nature portfolio

Peer Review File

Dysfunctional tumor-infiltrating Vδ1+ T lymphocytes in microsatellite-stable colorectal cancer

REVIEWER COMMENTS

Reviewer #1 (expert in immunology and genetics of CRC):

The study seeks to characterize the role of gd+ T cells in CRC and is primarily based on scRNAseq of 11,171 pan-leukocyte CD45+ cells from 6 treatment naïve colorectal cancer (CRC) tumors and patient matched distant normal colon tissue (HC) combined with TCRseq. Of these cells, 319 were gd+ T cells from HC and 50 were from CRC. Additional patients are analysed later in the study.

The paper is primarily descriptive, comparing the gd+ T cell populations in CRC and HC, but also seeks to functionally validate findings in CRC cell models.

The manuscript appears hastily prepared and would benefit from language review. For example, there is no need to describe the graph type in the legends, please focus on the results instead. The summary statements and conclusions are formulated weakly or vaguely throughout the manuscript. It is challenging to discern novelty from what is already known in the present manuscript.

R 256: Is there a statistical hypothesis test or similar underpinning the statement 'striking difference in CRC was the enhanced level of cell communication between gd+ T cells and fibroblasts 1'?

The patient number in the initial parts of the paper (Figs 1 and 2) (6 patients) appears low, particularly in light of the great variability of gd+ T cell number (Supp Table 2). In CRC, 36/50 gd+ T cells originate from the same patient, which skews the analysis. This means that several findings can be driven by this single sample. Statistical power analysis and reasoning is also lacking. I consider this low number of CRC samples with sufficient gd+ T cells to be the major weakness of this work.

The findings in Figures 1-3 should be validated in external data from other studies. There are several open access scRNAseq patient matched T/N datasets for CRC available.

Figures 5 and 6 are crucial for a core finding/novelty aspect of the work, namely that the dysfunctional gd+ T cells can be reactivated. In Fig 6, I would expect to see the gammadelta1+ T killing of CRC cells improve after treatment with PMA/ionomycin. However, d-f is not addressing this question but are rather of control character, demonstrating that gd1+ and 2+ T cells can kill CRC cell lines in vitro. This needs to be addressed through new experiments.

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R 288: What is the fluorescence microscopy finding relating specifically to gd T cells?

Supp Table 1: The normal HC specimens should be included in the table. The notation Sample is suboptimal, use CRC and HC instead.

Supp fig 2: In g, comparisons would be facilitated if HC and CRC were in the same graph.

Extended Data Fig 3: How many experiments were performed? Please show all experiments or an analysis of all performed experiments.

Reviewer #2 (expert in gamma delta T cells in cancer):

The study by Stary V et al. entitled: "Dysfunctional tumor infiltrating Vdelta1+ T lymphocytes in MSS colorectal cancer", aims to characterize a distinct population of Vd1 T cells in MSS CRC which displays a functionally impaired phenotype with a potential to be restored in vitro. In addition, they highlight the role of cancer-associated fibroblasts in the dysregulation of Vd1 T cells in CRC. The article is well-written, clear, well-organized and well-sourced. The authors have conducted numerous experiments with scientific criteria and appropriateness, the material and method section appropriately describe the techniques used. The statistical methods and bioinformatics tools are appropriate, the figures are well-represented and well-described in the legend. The

results are very interesting.
The only aspect of potentiating regards the cross-talk with CAF; are the authors sure that the interaction between TIGIT/NECTIN is sufficient to explain the dysfunctional state of Vd1 T cells??? In TME, different cells and soluble molecules are present and could interfere with cells. Further functional studies by blocking or enhancing the signals are required to identify the clear mechanism responsible for the dysfunction of cells.
Some figures are difficult to read; could the authors modify them (too small axis legend…..)

Reviewer #3 (expert in high-throughput sequencing):

NCOMMS-23-27985-T
Dysfunctional Tumor-infiltrating Vdelta1+ T-lymphocytes in MSS colorectal cancer

I enjoyed reading this well-written manuscript focusing on gamma-delta T-cells from microsatellite stable (MSS) tumors. The single-cell data are focused on better understanding this T-cell subset and could provide an explanation as to why this tumor entity poorly responds to checkpoint immunotherapy. Moreover, a strength of the manuscript is that some of its conclusions based on the scRNAseq data, were validated (although limited) with in vitro experiments.

I do have a couple of questions, highlighting some of the weaknesses in the manuscript, which I think should be addressed in a revised version of the paper.

Major comments

Very limited number of patients:
The authors first explore gamma-delta T-cells using scRNAseq and gamma-delta scTCRseq in 6 patients with two samples each (healthy colon and tumour) resulting in only 11 000 CD45+ cells.

This has a consequence for cell type identification: the authors identify 17 distinct cell clusters in CD45+ cells, but some clusters are very small (doublets?) and some clusters are characterized by cells originating from only 1 patient (cfr. Extended data figure 1a-b). The authors should demonstrate that there is no sample bias in their data.

Similar comment for the gamma-delta scTCRseq data : some samples (and most especially CRC samples) have a very small number of gamma-delta TCRs (Table S2).

One possible solution could be to access published scRNAseq data, in which gamma-delta T-cell subclusters can be further explored.

The same argument goes for the fibroblast subclusters. Four subclusters were identified, but they have been very poorly annotated. With a sample increase and with the help of some recent single-cell papers focusing on scRNAseq of fibroblasts, it should be possible to much better annotate these subclusters, in concordance with emerging literature. This should increase the impact and contribution of this manuscript to the field.

More in-depth analysis of scRNAseq data:
The comparative analysis in scRNAseq data are limited to differential gene expression/pathway analysis and interaction analysis. More in-depth analysis could be done, including trajectory analysis on gamma-delta T-cell subtypes, this would be informative to understand how these cells develop and how cells from healthy colon vs CRC are distributed along these trajectories.

Clinical relevance?
Overall, my impression is that the manuscript is very descriptive, but misses clinical relevance.

For example, is the presence of dysfunctional gamma-delta T-cells associated with worse prognosis (recurrence free survival?)? Is it possible to somehow deconvolute a dysfunctional gamma-delta T-cells from bulk RNA data with clinical annotation ?

Furthermore, while the description of gamma-delta T-cells in MSS colorectal tumours is interesting, a far more relevant comparison would be the comparison between MSS and MSI tumours (versus healthy colon). In the introduction the authors refer to de Vries et al. Nature

2023, a study that describes the role of gamma-delta T-cells in response to immunotherapy in MSI colon tumours. This cohort (or other?) would be of great interest to explore this most relevant question. The scRNAseq data from this study should be publicly available.
https://www.nature.com/articles/s41586-022-05593-1#data-availability

Novelty:
The authors essentially describe the enrichment of a subtype of gamma-delta T-cells in CRC with dysfunctional phenotype based on their expression of genes typically associated with exhaustion and conventional CD8 T-cells. This has however already been described in CRC and other cancer types.
The authors need to underline (in introduction, results and discussion) what is truly novel and exciting about their results. They do indeed demonstrate that the cytotoxic potential of these cell types can be restored in vitro, but again I am missing the link to clinical potential.

Minor comments
- Clinically the anatomically localization of the tumour (rectum vs ascending or transversal colon etc) is relevant. Currently, it seems the authors do not take this factor into account, though it could certainly influence the underlying biology. Did the authors consider this as a potential source of bias?

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Vienna, January 10, 2024

RE: MS# NCOMMS-23-27985-T, Dysfunctional tumor-infiltrating Vδ1+ T lymphocytes in MSS colorectal cancer

Point-to-point reply to the Reviewer's comments

Reviewer #1:

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The manuscript appears hastily prepared and would benefit from language review. For example, there is no need to describe the graph type in the legends, please focus on the results instead. The summary statements and conclusions are formulated weakly or vaguely throughout the manuscript. It is challenging to discern novelty from what is already known in the present manuscript.

We appreciate the Reviewer's detailed assessment of the manuscript. We obtained a professional language editorial revision. Regarding figure legends, we followed the guidelines published under "Brief guide for submission to Nature Communications", which state that results of the experiment should not be part of the legends. We revised figure legends wherever appropriate.
We also re-formulated the summary statements and paid emphasis to discern our findings from already published data (results section: page 7, ll. 142-143; page 8, ll. 170-173; page 10, ll. 218-219, ll. 234-235; page 11, l. 236: page 12, ll. 264-267; page 16; ll. 366-367; discussion section: page 21, ll. 471-480). We hope that this sufficiently acknowledges the Reviewer's concerns.

R 256: Is there a statistical hypothesis test or similar underpinning the statement 'striking difference in CRC was the enhanced level of cell communication between gd+ T cells and fibroblasts 1'?

We thank the Reviewer for pointing out this inaccuracy. We completely revised this part of the manuscript and focused on presenting statistically significant data only (Fig. 5a, 5c, S3a; results section: page 15, ll 350-357, page 16, ll. 358-364).

The patient number in the initial parts of the paper (Figs 1 and 2) (6 patients) appears low, particularly in light of the great variability of gd+ T cell number (Supp Table 2). In CRC, 36/50 gd+ T cells originate from the same patient, which skews the analysis. This means that several findings can be driven by this single sample. Statistical power analysis and reasoning is also lacking. I consider this low number of CRC samples with sufficient gd+ T cells to be the major weakness of this work.

We appreciate the Reviewer's feedback and agree that higher γδ T cell count would be beneficial. However, cell numbers in the γδ T cell sequencing field do not reach those of conventional αß T cells. Reis *et al*. analyzed a total of 1825 cells with final γδ T cell count of 716 cells from tumors and 1109 from healthy adjacent tissue of 5 individuals (Reis et al., 2022). The authors do not offer an explanation of the contribution of each individual sample to γδ T cells. Supplemental figure S1B shows a UMAP of γδ T cells colored by patient ID giving the impression that normal healthy is dominated by two (yellow and purple) and tumor γδ T cells are dominated by one (blue) sample. This shows that equal distribution is of course always desired but neither predictable nor always feasible.
De Vries *et al*. performed scRNA-sequencing of 5 microsatellite instable CRC and corresponding adjacent tissue with a total γδ T cell count of 4,442 (de Vries et al., 2023). In their data, patient distribution to the γδ T cell cell pool ranged from 992 cells to 195 cells. De Vries *et al*. focused on the immunologically interesting minority of CRC patients – those with microsatellite instable tumors. These patient cohort is characterized by a high infiltration of γδ T cell in the tumor which makes higher cell counts more feasible (de Vries et al., 2020). Rancan *et al*. performed RNA single cell and TCR-sequencing of six patients with renal cell carcinoma resulting in 3000 sorted γδ T cells. No information of the distribution of the individuals was made (Rancan et al., 2023).
However, in the mentioned papers above, only viable γδ T cells were sorted and sequenced contrasting to our data. We sorted for viable CD45+ and CD45- cells. The distribution of γδ T cells in our data reflects their overall numerically contribution in the colon and the tumor microenvironment in MSS CRC and allows us to study γδ T cell interactions with other cell subsets. Although this approach produces lower cell numbers of individual cell subsets, broad analysis of γδ T cells within their cellular microenvironment is only possible with an inclusive sequencing strategy. With our new sequencing data which we added for the revision of this manuscript, we enriched for viable TCRγδ+ CD3+ cells and supplemented with viable CD45+ and CD45- cells with equal contributions. We validated major findings using publicly available data sets (Fig. 2c, 5i, S2b-d, S2f, S3a) and changed the results section of the manuscript accordingly (page 8, ll. 173-184; page 9, ll. 185-187; page 10, ll. 216-221; page 16, ll. 367-369). We are aware of this limitation and added this to the discussion part of the manuscript (page 22, ll. 498-503).

The findings in Figures 1-3 should be validated in external data from other studies. There are several open access scRNAseq patient matched T/N datasets for CRC available.

We thank the Reviewer for the feedback and agree that it is highly beneficial to validate the major findings. As part of the revision, we have verified our data using publicly available data sets.
We gained access to a large 10X Genomics single cell sequencing data set with over 370.000 cells from colorectal tumors and adjacent normal tissues of 28 MSS and 34 MSI individuals

(Pelka et al., 2021). We included the adjacent normal tissue and MSS, because this patient cohort closely resembles the cohort in our data set. Further, we used information on MSI CRC for relevant comparison wherever appropriate. With this data set, we investigated exhaustion gene scores (Fig. 2c, S2f), gene expression of TRDV1+ cells (Fig. S2c, 2d) and receptor-ligand interactions using CellChat (Fig. S3a). We additionally included analysis of the TCGA-COAD (bulk RNA-sequencing data with clinical information) regarding the clinical relevance of the proposed findings (Fig. 5i). We updated the results section of the manuscript accordingly (page 8, ll. 173-184; page 9, ll. 185-187; page 10, ll. 216-221; page 16, ll. 367-369). We hope that this sufficiently addresses the Reviewer's comment.

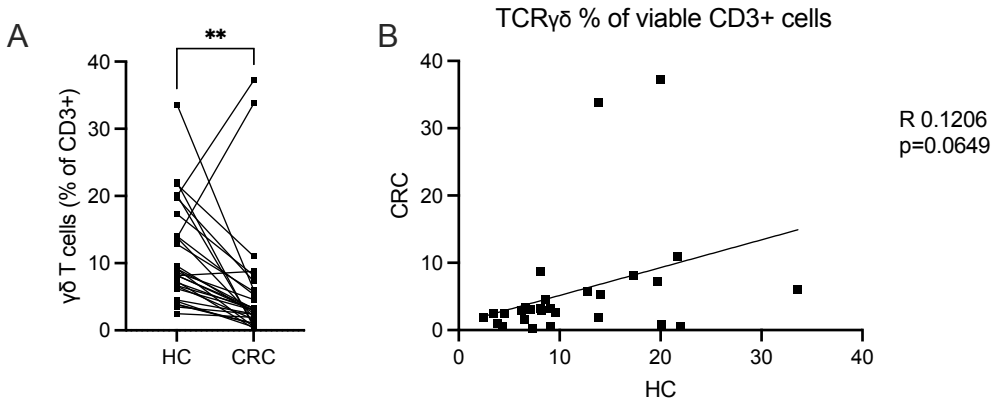
Figures 5 and 6 are crucial for a core finding/novelty aspect of the work, namely that the dysfunctional gd+ T cells can be reactivated. In Fig 6, I would expect to see the gammadelta1+ T killing of CRC cells improve after treatment with PMA/ionomycin. However, d-f is not addressing this question but are rather of control character, demonstrating that gd1+ and 2+ T cells can kill CRC cell lines in vitro. This needs to be addressed through new experiments.

We thank the Reviewer for this constructive feedback and suggestion to perform a killing assay after PMA/ionomycin stimulation. For this, we isolated viable Vδ1+ and Vδ2+ T cells from CRC and HC. Cells were stimulated with PMA/ionomycin without brefeldin. Killing as percentage of dead HT29 cancer cells compared to baseline (Fig. 4e) and CD107a expression (Fig. 4f) of Vδ1+ or Vδ2+ T cells were assessed after 4 hours of stimulation. Indeed, after stimulation we observed a significant increase of killed cancer cells by Vδ1+ T cells isolated from HC and CRC, and Vδ2+ isolated from HC supporting our previous results regarding enhanced cytotoxic potential after stimulation (Fig. 4a). This was partially mirrored by significant upregulation of CD107a on the surface of Vδ1+ T cells from CRC, as a marker of degranulation after the killing assay (Fig. 4f).
Given that the dysfunctional phenotype appeared reversible upon PMA/iono stimulation (Fig. 4e, 4f), we were prompted to explore the potential significance of the identified interaction between fibroblasts and Vδ1+ T cells via the TIGIT axis as uncovered by CellChat. For this, we isolated fibroblasts (viable CD45-, EpCAM-, CD31-, CD90+ cells) and Vδ1+ T cells from HC and CRC (Fig. 5f). We co-cultured HC Vδ1+ T cells with fibroblasts isolated from HC and CRC and CRC Vδ1+ T cells with fibroblasts from HC and CRC, respectively (as outlined in Fig. 5g). Neither co-culturing CRC Vδ1+ T cells with HC fibroblasts nor HC Vδ1+ T cells with CRC fibroblasts for 4 hours did significantly alter the phenotype of Vδ1+ T cells in such a way that it resulted in increased HT29 cell death (Fig. 5h). However, supplementation of a blocking antibody of TIGIT significantly elevated killing of HT29 by CRC Vδ1+ T cells indicative of an active TIGIT-NECTIN interaction (Fig. 5h).
We updated the results section of the manuscript accordingly (page 15, ll. 337-347; page 17, ll. 400-406; page 18, ll. 407-412), which strengthens the functional aspect of our work. Please find a detailed description of the experimental procedures in the updated method section (page 25, ll. 568-583).

