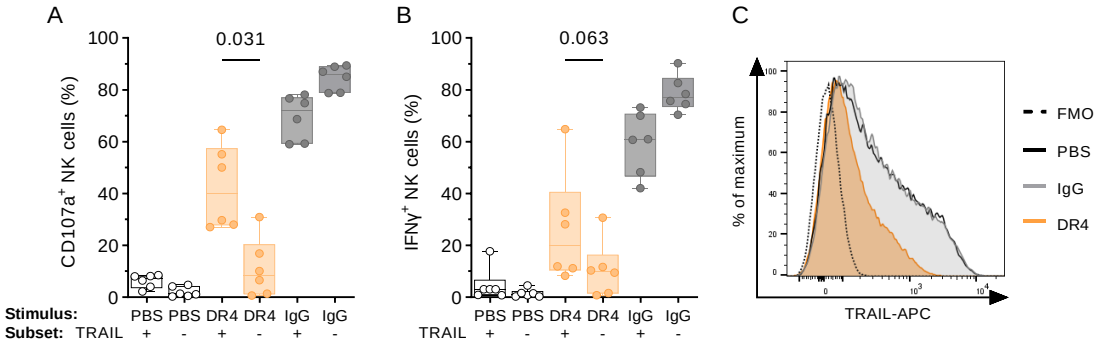

Figure R2-10 | TRAIL⁺ NK cells predominantly degranulate and produce IFNγ. NK cells from six different donors were incubated for 5 h in wells with immobilized DR4 protein, IgG or PBS in the presence of αCD107a-BV510 and Brefeldin A (added after 1 h). After incubation, TRAIL surface labeling and intracellular IFNγ staining was performed. **(A)** Relative frequency of CD107a⁺ NK cells, stratified by culture condition and subset. **(B)** Relative frequency of IFNγ⁺ NK cells, stratified by culture condition and subset. **(C)** Representative histogram of TRAIL expression for different culture conditions after 5 h incubation. **Data information:** Wilcoxon signed-rank test. Circles represent individual data points, box plots show the median and 25%/75% percentile, whiskers define min and max data points.

• They refer to a previous publication (PMID: 31742873) indicating that TRAIL-deficient murine NK cells do not show difference in CD107a expression compared to wild-type controls (presented in the discussion). It this same study, it was reported that these TRAIL-deficient murine NK cells produce more IFNg upon activation (not discussed in the current manuscript). This is in contrast with their findings indicating that TRAIL signaling promotes CD107a expression and IFNg production. The authors suggest that there may be differences in TRAIL signaling between mice and humans. This is possible, yet reverse TRAIL signaling apparently activates cytokine production both in murine (PMID: 11466352) and in human T cells (PMID: 14872508). These different aspects need to be more discussed.

11. Reply: The study mentioned by the referee (Cardoso Alves *et al.*, 2020, PMID: 31742873) did show an increased number and frequency of IFNγ⁺ NK cells in TRAIL-deficient mice after infection with LCMV. Given the differences between mice and humans and the complexity of the infection model, the inhibitory effect of TRAIL on IFNγ production may not be related to reverse signaling. Other indirect mechanisms during the antiviral immune response may be responsible for the observed increased number of IFNγ⁺ NK cells. Studies by Diehl *et al.* (PMID: 15589175) and Iyori *et al.* (PMID: 21832159) indicated that the TRAIL/TRAIL-R axis may serve as a negative regulator of immune responses altering NK cell-DC cross-talk. We reported and discussed these observations in the revised manuscript.

• The authors should better discuss how their findings are different from or extend the ones of Li et al., which they cite in the discussion (PMID: 30748035).

12. Reply: We revised the paragraph where we discuss the findings and conclusions of the study by Li *et al*.

Minor comments
• Fig 4A&C, Fig 5A&B: it would be helpful to see in these density plots were the cutoff was set to determine positivity versus negativity of CD107a expression.

13. Reply: We followed the referee's suggestion and now display gates in the density plots of figures 4A&C and 5A&B, indicating the cut-off for CD107a positivity.

• KIR-educated cells and NKG2A-educated cells should be defined in M&M in addition to the description in the legend of Fig. 2.

14. Reply: We followed the referee's recommendation and now define KIR-educated cells and NKG2A-educated cells in the material and method section. The included sentences read: "NK cell subsets subject to further analyses comprised CD56Dim, CD56Bright, KIR-educated, NKG2A-educated and uneducated NK cells. Definition of the education status of NK cells was based on the expression of the inhibitory receptors KIR2DL1/L2/L3, KIR3DL1 and NKG2A and the underlying HLA class I genotype. KIR-educated cells were defined as expressing at least one self-inhibitory KIR (2DL1/L2/L3, 3DL1) and negative for NKG2A, NKG2A-educated cells, expressing NKG2A but lacking self-inhibitory KIR, and uneducated cells, lacking self-inhibitory KIR and NKG2A altogether."

• They should indicate in the figure legends the number of independent experiments that were performed and produced data comparable to the ones presented. Alternatively, they should mention whether the data shown were pooled from several independent experiments.

15. Reply: We followed the referee's request and now indicate in the figure legends the number of independent experiments (e.g. different donors) that were performed.

Referee #3:

1. Does this manuscript report a single key finding? **YES** If YES, please describe it in one sentence:
TRAIL triggers degranulation, granzyme B release and IFNgamma production in NK cells.

2. Is the reported work of significance (YES), or does it describe a confirmatory finding or one that has already been documented using other methods or in other organisms etc (NO)?
YES. This is the first report of TRAIL reverse signaling in human NK cells. However, TRAIL reverse signaling has already been reported in murine NK cells and in T cells (as correctly discussed by the authors in lines 189-202 of this manuscript).

3. Is it of general interest to the molecular biology community? **NO**
If YES, please say why, in a single sentence. If NO, please state which more specialized community you feel it is aimed at (or none), in a single word or phrase. The main conclusion of this study is certainly of interest for immunologists and virologists but I am afraid that its appeal beyond these disciplines remains limited.

4. Is the single major finding robustly documented using independent lines of experimental evidence (YES), or is it really just a preliminary report requiring significant further data to become convincing, and thus more suited to a longer¬format article (NO)? NO As outlined in my comments below, I feel that the conclusion of "reverse signaling" is not yet sufficiently backed up by the presented data.

In their study, Höfle et al. report that reverse signaling of TRAIL triggers degranulation and IFNgamma production in human NK cells. While performing a screen for NK cell receptors involved in the recognition of HIV-infected cells, the authors found TRAIL as a candidate. They show that NK cells which recognize HIV-infected CD4 T cells display an elevated TRAIL expression on their surface. They further show that TRAIL is neither a surrogate nor an activation marker in this setting and that surface expression of TRAIL-R1 and TRAIL-R2 is increased on HIV-infected CD4 T cells. Höfle and colleagues furthermore report that antibodies against TRAIL or TRAIL-R1/TRAIL-R2 diminish NK cell degranulation in co-culture experiments. Coating plates with TRAIL antibodies or with recombinant TRAIL-R1/TRAIL-R2, they report that this coating induces an enhanced degranulation and release of granzyme B in NK cells. Two other coated TRAIL-binding receptors, OPG and TRAIL-R3, likewise increase NK cell degranulation (TRAIL-R4 is not tested in this study). In supernatants from the coculture experiments (antibodies against TRAIL or TRAIL-R1/TRAIL-R2), the authors find reduced IFNgamma levels, whereas increased levels are reported in supernatants collected from the experiments using plates coated with TRAIL antibodies or with recombinant TRAIL-R1 (supernatants from the other ligands are not tested). Finally, the authors investigate whether the observed induction of degranulation and IFNgamma production are caused by an increased ability of the NK cells to adhere to their target cells, but do not find evidence for this. Based on these results, the authors suggest that TRAIL contributes to the anti-HIV-1 activity of NK cells beyond receptor-mediated cytotoxicity through reverse signaling.
The manuscript is very well and carefully written, and the experiments are at a high technical level. I have no concerns with the experimental design or the integrity and quality of the presented data. It is obvious that the authors have great experience in flow cytometry (almost all experiments are designed to use flow cytometry), and this is reflected by the way the data are presented. Yet, given that EMBO Reports is targeted at a broader audience, I feel that the authors may wish to enhance the accessibility and clarity of their presentation by adding more explanations, if the character limit permits. As one example: in line 48, the authors should explain that CD107a is a marker for NK cell degranulation for readers from non-related fields.
I feel that this study is certainly of interest, but needs to be strengthened to provide the novel substantial physiological insight that EMBO reports is seeking for. For me, it is not convincingly demonstrated that reverse signaling by TRAIL actually takes place. If yes, the question remains how important this pathway is for the cytotoxic response of NK cells.

0. Reply: We are grateful for the overall positive assessment of the manuscript und the suggestions made to further improve the quality of the manuscript. In response to the referee's comments we conducted additional experiments including cytotoxicity assays. We also further investigated the intracellular signaling upon TRAIL engagement. Finally, we revised the manuscript in a way to enhance the accessibility and clarity as suggested by the referee. Please see our detailed point-by-point response to the referee's comments below.

Specific comments: Major:
1. Currently, the authors demonstrate the presence of effects that are statistically significant, but nevertheless only of modest nature (e.g., in Figures 5AB when compared to the positive controls, but also throughout the study). For me, it remains open to which extent the engagement of TRAIL on NK cells contributes to the total cytotoxic response of NK cells against their targets. Is this a major pathway for the elimination of pathogens or just a minor additional mechanism, if at all? This could, e.g., be addressed by deleting TRAIL from primary human NK cells or NK cell lines via CRISPR/Cas (such gene deletion has been established, e.g., for primary T cells, PMID: 29436394).

1. Reply: We concur with the referee that the effects on degranulation (% $CD107a^+$ NK cells), granzyme B release and IFNγ production were lower than induced by the positive controls. Activation of NK cells is controlled by the integration of activating and inhibitory signals and ultimately determined by the resulting net balance of signals. The positive controls, NKG2D, NKp46, and CD16 (IgG Fc receptor), are widely expressed on NK cells and are well known activating receptors which associate with adaptor proteins containing activating signaling motifs. All of those were intentionally selected to yield maximum effects and to ensure that the assays worked properly. The observed effects of TRAIL engagement in the various experiments were consistently higher than the negative controls and across all parameters we used to assess NK cell function. The TRAIL-mediated effects NK cell degranulation and IFNγ production are therefore considerable in our opinion.
Nevertheless, the referee raises an interesting point about the extent the engagement of TRAIL on NK cells contributes to the total cytotoxic response of NK cells against their targets. "TRAIL-mediated cytotoxicity has been shown to be important for NK cell-mediated control of viral infections and cancer, especially for liver NK cells." ("Mechanisms of natural killer cell-mediated cellular cytotoxicity", Prager & Watzl (2019), PMID: 31107565). In this context, multiple studies have demonstrated the contribution and importance of TRAIL-mediated cytotoxicity against various target cells (PMID: 22384114, PMID: 11257133, PMID: 11135622, PMID: 11745330). In addition, it was reported that NK cells switch from granule-mediated to death-receptors-mediated cytotoxicity in serial killing events (PMID: 31107565). Therefore, TRAIL-mediated cytotoxicity can be considered an important mechanism for NK cell-mediated killing of target cells.

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2. The study is currently limited to measurements of degranulation, granzyme B release and IFNgamma production. Yet, it is not shown whether the manipulation of TRAIL reverse signaling has also an effect on the cytotoxic activity of NK cells as the ultimately relevant response. In my opinion, such a demonstration would significantly strengthen the relevance and impact of this study.

2. Reply: The assessments of degranulation, granzyme B release and IFNγ production have been widely accepted as readouts for NK cell responses, generally covering the main effector functions of NK cells, cytotoxicity and cytokine production. Degranulation, GrzB release and even IFNγ have been shown to correlate with cytotoxicity (CD107a: PMID: 15604012, PMID: 18835598; GrzB: PMID: 15380049; IFNγ: PMID: 11145847). However, we do acknowledge that we did not directly measure cytotoxic activity of NK cells. Thus, we performed multiple cytotoxicity assays to confirm the impact of TRAIL on NK-cell-mediated cytotoxicity.

First, we generated a 721.221 double knockout cell line using CRISPR/Cas9 that featured the disruptions of the genes encoding DR4 and DR5 (.221-DR4/5KO) as well as a Raji cell line that overexpressed DR5 (Raji-DR5++). The altered expression levels of DR4 and DR5 in the transduced cell lines are in shown **Figure R3-2.1**.

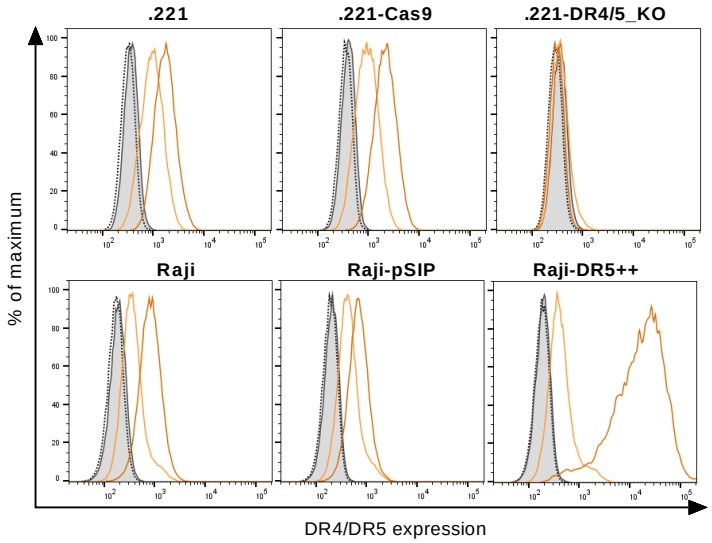

Figure R3-2.1 | Expression of TRAIL receptors on transduced 721.221 and Raji cells. The expression of DR4 and DR5 was assessed by flow cytometry. 721.221 and Raji cells were labelled with LIVE/DEAD Fixable Near-IR Stain, followed by incubation with biotin-conjugated mouse anti-human DR4 or DR5 and then labelled with Streptavidin-BV421. Expression was quantified as fluorescence intensity. Representative histogram of DR4 (light orange) and DR5 (dark orange) expression in comparison to the Streptavidin only control (grey) or the FMO control (dashed line). Upper panel (from left to right): untransduced 721.221 cells, Cas9-transduced .221s, DR4/5 double knockout .221s. Lower panel (from left to right): untransduced Raji cells, Raji cells transduced with an empty vector (pSIP), Raji cells overexpressing DR5.

Then we compared the ability of NK cells to lyse the respective cell lines in two competitive killing assays. In these assays, designated target cells and control cells were fluorescently labelled, mixed in a fixed ratio (app. 1:1) and then cultured in the presence or absence of NK cells (E:T ratio: 1:1) for five hours. Immediately after incubation, remaining cells were counted at a flow cytometer and normalized to counting beads. Specific lysis of designated target cells was calculated as previously described in Stary *et al.* (PMID: 33067380) with the following equation:

[1 − (#control cells/#target cells)$_{\text{no NK cells}}$ / (#control cells/#target cells)$_{\text{with NK cells}}$] × 100.

As shown in **Figure R3-2.2**, NK cells preferentially killed the designated target cells (.221-Cas9: p = 0.002, Raji-DR5++: p = 0.0005) compared to the control cells which either lack DR4 and DR5 (.221-DR4/5KO) or express a lower amount of DR5 (Raji-pSIP). The results of these experiments clearly showed that target cells expressing death receptors or higher amounts of death receptors are increasingly sensitive to NK-cell-mediated cytotoxicity.