R 282: The gd T cell fraction of CD45+ displays high variability between patients in both CRC and HC. Is this fraction more similar in CRC and HC from the same patient than in CRC and HC drawn from different patients?

This is an interesting question! As we understand the Reviewer asks if patients with high percentage of TCRγδ+ cells within the overall CD3+ population in distant healthy colon also show higher numbers in CRC compared to patients who already have a low TCRγδ+ infiltrate in HC. To address this, we first plotted the data with lines between HC and CRC of each individual (Reviewer Figure 1A). One may get the impression that indeed the higher the % of

TCRγδ+ cells within the CD3+ population in HC the higher this fraction is also in CRC. However, we further performed a simple linear regression (Reviewer Figure 1B) which demonstrated a weak positive relationship with an R of 0.1206 which did not quite reach statistically significance (p= 0.0649). With increased sample size, values would potentially reach statistical significance. It will be interesting to focus on factors driving TCRγδ+ infiltrate in a future project.



Reviewer Figure 1: (A) Fraction of γδ T cells as percentage of CD3+ T cells. Lines between the sample correspond to individual patients. (B) Linear regression analysis between percentage of γδ T cells in HC and CRC.

R 288: What is the fluorescence microscopy finding relating specifically to gd T cells?

Fluorescence microscopy imaging allows us to spatially validate our flow cytometry findings namely that TCRγδ+ CD3+ cells are present in HC and CRC lesions in close proximity to cancer cells. We observed increased infiltration of CRC lesions with CD3+ cells. We noticed that γδ T cells are distributed equally throughout the tissues and that a particular clustering of cells does not occur. Although we have not performed formal analysis we feel that fluorescence microscopy imaging is valid and beneficial. We changed the wording to accommodate TCRγδ+ CD3+ cell-related findings by immunofluorescence (page 11, ll. 250-253).

Supp Table 1: The normal HC specimens should be included in the table. The notation Sample is suboptimal, use CRC and HC instead.

We appreciate the Reviewer's detailed assessment and comment regarding the Supplementary Table S1. In the majority of samples, we were able to analyze HC and corresponding CRC. We included 2 columns (14th and 15th column) to specify if HC and /or CRC was used (Supplementary Table S1, Column HC and CRC).

Supp fig 2: In g, comparisons would be facilitated if HC and CRC were in the same graph.
9. Change graph.

We thank the Reviewer for this comment. To increase readability of the graph we combined receptor-ligand interactions for both, HC and CRC, in one figure (Fig. 5c) and did the same for the validation analysis (Fig. S3a).

Extended Data Fig 3: How many experiments were performed? Please show all experiments or an analysis of all performed experiments.

The Reviewer refers to a representative example of killing of HT29 by Vδ2+ T cells. This is a representative example for experiments that are summarized in Fig. 4d. In total, we performed 12 individual experiments. To avoid any further confusion, we removed the representative example of Fig. 4d. We added a representative example to the following PMA/ionomycin stimulation experiment, which has the same read-out (Fig. 4g).

Reviewer #2:

The study by Stary V et al. entitled: "Dysfunctional tumor infiltrating Vdelta1+ T lymphocytes in MSS colorectal cancer", aims to characterize a distinct population of Vd1 T cells in MSS CRC which displays a functionally impaired phenotype with a potential to be restored in vitro. In addition, they highlight the role of cancer-associated fibroblasts in the dysregulation of Vd1 T cells in CRC. The article is well-written, clear, well-organized and well-sourced. The authors have conducted numerous experiments with scientific criteria and appropriateness, the material and method section appropriately describe the techniques used. The statistical methods and bioinformatics tools are appropriate, the figures are well-represented and well-described in the legend. The results are very interesting.

The only aspect of potentiating regards the cross-talk with CAF; are the authors sure that the interaction between TIGIT/NECTIN is sufficient to explain the dysfunctional state of Vd1 T cells??? In TME, different cells and soluble molecules are present and could interfere with cells. Further functional studies by blocking or enhancing the signals are required to identify the clear mechanism responsible for the dysfunction of cells.

We would like to thank the Reviewer for this very positive overall evaluation of our manuscript, highlighting the importance of our findings. We appreciate the constructive feedback and address the specific concerns as follows. TIGIT is an inhibitory receptor expressed on cytotoxic T cells, T helper cells, regulatory T cells and NK cells (Joller et al., 2014; Stanietsky et al., 2009; Yu et al., 2009). Previous studies confirm our findings that TIGIT is expressed in γδ T cells in solid tumors (Rancan et al., 2023; Weimer et al., 2022). TIGIT it widely known to bind the two ligands CD155 and CD112 (Nectin2) which are expressed by CRC cancer cells (Masson et al., 2001) and various other cells in the tumor microenvironment such as epithelial cells and fibroblasts (Takai et al., 2008). Among other things, binding TIGIT regulates conventional αß T cell cytotoxicity and delivers inhibitory signals partially by preventing CD226 co-stimulation (Yu et al., 2009) and by diminishing TCR activation signals (Joller et al., 2011). In NK cells, TIGIT binding was shown to inhibit degranulation and cytokine production ultimately leading to decreased cytotoxicity (Stanietsky et al., 2009). Blocking Nectin4 led to enhanced tumor killing in vitro and in vivo (Reches et al., 2020) Antibody blockade of TIGIT as novel immunotherapeutic strategy is under investigation in clinical studies (Ge et al., 2021).

The TIGIT-NECTIN pathway has so far not been addressed in γδ T cells. We agree with the Reviewer that further functional studies are needed to sufficiently explain the dysfunctional state of Vδ1+ T cells. Our analyses hint to an interaction of fibroblasts with γδ T cells via TIGIT/NECTIN2 (Fig. 5c) leading to a dysregulated state of γδ T cells, which we confirm in the revised version of the manuscript in a publicly available data set (Fig. S3a). We further tested the relevance of the interaction of fibroblasts and Vδ1+ T cell via the TIGIT axis as uncovered by CellChat. For this we isolated fibroblasts (viable CD45-, EpCAM-, CD31-, CD90+ cells) and Vδ1+ T cells from HC and CRC. We co-cultures HC and CRC Vδ1+ T cells with fibroblasts isolated from HC and CRC (Fig. 5g). Neither co-culturing CRC Vδ1+ T cells with HC fibroblasts nor HC Vδ1+ T cells with CRC fibroblasts for 4 hours significantly changed the phenotype of Vδ1+ T cells. Adding a blocking antibody for TIGIT significantly elevated the killing of HT29 by CRC Vδ1+ T cells confirming an active TIGIT-NECTIN pathway responsible for dysfunctional Vδ1+ T cells (Fig. 5h). However, despite the recent novel results we agree with the Reviewer that the

tumor microenvironment is in an interplay with a variety of cells, molecules and other soluble factors combined with high patient heterogeneity. Care must be taken to corroborate these findings in further studies.
To accommodate the latest observations, we made the following changes on the manuscript results section (page 15, ll. 350-357; page 16, ll. 358-361; page 17, ll. 400-406; page 18, ll. 407-412), methods (page 25, ll. 575-583) and discussion (page 22, ll. 494-497) and think that the new functional assays and discussion sufficiently address the Reviewer's concern.

Some figures are difficult to read; could the authors modify them (too small axis legend…..)

We thank the Reviewer for pointing this out and paid attention in the revised version of the figures to increase font sizes wherever appropriate.

Reviewer #3:

I enjoyed reading this well-written manuscript focusing on gamma-delta T-cells from microsatellite stable (MSS) tumors. The single-cell data are focused on better understanding this T-cell subset and could provide an explanation as to why this tumor entity poorly responds to checkpoint immunotherapy. Moreover, a strength of the manuscript is that some of its conclusions based on the scRNAseq data, were validated (although limited) with in vitro experiments.

I do have a couple of questions, highlighting some of the weaknesses in the manuscript, which I think should be addressed in a revised version of the paper.

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The authors first explore gamma-delta T-cells using scRNAseq and gamma-delta scTCRseq in 6 patients with two samples each (healthy colon and tumour) resulting in only 11 000 CD45+ cells.

This has a consequence for cell type identification: the authors identify 17 distinct cell clusters in CD45+ cells, but some clusters are very small (doublets?) and some clusters are characterized by cells originating from only 1 patient (cfr. Extended data figure 1a-b). The authors should demonstrate that there is no sample bias in their data.

Similar comment for the gamma-delta scTCRseq data : some samples (and most especially CRC samples) have a very small number of gamma-delta TCRs (Table S2).

One possible solution could be to access published scRNAseq data, in which gamma-delta T-cell subclusters can be further explored.

We thank the Reviewer for the detailed assessment and agree that we work with a limited number of γδ T cells. Our numbers are the result of low γδ T cell count in general in MSS CRC as compared to MSI CRC. Our experience and published reports demonstrate much higher numbers of γδ T cells infiltrating MSI CRC compared to MSS CRC (de Vries et al., 2020). We conducted additional 10X single-cell sequencing experiments including αβ- and γδ-T cell receptor sequencing with a similar sorting strategy as before, allowing for interaction analysis of γδ T cells with other immune cells and structural cells. Moreover, we carried out the following analysis of publicly available CRC data sets to verify the most important findings of this study.

We compared the gene expression of receptors and effector molecules in TRDV1+ cells in a data set with MSI and MSS CRC (Fig. S2c, S2d). We identified the exhaustion gene signatures of γδ T cells in this data set (Fig. 2c, S2f). Additional analysis also confirmed the TIGIT-NECTIN2 crosstalk between fibroblasts and γδ T cells as a targetable interaction in CRC (Fig. S3a). We exploited bulk sequencing data with clinical annotation of the TCGA-COAD to address the relevance of γδ T cells in MSS CRC (Fig. 5i).
We changed the results section accordingly (page 8, ll. 170-184; page 9, ll. 185-189, page 10, ll. 213-221; page 16, ll. 366-369; page 18, ll. 410-412).

The same argument goes for the fibroblast subclusters. Four subclusters were identified, but they have been very poorly annotated. With a sample increase and with the help of some recent single-cell papers focusing on scRNAseq of fibroblasts, it should be possible to much better annotate these subclusters, in concordance with emerging literature. This should increase the impact and contribution of this manuscript to the field.

We highly appreciate the Reviewer's objection to better annotate fibroblasts subsets to increase the relevance of the manuscript. Previous studies have identified two major fibroblast subsets in CRC based on the gene signature at single-cell level. Li *et al*. (Li et al., 2017) investigated two types of fibroblasts in CRC: CAF-A which expressed genes related to extracellular matrix remodeling (*MMP2, DCN, COL1A2*); and CAF-B which were distinguished by markers of activated myofibroblasts (*ACTA2, TAGLN, PDGFA*). The observation of two CAF subsets was confirmed in pancreatic (Elyada et al., 2019) and bladder cancer (Chen et al., 2020). Elyada *et al*. termed the two subsets myofibroblastic CAF (myCAF) and inflammatory CAF (iCAF) based on their distinct gene expression. ICAF expressed immune modulating genes such as *IL6, IL8*, chemokines (*CXCL1, CXCL2, CCL2, CXCL12*) and *PDGFRA*. In accordance with the gene expression, inflammatory and complement pathways were upregulated in iCAFs. MyCAF expressed high levels of *ACTA2* and genes for contractile proteins such as *TAGLN, MYL9, TPM1, TPM2, MMP11, POSTN* and *HOPX*. Pathways associated with smooth muscle contraction, adhesion, extracellular matrix remodeling and collagen synthesis were significantly upregulated in myCAFs.
We analyzed our sequencing data including the newly added sample and compared them with currently published papers on fibroblast subsets in the colon. We identified 4 fibroblast clusters (clusters 8, 11, 13, 14 in Fig. 5b) expressing markers for fibroblasts, such as *COL1A1*, *S100A4* and *VIM* (Li et al., 2022). We found the most recent classification of iCAF and myCAF by Elyada *et al*. (Elyada et al., 2019) being in line with our data. However, the results did not demonstrate black and white pattern of two opposing subsets but rather a predominance of one phenotype over the other. All clusters expressed genes previously associated with myCAFs (*ACTA2, TAGLN, MYL9, TPM1, TPM2*). However, cells in cluster 11 and 14 were positive for several chemokines (*CCL2, CXCL1, CXCL2, CXCL3, CXCL8*) and for *IL6* which were attributed to iCAFs. Cluster 8 and 13 expressed myofibroblastic markers but were not associated with immunoregulatory markers to an extent we have seen in the other clusters.
We added several new figures to underline the importance and influence of fibroblasts subsets on γδ T cells (Fig. 5d-i). We revised the manuscript according to the new findings (results section: page 16, ll. 369-382; page 17, ll. 383-406; page 18, ll. 407-412; methods section: page 25, ll. 575-583; page 28, ll. 639-645) and hope that these additions answer the Reviewer's questions sufficiently.

More in-depth analysis of scRNAseq data:
The comparative analysis in scRNAseq data are limited to differential gene expression/pathway analysis and interaction analysis. More in-depth analysis could be done, including trajectory

analysis on gamma-delta T-cell subtypes, this would be informative to understand how these cells develop and how cells from healthy colon vs CRC are distributed along these trajectories.

We thank the Reviewer for the recommendation to perform trajectory analysis facilitating a more in-depth exploration of the data. We initiated trajectory pseudotime analysis of TRDV1+ cells in HC and CRC (Fig. 2d, 2e). TRDV1+ cells from HC and CRC demonstrated great similarities in their movement along the trajectory (Fig. 2d). We specified *GZMB+, PRF1+* cells as the root which resulted in PTPRC+, CXCR4+, LIME1+ and ANXA+ cells as the terminal states. We overlaid pseudotime analysis with the gene expression of *TOX* and *GNLY* which we previously found to be altered in CRC (Fig. 2b). The expression of *GLNY* increased over time with the highest point at the terminal state. *TOX* was almost absent in HC. In CRC, both genes displayed the highest expression at the same pseudotime when genes associated with TCR stimulation were upregulated indicating a common cause of simultaneous dysregulation. We updated the results (page 10, ll. 221-232, ll. 234-235; page 11, ll. 238-240) and methods sections (page 28, ll. 638-639) accordingly and think that our new data provide information to understand the development and distribution of γδ T cells in HC and CRC.

Overall, my impression is that the manuscript is very descriptive, but misses clinical relevance.

For example, is the presence of dysfunctional gamma-delta T-cells associated with worse prognosis (recurrence free survival?)? Is it possible to somehow deconvolute a dysfunctional gamma-delta T-cells from bulk RNA data with clinical annotation ?