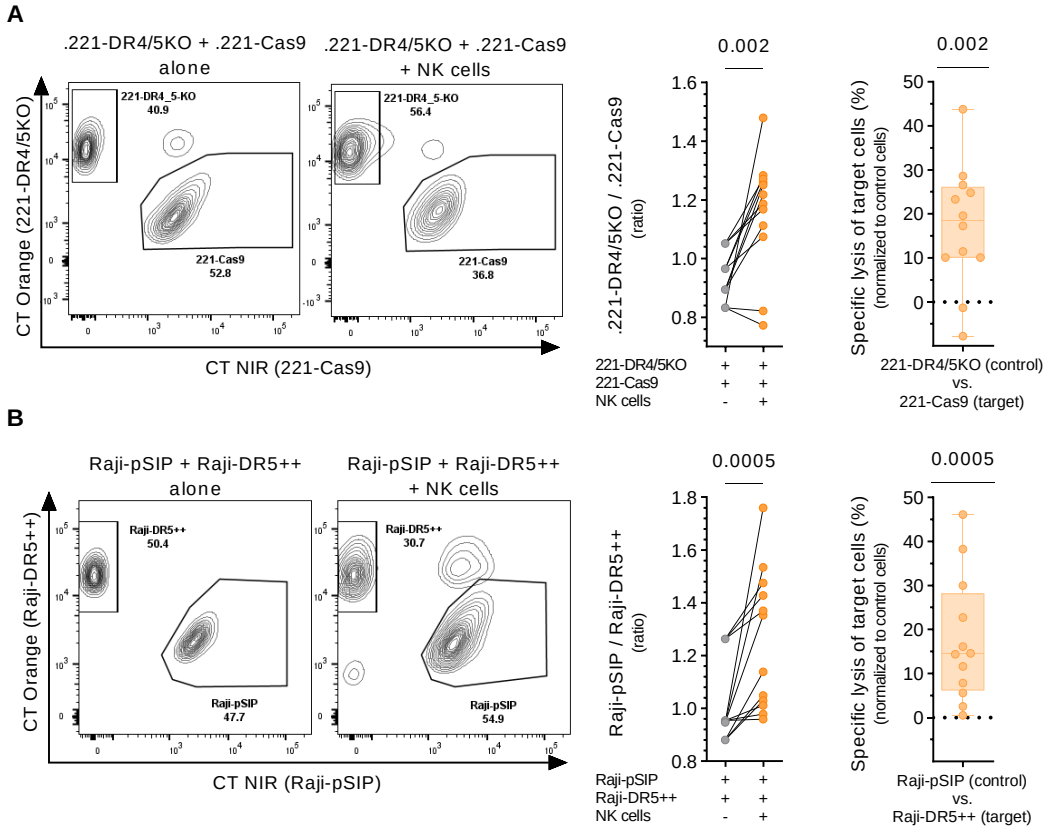

Figure R3-2.2 | Preferential killing of TRAIL receptor-positive target cells by NK cells. (A) Left panel: Representative contour plots displaying .221-DR4/5KO control cells (top left) and .221-Cas9 target cells (right). Numbers represent the relative frequency of the gated population. Middle panel: Ratio of .221-DR4/5KO and .221-Cas9 target cells after 5 h incubation in the absence or presence of NK cells (n = 12). Right panel: Specific lysis (in %) of .221-Cas9 cells (n = 12). **(B)** Left panel: Representative contour plots displaying Raji-DR5++ target cells (top left) and Raji-pSIP control cells (right). Numbers represent the relative frequency of the gated population. Middle panel: Ratio of Raji-pSIP and Raji-DR5++ target cells after 5 h incubation in the absence or presence of NK cells (n = 12). Right panel: Specific lysis (in %) of Raji-DR5++ cells (n = 12). **Data information:** Wilcoxon signed-rank test. Experiments were performed in four batches with three different donors each. No NK cell control served for all donors in each batch. Lines connect each data value of the NK cell condition with their designated No NK cell control. Each data point represents the mean of at least 3 technical replicates. Box plots represent the median and 25%/75% percentile. Whiskers indicate minimum and maximum data points.

Lastly, we cultured 721.221 cells alone or in co-culture with NK cells in the presence of either αTRAIL (10ug/ml) or the respective isotype control (10ug/ml). After 5-hour incubation, remaining cells were counted using a flow cytometer and then normalized to counting beads. Our results showed a generally strong ability of NK cells to lyse .221 cells (**Figure R3-2.3**). More importantly, presence of αTRAIL reduced NK-cell-mediated lysis compared to the isotype control (p = 0.012).

Blockade of TRAIL interactions resulted in an increased number of remaining target cells (median 5.1 p.p. compared to isotype, p = 0.012). While the effect of the TRAIL blockade on target cell lysis seems modest in our experimental setup at first, it should be noted that .221 cells are HLA class I devoid and are therefore highly sensitive to NK cell mediated cytotoxicity. Given a certain redundancy of activating NK cell receptors that are triggered after exposure to .221 cells, the contribution of TRAIL to target cell lysis is therefore considerable.

Collectively, our results showed that i) target cells with increased DR4 or DR5 expression are preferentially killed by NK cells and ii) that blockade of TRAIL interactions using soluble αTRAIL led to reduced killing by NK cells. We included and discussed these results in the revised manuscript.

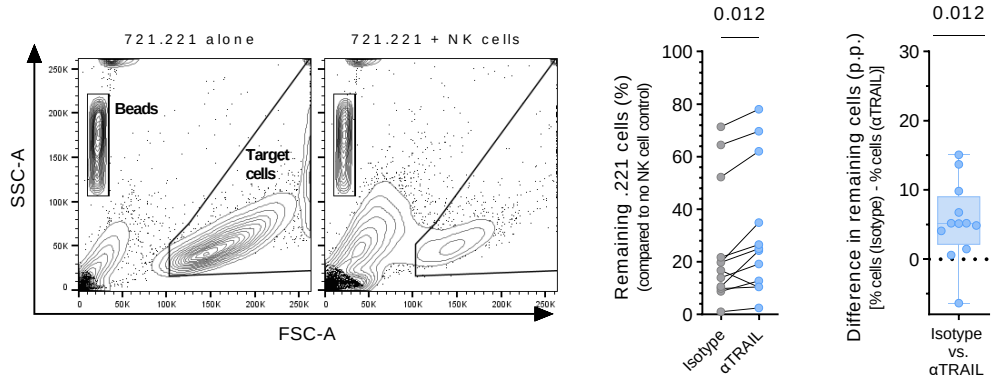

Figure R3-2.3 | Blocking of TRAIL interactions decreases NK-cell-mediated killing of .221 cells. Left panel: Representative contour plots displaying counting beads (top left), target cells (right) and NK cells (bottom left) based on size (FSC-A) and granularity (SSC-A). Middle panel: Percentage of remaining target cells in the presence of αTRAIL or isotype control compared to the culture condition without NK cells. Each data point represents one donor (n = 12). Right panel: Difference in remaining cells (percentage points, p.p.) between αTRAIL or isotype condition. **Data information:** Wilcoxon signed-rank test. Each data point represents the mean at least 2 technical replicates. Lines connect data points of the same donor. Box plots represent the median and 25%/75% percentile. Whiskers indicate minimum and maximum data points.

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3. The existence of TRAIL reverse signaling is controversial due to the very short cytoplasmic moiety of TRAIL (PMID: 33215853). Therefore, the study would gain impact if the authors provided more conclusive evidence for intracellular signaling in response to TRAIL engagement (e.g., p38 activation, as discussed in line 231) beyond solely describing cellular responses after inhibition or engagement of TRAIL.

3. Reply: We are grateful to the referee for this suggestion in order to strengthen the manuscript. Accordingly, we performed additional experiments investigating intracellular signaling in response to TRAIL engagement. For this, we incubated NK cells from 3 different donors with IL-15 and IL-2 for 4 days and then co-cultured NK cells with various immobilized antibodies and proteins for 30 min. Immediately after we measured the phosphorylation of the following molecules: PLC-γ2 (pY759), Syk (pY348), p38 MAPK (pT180/pY182) and Akt (pS473). Given the rapid speed of NK cell receptor signaling and the associated phosphorylation state of signaling molecules we sought to quantify elapsed signaling of NK cells, by measuring the decrease of phosphorylated signaling proteins after receptor engagement. Data analysis showed a high baseline phosphorylation of the examined

molecules in the majority of NK cells for the unstimulated conditions, PBS and isotype control (**Figure R3-3**). This is most likely due to pro-longed exposure to IL-15 and IL-2 (PMID: 33593878) and that NK cells were not starved before the stimulation.

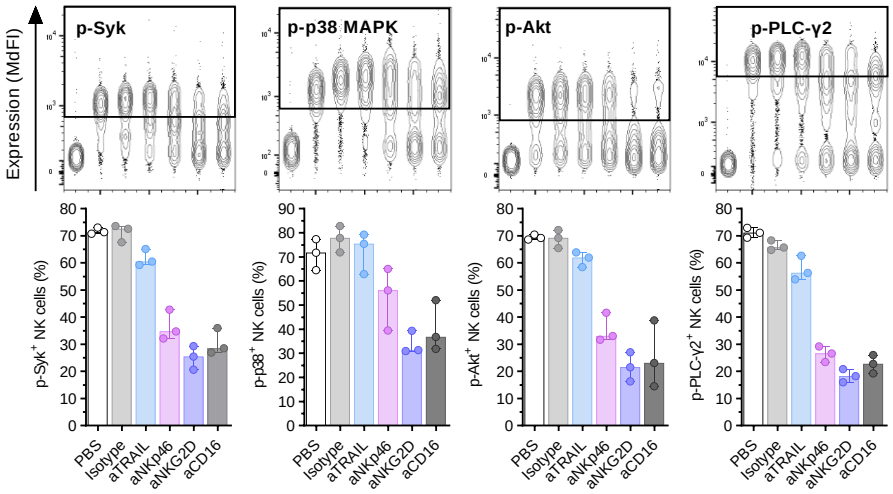

Figure R3-3 | Expression levels of phosphorylated signaling proteins. Upper panel: Concatenated contour plots of one donor depicting the expression of phosphorylated signaling proteins Syk, p38 MAPK, Akt and p-PLC-γ2 for the following culture conditions and controls (x-axis: left to right): FMO, PBS, isotype, αTRAIL, αNKp46, αNKG2D, αCD16. Lower panel: Bar graphs showing the relative frequency of p-Syk+, p-p38 MAPK+, p-Akt+ and p-PLC-γ2+ NK cells after 30 min of stimulation (n = 3 different donors). **Data information:** Bar graphs represent the median and the associated whiskers display min and max.

Either engagement of the activating receptors NKp46, NKG2D or CD16 led to a marked reduction of NK cells expressing phosphorylated signaling proteins. In contrast, observed effects after the engagement of TRAIL through immobilized αTRAIL proteins were less pronounced. NK cells co-cultured with αTRAIL did exhibit a reduction of phosphorylated Syk$^+$, Akt$^+$, and PLC-γ2$^+$ NK cells, but not for p-p38 MAPK. These results seem to reflect the hierarchy of NK degranulation levels we observed for similar experiments in Figure 5. Altogether, these results suggest that limited NK cell signaling takes place after the engagement of TRAIL on NK cells. Signaling, however, seems to be less effective compared to the activation of NK cells through the activating NK cell receptors which have the ability to associate with activating adapter proteins through positively charged amino acids in their transmembrane domains. The results of these experiments have been included and discussed in the revised manuscript. Further and more focused investigation of TRAIL signaling is certainly warranted but currently exceeds the scope of the study and the available resources.

Minor:

1. The authors use OPG and TRAIL-R3 as engagers of TRAIL and should mention whether they are expressed on CD4 T cells, i.e., whether they have physiological relevance in this system.

4. Reply: The initial attempt of testing OPG and TRAIL-R3 was to see whether the induction of degranulation by engaging TRAIL was only limited to certain TRAIL interaction partners. Since

osteoprotegerin is a soluble protein (PMID: 9108485) it is not expressed on the surface of CD4 T cells. However, two publications reported that human CD4 T cells can produce and secrete osteoprotegerin (PMID: 18040268, PMID: 29424771). Furthermore, its production seemed to be reduced by HIV infection. The results regarding TRAIL-R3 expression on CD4 T cells are contradictory. In principle, CD4 T cells appear to be able to express TRAIL-R3, but the extent described is highly variable. Overall, the expression level is rather low. In two publications, no (PMID: 15919363) or negligible (PMID: 19690337) expression was detected on freshly isolated CD4 T cells. In another study, expression was detected in HIV negative as well as HIV positive CD4 T cells (PMID: 11602752). Our assessment of DcR1 is in line with the latter report, showing DcR1 expression on CD4 T cells (**Figure R3-4**). We followed the referee's advice and included our findings in the results section of the revised manuscript.

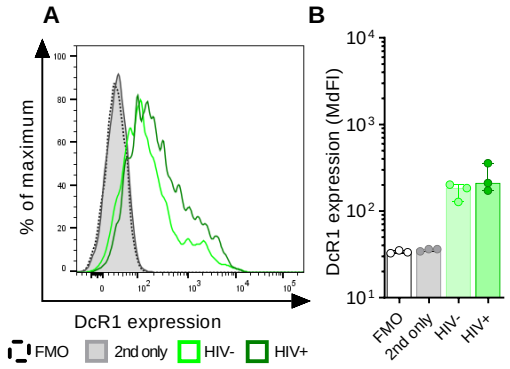

Figure R3-4 | DcR1 is expressed on CD4 T cells. Primary enriched CD4 T cells were stimulated with αCD3/CD28 for 3 days and then infected with the HIV strain NL4-3. Four days post infected cells were labelled with LIVE/DEAD Fixable Near-IR Stain, followed by incubation with CD4 APC, p24-FITC, goat anti-human DcR1 and then labelled with anti-goat-PE. Expression was measured as median fluorescence intensity (MdFI) by flow cytometry. **(A)** Representative flow cytometry histogram of DcR1 expression on uninfected (HIV-: $CD4^+/p24^-$) and infected (HIV+: CD4-/p24+) cells in comparison to the anti-goat-PE only control (grey) or FMO control (dashed line). **(B)** Cumulative data of DcR1 expression displayed as MdFI. Data points represent the mean of two technical replicates per condition of 3 different donors (n = 3). Columns show the median. Error bars show IQR.

In Figure. 4F, G, degranulation is reduced by antibodies to TRAIL-R1/TRAIL-R2. Have the authors excluded that this is due to agonistic killing of the target cells by the antibodies (i.e., less target cells, less engagement of NK cells, less degranulation)?

5. Reply: The referee raises an interesting question. Given the information provided by the vendor AdipoGen, we did not anticipate agonistic killing of the target cells by the antibodies. For both antibodies, anti-TRAIL-R1 (αDR4, clone HS101, Cat# AG-20B-0022PF) and anti-TRAIL-R2 (αDR5, clone HS201, Cat# AG-20B-0023PF), the functional application notes read:

- anti-TRAIL-R1: Inhibition (blocks TRAIL-R1 mediated killing if applied in solution)
- anti-TRAIL-R2: Inhibition (blocks TRAIL-R2 mediated killing if applied in solution)

The majority of studies previously using these antibodies, reported antagonistic effects (blocking induction of apoptosis) by the antibodies in question (PMID: 12198154, PMID: 12839575, PMID: 14726404, PMID: 16638878). A potential agonistic side effect caused by αDR5 but not αDR4 was reported in one study (PMID: 16297203). Another study reported a minor agonistic effect against TRAIL-resistant Hep3b cells when αDR5 and αDR4 were used together (PMID: 15105837). Based on these studies we tested the potential agonistic effect of αDR4 and αDR5 against 721.221 cells. Co-culture of 721.221 cells with soluble anti-DR4/DR5 had no detrimental effect on cell numbers and viability (Table R3-5). Based on our findings we can now exclude agonistic killing of the target cells by the respective antibodies in our experimental conditions.

Condition	**Cell conc.**	**Viability**
Media	$0.98*10^6$	71%
Isotype (20 ug/ml)	$0.90*10^6$	70%
αDR4 (10ug/ml) + αDR5 (10ug/ml)	$1.09*10^6$	76%

721.221 cells were cultured at $1*10^6$ cells/ml for 5 ½ hours. Cell concentration and viability were measured at Bio-Rad T20 cell counter. N = 3 technical replicates per condition. Values represent the mean of technical replicates.

In Figure 6A, plate-coated anti-TRAIL antibodies induce IFNgamma, whereas in Figure 6B, soluble anti-TRAIL antibodies reduce its production in the coculture system. Please explain this discrepancy to the reader (no crosslinking of TRAIL by the soluble antibodies?).