We agree with the Reviewer that finding clinical relevance behind the investigated research topic is of great importance. For the revised version of the manuscript, we performed additional analysis comparing Vδ1+ T cells in MSI and MSS CRC. We observed distinctions between Vδ1+ T cells in MSI and MSS CRC. In MSI CRC, Vδ1+ T cells displayed significantly increased exhaustion scores (Fig. 2b) in contrast to Vδ1+ T cells in MSS CRC. Despite enhanced exhaustion, Vδ1+ T cells in MSI retain effector molecules determined by *IFNG* and *GNLY* expression (Fig 2Sd). These findings imply functional differences and underline the clinical relevance.
Furthermore, we analyzed bulk sequencing data of the TCGA-COAD. We identified MSS CRC samples according to a previously published paper (Liu et al., 2018). Based on our findings of iCAF markers in our cohort (Fig. 2d), we defined an iCAF signature according to the expression of *VIM, S100A4, COL1A2, IL6.* Since *TOX* was significantly upregulated in TRDV1+ cells isolated from CRC compared to HC (Fig. S2e) and TOX known role in exhaustion, we determined a dysfunctional Vδ1 signature by *TRDV1* and *TOX* expression. We observed a significant positive correlation between iCAF and dysfunctional Vδ1+ T cells in the MSS cohort of the TCGA-COAD (Fig. 5i). However, in our preliminary data we did not find a significant difference in the overall survival based on a dysfunctional Vδ1 signature.
We updated the results (page 18, ll. 407-412) and method section (page 28, ll. 638-645) of the manuscript accordingly.

Furthermore, while the description of gamma-delta T-cells in MSS colorectal tumours is interesting, a far more relevant comparison would be the comparison between MSS and MSI tumours (versus healthy colon). In the introduction the authors refer to de Vries et al. Nature 2023, a study that describes the role of gamma-delta T-cells in response to immunotherapy in MSI colon tumours. This cohort (or other?) would be of great interest to explore this most relevant question. The scRNAseq data from this study should be publicly available. https://www.nature.com/articles/s41586-022-05593-1#data-availability

We agree with the Reviewer that the comparison of MSS and MSI tumors is of great interest. Although we feel that the analysis of distinction and similarities of γδ T cells in MSI vs MSS CRC is not the main scope of the paper, we performed the following additional analysis and added them to the manuscript wherever we found it appropriate.
In analysis of bulk sequencing data of the TCGA-COAD data base, we observed a significant upregulation of TRDV1 expression (Fig. S2b). We additionally gained access to the data set of Pelka *et al*. *(Pelka et al., 2021),* who performed single-cell sequencing on 28 MSS and 34 MSI CRC with adjacent tissue. In summary, they profiled over 370.000 cells. In this data set we found significantly higher numbers of TRDV1+ in MSI CRC validating our findings of the TCGA-COAD (Fig. S2c). We investigated the expression of genes associated with inhibiting and activating receptors, cytotoxicity and tissue residency of TRDV1+ cells in MSI vs MSS CRC (Fig. S2d). We found no relevant differences in known γδ T cell receptors or tissue markers reflective of a shared gene signature of TRDV1+ cells in MSS and MSI. However, we observed that the expression of *IFNG* and *GNLY* is significantly elevated in MSI CRC arguing that γδ T cells potentially retain effector functions and contribute to the previously proposed "hot" tumor microenvironment in MSI.
Furthermore, we applied two previously published sets of exhaustion genes and calculated a score (Fig. 2c, Fig. S2f). Using the Pelka *et al*. data set, we observed no significant upregulation in both exhaustion scores in MSS CRC similar to the findings in our study. However, interestingly in MSI we found upregulation of the exhaustion scores (Fig. 2c, $p<0.05$; Fig S2f, $p=0.064$) demonstrating relevant distinctions in TRDV1+ cells between MSS and MSI.
We changed the manuscript in accordance to the new findings in the results (page 8, ll. 170-184; page 9, ll. 185-189; page 10, ll. 216-221; page 16, ll. 367-369) and method section (page 28, ll. 637-638). We think that the comparison of MSI and MSS CRC is a very relevant clinical research topic that needs to be further addressed in a comprehensive study and thank the Reviewer for this interesting question.

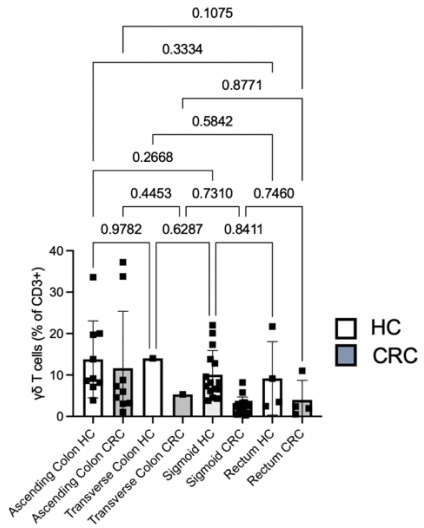
Novelty:
The authors essentially describe the enrichment of a subtype of gamma-delta T-cells in CRC with dysfunctional phenotype based on their expression of genes typically associated with exhaustion and conventional CD8 T-cells. This has however already been described in CRC and other cancer types.
The authors need to underline (in introduction, results and discussion) what is truly novel and exciting about their results. They do indeed demonstrate that the cytotoxic potential of these cell types can be restored in vitro, but again I am missing the link to clinical potential.

We thank the Reviewer for the constructive feedback. We are aware that γδ T cells with an exhausted phenotype have been described previously. In ovarian cancer, Vδ1+ T cells were found to display co-expression of TIGIT and PD-1 (Weimer et al., 2022). Diminished effector functions of γδ T cells have been observed in acute myeloid leukemia (Tang et al., 2020; Wu et al., 2020). After initial submission of the manuscript, Rancan *et al*. published a study on exhausted Vδ2- cells in renal cancer with clinical relevance based on single cell sequencing data (Rancan et al., 2023). In human MSI CRC, de Vries et al. reported in an earlier study the enrichment of PD1+ γδ T cells using mass cytometry data (de Vries et al., 2020). After the initial submission of our manuscript, Yu *et al*. reported on γδ T cells with an exhausted phenotype analyzing single-cell sequencing data of a CRC mouse model (Yu et al., 2023). In the same year, de Vries *et al*. reported that γδ T cells are the main effector cells in HLA class I-negative CRC after checkpoint inhibition using single cell sequencing on MSI CRC (de Vries et al., 2023). The latest studies demonstrate rising interest in this topic. However, the observations of de Vries *et al*. are only applicable to the small percentage of patients who present with an MSI tumor which is on top of that HLA-negative (approximately 5% of all

CRC, 10% MSI in CRC of which 30-70% have loss of HLA class I (Cabrera et al., 1998; Moller et al., 1991)). In the routine clinical setting, these patients usually receive anti-checkpoint blockade regardless of the HLA status. For MSI CRC patients, immunotherapy is already established and therapy regime does not change based on this novel finding. On the other side, the majority of CRC patients (around 90%) present with an MSS CRC. Currently in the routine setting, immunotherapy is not yet an option for these patients. We did not find any other study which combines single-cell sequencing analysis on MSS CRC including TCRγδ- and TCRαß-receptor with functional assays on γδ T cells.
As suggested in a previous question of the Reviewer and in order to draw conclusions to the clinical potential, we added an analysis performed on bulk sequencing data on the TCGA-COAD data set (Fig. 5i). Furthermore, we changed the manuscript and paid emphasis on novel findings (results section: page 7, ll. 142-143; page 8, ll. 170-173; page 10, ll. 218-219, ll. 234-235; page 11, l. 236: page 12, ll. 264-267; page 16; ll. 366-367; discussion section: page 21, ll. 471-480).

Minor comments
- Clinically the anatomically localization of the tumour (rectum vs ascending or transversal colon etc) is relevant. Currently, it seems the authors do not take this factor into account, though it could certainly influence the underlying biology. Did the authors consider this as a potential source of bias?

We appreciate the Reviewer's thoughtful comment concerning the relevance of the anatomical localization. We did not take this factor into account so far. The maximum follow-up of the patients is three years. Therefore, there is limited outcome related data of this cohort available. We agree with the Reviewer that clinically the anatomical location of the tumor is potentially of relevance. Microbiome, mutational status and metastatic spread are known factors which differ at least between left and right sided tumor (Baran et al., 2018). As our patient cohort is relatively small and the localization of the tumor is unevenly distributed, we can only speculate but not draw significant conclusion. The distribution of tumor localization reflects the traditionally reported rates of incidence with the majority of colorectal cancers in the right sided colon (sigmoid, rectum) followed by ascending colon and transverse colon. We analyzed the percentage of γδ T cells within the CD3+ population per localization and found no significant differences of γδ T cell distribution (Reviewer Figure 2). At the most, we observed a tendency of decreasing γδ T cells from the proximal parts (ascending and transverse colon) towards the distal parts of the colon (sigmoid and rectum). This might be in line with papers that reported a significant reduction of γδ T cells in the descending colon and rectum compared to the transverse colon which may indicate changing functional characteristics. No significant difference was observed between the ascending colon and descending colon/rectum (Tyler et al., 2020). In recent years, gut atlases based on single-cell RNA expression have been published (Elmentaite et al., 2021; Hickey et al., 2023). However, since γδ T cells are still an underrepresented immune cell population, most do not include the description of γδ T cells.
We now consider this issue within in the discussion part of the manuscript (page 22, ll. 503-506) and hope that this sufficiently addresses the Reviewer's comment.



Reviewer Figure 2: γδ T cells as percentage of CD3+ cells subdivided per location in HC and CRC.

We would like to conclude by thanking the Reviewers for the excellent feedback, which guided us through a careful revision that, in our view, substantially increased the relevance, interest, and anticipated impact of this paper.

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

My comments and concerns have been satisfactorily addressed.

Reviewer #2 (Remarks to the Author):

The authors have exhaustively improved the quality and the significance of their results. The manuscript is acceptable for publication in the new form, considering its potential implications in therapy.

Reviewer #4 (Remarks to the Author):

[Note from editor: Reviewer 3 was no longer available for review and was therefore replaced by Reviewer 4.]

The manuscript by Stary et al reports a single cell characterization of MSS CRC tumor-infiltrating immune cells coupled with ab/gd TCR sequencing. The authors specifically zoom in on Vd1 cells, which represent an important and understudied immune subset in cancer immunotherapy. The analyses are particularly focusing on the expression of their activating and inhibiting receptors and molecules. While the manuscript is mainly descriptive in nature, the authors provide some (limited) functional experiments to investigate the cytotoxic capacity of this immune subset and provide an early suggestion on how to further enhance this type of antitumor immunity.

I have read this manuscript with mixed feelings. A key strength of the manuscript is the interesting scope: an important but understudied immune subset is studied in MSS colorectal cancer, a tumor type with known immune dysregulation and surprising resistance to immunotherapy considering its medium-range tumor mutational burden. Also, the combination of gdTCR-seq with scRNA-seq is a clear asset of the study, especially as much larger scRNA-seq studies of CRC have been published but lack TCR sequencing (e.g. Pelka et al, 2021). While the combination of descriptive single cell analyses with functional experiments could be seen as a strength of the study, the depth of these experiments is very limited. In general, the manuscript would greatly benefit from a more streamlined and focused writeup, as the story line currently has many side stories and less relevant results on details which distract the reader from the key (clinically relevant?) message of the manuscript. Also, there is only limited integration and correlation of the scRNA-seq results, the flow cytometry results, and the functional experiments; it almost reads as three separate stories. Finally, it is a missed opportunity to not functionally dissect the broad tumor reactivity of the expanded gdTCRs, especially because the availability of (gd)TCR data is the key strength of the study.

As per request of the Editor, I have specifically focused on the Author's response to the (important) points raised by Reviewer 3; my point-by-point assessment follows below.

1. Limited patient/cell numbers and the robustness of cell clusters/cell type identification, including the risk of sample bias.

The authors' response to this point is not entirely clear to me, as they merely provide an enumeration of the additional analyses that were performed, rather than providing an integrated discussion of the new results and how their additional work proofs that their patient/cell numbers are sufficient to get robust cell clusters and cell type identifications. If I understood it correctly, the authors increased the cell count from 11,000 to 12,962 cells, which is only a marginal increase and it remains unclear if this does result in robust clusters (without sample bias). Indeed, the number of samples for the selected ab/gd T cells clusters are still very homogeneous, with some only consisting of 1 or 2 samples. Furthermore, the authors mention that they have included 7 additional patients for the gdT cell-based analysis (figure 2), but it is unclear to me why these same patients were not included in the analyses presented in figure 1 to increase sample size? If there is a good reason, this should be clear from the manuscript.

Besides the limited sample size (which is a key point), there are some specific aspects of the cell

clusters that concern me. For example:
- Why are the gdT cells not clearly clustering based on their delta chain usage, while Vd1 and Vd2 cells are expected to show very different biology and expression profiles (and, indeed, form clearly separate clusters in Fig 2A of the publication of de Vries et al, Nature, 2023)?
- For the fibroblast clusters, the authors now provide a comparison with Elyada et al, 2019 in the rebuttal document. However, the authors conclude that their clusters are inconsistent with those described bu Elyada et al. Why is this the case? Is this biological or technical?
- The authors now included a CellTypist analyses to annotate the clusters via a standardized procedure. However, CellTypist annotated the fibroblast clusters as endothelial cells, NK cells, fibroblasts, Tcm/Naïve helper T cells and double-positive thymocytes (fig S1b). Why?

Taken together, more work is needed to address the concerns raised by Reviewer 3 regarding the robustness of cell clusters/types, especially given the limited sample/cell size.

2. More in-depth analysis of scRNA-seq data, including trajectory analysis

As suggested, the authors included a pseudotime-based trajectory analysis; however, it is a bit unclear how these results contribute to the story without further alignment with the other sections (especially the functional experiments). E.g., can it be confirmed experimentally that the terminal state indeed is the terminal state?

Furthermore, how robust are the trajectories to patient bias and (low) cell counts? This is important, e.g. to understand how reliable the finding of different trajectories in CRC vs healthy control truly is. Is this true biology or technical noise?

3. Clinical relevance of the findings

The clinical relevance of the findings remains unclear to me, and this remains a key limitation of the study.

In the rebuttal document, the authors state that the observation that Vd1 cells in MSI cancers have an exhausted phenotype whereas this is not the case in MSS cancers is a clinically relevant finding. However, why does this underscore clinical relevance? For example, I believe that the lack of exhaustion in MSS cancers could also be observed if Vd1 cells are bystanders with no or only weak tumor recognition in most MSS cancers? MSI cancers frequently lose HLA class I expression which contributes to the activation of Vd1 cells (de Vries et al, Nature, 2023), so perhaps this underlies the key difference between the levels of exhaustion between MSS and MSI?

Furthermore, the authors create a signature of exhausted Vd1 cells and iCAF cells. Signature design for bulk RNA-seq is challenging as, for specificity, the genes in the signature should only be expressed by the cell type of interest; here, that seems not to be the case:
- The exhausted Vd1 signature is composed of TRDV1 (which is expressed by all Vd1 cells and not only exhausted Vd1 cells) and TOX (which is expressed by many exhausted immune cells and not only exhausted Vd1 cells). This makes it unlikely that this signature reliably captures the levels of exhausted Vd1 cells; at the same time, the authors do not rigorously validate that this would be the case.
- The iCAF signature is composed of VIM (which is, according to the scRNA-seq data in the Human Protein Atlas, also expressed by immune cells, muscle cells, neurons, etc), S100A4 (which is also expressed by immune and muscle cells), COL1A2 (which is also expressed by smooth muscle cells), and IL6 (which is also expressed by endothelial cells, cancer cells, smooth muscle cells and (mostly myeloid) immune cells). Again, this makes it unlikely that this signature reliably captures the levels of iCAF cells and there is no validation to show otherwise.
Hence, these signature analyses on bulk data are unconvincing and in their current form a liability of the study.

That being said, I do agree with Reviewer 3 that it would greatly improve the manuscript if the authors could provide convincing evidence of clinical relevance. For example, by showing (A) the in vivo potential of reinvigorating Vd1 cell-based antitumor immunity in MSS CRC (e.g. by patient or mouse models), (B) the association of Vd1 T cell states with disease progression in MSS CRC, or (C) the association of Vd1 T cell infiltration with response to immune checkpoint blockade of MSS CRC, etc.