6. Reply: The referee is correct that soluble antibodies generally do not cross-link TRAIL on NK cells in contrast to its plate-bound form. Immobilized (plate-bound) antibodies have been widely used to induce cross-linking of the targeted antigen and subsequent signaling, while soluble antibodies are regularly used to block the interaction between receptors and its respective ligands. The rigged orientation and the high density of immobilized antibodies facilitate clustering of the recognized receptors and subsequent signaling. Soluble antibodies in most cases do not have the capacity to induce cross-linking of the receptor and therefore show antagonistic effects. The induction of agonistic (triggering of degranulation) and antagonistic effects (blocking) by the same antibodies was applied by Niehrs *et al.* (Nat Immunol., 2019, PMID: 31358998) for the activating NK cell receptor NKp44 and by Garcia-Beltran *et al.* (Nat Immunol,. 2016, PMID: 27455421) for the activating NK cell receptor KIR3DS1. We added the following sentences in the methods section of the revised manuscript to explain the different mode of actions.

"Blocking of TRAIL interactions was conducted by using a soluble TRAIL antibody (BioLegend, clone RIK-2)."

"Immobilized (plate-coated) antibodies and proteins were used to induce cross-linking of various NK cell receptors and to trigger subsequent degranulation."

Dear Dr. Körner,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that were asked to re-evaluate your study, you will find below. As you can see, referees #1 is satisfied by the revisions and supports publication. However, referees #2 and #3 still have concerns and do not support publication of the study in its current form. Both referees indicate that major aspects of the study are still unclear, in particular they note that a pro-apoptotic role of TRAIL on target cells in this study cannot be excluded and that the involvement of reverse signaling by TRAIL is not convincingly shown.

Both referees indicate, though, that these points can be addressed in a further revision by adjusting the phrasing of the title, repharsing or discussing the interpretation of some of the results and by changing the conclusions so that they more accurately reflect the current data. I thus ask you to do that in a further revised version of the manuscript. Please also provide a detailed p-b-p-response to the remaining concerns of the referees.

Moreover, I have these editorial requests:

- Please provide the abstract written in present tense.

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

- Please order the manuscript sections like this:
Title page - Abstract - Introduction - Results - Discussion - Materials and Methods - Data Availability Section - Acknowledgements - Author contributions - 'Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main, EV and Appendix figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams. Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant.

- Please upload the information in Table 1 as 'Reagents and Tools table'. I have attached templates for that in word or excel format. Please upload the filled in table to the manuscript tracking system as a 'Reagent Table' file. Then remove the table from the final manuscript file. The example linked below shows how the table will display in the published article and includes examples of the type of information that should be provided for the different categories of reagents and tools. Please list your reagents/tools using the categories provided in the template and do not add additional subheadings to the table. Reagents/tools that do not fit in any of the specific categories can be listed under "Other":
https://www.embopress.org/pb%2Dassets/embo-site/msb_177951_sample_FINAL.pdf

- Please upload tables EV1 and EV2 as separate files and call these out with this name in the text (Table EV1 and Table EV2).

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

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (around 35 words).
- three to four short bullet points highlighting the key findings of your study
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

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

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The authors have carefully addressed all the points I had raised in the previous review and have modified their manuscript accordingly. I have no more comments.

Referee #2:

The authors have reasonably addressed more of my comments and performed additional experiment that strengthen their manuscript. Yet I still have some comments, which are detailed below.
In general, certain aspects are still unclear; at least, the phrasing of the title, the interpretation of some of the results and the conclusion should be changed to more accurately reflect the current data.

Major comments:

1. The authors show that there is a correlation between TRAIL expression on NK cells and CD107 positivity / degranulation, and that DR4/5-expressing targets are more susceptible to NK cell-mediated elimination. However, it is not demonstrated that degranulation of lytic granules is per se (solely) responsible of the cytolysis of DR4/5-expressing targets. In other terms, based on the data presented in the manuscript, it cannot be excluded that cell killing / apoptosis in their system occurs via engagement of NK cell-derived TRAIL ligand on DR4/DR5-expressing target cells. Therefore, with the current data it would be more accurate to refer to TRAIL-induced apoptosis / killing of target cells.
Additional experiments would be necessary to exclude a pro-apoptotic role of TRAIL on target cells in this study. This could be addressed for instance via inhibition of the perforin / GZMB pathway (e.g. pharmacologically; or via expression of a serine protease inhibitor in target cells); or by reconstituting 721.221-DR4/5 DKO cells (Fig. 7) with DR4/5 receptors harboring a deleted death domain; or by overexpressing DcR1/2 on target cells to impair engagement of dead-inducing DR4/5 receptors.
Alternatively, the authors should show that the NK target cells they use are resistant to TRAIL-induced cell apoptosis.

At least, this aspect needs to be clearly and better presented and discussed in their manuscript.

2. While the authors convincingly present an apoptosis-independent role of TRAIL for NK cells in their system, there is not clear or definitive evidence that this occurs via reverse signaling. Indeed, although the experiments are well executed, there are several datasets that do not permit to rule out that TRAIL activates DR4/5 on NK cells either via a cis- or trans-mechanism:
-there is no apparent correlation between NK cell degranulation and the affinity for TRAIL of the different TRAIL receptors (Fig. 5). Among others, there is no dose-dependent effect of DcR1 and NK cells degranulation (Fig 5E).
-Although their level is not high, DR4/5 are still clearly expressed on NK cells (Figure R2-5).
-Although phospho flow cytometry staining requires some expertise and the data in Fig. 6E are well performed, there is only a minor effect of TRAIL on the phosphorylation level of phospho -Syk, -Akt and -PLC-gamma2. In addition, this experiment was apparently only performed once (with 3 different NK cell donors).
Stronger evidence for reverse TRAIL signaling in their model could be obtained for instance by addressing the effect of agonist DR5/DR4 ligands on NK cells on the phosphorylation level of the signaling effectors tested in Fig. 6E; or by performing the assays shown in Fig 6E (or in other figures) using antagonistic DR5/DR4 ligands. Alternatively, the authors should use a more careful / general wording (e.g. non-apoptotic / apoptosis-independent TRAIL signaling...) and remove the mention of TRAIL reverse signaling from their title and result part.

Referee #3:

In my original assessment, I had raised a number of concerns to which the authors have now responded with a revised version of their manuscript and a rebuttal letter. Below, I have listed my respective original concerns and my impressions of the authors' responses:
- General comment: "As one example: in line 48, the authors should explain that CD107a is a marker for NK cell degranulation for readers from non-related fields."
This has been done, this comment is fully addressed.
- Minor comment 1: "The authors use OPG and TRAIL-R3 as engagers of TRAIL and should mention whether they are expressed on CD4 T cells, i.e., whether they have physiological relevance in this system."
This has been done, this comment is fully addressed.