4. The comparison of gdT cells in MSS vs MSI CRC

The authors included an in-depth analysis comparing gdT cells between MSS vs MSI CRC into their revised manuscript, which is a notable effort and strengthens the manuscript. I do have some suggestions regarding these analyses:
- A key observation of de Vries et al (Nature, 2023) was that tumor reactivity was largely contained within the PD-1-expressing gdT cell compartment. Earlier work of de Vries et al (2020) has suggested that PD-1-positive gdT cells are largely absent in MSS CRC, whereas they are abundant in MSI cancers. In light of these findings, it is highly relevant to:
o Also include PD-1 to figure S2d. Here, it would be good to also add CD39 (marking activated gdT cells). For alignment with the rest of the manuscript, it would be informative to also show NKG2A, FasL, and TRAIL expression in fig S2d.
o It would be informative to compare the clonality/expansion of PD-1 positive vs negative gdT cells.
o If possible, functionally test if tumor reactivity of Vd1 cells against MSS cancers is also restricted to PD-1 positive cells, to get a sense if all other gdT cells in MSS cancers are bystanders (as PD-1-negative gdT cells seem to be in MSI cancers).
- It would be helpful to integrate the MSS vs MSI comparison better into the general story line. Currently it is presented as a bit of a separate story, whereas (also in light of the remarks below) this comparison provides important fundamental insights about the role of the potential role of gdT cells in MSS cancers. Are they bystanders or functional? If they are bystanders, can they be activated? If there already is baseline tumor reactivity, how can this be amplified and which gdT cell subsets should be targeted?

5. Novelty

To explain what is truly novel, the authors mainly stress the uniqueness of their dataset, rather than explaining the novelty of their conclusions/findings. Rather than providing a descriptive summary of the data, the Discussion of the manuscript would benefit from a more to the point discussion of the key novel conclusions/findings. Furthermore, we agree with the authors that the addition of (gd)TCR-seq to single cell analyses in MSS CRC is very interesting and novel, but the authors do not clearly translate this advantage of the dataset into novel biological insights (e.g. are clonally expanded gdTCRs broadly reactive to MSS CRC? Do gdT cells expressing activation markers such as PD-1, CD39 and Ki67 show clonal expansion? Are specific clonotypes associated with specific phenotypes?). Also, linking the single cell analyses to functional experiments is potentially powerful, but the functional experiments are very limited and the interaction/correlation of the single cell findings with the functional experiments is limited. In conclusion, the manuscript could be greatly improved if the authors would more effectively exploit the uniqueness of their dataset to discover uncharted territory.

6. Anatomical localization of the tumors

This point has been well addressed by the authors and provides interesting insights.

Minor additional points:
- Fig1d: it seems like TRDV1 mainly pairs with TRDV2, which I presume is a (mis)labelling issue?
- Line 191-208: It is unclear what statistics or quantification underlies "altered"
- Figure 3j,k,l: the PD-1 gating in the gating strategy is missing, because that was strongly varying between the HC and CRC, so that may affect the populations and the "number" of cells in the populations
- Figure 4a: this is a bit of a chaotic and not clearly readable figure
- Figure 5h: the key control that I'm missing here is the Vd1/Vd2-TIGIT alone condition

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Vienna, June 4th, 2024

RE: MS# NCOMMS-23-27985-T, Dysfunctional tumor-infiltrating Vδ1+ T lymphocytes in MSS colorectal cancer

Point-to-point reply to the Reviewer's comments

Reviewer #1 (Remarks to the Author):

My comments and concerns have been satisfactorily addressed.

We thank the Reviewer for the positive reply.

Reviewer #2 (Remarks to the Author):

The authors have exhaustively improved the quality and the significance of their results. The manuscript is acceptable for publication in the new form, considering its potential implications in therapy.

Pointing out potential implications in therapy, the Reviewer's feedback is highly appreciated.

Reviewer #4:

The manuscript by Stary et al reports a single cell characterization of MSS CRC tumor-infiltrating immune cells coupled with ab/gd TCR sequencing. The authors specifically zoom in on Vd1 cells, which represent an important and understudied immune subset in cancer immunotherapy. The analyses are particularly focusing on the expression of their activating and inhibiting receptors and molecules. While the manuscript is mainly descriptive in nature, the authors provide some (limited) functional experiments to investigate the cytotoxic capacity of this immune subset and provide an early suggestion on how to further enhance this type of antitumor immunity.

I have read this manuscript with mixed feelings. A key strength of the manuscript is the interesting scope: an important but understudied immune subset is studied in MSS colorectal cancer, a tumor type with known immune dysregulation and surprising resistance to immunotherapy considering its medium-range tumor mutational burden. Also, the combination of gdTCR-seq with scRNA-seq is a clear asset of the study, especially as much larger scRNA-seq studies of CRC have been published but lack TCR sequencing (e.g. Pelka et

al, 2021). While the combination of descriptive single cell analyses with functional experiments could be seen as a strength of the study, the depth of these experiments is very limited. In general, the manuscript would greatly benefit from a more streamlined and focused writeup, as the story line currently has many side stories and less relevant results on details which distract the reader from the key (clinically relevant?) message of the manuscript. Also, there is only limited integration and correlation of the scRNA-seq results, the flow cytometry results, and the functional experiments; it almost reads as three separate stories. Finally, it is a missed opportunity to not functionally dissect the broad tumor reactivity of the expanded gdTCRs, especially because the availability of (gd)TCR data is the key strength of the study.

As per request of the Editor, I have specifically focused on the Author's response to the (important) points raised by Reviewer 3; my point-by-point assessment follows below.

1. Limited patient/cell numbers and the robustness of cell clusters/cell type identification, including the risk of sample bias.

The authors' response to this point is not entirely clear to me, as they merely provide an enumeration of the additional analyses that were performed, rather than providing an integrated discussion of the new results and how their additional work proofs that their patient/cell numbers are sufficient to get robust cell clusters and cell type identifications.

If I understood it correctly, the authors increased the cell count from 11,000 to 12,962 cells, which is only a marginal increase and it remains unclear if this does result in robust clusters (without sample bias). Indeed, the number of samples for the selected ab/gd T cells clusters are still very homogeneous, with some only consisting of 1 or 2 samples. Furthermore, the authors mention that they have included 7 additional patients for the gdT cell-based analysis (figure 2), but it is unclear to me why these same patients were not included in the analyses presented in figure 1 to increase sample size? If there is a good reason, this should be clear from the manuscript.

We appreciate the Reviewer's detailed assessment of the manuscript. We had conducted single-cell RNA-sequencing on seven patients, including both αβ- and γδ-T cell receptor (TCR) sequencing. In the initial figures, in addition to UMAP and gene marker expression analysis, we presented the TCR analysis. Consequently, we had analyzed these patient cohorts separately based on whether TCR sequencing was performed or not. However, we agree with the Reviewer that this strategy is not optimal, as we end up with a lower cell count for cell cluster analysis. To increase the robustness of cell clustering, we followed the Reviewer's suggestion and integrated all available sequencing data. We additionally included three newly sequenced samples (Sample 31-33, Supplementary Table S1). By integrating all samples, we increased the cell numbers substantially from 12,962 to 46,491 cells (Fig. 1b/c). Subsequently, we reanalyzed the entire dataset with this integrated cohort. The updated sample to cluster distribution subsequent to integration now showcases a comprehensive representation of samples across diverse clusters (Fig. S1b). On average, each cluster is depicted by 13.9 samples, with a minimum of 7 and a maximum of 17 samples. We updated the figures (Fig. 1b, 1c, 2a-h, 5a-e, S1a-d, S2a, S2b, S4c), results (page 5, ll.: 94-107; pages 7-8, ll.: 145-164; page 8, ll.: 174-178; pages 8-9, ll.: 183-201; pages 9-10, ll.: 210-214; page 15, ll.: 348-354; page 16, ll.: 359-368; page 17, ll.: 380-382; page 17, ll.: 385-402) and method section (page 27, ll.: 608-613)

accordingly. We thank the Reviewer for this important remark and think that based on the Reviewer's comment, we substantially increased the robustness of the manuscript.

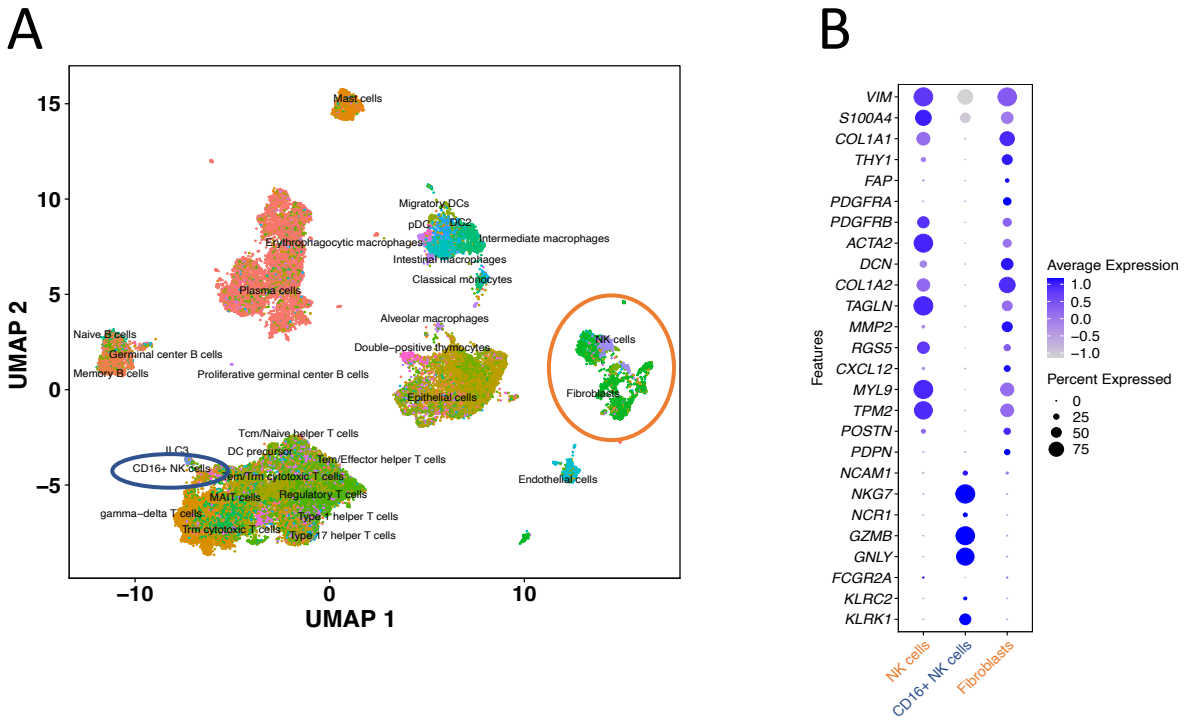
Besides the limited sample size (which is a key point), there are some specific aspects of the cell clusters that concern me. For example:
- Why are the gdT cells not clearly clustering based on their delta chain usage, while Vd1 and Vd2 cells are expected to show very different biology and expression profiles (and, indeed, form clearly separate clusters in Fig 2A of the publication of de Vries et al, Nature, 2023)?

We appreciate the Reviewer's intriguing question. We conducted a reanalysis of the clustering of *TRDV1-3*+ cells using the same FASTMNN integration method as described in the de Vries *et al*. paper (as mentioned by the Reviewer), using varying resolutions and dimensions (de Vries et al., 2023). However, unlike the findings in the de Vries *et al.* paper, γδ T cells of MSS CRC did not clearly cluster by their delta chain usage. We believe the reasons for this lack of distinct clustering are multifactorial, involving technical and biological components.
In comparison to our initial submission, we have increased the number of γδ T cells by almost two-fold, now covering 1838 *TRDV1-3*+ cells (compared to the total count of 4,442 γδ T cells in the de Vries' study). De Vries *et al.* focused their single-cell RNA-sequencing on five microsatellite instable CRC samples along with adjacent tissues, highlighting the subset of CRC patients with MSI tumors known for heightened γδ T cell infiltration. However, the majority of patients (approximately 85% of all CRC) have MSS tumors, where γδ T cells are reduced rather than increased. Thus, we needed to include more patients in our study, leading to increased heterogeneity, potentially contributing to the lack of clear clustering.
Despite the increased sample size and use of the same integration method, γδ T cells did not cluster separately based on their delta chain usage, suggesting that the difference may also be due to biological differences between MSI and MSS CRC. The reason for the sparse presence of γδ T cells in MSS CRC is not fully understood. We discovered 111 genes with significant differential expression across the three subsets and included a figure in the manuscript showcasing the top genes that exhibit significant differences among the three subsets (Fig. 2b). Among those are known marker genes for γδ T cells and genes that have been previously linked to γδ T cell biology. Although *TRDV1* expression was significantly upregulated in these cells, the expression of *TRDV1* was in the lower average compared to the other cells which potentially contributed to the fact that the cell subsets did not cluster separately.
We acknowledge that while the lack of separate clustering is an interesting observation, it does not impact our downstream analyses, since our efforts are based on quantitative assessments rather than the separate clustering itself. We updated figure 2, methods (page 27, ll.: 609-613) and results section accordingly (page 7, ll.: 151-154).

- For the fibroblast clusters, the authors now provide a comparison with Elyada et al, 2019 in the rebuttal document. However, the authors conclude that their clusters are inconsistent with those described bu Elyada et al. Why is this the case? Is this biological or technical?
- The authors now included a CellTypist analyses to annotate the clusters via a standardized procedure. However, CellTypist annotated the fibroblast clusters as endothelial cells, NK cells, fibroblasts, Tcm/Naïve helper T cells and double-positive thymocytes (fig S1b). Why? Taken together, more work is needed to address the concerns raised by Reviewer 3 regarding the robustness of cell clusters/types, especially given the limited sample/cell size.

We express our gratitude to the Reviewer for the insightful feedback and recommendations. In response to the Reviewer's concerns regarding potential technical discrepancies, we conducted a re-analysis using CellTypist following the updated integration process described earlier. Our

findings affirm the presence of fibroblast clusters; however, CellTypist once again categorized a subset of cells as NK cells (Reviewer Fig. 1A). To address this discrepancy, we filtered all cells within the fibroblast clusters labeled as NK cells (as circled in orange Reviewer Fig. 1A) and assessed their expression of established fibroblast and NK cell markers (Reviewer Fig. 1B). For comparison, we performed the same analysis with fibroblasts and CD16+NK cells (circled in blue, Reviewer Fig. 1A) clustering in close proximity to T cells side by side. Cells within the fibroblast cluster labeled as "NK cells" exhibited expression of common fibroblast markers akin to other fibroblasts (*VIM, COL1A1, PDGFRB, COL1A2*). Notably, these cells lacked expression of typical NK cell marker, including *NCAM1, NCR1, NKG6, GZMB, GNLY, KLRC2,* and *KLRK1,* which are usually observed in NK cells. Upon comparing another subset of NK cells identified by CellTypist as CD16+ NK cells, which cluster in close proximity to T cells exhibiting common NK cell marker expression, the distinctions between the two cell subsets became even more obvious. Specifically, these "NK cells" bear a closer resemblance to fibroblasts rather than CD16+ NK cells that express the typical NK cell marker. Hence, given that the "NK cells" designated by CellTypist lack the expression of established NK cell marker genes while displaying markers similar to fibroblasts, we conclude that CellTypist misclassified these cells as NK cells, affirming their true identity as fibroblasts. We have accordingly updated figure S1c and corresponding figure legend to reflect these findings.



Reviewer Figure 1: (A) Celltypist cluster annotation after re-integration. **(B)** Dot plot of fibroblast and NK cell marker genes expressed by "NK cells", CD16+ NK cells and fibroblasts.

Regarding the comparison of fibroblast phenotype to the work of Elyada *et al.*, Elyada *et al.* conducted the analysis on fibroblasts in pancreatic ductal adenocarcinoma, thus prompting us to consider that the biological disparities are attributable to the distinct tissue contexts (pancreas vs colon). In recent years, other studies utilizing single-cell RNA-sequencing analysis of CRC specimens have referenced Elyada's research to delineate fibroblast subtypes (Berlin et al., 2023; Koncina et al., 2023; Martinez-Ordonez et al., 2023; Nicolas et al., 2022; Peng et al., 2022). However, in our revised work, we endeavored to refine our approach by comparing fibroblast clusters in our manuscript with those identified in other studies using single-cell

RNA-sequencing data from CRC specimens, which offers a more relevant comparison. Similar to the insights from Elyada's study, investigations in CRC have identified comparable subsets, including inflammatory fibroblasts and a myofibroblastic subset. Additionally, Pelka *et al*. have introduced further subsets such as *CXCL14+* fibroblasts and those expressing stem cell niche factors (Pelka et al., 2021). Despite the new re-integration of all single-cell RNA-sequencing data, the inflammatory fibroblast subset previously described by us persists in the updated analysis (Fig. 5b, 5d). Consequently, we have revised the manuscript to facilitate a comprehensive comparison with other studies investigating CRC fibroblasts and single-cell RNA-sequencing data. Furthermore, we have omitted the terms "iCaf" and "myCAF" as they are closely associated with Elyada *et al.*'s work in pancreatic cancer, which we acknowledge does not entirely correspond to the findings in CRC.
We hope that the clarifications, supplementary efforts and alterations adequately address the Reviewer's inquiries. We updated the results section accordingly (page 15, ll.: 348-354).

2. More in-depth analysis of scRNA-seq data, including trajectory analysis

As suggested, the authors included a pseudotime-based trajectory analysis; however, it is a bit unclear how these results contribute to the story without further alignment with the other sections (especially the functional experiments). E.g., can it be confirmed experimentally that the terminal state indeed is the terminal state?

Furthermore, how robust are the trajectories to patient bias and (low) cell counts? This is important, e.g. to understand how reliable the finding of different trajectories in CRC vs healthy control truly is. Is this true biology or technical noise?

We thank the Reviewer for the insightful feedback. After careful consideration of the Reviewer's comments, we have chosen to exclude the trajectory analysis for several reasons. By omitting this analysis, we can allocate more narrative focus to the distinction between MSS/MSI status, as specifically suggested by the Reviewer. This decision enables a more streamlined storytelling approach without introducing additional narratives that do not add substantial information to the main findings. Furthermore, we acknowledge that we did not endeavor to experimentally validate the terminal state. We trust that the Reviewer agrees with our rationale behind this decision.

3. Clinical relevance of the findings

The clinical relevance of the findings remains unclear to me, and this remains a key limitation of the study.
In the rebuttal document, the authors state that the observation that Vd1 cells in MSI cancers have an exhausted phenotype whereas this is not the case in MSS cancers is a clinically relevant finding. However, why does this underscore clinical relevance? For example, I believe that the lack of exhaustion in MSS cancers could also be observed if Vd1 cells are bystanders with no or only weak tumor recognition in most MSS cancers? MSI cancers frequently lose HLA class I expression which contributes to the activation of Vd1 cells (de Vries et al, Nature, 2023), so perhaps this underlies the key difference between the levels of exhaustion between MSS and MSI?

The recent study by de Vries *et al*., as referenced by the Reviewer, underscores the rising interest in γδ T cells in CRC (de Vries et al., 2023). Nonetheless, the observations made by de Vries *et al*. are relevant to the minority of patients presenting with an MSI tumor, which on top are HLA-negative (approximately 5% of all CRC, with 10% being MSI in CRC, of which 30-70% exhibit loss of HLA class I (Cabrera et al., 1998; Moller et al., 1991)). In routine clinical practice, MSI CRC patients typically receive anti-checkpoint blockade therapy irrespective of their HLA status. Therefore, for MSI CRC patients, immunotherapy is already established, and the therapeutic regimen remains unchanged based on these novel findings. We contend that shedding light on the other facet of CRC is clinically relevant. The majority of patients do not present with MSI CRC (approximately 85%) and are not eligible for immunotherapy. This patient cohort faces an unmet need for participation in novel immunotherapeutic approaches. Hence, evaluating hitherto unexplored facets of the immune infiltrate in MSS CRC holds clinical significance as it can propel our understanding forward. While we acknowledge the inability to definitively demonstrate the full potential of revitalized Vδ1+ T cells in MSS CRC through mouse models and clinical trials, we firmly believe that the modifications made during the initial and subsequent rounds of revisions have significantly improved the clinical relevance of the manuscript. Nonetheless, our manuscript contributes substantially to the body of knowledge concerning MSS CRC and may serve as a catalyst for further research utilizing γδ T cells and their interactions with fibroblasts (e.g., via TIGIT blockade) as indicated in our *ex vivo* experiments. Key limitations have been included to the discussion section of the manuscript (page 19, ll.: 442-445; page 20, ll.: 467-471; page 21, ll.: 481-486, ll.: 490-492).

Furthermore, the authors create a signature of exhausted Vd1 cells and iCAF cells. Signature design for bulk RNA-seq is challenging as, for specificity, the genes in the signature should only be expressed by the cell type of interest; here, that seems not to be the case:

- The exhausted Vd1 signature is composed of TRDV1 (which is expressed by all Vd1 cells and not only exhausted Vd1 cells) and TOX (which is expressed by many exhausted immune cells and not only exhausted Vd1 cells). This makes it unlikely that this signature reliably captures the levels of exhausted Vd1 cells; at the same time, the authors do not rigorously validate that this would be the case.
- The iCAF signature is composed of VIM (which is, according to the scRNA-seq data in the Human Protein Atlas, also expressed by immune cells, muscle cells, neurons, etc), S100A4 (which is also expressed by immune and muscle cells), COL1A2 (which is also expressed by smooth muscle cells), and IL6 (which is also expressed by endothelial cells, cancer cells, smooth muscle cells and (mostly myeloid) immune cells). Again, this makes it unlikely that this signature reliably captures the levels of iCAF cells and there is no validation to show otherwise.
Hence, these signature analyses on bulk data are unconvincing and in their current form a liability of the study.

We thank the Reviewer for the critical feedback. We have been specifically asked by Reviewer 3 to design a signature using bulk sequencing with outcome data (refer to Fig. 5j). We acknowledge that this approach may not entirely exclude the possibility that the observed expression could originate from cell types beyond our designated cluster of interest, given the inherent limitations of bulk sequencing data. However, due to the absence of comprehensive single-cell RNA-sequencing datasets for MSS CRC with clinical and outcome data, we determined that utilizing the TCGA-COAD dataset was the most feasible option for correlating gene expression with patient outcomes. We attempted to utilize deconvolution pipelines as a computational method that aims to estimate the cell type-specific gene expression profiles from

bulk RNA-sequencing data. However, it appears that these are only feasible analyzing abundant cell subsets. In order to improve the validation of our approach, we analyzed the expression levels of the selected genes (*COL1A2, IL6, TRDV1, TIGIT*) within our defined cell clusters (refer to Fig. S4c). Our findings revealed that *TRDV1* expression was specific to the cluster housing γδ T cells (Cluster 2), while TIGIT was expressed in both Cluster 2 and to a similar extent in Cluster 14, which we identified as regulatory T cells. *COL1A2* exhibited notable expression in clusters previously identified as fibroblasts (Cluster 16, 17, 20), as well as in a small subset of enteric glial cells. *IL6* demonstrated heightened expression in the inflammatory fibroblast cluster (Cluster 17), with moderate expression in fibroblast cluster 16, while cluster 20 displayed minimal *IL6* expression. In an effort to delineate gene expression specific to cell types, we only included samples with *TIGIT* and *TRDV1* co-expression. Consequently, samples expressing only *TIGIT* were excluded, suggesting that *TIGIT* originates from a cell subset distinct from γδ T cells.
Based on these observations, we conclude that the predominant signals in the TCGA-COAD dataset likely stem from inflammatory fibroblasts and *TIGIT*-expressing *TRDV1*+ cells. We updated Fig. 5j and S4c accordingly. Changes were made to the following sections of the manuscript to reflect the updated findings (page 17, ll.: 385-394; page 28, ll.: 641-642).

That being said, I do agree with Reviewer 3 that it would greatly improve the manuscript if the authors could provide convincing evidence of clinical relevance. For example, by showing (A) the in vivo potential of reinvigorating Vd1 cell-based antitumor immunity in MSS CRC (e.g. by patient or mouse models), (B) the association of Vd1 T cell states with disease progression in MSS CRC, or (C) the association of Vd1 T cell infiltration with response to immune checkpoint blockade of MSS CRC, etc.

We agree with the Reviewer that indications for the clinical significance are important for the translation of our findings. We agree that establishing a proof of concept for revitalized Vδ1+ T cell-based immunotherapy, whether through murine models or ultimately via clinical trials involving patients, represents a pivotal next step in this process.
Nevertheless, prior to this, we have undertaken several measures to advance the field of γδ T cell research and underscore the clinical relevance of Vδ1+ T cells in MSS CRC. At both the gene and protein levels, we have demonstrated the impaired cytotoxic effector mechanisms of Vδ1+ T cells in MSS CRC and validated this observation by functional *ex vivo* assays. We have also presented *ex vivo* evidence supporting the potential rejuvenation of these cells, as evidenced by enhanced killing of HT29 cancer cell lines following activation with broad cellular stimuli (PMA/ionomycin), as requested by the Reviewers during the initial round of revisions (Fig. 4e-g). Furthermore, we have demonstrated the restored killing capacity of Vδ1+ T cells following TIGIT blockade in a coculture assay with fibroblasts (Fig. 5g, 5h). In response to this round of revisions, we have i) increased the number of experiments for functional assays involving TIGIT blockade, ii) introduced a control experiment (Fig. S4b), as suggested by Reviewer 4), and iii) computationally assessed a correlation of disease stages with *TRDV1* expression, highlighting the significant association of Vδ1+ T cell depression with advanced stage IV MSS CRC (Fig. 5i). These enhancements have been updated in the results section (page 17, ll.: 385-389).
Unfortunately, the evaluation of the association between Vδ1+ T cell infiltration and response to immune checkpoint inhibitors in patients with MSS CRC as shown by de Vries *et al.* (de Vries et al., 2023) for MSI CRC remains unfeasible at present, since these patients typically do not receive checkpoint inhibitors. We have addressed the limitations within the discussion of the manuscript (page 21, ll.: 483-488). We hope for the Reviewer's recognition of our efforts to address queries regarding the clinical relevance of our work. While we acknowledge that further

research is warranted in this field, it is noteworthy that Reviewers 1 and 2 have underlined the clinical significance of the manuscript.

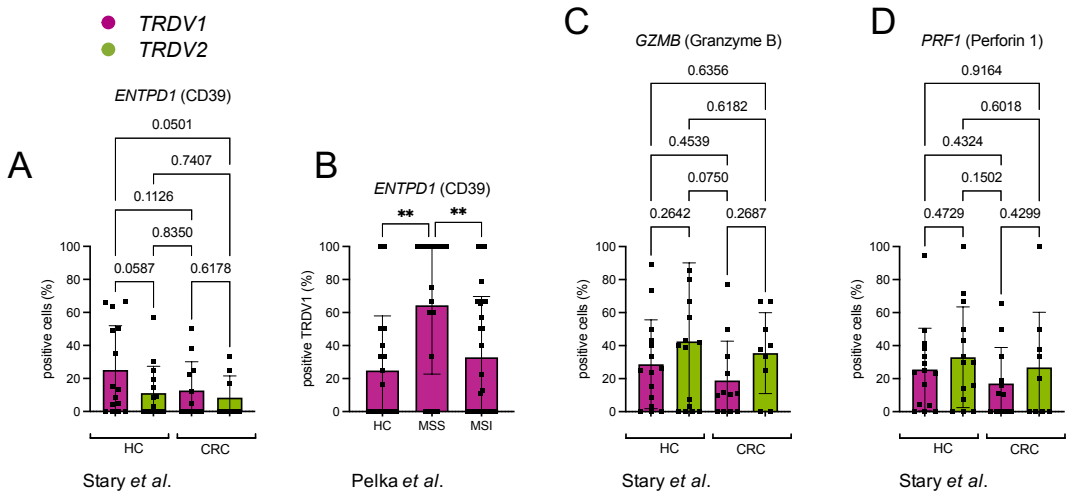
4. The comparison of gdT cells in MSS vs MSI CRC

The authors included an in-depth analysis comparing gdT cells between MSS vs MSI CRC into their revised manuscript, which is a notable effort and strengthens the manuscript. I do have some suggestions regarding these analyses:
- A key observation of de Vries et al (Nature, 2023) was that tumor reactivity was largely contained within the PD-1-expressing gdT cell compartment. Earlier work of de Vries et al (2020) has suggested that PD-1-positive gdT cells are largely absent in MSS CRC, whereas they are abundant in MSI cancers. In light of these findings, it is highly relevant to:

o Also include PD-1 to figure S2d. Here, it would be good to also add CD39 (marking activated gdT cells). For alignment with the rest of the manuscript, it would be informative to also show NKG2A, FasL, and TRAIL expression in fig S2d.

We thank the Reviewer for the constructive feedback. We agree with the Reviewer that comparing γδ T cells between MSS and MSI CRC is of clinical relevance and scientific interest. To streamline the narrative, as suggested by the Reviewer, we have incorporated the comparison into the main figures of our study (Fig. 2d-f). Additionally, we conducted further analysis on the genes *PDCD1*, *ENTPD1* (CD39), *KLRC1* (NKG2A), *FASLG* (FASL) and *TNFSF10* (TRAIL), as per the Reviewer's recommendations.
Our findings reveal significant upregulation of *PDCD1* (PD-1) in *TRDV1*+ cells within our cohort and reflects our results at the protein levels (Fig. 2b). A tendency towards upregulation of *PDCD1* is also noted in MSS when comparing with the Pelka *et al*. dataset (Fig. 2f). Significant upregulation is observed in MSI CRC, aligning with de Vries *et al*. (de Vries et al., 2023). However, discrepancies in *ENTPD1* (CD39) expression between the two datasets are noted. While some downregulation is observed in *TRDV1*+ cells within our cohort, significant upregulation is observed in the Pelka *et al*. dataset (Reviewer Fig. 2A+B). However, values for MSI CRC are similar to what de Vries *et al*. reported (approximately 30%). Hence, we decided to not present *ENTPD1* in the main figures.
Regarding *KLRC1* (NKG2A), MSS CRC demonstrate a significant downregulation (Fig. 2c). However, the opposite is true for MSI CRC (Fig. 2f), suggesting a regulatory mechanism to modulate γδ T cell activity in response to the tumor microenvironment. *FASLG* (FASL) displays a tendency of downregulation in MSS CRC in our cohort, consistent with our flow cytometry results. Interestingly, *TNFSF10* expression in our cohort contradicts the downregulation observed in flow cytometry data. Notably, there is an almost significant upregulation of *TNFSF10* (TRAIL) in MSI CRC in the Pelka *et al.* dataset.
The data presented in Reviewer Fig. 2C and 2D suggest a trend indicating reduced levels of the effector molecules granzyme B and perforin (approximately 20% in MSS CRC), reflective of decreased cytotoxicity in *TRDV1*+ cells. It's worth mentioning that de Vries *et al*. reported that roughly 70% of *TRDV1*+ cells expressed granzyme B, and approximately 60% exhibited positivity for perforin.
In summary, γδ T cells in MSI tumors tend to be skewed towards cytotoxicity (significantly upregulated genes: *TNFSF10* (TRAIL), *IFNG* (IFN-γ), *GNLY, KLRC1* (NKG2A)) whereas the same genes remain unaffected or downregulated in MSS CRC (significantly downregulated: *KLRC1* (NKG2A), *KLRC2* (NKG2C), *IFNG* (IFN-γ), *GNLY*).
Discrepancies in expression in HC tissue in the Pelka *et al*. dataset may arise from unmatched HC samples (36 HC vs 28 MSS vs 34 MSI). In our experience, HC tissue exhibits differences

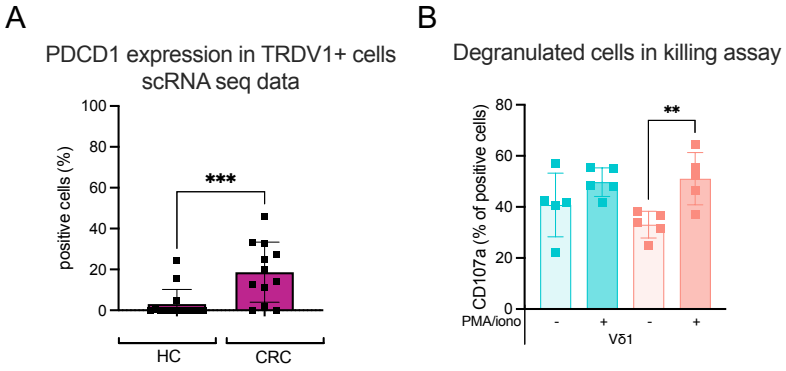
between patients with MSI and MSS. Therefore matching of HC is crucial. Consequently, we have revised Fig. 2 and adjusted the results section accordingly (page 8, ll.: 174-178; pages 8-9, ll.: 183-187, pages 9-10, ll.: 210-214).