- Minor comment 2: "In Figure. 4F, G, degranulation is reduced by antibodies to TRAIL-R1/TRAIL-R2. Have the authors excluded that this is due to agonistic killing of the target cells by the antibodies (i.e., less target cells, less engagement of NK cells, less degranulation)?"
This comment has been satisfactorily addressed in the rebuttal letter.
- Minor comment 3: "In Figure 6A, plate-coated anti-TRAIL antibodies induce IFNgamma, whereas in Figure 6B, soluble anti-TRAIL antibodies reduce its production in the coculture system. Please explain this discrepancy to the reader (no crosslinking of TRAIL by the soluble antibodies?).
This has been done, this comment is fully addressed.
- Major comment 2: " The study is currently limited to measurements of degranulation, granzyme B release and IFNgamma production. Yet, it is not shown whether the manipulation of TRAIL reverse signaling has also an effect on the cytotoxic activity of NK cells as the ultimately relevant response. In my opinion, such a demonstration would significantly strengthen the relevance and impact of this study."
The authors have now included data showing that "i) target cells with increased DR4 or DR5 expression are preferentially killed by NK cells and ii) that blockade of TRAIL interactions using soluble αTRAIL led to reduced killing by NK cells." While these experiments address my request for cytotoxicity assays, they do not answer my concern "whether the manipulation of TRAIL REVERSE SIGNALING has also an effect on the cytotoxic activity of NK cells". While the data show that the killing depends on the expression levels of DR4/5, this killing may also be caused by direct cytotoxic signaling through DR4/5 rather than by reverse signaling. Likewise, the addition of anti-TRAIL antibodies may just block direct rather than reverse cytotoxic signaling. Overall, this major concern has been partially addressed by the authors, but the new data do not - at least in my opinion - provide further evidence for an importance of TRAIL reverse signaling in the cytotoxic response of human NK cells.
- Major comment 3: "The existence of TRAIL reverse signaling is controversial due to the very short cytoplasmic moiety of TRAIL (PMID: 33215853). Therefore, the study would gain impact if the authors provided more conclusive evidence for intracellular signaling in response to TRAIL engagement (e.g., p38 activation, as discussed in line 231) beyond solely describing cellular responses after inhibition or engagement of TRAIL."
Following my suggestion, Höfle et al. have investigated changes in the phosphorylation of PLC-γ2, Syk, p38, and Akt. While this is per se commendable, it puzzles me that the authors observe a markedly DECREASED phosphorylation of these molecules after engagement of the activating receptors NKp46, NKG2D or CD16. Since the very first experiments in signal transduction, it has been firmly established in thousands of publications that activation of proteins by phosphorylation is almost always caused by an increase rather than a decrease of phosphorylation. Accordingly, (NKG2D-mediated) NK cell cytotoxicity has been shown to depend on an increased, not decreased phosphorylation of PLC-γ2 (e.g., PMID: 12740575), Syk (e.g., PMID: 9396765) or Akt (e.g., PMID: 28360428). However, this discrepancy is not adequately discussed or resolved in the revised manuscript. Rather, the authors state that "Here we provide further indications that TRAIL engagement may activate signaling proteins, such as Syk, Akt and PLC-γ2, which also play an important role in the signaling pathways of known activating NK cell receptors...". In my understanding, the shown results are in contradiction, not in support of an activation of PLC-γ2, Syk and Akt by activating NK cell receptors.
As another point, and as stated by the authors, "these results suggest that limited NK cell signaling takes place after the engagement of TRAIL on NK cells. Signaling, however, seems to be less effective compared to the activation of NK cells through the activating NK cell receptors...". In consequence, this means that reverse signaling by TRAIL has only a minor role compared to the signaling events triggered by activating NK cell receptors, reducing the impact of the study.
In summary, although the authors have formally addressed my concern and performed the requested experiments, the observed results do not clarify but rather confuse the picture, and this is not satisfactorily discussed and explained to the reader in the revised manuscript.
- Major comment 1a: "Currently, the authors demonstrate the presence of effects that are statistically significant, but nevertheless only of modest nature"
The authors now show normalized vs. representative and cumulative data, and I consider this as a satisfactory solution.
- Major comment 1b: "For me, it remains open to which extent the engagement of TRAIL on NK cells contributes to the total cytotoxic response of NK cells against their targets. Is this a major pathway for the elimination of pathogens or just a minor additional mechanism, if at all? This could, e.g., be addressed by deleting TRAIL from primary human NK cells or NK cell lines via CRISPR/Cas (such gene deletion has been established, e.g., for primary T cells, PMID: 29436394)."
Here, the authors cite from the literature, e.g., that "TRAIL-mediated cytotoxicity has been shown to be important for NK cell-mediated control of viral infections and cancer", that "NK cells switch from granule-mediated to death-receptors-mediated cytotoxicity in serial killing events" and that "TRAIL-mediated cytotoxicity can be considered an important mechanism for NK cell-mediated killing of target cells". While all these literature data are correct by themselves, they do not address my concern of whether these effects depend on reverse signaling, i.e., "the engagement of TRAIL on NK cells", as they may also be caused by direct signaling of TRAIL via the TRAIL receptors. This is exactly why I had suggested to use a genetic system, i.e., deletion of TRAIL from human NK cells via CRISPR/Cas. Given that a similar suggestion has been made by referee #1, it surprises me that the authors have chosen to completely ignore this suggestion in their revision. The manuscript lacks corresponding experiments, and not even an explanation is given in the rebuttal letter or the manuscript why the authors deemed it unnecessary to perform these experiments. Therefore, this comment remains completely unaddressed.

In summary, this revision does not address my concerns to the extent that I had hoped for.

Authors' response to the Referee Reports for EMBOR-2021-54133V2

Dear editor,

Please find below our point-by-point response to your and the referees' comments.

Sincerely,

Christian Körner

Referee #1

The authors have carefully addressed all the points I had raised in the previous review and have modified their manuscript accordingly. I have no more comments.

Reply: We would like to thank referee #1 for their ultimately positive assessment of our revised

manuscript.

Referee #2

The authors have reasonably addressed more of my comments and performed additional experiment that strengthen their manuscript. Yet, I still have some comments, which are detailed below. In general, certain aspects are still unclear; at least, the phrasing of the title, the interpretation of some of the results and the conclusion should be changed to more accurately reflect the current data.

Reply: We followed the referee's recommendation and revised the phrasing of the title, the interpretation of some of the results and the conclusion to more accurately reflect the current data. We also added a section in the discussion of the manuscript addressing remaining open questions and limitations of the study.

Major comments:
1. The authors show that there is a correlation between TRAIL expression on NK cells and CD107 positivity / degranulation, and that DR4/5-expressing targets are more susceptible to NK cell-mediated elimination. However, it is not demonstrated that degranulation of lytic granules is per se (solely) responsible of the cytolysis of DR4/5-expressing targets. In other terms, based on the data presented in the manuscript, it cannot be excluded that cell killing / apoptosis in their system occurs via engagement of NK cell-derived TRAIL ligand on DR4/DR5-expressing target cells. Therefore, with the current data it would be more accurate to refer to TRAIL-induced apoptosis / killing of target cells.
Additional experiments would be necessary to exclude a pro-apoptotic role of TRAIL on target cells in this study. This could be addressed for instance via inhibition of the perforin / GZMB pathway (e.g. pharmacologically; or via expression of a serine protease inhibitor in target cells); or by reconstituting 721.221-DR4/5 DKO cells (Fig. 7) with DR4/5 receptors harboring a deleted death domain; or by overexpressing DcR1/2 on target cells to impair engagement of dead-inducing DR4/5 receptors. Alternatively, the authors should show that the NK target cells they use are resistant to TRAIL-induced cell apoptosis.
At least, this aspect needs to be clearly and better presented and discussed in their manuscript.

Reply: We are grateful for the referee's suggestions to address this specific matter. Indeed, inhibition of either granule- or death-receptor mediated apoptosis would provide an indication of the proportion of either mode of action associated with target cell lysis. However, this was not the aim of the study and we are not disputing that TRAIL-induced death-receptor-mediated apoptosis may take place in our experimental setup. Collectively, our data demonstrates that the direct engagement of TRAIL on NK cells leads to degranulation as measured by CD107a (Figure 5). CD107a expression strongly correlates with target cell lysis (PMID: 15604012). In our opinion, it is therefore highly likely that TRAIL engagement contributes to granule-mediated induction of apoptosis independent of and in addition to death-receptor-mediated induction of apoptosis.

The referee's suggestions are certainly exciting ideas for subsequent studies investigating this interesting aspect of TRAIL biology but are, as already mentioned, beyond the scope of this study. Some potential limitations of the recommended experiments come to mind though. In the case of inhibiting the perforin / GZMB pathway, we would not be able to identify the responsible NK cell receptors preceding the activation of NK cells, let alone whether it is triggered by TRAIL. On the other hand, blocking of death-receptor-mediated apoptosis by reconstituting 721.221-DR4/5 DKO cells with DR4/5 receptors harboring a deleted death domain would allow us in theory to measure the contribution of TRAIL on granule-mediated cytotoxicity (i.e. in presence or absence of anti-TRAIL). Altogether, we included these suggestions as an outlook in the discussion and further clarified the above mentioned aspects in a revised version of the manuscript.