Reviewer Figure 2: (A) Percentage of positive cells for *ENTPD1*. **(B)** Percentage of positive *TRDV1* cells expressing *ENTPD1* in HC, MSS and MSI. **(C)** Percentage of *GZMB* positive cells. **(D)** Percentage of *PRF1* positive cells.

o It would be informative to compare the clonality/expansion of PD-1 positive vs negative gdT cells.
o If possible, functionally test if tumor reactivity of Vd1 cells against MSS cancers is also restricted to PD-1 positive cells, to get a sense if all other gdT cells in MSS cancers are bystanders (as PD-1-negative gdT cells seem to be in MSI cancers).

We appreciate the intriguing question posed by the Reviewer. Our examination revealed that only a minority of Vδ1+ T cells displayed positivity for PD-1 in HC. Interestingly, we observed a notable increase in PD-1 expression within CRC (as depicted in Reviewer Fig. 3A). If we follow the hypothesis of the Reviewer, namely that only PD-1 positive cells are capable to recognize tumor cells, this would exclude that Vδ1+ T cells in MSS CRC are solely bystanders. Because, when considering CD107a expression as a surrogate marker for degranulated cells that have recognized tumor cells, we found that a substantial proportion, ranging from 33% to 51%, of all Vδ1+ cells were CD107a positive, indicative of antigen recognition and degranulation (Reviewer Fig. 3B). These percentages exceeded the proportion of PD-1-positive cells observed in HC and CRC. It remains plausible that during antigen recognition, Vδ1+ cells upregulate PD-1, potentially contributing to the higher values of CD107a positivity observed. Consequently, we cannot conclusively assert that tumor reactivity is solely confined to PD-1-positive cells. We have included a discussion of these findings in the relevant section of the manuscript (page 20, ll.: 469-473).



Reviewer Figure 3: (A) Percentage of positive TRDV1+ cells for *PDCD1* using single-cell RNA-sequencing. **(B)** Percentage of CD107a positive VD1 T cells, as a marker of degranulation in a killing assay with HT29 CRC line.

- It would be helpful to integrate the MSS vs MSI comparison better into the general story line. Currently it is presented as a bit of a separate story, whereas (also in light of the remarks below) this comparison provides important fundamental insights about the role of the potential role of gdT cells in MSS cancers. Are they bystanders or functional? If they are bystanders, can they be activated? If there already is baseline tumor reactivity, how can this be amplified and which gdT cell subsets should be targeted?

We agree with the Reviewer's assertion regarding the timeliness and potential significance of comparing MSI and MSS, which can offer valuable insights. We have integrated our analysis and comparison between MSI/MSS CRC into the general storyline (Fig. 2). Specifically, we observed a significant upregulation of *TRDV1*+ expression and consequently an increase in the abundance of Vδ1+ T cells in MSI CRC, as evidenced by analyses of the TCGA-COAD and Pelka *et al.* datasets and compared to MSS CRC (Fig. 2d, 2e). This upregulation correlated with a significant increase of Ki-67 in MSI CRC which was not the case in the MSS cohort. Furthermore, while we did not observe clonal expansion of γδ T cells in MSS, we did note an increase in a similar pattern within the CDR3 region, responsible for antigen recognition, although caution is warranted due to limited information on γδ T cells with TCR sequencing data. Our findings indicate that γδ T cells in MSS exhibit a lesser degree of exhaustion compared to those in MSI (Fig. 2g, 2h). Additionally, despite being equipped with a killing machinery in the normal adjacent colon, γδ T cells significantly downregulate effector molecules in MSS CRC. Specifically, compared to MSI, γδ T cells from MSS demonstrate significant downregulation of the effector molecules *IFNG* and *GNLY*, corroborated by functional data showing a diminished killing capacity of Vδ1+ T cells in MSS CRC. Hence, it is conceivable that γδ T cells in the development of MSS CRC undergo a transition from potential killers to bystanders. However, their functional capacity could be reactivated through stimulation, as demonstrated by our experiments using PMA/iono and TIGIT blockade. We acknowledge the possibility of a low neoantigen count in MSS CRC, potentially resulting in decreased recognition of tumor cells by γδ T cells. Additionally, our findings suggest a crosstalk between inflammatory fibroblasts and γδ T cells in shaping their fate in MSS CRC. Nonetheless, we contend that this process is likely multifactorial and needs to be elucidated in follow-up studies. We updated the results section to reflect the findings (page 8, ll.: 174-178; pages 8-9, ll.: 183-187; pages 9-10, ll.: 210-214).

5. Novelty

To explain what is truly novel, the authors mainly stress the uniqueness of their dataset, rather than explaining the novelty of their conclusions/findings. Rather than providing a descriptive summary of the data, the Discussion of the manuscript would benefit from a more to the point discussion of the key novel conclusions/findings. Furthermore, we agree with the authors that the addition of (gd)TCR-seq to single cell analyses in MSS CRC is very interesting and novel, but the authors do not clearly translate this advantage of the dataset into novel biological insights (e.g. are clonally expanded gdTCRs broadly reactive to MSS CRC? Do gdT cells expressing activation markers such as PD-1, CD39 and Ki67 show clonal expansion?

We express our gratitude to the Reviewer for the feedback on how to best phrase the novelty of the manuscript. In response to this, we have made revisions and included a concise discussion section highlighting the key novel findings (refer to page 18, ll.: 404-424). Notably, we have demonstrated the reactivity of γδ T cells isolated from MSS CRC to MSS CRC cell lines, providing indirect evidence of γδ T cell recognition of CRC *ex vivo*. While we cannot definitively confirm clonal expansion during the transition to CRC, we have observed a significant increase in shared patterns in the CDR3 region of γδ T cells, suggestive of shared antigen recognition. However, further research is warranted to validate these findings due to limited data availability.
Additionally, we have observed an upregulation in TCR-associated genes in CRC, indicating antigen recognition and downstream TCR activation in this context. Despite this, the overall γδ T cell count in MSS CRC is lower compared to MSI CRC, and cytotoxicity is impaired, as evidenced by gene and protein expression data validated with functional assays. While MSS γδ T cells exhibit some upregulation of exhaustion genes, they do not meet the classical definition of exhaustion observed in MSI CRC.
Consequently, γδ T cells in MSS CRC do not respond to checkpoint inhibitors targeting PD-1, which are efficacious in MSI CRC therapy. The trajectory of γδ T cell fate likely mirrors that of conventional T cells in both tumor identities, being responsive to PD-1 inhibition in MSI but unresponsive in MSS CRC. The underlying reasons for this disparity in conventional T cells of MSS CRC may also apply to γδ T cells, including lower mutational burden, reduced neoantigen recognition, diminished overall immune cell numbers, an immunosuppressive microenvironment, and alternative immune escape mechanisms. Our findings corroborate these hypotheses at the γδ T cell level, highlighting the absence of clear signs of clonal expansion, diminished γδ T cell counts, the presence of immunosuppressive fibroblast subsets, and the responsiveness of γδ T cells to TIGIT blockade as an alternative mechanism of immune evasion.

Are specific clonotypes associated with specific phenotypes?). Also, linking the single cell analyses to functional experiments is potentially powerful, but the functional experiments are very limited and the interaction/correlation of the single cell findings with the functional experiments is limited. In conclusion, the manuscript could be greatly improved if the authors would more effectively exploit the uniqueness of their dataset to discover uncharted territory.

We value the Reviewer's thoughtful examination of the matter. Our study encompassed a range of analyses, spanning from single-cell RNA sequencing to functional assessments of γδ T cells. We conducted these investigations wherever applicable and feasible, ensuring a comprehensive approach. Our findings were substantiated through validation at both the RNA and protein levels, confirming the ability of γδ T cells to recognize and eliminate CRC. However, we observed that γδ T cells from CRC exhibited reduced efficacy in this regard, potentially due to

decreased cytokine secretion, as indicated in our single-cell data. Notably, we identified significant transcriptomic differences between MSS and MSI CRC, with potential implications for clinical responsiveness to PD-1 inhibition, a topic of current relevance. Furthermore, we endeavored to elucidate the factors contributing to dysfunctional Vδ1+ T cells, successfully demonstrating *ex vivo* the rescue of their killing capability. While acknowledging that many aspects of γδ T cell biology remain enigmatic, our study represents a notable effort to unravel their role in MSS CRC to an unprecedented extent. We hope the Reviewer recognizes the diligence invested in our work. In response to the Reviewer's suggestion, we focus the discussion more on the novelty of the findings and have undertaken significant revisions to the discussion part (page 18, ll.: 404-424, page 21-22, ll.: 498-509). Limitations have been included in the discussion section for transparency (page 19, ll.: 444-447; page 20, ll.: 469-473; page 21, ll.: 483-488, ll.: 492-494).

6. Anatomical localization of the tumors

This point has been well addressed by the authors and provides interesting insights.

Minor additional points:
- Fig1d: it seems like TRDV1 mainly pairs with TRDV2, which I presume is a (mis)labelling issue?

Figure 1d illustrates the pairing of TRDV with TRGV in HC compared to CRC. We are grateful for the Reviewer's meticulous evaluation and apologize for the mislabeling. The error has been rectified. To enhance clarity and readability, we have now highlighted TRDV in bold within the figure.

- Line 191-208: It is unclear what statistics or quantification underlies "altered"

As additional sequencing data was incorporated, we have revised Figure S2b. Here, the manuscript discusses a heatmap depicting the top differentially overexpressed genes between *TRDV1* in CRC and HC. The term "altered gene expression" pertains to statistical significance, defined by a p-value < 0.05 using the Wilcoxon Rank Sum test. We have revised the wording to clearly indicate statistical significance for the reader's understanding (page 9, line 188).

- Figure 3j,k,l: the PD-1 gating in the gating strategy is missing, because that was strongly varying between the HC and CRC, so that may affect the populations and the "number" of cells in the populations

We appreciate the Reviewer's thorough evaluation of the figure. We apologize for the oversight in not initially displaying all the assessed markers in the representative examples used to calculate the values in Fig. 3i. In the revised version of the figures, we have now included the correct gating strategy for the representative examples in Fig. S3.

- Figure 4a: this is a bit of a chaotic and not clearly readable figure

We extend our appreciation to the Reviewer for the comprehensive evaluation. To enhance readability, several adjustments have been made to Fig. 4a. Font sizes for labels and axis titles have been increased, and labeling has been refined for clarity and descriptive accuracy. Contrast in colors has been heightened to improve visibility, and consistent formatting has been maintained throughout the figure. Additionally, increased white space has been incorporated to

delineate elements and enhance overall clarity, thereby preventing overcrowding and facilitating easier interpretation of the figure. We trust that these enhancements have effectively improved the figure's readability.

- *Figure 5h: the key control that I'm missing here is the Vd1/Vd2-TIGIT alone condition*

This comment refers to the figure displaying the percentage of dead HT29 cells in the killing assay involving V δ 1 and fibroblasts. In response to the Reviewer's request, we have included the requested control condition of Vδ1+TIGIT antibody+HT29 without fibroblasts in Figure S4b of the supplementary data for four additional experiments and added the results section accordingly (page 16, ll.: 380-382).

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REVIEWERS' COMMENTS

Reviewer #4 (Remarks to the Author):

The revised manuscript of Stary et al has substantially improved, both in terms of its clarity/story line as well as in terms of content, with the addition of important extra data and several interesting additional analyses. Multiple of my points are well addressed by the authors. However, I do still have major concerns, especially related to the interpretation of the data and accuracy of multiple main claims.

In summary, the story line is based on the next set of key claims:
1. gd T cells in MSS CRC show favored delta chain usage with expanded CDR3 identity patterns (Fig 1)
2. TRDV1+ cells demonstrate profound dysregulation but not exhaustion in MSS CRC (Fig 2). This dysfunctional state is characterized by low expression of genes associated with cytotoxicity and the inability to proliferate.
3. Such dysfunction results in reduced Vd1 T cell numbers in MSS CRC vs healthy control (Fig 3)
4. This dysfunctional state is reversible in vitro by treatment with PMA/ionomycin (fig 4)
5. TIGIT blockade is also able to rescue the Vd1 cells from their dysfunctional state in tumor cell-fibroblast-gdT cell triple co-cultures. This works by breaking the inhibitory effects of NECTIN2-expressing fibroblasts triggering the TIGIT receptor on Vd1 cells (fig 5h & fig S4b)

Attn claim 1:
- Adding additional scRNA-seq data has substantially improved the reliability of the cell clusters. The labeling of the cell clusters has also been corrected. However, regarding the overlay of scRNA-seq with TCR sequencing, it remains surprising that the gdT cells do not cluster according to delta chain usage given the vastly different biology of Vd1/3 cells vs Vd2 cells. As mentioned earlier, clustering by delta chain usage was clearly observed in MSI CRC infiltrating gdT cells (de Vries et al, Nature, 2023). Furthermore, also in kidney cancer clustering according to delta chain usage is observable (figure 5d of Rancan et al, Nature Immunology, 2023). In their rebuttal, the authors hypothesize that this is due to the disease under study: MSS CRC, where for some reason the biology of Vd1/3 and Vd2 cells would show more overlap than in other contexts. This is possible but other explanations are also plausible, including technical issues with the data. Therefore, I think it is important to confirm this hypothesis with a clustering analysis of the data of Reis et al (Science, 2022), which provides another dataset with scRNA-seq + gdTCR-seq of gdT cells in MSS CRC.
- In the previous round of review, I suggested more integration of the scRNA-seq and TCR-seq results. Despite the fact that the gdTCRs did not show clear signs of clonal expansion, the authors suggest that the shared identity patterns of the CDR3 region reflect antigen recognition / tumor reactivity. However, evidence to substantiate this claim is not being provided. Functional evidence would be ideal, but showing enrichment of an activated phenotype in gdT cells with vs without the key shared identify patterns seems possible with the data available; In line with my earlier suggestions, the latter would offer a clear opportunity to exploit the strength of the author's dataset (scRNA-seq + TCR-seq) and add considerably more depth to the story.
- If I understand it correctly, the GLIPH2 analysis (fig 1G) was run on all gdTCRs at the same time, but I believe it is important to run this separately for Vd1, Vd2 and (if possible) Vd3 TCRs. The reason is that these three classes of TCRs likely have very different identity patterns and at the same time show very different frequencies between CRC and healthy control. This makes it hard to interpret the results. Perhaps the differences between tumor vs normal are primarily driven by different frequencies of the three main classes in tumor vs normal, rather than true differences regarding identity patterns? This is a relatively easy but important fix.
- At several instances in the manuscript, the authors discuss that their results are in line with Reis et al (Science, 2022). The main finding of Reis et al is that the gamma-chain determines if MSS CRC infiltrating gdT cells have pro-tumorigenic vs anti-tumorigenic functions. Do the authors see evidence for this hypothesis? As mentioned in the previous review round, it seems a missed opportunity to not exploit the fact that the authors have TCR-seq + scRNA-seq in order to gain more functional insights about gdTCR's in MSS CRC.
- Despite the correction of Figure 1D there are remaining issues with this figure: in many cases the coloring of the outer ring is flipped (in comparison to the coloring of the inner side of the figure).

Attn claim 2:
- The authors describe the dysfunctional state of gdT cells in MSS CRC is being characterized by low expression of genes associated with cytotoxicity and the inability to proliferate (where the

comparison with healthy control tissue is key). However, the data in the revised manuscript to substantiate this is inconsistent. Although the data of the author's own dataset is in line with their interpretation of the dysfunctional state (fig 2c), the revised manuscript also contains analysis of data of a (large) cohort of Pelka et al, which shows a different picture (fig 2f). Here, IFNG, GNLY, and FASL are not downregulated in MSS CRC vs healthy control and hence is not in line with a reduction of cytotoxicity markers in MSS CRC infiltrating gdT cells as compared to healthy control. The latter does align with figure 4, which shows (with immunophenotyping data of the authors themselves) that expression of the key cytotoxic molecules granzyme B and perforin is not reduced in gdT cells in MSS CRC vs healthy control tissue. Furthermore, the TRDV1 cells in the Pelka et al dataset show increased expression of MKI67 in MSS CRC versus healthy control and the author's own data presented in figure 4b shows that gdT cells in MSS CRC frequently express ki-67 (and thus are happily proliferating). In line 185-187 of the manuscript, the authors bring up a potential explanation for inconsistencies of their data with Pelka et al but this explanation if purely hypothetical and proof is missing; At the same time this is not addressing inconsistencies between RNA and protein-based analyses of the authors themselves. A more likely explanation of the data seems to be that the "dysfunctional" state needs a more refined definition. Given that the description of the "dysfunctional" state of gdT cells in MSS CRC is really at the core of this story I think a more refined definition/description is essential. What exactly is this "dysfunctional" state? What is consistent across all datasets and across RNA and protein analyses? It doesn't seem to be a consistent downregulation of cytotoxic programs and the inability to profilerate.
- Related to the above: based on the RNA-seq data, the authors claim that gdT cells in MSS CRC do not show signs of exhaustion. However, in the next section they show the opposite (Fig 3i): an enrichment of PD-1+CTLA-4+LAG-3 or PD-1+CTLA-4+TIGIT triple positive cells (all exhaustion markers) in MSS CRC vs healthy control. Why are the gdT cells dysfunctional and not exhausted? Given that the distinction of these two phenotypes is central to the paper I believe this should be addressed.
- I feel that some discussion / analysis is needed to put the "dysfunctional" state that is described by the authors into context with known pro-tumorigenic states which are also characterized by lower cytotoxicity (e.g. IL-17 producers). How novel is the discovered "dysfunctional" state?

Attn claim 5:
- In the revised document the authors provide a new figure S4b containing an essential control condition (the Vd1 +/- anti-TIGIT without fibroblasts conditions) for the key functional data supporting the claim that TIGIT blockade works by breaking fibroblast-gdT cell interactions (figure 5h). However, this means that this key data was generated in two separate experiments. I have two major concerns with this:
o The key control (fig S4b) is being tested post-hoc in separate experiments, rather than being tested in the same experiment than the other conditions. As variability of in vitro killing capacity of gdT cells is quite variable between biological replicates, it is unclear if the "true signal" (that is, the enhanced killing with anti-TIGIT in the presence of fibroblasts) was detectable in these experiments. In other words, figure S4b contains the missing negative control but lacks the essential positive control. Therefore, I am still not convinced about this claim.
o The control experiment in fig S4b was performed with only 4 replicates (instead of 8 replicates as shown in figure 5h). Given the relatively large variability per condition in figure 5h, I believe the control experiment is underpowered and hence the "negative" result does not rule out a biologically relevant effect of TIGIT blockade in the absence of fibroblasts.
I believe these issues need to be resolved with great care before claim 5 can be made.
- Given the CellChat result, I assume that the fibroblasts in MSS CRC express NECTIN2, but this is not shown. For proper interpretability of the NECTIN2-TIGIT axis and to further substantiate that this axis depends on the interaction of fibroblasts with Vd1 cells in this context, it is essential that the authors show which fraction of fibroblasts express NECTIN2 and if fibroblasts are the key source of NECTIN2 in the tumor micro-environment. Which other stromal cells are expressing NECTIN2? Are tumor cells expressing NECTIN2?
- The revised gene set analysis (now using TRDV1 + TIGIT to mark Vd1 cells and COL1A2 + IL6 for fibroblasts) is still problematic:
o TIGIT is also highly expressed in cluster 14 (see fig S4c), which consists of abT cells, which makes this marker nonspecific for gdT cells.
o IL6 is just lowly expressed in the fibroblast clusters; at the same time expression is not zero in many other cell types which (in total) are much more abundant than fibroblasts, making it likely that IL6 expression largely originates from non-fibroblasts. Hence, it seems unlikely that IL-6 expression levels reliably reflects (immuno)fibroblast infiltration.
o The correlation is weak with a correlation coefficient of 0.24, which translated in approximately only 5% of the (rank) variance of TRDV1 + TIGIT is explained by COL1A2 + IL6. So, in addition to

the points raised above, it is questionable if this association is biologically relevant.
- Minor: For further generalizability, it would be interesting to see the frequency of NECTIN2 expressing fibroblasts in MSI CRC as well (given the relevance of gdT cells in this disease).
- Minor: The x-axis order of fig 5h seems off (HC, CRC, CRC, CRC, HC, HC; more logical is CRC, CRC, CRC, HC, HC, HC (or the reverse)).

Point-to-point reply to the Reviewer's comments

Reviewer #4:

The revised manuscript of Stary et al has substantially improved, both in terms of its clarity/story line as well as in terms of content, with the addition of important extra data and several interesting additional analyses. Multiple of my points are well addressed by the authors. However, I do still have major concerns, especially related to the interpretation of the data and accuracy of multiple main claims.

In summary, the story line is based on the next set of key claims:
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We are pleased to hear that the Reviewer found the improvements in clarity, storyline, and content substantial, and that the additional data and analyses were valuable. We greatly appreciate the Reviewer's thoughtful comments and suggestions, which have significantly contributed to enhancing our work.

Attn claim 1:
- Adding additional scRNA-seq data has substantially improved the reliability of the cell clusters. The labeling of the cell clusters has also been corrected. However, regarding the overlay of scRNA-seq with TCR sequencing, it remains surprising that the gdT cells do not cluster according to delta chain usage given the vastly different biology of Vd1/3 cells vs Vd2 cells. As mentioned earlier, clustering by delta chain usage was clearly observed in MSI CRC infiltrating gdT cells (de Vries et al, Nature, 2023). Furthermore, also in kidney cancer clustering according to delta chain usage is observable (figure 5d of Rancan et al, Nature Immunology, 2023). In their rebuttal, the authors hypothesize that this is due to the disease under study: MSS CRC, where for some reason the biology of Vd1/3 and Vd2 cells would show more overlap than in other contexts. This is possible but other explanations are also plausible, including technical issues with the data. Therefore, I think it is important to confirm this hypothesis with a clustering analysis of the data of Reis et al (Science, 2022), which provides another dataset with scRNA-seq + gdTCR-seq of gdT cells in MSS CRC.

In our previous revision, we posited that the absence of clustering by the delta chain could be attributed to several factors. These include increased heterogeneity from a larger patient sample and the generally low expression of *TRDV1* in MSS CRC. Given that γδ T cell counts are diminished in MSS CRC - contrary to the increased counts in MSI CRC as reported by de Vries

et al.[1] - and considering our sorting strategy (CD45+/CD45- versus γδ T cells only in de Vries et al.), we were compelled to include a larger patient cohort in our study. This expansion in sample size likely introduced additional heterogeneity, impacting the clustering outcome. Furthermore, we identified significantly differentially expressed marker genes for γδ T cells, along with genes associated with γδ T cell biology. While *TRDV1* expression was markedly upregulated in these cells, its comparatively lower average expression relative to other cell types likely contributed to the absence of distinct clustering of the cell subsets. We acknowledge that this lack of distinct clustering is an intriguing observation. However, it does not affect our downstream analyses, as our study does not depend on the distinct clustering of cell subsets itself.
The Reviewer contends that in Rancan *et al*.'s study on γδ T cells in renal cancer, these cells also exhibit distinct clustering by their delta chain (Fig. 5d,[2]). Rancan *et al*. sorted over 3,000 γδ T cells from six renal cell cancer patients. Upon a detailed examination of the figures, we believe that distinct clustering is not entirely evident. While there is an area where *TRDV2* cells are concentrated, *TRDV1* and *TRDV3* cells are dispersed and do not form distinct clusters. We propose that the minor indications of clustering in Rancan *et al*.'s study are likely due to reduced heterogeneity from the smaller sample size of six patients and the larger number of cells sorted. We believe these factors similarly influence the dataset of de Vries *et al*. Prior to our revisions, we accessed the data set of Reis *et al*. as recommended by the Reviewer [3]. Unfortunately, the clinical information regarding the microsatellite status of the patients is either missing or inconclusive. We contacted the authors for clarification but did not receive a response. We cannot definitively determine whether these patients are MSI or MSS, and therefore cannot confirm the lack of clustering in γδ T cells from MSS CRC.

- In the previous round of review, I suggested more integration of the scRNA-seq and TCR-seq results. Despite the fact that the gdTCRs did not show clear signs of clonal expansion, the authors suggest that the shared identity patterns of the CDR3 region reflect antigen recognition / tumor reactivity. However, evidence to substantiate this claim is not being provided. Functional evidence would be ideal, but showing enrichment of an activated phenotype in gdT cells with vs without the key shared identify patterns seems possible with the data available; In line with my earlier suggestions, the latter would offer a clear opportunity to exploit the strength of the author's dataset (scRNA-seq + TCR-seq) and add considerably more depth to the story.

We agree with the Reviewer's suggestion to fully exploit the TCR sequencing data. However, due to the limited availability of data, we are concerned that further analysis may be underpowered. Consequently, we have decided not to pursue this line of investigation further. Using TCR sequencing, we found that γδ T cells do not exhibit clear signs of clonal expansion. Analysis of the CDR3 region indicated that certain patterns appear to be more prevalent in CRC, which suggests some form of shared antigen recognition. Experimentally, we demonstrated *ex vivo* that isolated γδ T cells from both HC and CRC patients are in principle capable of proliferating. However, the γδ T cell count in CRC is significantly decreased, which typically does not support clonal expansion. We acknowledge that we did not conduct further experimental validation of clonal expansion and trust that the Reviewer understands our rationale for this decision.

- If I understand it correctly, the GLIPH2 analysis (fig 1G) was run on all gdTCRs at the same time, but I believe it is important to run this separately for Vd1, Vd2 and (if possible) Vd3 TCRs. The reason is that these three classes of TCRs likely have very different identity patterns and at the same time show very different frequencies between CRC and healthy control. This makes it hard to interpret the results. Perhaps the differences between tumor vs normal are primarily

driven by different frequencies of the three main classes in tumor vs normal, rather than true differences regarding identity patterns? This is a relatively easy but important fix.

The GLIPH2 analysis was conducted on all γδ T cells. Given that the majority of tested γδ T cells are Vδ1 in both HC and CRC patients, we anticipate that Vδ1 cells primarily drive the results. Due to the low numbers of Vδ2 and Vδ3 cells, we do not expect these minority subsets to significantly influence the outcome.

- At several instances in the manuscript, the authors discuss that their results are in line with Reis et al (Science, 2022). The main finding of Reis et al is that the gamma-chain determines if MSS CRC infiltrating gdT cells have pro-tumorigenic vs anti-tumorigenic functions. Do the authors see evidence for this hypothesis? As mentioned in the previous review round, it seems a missed opportunity to not exploit the fact that the authors have TCR-seq + scRNA-seq in order to gain more functional insights about gdTCR's in MSS CRC.

Reis *et al*. illustrated in Figure 1 a preferential usage of specific γ- and δ-chains in human CRC, a finding we concur with[3]. They further demonstrated, using mouse models, that these chains are linked to either pro- or anti-tumorigenic functions. Although γδ T cell biology differs significantly between humans and mice, our study was not designed to verify the findings of Reis *et al.* We previously attempted to integrate our dataset with that of Reis *et al*. However, the cells in Reis *et al.*'s study were highly stimulated with PMI/ionomycin, which prevented successful integration with our data. We have noted several instances where our results align with their observations in human CRC. However, we did not explore whether particular γ-chains are associated with the pro- or antitumorigenic functions of γδ T cells in human MSS CRC. While this is an intriguing area for future research, addressing it within the context of human MSS CRC poses challenges. In γδ T cells, δ-delta chains can pair with various γ-chains. The availability of antibodies for flow cytometry and cell isolation kits of human γδ T cells are limited. Furthermore, the material for cell subset isolation is sparse, and the overall low infiltration of γδ T cells in MSS CRC makes functionally assessing different cell subsets even more difficult. We appreciate the Reviewer's insightful suggestion but propose this topic for future research endeavors.

- Despite the correction of Figure 1D there are remaining issues with this figure: in many cases the coloring of the outer ring is flipped (in comparison to the coloring of the inner side of the figure).

Figure 1d presents a Circos plot illustrating the pairing of Vδ1-3 with various γ- chains in HC and CRC samples from our TCR sequencing data. The outer ring of the Circos plot displays the individual γ- and δ-chains, with each segment color-coded for easy distinction. The γ-chains occupy one half of the circle, while δ-chains occupy the other, allowing for clear differentiation and comparison. The Reviewer mentions that the coloring of the outer ring appears flipped. However, the segments are arranged by quantity, starting with the most common pairing. This causes the colors to be in a different order. The inner ribbons of the plot depict the pairings between γ- and δ-chains, with each ribbon connecting a specific γ-chain to its corresponding δ-chain. The thickness of these ribbons indicates the frequency of the pairings, with thicker ribbons representing more frequent pairings. This figure was created using the online Circos plot tool (http://circos.ca/circos_online), as described in the methods section of our manuscript. We enlarged the font size to improve readability. After carefully considering the Reviewer's concerns, we found no further issues with this figure. We trust this description clarifies the utility and interpretation of the Circos plot in our study.

Attn claim 2:
- The authors describe the dysfunctional state of gdT cells in MSS CRC is being characterized by low expression of genes associated with cytotoxicity and the inability to proliferate (where the comparison with healthy control tissue is key). However, the data in the revised manuscript to substantiate this is inconsistent. Although the data of the author's own dataset is in line with their interpretation of the dysfunctional state (fig 2c), the revised manuscript also contains analysis of data of a (large) cohort of Pelka et al, which shows a different picture (fig 2f). Here, IFNG, GNLY, and FASL are not downregulated in MSS CRC vs healthy control and hence is not in line with a reduction of cytotoxicity markers in MSS CRC infiltrating gdT cells as compared to healthy control. The latter does align with figure 4, which shows (with immunophenotyping data of the authors themselves) that expression of the key cytotoxic molecules granzyme B and perforin is not reduced in gdT cells in MSS CRC vs healthy control tissue. Furthermore, the TRDV1 cells in the Pelka et al dataset show increased expression of MKI67 in MSS CRC versus healthy control and the author's own data presented in figure 4b shows that gdT cells in MSS CRC frequently express ki-67 (and thus are happily proliferating). In line 185-187 of the manuscript, the authors bring up a potential explanation for inconsistencies of their data with Pelka et al but this explanation if purely hypothetical and proof is missing; At the same time this is not addressing inconsistencies between RNA and protein-based analyses of the authors themselves. A more likely explanation of the data seems to be that the "dysfunctional" state needs a more refined definition. Given that the description of the "dysfunctional" state of gdT cells in MSS CRC is really at the core of this story I think a more refined definition/description is essential. What exactly is this "dysfunctional" state? What is consistent across all datasets and across RNA and protein analyses? It doesn't seem to be a consistent downregulation of cytotoxic programs and the inability to profilerate.