2. While the authors convincingly present an apoptosis-independent role of TRAIL for NK cells in their system, there is not clear or definitive evidence that this occurs via reverse signaling. Indeed, although the experiments are well executed, there are several datasets that do not permit to rule out that TRAIL activates DR4/5 on NK cells either via a cis- or trans-mechanism:
-there is no apparent correlation between NK cell degranulation and the affinity for TRAIL of the different TRAIL receptors (Fig. 5). Among others, there is no dose-dependent effect of DcR1 and NK cells degranulation (Fig 5E).
-Although their level is not high, DR4/5 are still clearly expressed on NK cells (Figure R2-5).

-Although phospho flow cytometry staining requires some expertise and the data in Fig. 6E are well performed, there is only a minor effect of TRAIL on the phosphorylation level of phospho -Syk, -Akt and -PLC-gamma2.
In addition, this experiment was apparently only performed once (with 3 different NK cell donors). Stronger evidence for reverse TRAIL signaling in their model could be obtained for instance by addressing the effect of agonist DR5/DR4 ligands on NK cells on the phosphorylation level of the signaling effectors tested in Fig. 6E; or by performing the assays shown in Fig 6E (or in other figures) using antagonistic DR5/DR4 ligands. Alternatively, the authors should use a more careful / general wording (e.g. non-apoptotic / apoptosis-independent TRAIL signaling...) and remove the mention of TRAIL reverse signaling from their title and result part.

Reply: We agree with the referee that this study does not provide definitive evidence of reverse signaling since the exact mechanism of how engagement of TRAIL leads to the activation of NK cells (i.e. degranulation and IFNγ production) has yet to be identified. In our opinion, the elucidation of the underlying mechanisms are beyond the scope of the current study which sought to describe the effect as well as possible and to rule out other explanations (e.g. TRAIL serving as an activation marker). We added a sentence to the discussion clarifying this.

Here, the referee suggested an additional hypothesis i.e. that TRAIL activates DR4/5 on NK cells either via a cis- or trans-mechanism. In this context, it should be noted that indeed multiple non-canonical effects of TRAIL beyond induction of apoptosis have been reported in the past (PMID: 23579241). Yet, to our knowledge, there were no reports nor comparable observations so far that the engagement of DR4 or DR5 leads to degranulation or production of IFNγ in NK cells.

We are thankful for the opportunity to discuss the results of our study highlighted by the referee, as we do not believe that they necessarily support cis-/trans signaling of TRAIL via DR4/5 on NK cells. The fact, that "there is no apparent correlation between NK cell degranulation and the affinity for TRAIL of the different TRAIL receptors (Fig. 5)." could be due to multiple others reasons. The referee previously referred to a study by Truneh and colleagues which determined binding affinities, using isothermal titration calorimetry (ITC). ITC is a highly sensitive and accurate physical technique, intended to use thermodynamics for the calculation of binding affinity between molecules, among other things. In the present study, we assessed the cellular response of NK cells after TRAIL engagement. In contrast to ITC, our experimental setup contains a cellular system with all its complexity. Therefore, sole binding affinities between TRAIL and its interaction partner may not coincide with the functional readouts we conducted.

While one could argue that NK cells exhibit a low density expression of TRAIL receptors on their surface, this does not translate into increased activation of NK cells in our experiments despite high TRAIL expression levels. TRAIL-mediated activation of NK cells through engagement of TRAIL receptors in cis or trans was not observed in our negative controls, as sole NK cells did not show a high baseline level of degranulation and the latter was not affected by the presence of soluble anti-TRAIL (Figure 4).

Lastly, it is true that the phosphorylation experiment has been performed with 3 different donors, which is a lower number compared to other experiments in the study. Limitations in time and

availability of donors were unfortunately the reason for this. However, the fact that that experiment has been performed in one batch, shouldn't be held against us, as it was intended to reduce inter-assay variation.

Altogether, it is our opinion that there's no evidence for TRAIL-mediated activation of NK cells through engagement of TRAIL receptors in cis or trans on NK cells. In turn, we provided experimental evidence that the direct engagement of TRAIL on NK cells itself led to degranulation (Figure 5) and cytokine production (Figure 6) in NK cells. However, since the exact mechanism of TRAIL signaling on NK cells remains elusive for now, we revised the manuscript accordingly and use the alternative wording as suggested by the referee.

Referee #3

In my original assessment, I had raised a number of concerns to which the authors have now responded with a revised version of their manuscript and a rebuttal letter. Below, I have listed my respective original concerns and my impressions of the authors' responses:

- General comment: "As one example: in line 48, the authors should explain that CD107a is a marker for NK cell degranulation for readers from non-related fields."
This has been done, this comment is fully addressed.

- Minor comment 1: "The authors use OPG and TRAIL-R3 as engagers of TRAIL and should mention whether they are expressed on CD4 T cells, i.e., whether they have physiological relevance in this system."
This has been done, this comment is fully addressed.

- Minor comment 2: "In Figure. 4F, G, degranulation is reduced by antibodies to TRAIL-R1/TRAIL-R2. Have the authors excluded that this is due to agonistic killing of the target cells by the antibodies (i.e., less target cells, less engagement of NK cells, less degranulation)?"
This comment has been satisfactorily addressed in the rebuttal letter.

- Minor comment 3: "In Figure 6A, plate-coated anti-TRAIL antibodies induce IFNgamma, whereas in Figure 6B, soluble anti-TRAIL antibodies reduce its production in the coculture system. Please explain this discrepancy to the reader (no crosslinking of TRAIL by the soluble antibodies?).
This has been done, this comment is fully addressed.

- Major comment 2: " The study is currently limited to measurements of degranulation, granzyme B release and IFNgamma production. Yet, it is not shown whether the manipulation of TRAIL reverse signaling has also an effect on the cytotoxic activity of NK cells as the ultimately relevant response. In my opinion, such a demonstration would significantly strengthen the relevance and impact of this study."

The authors have now included data showing that "i) target cells with increased DR4 or DR5 expression are preferentially killed by NK cells and ii) that blockade of TRAIL interactions using soluble αTRAIL led to reduced killing by NK cells." While these experiments address my request for cytotoxicity assays, they do not answer my concern "whether the manipulation of TRAIL REVERSE SIGNALING has also an effect on the cytotoxic activity of NK cells". While the data show that the killing depends on the expression levels of DR4/5, this killing may also be caused by direct cytotoxic signaling through DR4/5 rather than by reverse signaling. Likewise, the addition of anti-TRAIL antibodies may just block direct rather than reverse cytotoxic signaling.

Overall, this major concern has been partially addressed by the authors, but the new data do not - at least in my opinion - provide further evidence for an **importance of TRAIL reverse signali**ng in the cytotoxic response of human NK cells.

Reply: We agree with the reviewer that the cytotoxicity experiments conducted does not allow any distinction between the granule-mediated and death-receptor-mediated cytotoxicity. In this context, it is in fact highly likely that the addition of anti-TRAIL antibodies or the increased expression of death receptors affected death-receptor-mediated induction of apoptosis. However, the provided evidence in this manuscript [e.g. Figure 4 (blocking), Figure 5 (induction)] clearly indicated that the direct engagement of TRAIL contributes significantly to granule exocytosis which in itself is an important mechanism for NK-cell-mediated cytotoxicity and the pre-dominant mode of action for initial target cell killing executed by NK cells. To determine the "importance of TRAIL reverse signaling in the cytotoxic response of human NK cells" was not within the scope of this study, however interesting this question may be. This study will be important to initiate future research addressing this question in subsequent studies. The potential contribution of TRAIL reverse signaling to the cytotoxic response of NK cells will be a challenging task requiring a new experimental study as multiple confounding factors are required to be taken into account. These include the expression levels of TRAIL receptors on target cells, the expression of other ligands for NK cell receptors and the expression profile of the individual NK cell itself. Nevertheless, we addressed this matter as a remaining open question in the discussion section of the manuscript.

- Major comment 3: "The existence of TRAIL reverse signaling is controversial due to the very short cytoplasmic moiety of TRAIL (PMID: 33215853). Therefore, the study would gain impact if the authors provided more conclusive evidence for intracellular signaling in response to TRAIL engagement (e.g., p38 activation, as discussed in line 231) beyond solely describing cellular responses after inhibition or engagement of TRAIL."
Following my suggestion, Höfle et al. have investigated changes in the phosphorylation of PLC-γ2, Syk, p38, and Akt. While this is per se commendable, it puzzles me that the authors observe a markedly DECREASED phosphorylation of these molecules after engagement of the activating receptors NKp46, NKG2D or CD16. Since the very first experiments in signal transduction, it has been firmly established in thousands of publications that activation of proteins by phosphorylation is almost always caused by an increase rather than a decrease of phosphorylation. Accordingly, (NKG2D-mediated) NK cell cytotoxicity has been shown to depend on an increased, not decreased phosphorylation of PLC-γ2 (e.g., PMID: 12740575), Syk (e.g., PMID: 9396765) or Akt (e.g., PMID: 28360428). However, this discrepancy is not adequately discussed or resolved in the revised manuscript. Rather, the authors state that "Here we provide further indications that TRAIL engagement may activate signaling proteins, such as Syk, Akt and PLC-γ2, which also play an important role in the signaling pathways of known activating NK cell receptors...". In my understanding, the shown results are in contradiction, not in support of an activation of PLC-γ2, Syk and Akt by activating NK cell receptors.
As another point, and as stated by the authors, "these results suggest that limited NK cell signaling takes place after the engagement of TRAIL on NK cells. Signaling, however, seems to be less effective compared to the activation of NK cells through the activating NK cell receptors...". In consequence, this means that reverse signaling by TRAIL has only a minor role compared to the signaling events triggered by activating NK cell receptors, reducing the impact of the study.

In summary, although the authors have formally addressed my concern and performed the requested experiments, the observed results do not clarify but rather confuse the picture, and this is not satisfactorily discussed and explained to the reader in the revised manuscript.

Reply: We apologize for the missing context and the misleading interpretation of the results of the phosphorylation experiments. We completely agree with the reviewer, that engagement of activating receptors such as NKG2D leads to the phosphorylation of signaling molecules in a timely fashion. The majority of the experiments assessing the phosphorylation state of signaling molecules in NK cells such as PLC-γ2, Syk or Akt chose incubation times of less than 10 minutes down to 1 minute. However, due to limited time and resources during the revision we were not able to adequately optimize and adapt our experimental setup to accommodate these short time frames and also wanted to keep a certain consistency with the previous experimental setups. Therefore, we decided to observe an elapsed signaling cascade by assessing phosphorylation after 30 minutes. The rationale behind this was that the phosphorylation signaling of molecules occurs rapidly and transiently with dephosphorylation following shortly thereafter (PMID: 33123121, PMID: 22340367; PMID: 10712927, PMID: 19740675; PMID: 28431241). In this context, it was previously shown that pre-treatment of NK cells with IL-15 leads to increased baseline phosphorylation of signaling molecules in murine NK cells (PMID: 33593878); an effect that we also observed in our negative controls. Altogether, this was indeed an unorthodox approach. Therefore, we provided further details in the result section of the revised manuscript.

Since, we do not have the resources to further investigate the signaling after TRAIL engagement at this point in time, we would like to leave this matter to the discretion of the referee. We are willing to remove the data in question if it is not deemed to add additional value. For the time being we provided additional context to the experimental setup and revised the conclusions drawn from it accordingly.

The overall impact of TRAIL-medicated granule exocytosis still needs to be determined and may play "minor role compared to the signaling events triggered by activating NK cell receptors" as suggested by the referee. But NK cell activation is tightly regulated and determined by the net balance of all signals integrated. The impact of TRAIL engagement on the responsiveness of a given NK cell is therefore highly dependent on the presence of receptor/ligand pair on the NK cell and the encountered target cell.

- Major comment 1a: "Currently, the authors demonstrate the presence of effects that are statistically significant, but nevertheless only of modest nature".
The authors now show normalized vs. representative and cumulative data, and I consider this as a satisfactory solution.

- Major comment 1b: "For me, it remains open to which extent the engagement of TRAIL on NK cells contributes to the total cytotoxic response of NK cells against their targets. Is this a major pathway for the elimination of pathogens or just a minor additional mechanism, if at all? This could, e.g., be addressed by deleting TRAIL from primary human NK cells or NK cell lines via CRISPR/Cas (such gene deletion has been established, e.g., for primary T cells, PMID: 29436394)."

Here, the authors cite from the literature, e.g., that "TRAIL-mediated cytotoxicity has been shown to be important for NK cell-mediated control of viral infections and cancer", that "NK cells switch from granule-mediated to death-receptors-mediated cytotoxicity in serial killing events" and that "TRAIL-mediated cytotoxicity can be considered an important mechanism for NK cell-mediated killing of target cells". While all these literature data are correct by themselves, they do not address my concern of whether these effects depend on reverse signaling, i.e., "the engagement of TRAIL on NK cells", as they may also be caused by direct signaling of TRAIL via the TRAIL receptors. This is exactly why I had suggested to use a genetic system, i.e., deletion of TRAIL from human NK cells via CRISPR/Cas. Given that a similar suggestion has been made by referee #1, it surprises me that the authors have chosen to completely ignore this suggestion in their revision. The manuscript lacks corresponding experiments, and not even an explanation is given in the rebuttal letter or the manuscript why the authors deemed it unnecessary to perform these experiments. Therefore, this comment remains completely unaddressed.

Reply: We apologize for this misunderstanding. We would like to ensure the referee that we did not avoid to address this matter on purpose. In our initial assessment of the proposed additional experiments we deemed the suggestion to delete TRAIL on NK cells a lower priority since our data already provided us with the comparison between $TRAIL^+$ and $TRAIL^-$ NK cells that clearly showed that NK cells with high TRAIL expression have a higher likelihood for degranulation. We therefore chose to pursue the other alternative proposed by referee #1, namely the generation of genetically modified target cells. This latter approach also seemed to overlap with suggestions and comments of referee #2 and #3.

While we could not ultimately identify the exact mechanism by which engagement of TRAIL triggers degranulation and IFNγ production, our results do not support the hypothesis that the observed effects "may also be caused by direct signaling of TRAIL via the TRAIL receptors" as suggested by the referee. First, a literature search has not yielded any reporting of death-receptor-mediated induction of degranulation or production of IFNγ in human NK cells. Given that multiple non-canonical effects of TRAIL beyond induction of apoptosis have been reported in the past (PMID: 23579241), we would most certainly reconsider this hypothesis and discuss the possibility if the referee would provide any studies supporting this hypothesis. So far, our own results also do not necessarily support this hypothesis either. For one, expression of TRAIL receptors were at very low levels at best, and those did not translate into increased activation of NK cells in our experiments despite high TRAIL expression levels. Most importantly in this context, we did not observed increased baseline levels of degranulation in NK cells without target cells and in this condition degranulation was not affected by the presence of soluble anti-TRAIL (Figure 4). In turn, exposure to immobilized anti-TRAIL or DR4 protein directly induced degranulation in NK cells (Figure 5), strongly indicating that activation of NK cells does not require TRAIL receptors on NK cells. Altogether, it is our opinion that there's no evidence for TRAIL-mediated activation of NK cells through engagement of TRAIL receptors in cis or trans on NK cells.

Independent of this hypothesis, the referee raised in an interesting question about the contribution of TRAIL-mediated granule-mediated apoptosis "to the total cytotoxic response of NK cells". While

this question is beyond the scope of the current study, we addressed this as a remaining open question in the discussion section of the manuscript.

In summary, this revision does not address my concerns to the extent that I had hoped for.

Reply: We hope that the provided arguments and further explanations now address the referee's concerns to a satisfactory level. We are aware that some observations remain puzzling and are certainly warranted to be investigated in future studies. However, the exact mechanisms of TRAIL-induced degranulation as well as its contribution to the overall cytotoxicity go beyond the scope of this study which intended i) to identify NK cell receptors that were involved in NK cell degranulation in the context of HIV-1 infection, ii) to validate the observed effects and iii) to describe the effect as accurate as possible and rule out other explanations (e.g. TRAIL serving as an activation marker).

Christian Körner
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Dear Dr. Körner,

Thank you for the submission of your further revised manuscript. I now went through your detailed p-b-p-response and I consider the remaining concerns of the referees as adequately addressed by the textual changes of the manuscript. I am thus very pleased to accept your study for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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