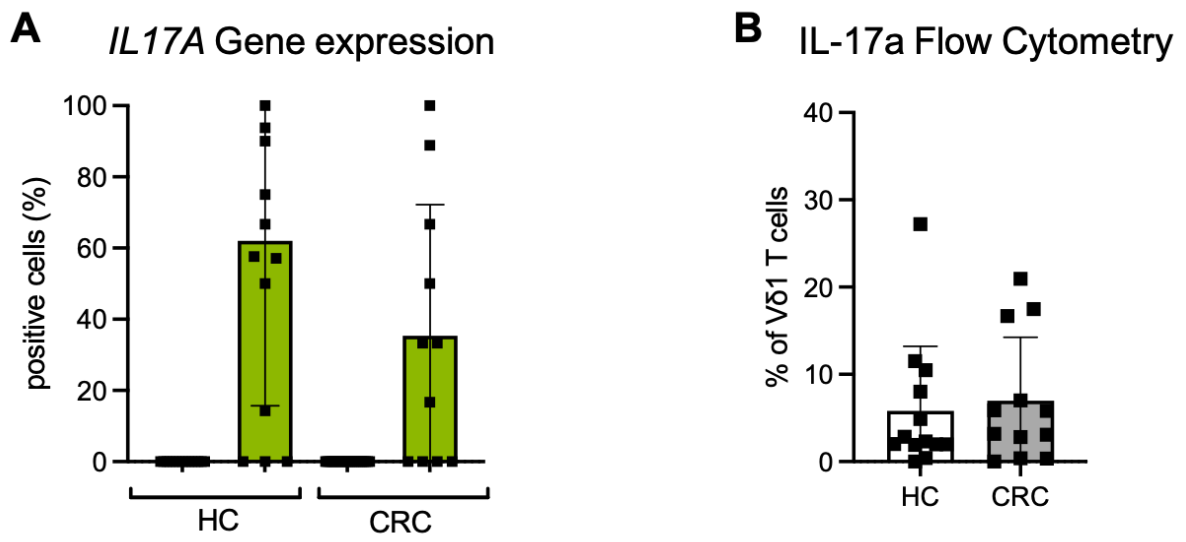
We recognize the Reviewer's observation regarding the inconsistencies between RNA and protein levels identified through single-cell sequencing and flow cytometry. The reasons for this are likely multifactorial including technical variability between the data sets, transcriptional and post-transcriptional regulations and temporal dynamics. These discrepancies can be attributed to technical variability inherent in both methods. Single-cell RNA sequencing measures the transcriptome, capturing mRNA levels, while flow cytometry assesses protein expression on the cell surface or intracellularly. The sensitivity, resolution, and specific technical limitations of each technique can lead to differences in detected levels.
Gene expression is regulated at multiple levels, including transcription, mRNA stability, translation, and post-translational modifications. As a result, mRNA levels measured by single-cell sequencing may not directly correlate with protein levels measured by flow cytometry. For example, mRNA might be transcribed but not translated efficiently into protein, or proteins might be rapidly degraded. We would like to point out that RNA and protein levels can vary over time due to dynamic cellular processes. Single-cell sequencing provides only a snapshot of the transcriptome at a particular moment, whereas protein levels detected by flow cytometry may reflect cumulative expression over time or more immediate cellular states. Previous studies have documented similar discrepancies between mRNA and protein levels, suggesting that these differences are a common and well-recognized phenomenon in biological research[4,5]. Integrating data from both single-cell sequencing and flow cytometry provides a comprehensive view of cellular states and functions. While each method has its limitations, their combination allows for a more holistic understanding of the biological processes under investigation. To further elucidate the observed discrepancies, future studies could employ additional methods such as protein mass spectrometry, single-cell proteomics, or longitudinal studies to capture the dynamic relationship between RNA and protein levels over time.

- Related to the above: based on the RNA-seq data, the authors claim that gdT cells in MSS CRC do not show signs of exhaustion. However, in the next section they show the opposite (Fig 3i): an enrichment of PD-1+CTLA-4+LAG-3 or PD-1+CTLA-4+TIGIT triple positive cells (all exhaustion markers) in MSS CRC vs healthy control. Why are the gdT cells dysfunctional and not exhausted? Given that the distinction of these two phenotypes is central to the paper I believe this should be addressed.

Much of the research on immune cell exhaustion has focused on conventional T cells[6]. However, the concept of exhaustion has also been extended to other leukocytes such as NK cells[7], macrophages[8], γδ T cells[2], B cells[9] and dendritic cells[10]. Exhausted T cells are a unique subset of conventional T cells that emerge in the context of chronic infections and cancer, where they are subjected to prolonged antigen exposure. Unlike functional T cells, exhausted T cells are characterized by a distinct set of marker genes, impaired effector functions, reduced cytokine production, diminished proliferative capacity and increased expression of inhibitory receptors[11]. Interestingly, although γδ T cells in MSS CRC exhibit certain features of exhaustion—such as impaired effector function, reduced cytokine expression, and increased co-expression of checkpoint receptors at the protein level—they do not display the expected exhaustion associated profile at the transcriptional level. Notably, when we analyzed previously published exhaustion marker gene sets and compared γδ T cells from MSS to those from MSI CRC, we observed a clear increase of an exhaustion associated phenotype from HC to MSI. In contrast, γδ T cells from MSS lacked this clear distinction, suggesting a differential profile between γδ T cells from MSS and MSI CRC. While we acknowledge the Reviewer's observation that γδ T cells in MSS exhibit certain characteristics of exhaustion, they do not meet all criteria. While we concede that this remains a topic of debate, we trust the Reviewer appreciates our rationale for not definitively categorizing γδ T cells from MSS as exhausted, opting instead for the term "dysfunctional." We provided an explanation in the discussion section regarding our rationale behind this decision (page 15, ll.: 411-418).

- I feel that some discussion / analysis is needed to put the "dysfunctional" state that is described by the authors into context with known pro-tumorigenic states which are also characterized by lower cytotoxicity (e.g. IL-17 producers). How novel is the discovered "dysfunctional" state?

The study by Reis *et al*. primarily evaluates the functional aspects of γδ T cells in mice. Their analysis of human CRC (Figure 1) does not clearly demonstrate significant overexpression of *IL-17*, despite noting the overexpression of *CD9* and *LGALS3*, which they attributed to an IL-17-producing γδ T cell signature [3]. However, evidence supporting IL-17 production by human γδ T cells in this work remains limited. In our single-cell RNA-seq data we observed no relevant expression of *IL17A* in *TRDV1* positive cells (Reviewer Fig. 1A). In preliminary studies leading up to this paper, we discovered that approximately 10% of all γδ T cells after PMA/ionomycin stimulation are able to produce IL-17A, as determined by flow cytometry (Reviewer Fig. 1B).



Reviewer Figure 1: (A) Percentage of *IL17a* expressing *TRDV1* and *TRDV2* (green) *c*ells in HC and CRC. **(B)** IL-17a expression of Vδ1+ T cells in HC and CRC after PMA/iono.

However, the biological significance of this finding remains unknown to us. Literature also indicates that human γδ T cells are less prone to IL-17 production compared to their murine counterparts[12,13]. Furthermore, given the significant biological differences between human and murine γδ T cells, extrapolating findings from mice to humans are challenging[12]. Notably, the primary γδ T cell subsets in humans do not have direct orthologues in mice. A major obstacle in γδ T cell research is the divergence in TCR genes between humans and mice, since most studies have focused on pro-tumorigenic γδ T cells in mouse models[14-16]. It is also important to note that IL-17 production by γδ T cells has been associated not just with pro- but also antitumor properties, the latter due to the recruitment of neutrophils and αβ T cells[17,18]. In the initial reports on IL-17 producing γδ T cells in CRC a decade ago, it was discovered that IL-17 production was significantly elevated compared to normal tissue[19]. These studies identified Vδ1+ T cells as the primary source of IL-17. Moreover, they demonstrated that a high concentration of Il-17 producing γδ T cells within the tumor was associated with poor patient prognosis, largely due to the recruitment of myeloid suppressor cells. However, in the light of the reports of Reis *et al*., a recent review has questioned the significance of IL-17-producing γδ T cells in human CRC[20]. In contrast to pro-tumorigenic IL-17 producing γδ T cells in human, antitumor properties of γδ T cells are well-established and have been repeatedly confirmed[1,2,21-23]. Therefore, we propose that the antitumor properties of γδ T cells outweigh the evidence and reports on their pro-tumorigenic roles in the human context. We address these considerations in the discussion (page 14, ll.: 386-389).

Attn claim 5:
- In the revised document the authors provide a new figure S4b containing an essential control condition (the Vd1 +/- anti-TIGIT without fibroblasts conditions) for the key functional data supporting the claim that TIGIT blockade works by breaking fibroblast-gdT cell interactions (figure 5h). However, this means that this key data was generated in two separate experiments. I have two major concerns with this:
o The key control (fig S4b) is being tested post-hoc in separate experiments, rather than being tested in the same experiment than the other conditions. As variability of in vitro killing capacity of gdT cells is quite variable between biological replicates, it is unclear if the "true signal" (that is, the enhanced killing with anti-TIGIT in the presence of fibroblasts) was detectable in these experiments. In other words, figure S4b contains the missing negative control but lacks the essential positive control. Therefore, I am still not convinced about this

claim.
o The control experiment in fig S4b was performed with only 4 replicates (instead of 8 replicates as shown in figure 5h). Given the relatively large variability per condition in figure 5h, I believe the control experiment is underpowered and hence the "negative" result does not rule out a biologically relevant effect of TIGIT blockade in the absence of fibroblasts.
I believe these issues need to be resolved with great care before claim 5 can be made.

We appreciate the Reviewer's concerns regarding the absence of the additional requested control in our initial experiments. Originally, we conducted four experiments and subsequently included four more, incorporating the requested control experiments. While we concur that all tested samples should ideally include the necessary controls, it is important to note that we are working with scarce human samples that are often difficult to obtain. Nonetheless, we believe the primary outcome of our study is the partial rescue effect achieved with the TIGIT blocking antibody. We recognize that further research is essential to substantiate this finding and added this to the discussion section (page 16, ll.: 445-446).

- Given the CellChat result, I assume that the fibroblasts in MSS CRC express NECTIN2, but this is not shown. For proper interpretability of the NECTIN2-TIGIT axis and to further substantiate that this axis depends on the interaction of fibroblasts with Vd1 cells in this context, it is essential that the authors show which fraction of fibroblasts express NECTIN2 and if fibroblasts are the key source of NECTIN2 in the tumor micro-environment. Which other stromal cells are expressing NECTIN2? Are tumor cells expressing NECTIN2?

We appreciate the Reviewer for raising this insightful query. Recently, a newly published study highlighted the role of colorectal cancer-associated fibroblasts in suppressing effector T cells through the Nectin 2 pathway indicating that the same pathway is potentially operative not just in γδ T cells (as we have shown) but also conventional T cells[24]. This inhibition was linked to the expression of immune-modulating receptors by fibroblasts. The research work demonstrated that Nectin 2 protein expression was exclusively localized within the tumor microenvironment, particularly co-localizing with a subset of fibroblasts expressing CD90, rather than epithelial tumor cells.
Furthermore, investigations into liver metastases of MSS colorectal cancer revealed predominant *Nectin2* expression in cancer-associated fibroblasts and endothelial cells[25]. Additionally, another study observed varying levels of *Nectin2* expression in ovarian metastases of colorectal cancer[26]. We re-examined our single-cell RNA-sequencing data with respect to *NECTIN2* expression and found that, in CRC, *NECTIN2* was expressed by cells in cluster 17 (Fig. S4a). Within this cluster, we also identified increased interaction of γδ T cells via the TIGIT/NECTIN2 pathway. We updated the manuscript accordingly (page 11, ll.: 305-306).

- The revised gene set analysis (now using TRDV1 + TIGIT to mark Vd1 cells and COL1A2 + IL6 for fibroblasts) is still problematic:
o TIGIT is also highly expressed in cluster 14 (see fig S4c), which consists of abT cells, which makes this marker nonspecific for gdT cells.
o IL6 is just lowly expressed in the fibroblast clusters; at the same time expression is not zero in many other cell types which (in total) are much more abundant than fibroblasts, making it likely that IL6 expression largely originates from non-fibroblasts. Hence, it seems unlikely that IL-6 expression levels reliably reflects (immuno)fibroblast infiltration.
o The correlation is weak with a correlation coefficient of 0.24, which translated in approximately only 5% of the (rank) variance of TRDV1 + TIGIT is explained by COL1A2 + IL6. So, in addition to the points raised above, it is questionable if this association is biologically relevant.

We appreciate the critical feedback from the Reviewer. We acknowledge that designing a signature using bulk sequencing data may not definitively exclude contributions from cell types beyond our specified cluster of interest, given the inherent limitations of such data. However, due to the lack of comprehensive single-cell RNA-sequencing datasets for MSS CRC with associated clinical and outcome data, we determined that leveraging the TCGA-COAD dataset represented the most viable approach for correlating gene expression with patient outcomes.
In attempting to enhance the robustness of our approach, we explored deconvolution pipelines as a computational tool aimed at estimating cell type-specific gene expression profiles from bulk RNA-sequencing data. Nonetheless, it became evident that these methods are primarily effective for analyzing abundant cell subsets. To strengthen the validation of our methodology, we evaluated the expression levels of selected genes (*COL1A2, IL6, TRDV1, TIGIT*) within defined cell clusters (refer to Fig. S4c).
Our analysis revealed distinct patterns: *TRDV1* expression was notably specific to the cluster housing γδ T cells (Cluster 2), while *TIGIT* showed expression in both Cluster 2 and, to a comparable degree, in Cluster 14, identified as regulatory conventional T cells. Thus, we concur with the Reviewer that *TIGIT* expression is not exclusive to γδ T cells. *IL6* exhibited elevated expression in the inflammatory fibroblast cluster (Cluster 17), moderate expression in fibroblast cluster 16, and minimal expression in cluster 20.
We would like to emphasize to the Reviewer that even weak correlations can be meaningful in biological contexts, where outcomes are shaped by numerous factors. While strong correlations are typically easier to interpret, weaker correlations can still shed light on important underlying biological processes and interactions. Thus, we believe that this correlation merits inclusion in our study, as it provides relevant insights and aids in understanding the mechanisms at work.

- Minor: For further generalizability, it would be interesting to see the frequency of NECTIN2 expressing fibroblasts in MSI CRC as well (given the relevance of gdT cells in this disease).

In the Pelka *et al*. data set, we observed a significant overexpression of *NECTIN2* in fibroblasts in both, MSS and MSI (Fig. S4c). We included a graph displaying the expression of *NECTIN2* in MSS and MSI in the supplementary data figures and updated the manuscript accordingly (page 11, ll.: 310-311).

- Minor: The x-axis order of fig 5h seems off (HC, CRC, CRC, CRC, HC, HC; more logical is CRC, CRC, CRC, HC, HC, HC (or the reverse)).

To optimize the figure's legibility, we revised the order of the x-axis as advised by the Reviewer.

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