nature portfolio

Peer Review File

Cancer-specific innate and adaptive immune rewiring drives resistance to PD-1 blockade in classic Hodgkin lymphoma

Corresponding Author: Dr Margaret Shipp

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript, the authors conducted single-cell RNA-seq of peripheral blood samples from Hodgkin lymphoma (cHL), which had different responses to PD-1 blockade treatment. They identify that CD4+ T cells and B cells are enriched in responders and IL-1β+ monocytes are enriched in nonresponders. Overall the findings are interesting and expected from the immunological perspective. As the authors discussed, these features can be captured with a peripheral blood test. My major concern is how the blood test should be established.

First, the authors separated CD3+ from CD3- cells before single-cell RNA-seq. Is such separation necessary to set up a blood test predicting the responses of PD-1 blockade?

Second, protein-level analysis is absent in the current study. The authors should clarify whether the future blood test should be protein-based or RNA-based.

Third, the authors only demonstrated the statistical trends of the predictive values, without providing an evaluation of the real sensitivity, and specificity. Machine learning algorithms should be applied to demonstrate the predictive power of such testing.

Overall, this manuscript provides important data on the predictive value of peripheral blood testing on PD-1 blockade responses. If this point is further enhanced, I am very glad to recommend its publication.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
In this manuscript by Paczkowska et. al, the authors described immune cells features associated with response and lack or response to PD1 inhibition in cHL. By using SC RNA seq and spatial analysis they described circulating and TME features of the immune response to anti-PD1 That include more circulating CD4+ naïve/ TCM and B cells, that showed more diverse TCR and BCR repertoires. Also, IL1B+ monocytes were more abundant in non-responders, features previously described in other tumors. Overall, this a descriptive manuscript with little insight into mechanisms apart from what was extensively reported by this (e.g., Nat Med 2020) and other groups, decreasing the impact of the findings. Most findings remain speculative leading to overstated conclusions. In general, there is not consideration of intra- and interpatient variability in the proportion of a particular cell population that question the strength and statistical validity of the reported associations with outcomes.

Specific comments:
1. Comparison with healthy donors and/or treatment naïve cHL are biased for the apparent lack of matching of sex and age with the PD1 treatment samples. Given that many of these cell populations and transcriptional profiles of cell subsets are affected by sex and, to a greater extend, by age, (and comorbidities, non-cancer treatments, etc.) corrections should be applied when comparing very small datasets.

2. In Figure 1, the authors describe several T cell populations with potential cytotoxic functon(clusters 5, 22, 16, 7b and 23). Given their relevance for anti PD-1 therapy efficacy, authors should provide functional evidence of the cytotoxic capacity of these cells
3. There are certain inconsistencies in several populations in relation with response, for example, cluster 19 T cells (Fig 2g): C1D1 cluster 19 cells are low in PR, high in PD and intermediate in CR. This could suggest that measurements are in fact very variable with minimal differences in percentages very likely falling within the interpatient variability. Moreover, cluster 19 T cells measurements at C1D1 are not consistent with C4D1 measurements. This is also seen for the data shown in Fig. 4e.
4. What is the correlation between measurements in peripheral blood vs. TME, for example, for cluster 19 cells, in the same patients and sampling time? Are cells in circulation and TIME functionally equivalent?
5. In Figure 2I, the definition of “proximal” and “distal” is rather arbitrary. Instead, the authors should compare the distribution of distances separating HRS cells from CD4+ CTLA4+ Ki67+ and from ‘other’ cells.
6. Similarly, in Figure 3 the authors describe multiple CD3- cell populations, including B cells, NK and dendritic cells. Given the apparent association of some of these populations with anti-PD1 therapy response, the authors should provide evidence that circulating populations are equivalent to the ones found in the TIME.
7. The proportion of circulating cells, like B cells shown in Fig. 4d and myeloid in Figs 5b (IL1B+ monocytes) and 6 should be validated by flow-cytometry that is a more reliable methodology to identify cell populations since processing of cells for single cell RNA-seq usually affect the proportion of these cells. In addition, this should be done indicating individual patient contributions to the mean proportions. This is important to establish interpatient variability.
8. Data shown in Fig 5e is irrelevant for this manuscript. There are plenty manuscripts showing the relevance of IL1B monocytes/TAMs in solid tumors in prognosis and response to immunotherapies, including anti-PD1.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
Summary

In this manuscript by Paczkowska et al, the authors leverage PBMC from healthy donors and 20 cHL patients that received anti-PD1 therapy as part of Checkmate 205 plus additional FFPE tissues to evaluate MHC-I independent mechanisms underlying response or resistance to anti-PD1 blockade. The authors find several intriguing cell subsets including cytotoxic CD4+ T cells and B cell features that are associated with response to anti-PD1 and CD4+ Treg and IL1B+ monocytes that were associated with resistance to anti-PD1. The authors also leveraged several external datasets to corroborate their findings with respect to IL1B+ monocytes. Overall, the authors are to be commended for a thorough and clearly presented analysis of their scRNAseq cohort and for including external validation experiments and datasets. The findings are compelling, but several major points outlined below related to the biological interpretation should be addressed. MHC-I independent effects of PD1 blockade are an important topic with broad implications, but there are several important points that would help better explain the underlying mechanisms.

Major Comments

1) The authors should include a table of patient demographics and relevant clinical characteristics for the Checkmate 205 cohort, the cohort of FFPE samples, and the cohort of healthy donors that they utilize for this study.
2) This paper reports several immunologic features from blood that are associated with response or resistance to therapy in cHL, which is to be commended. However, it seems that authors have missed the opportunity to address another key question: how do immunologic states change from prior to and following anti-PD1 therapy? It would be important to understand these changes especially in the context of the MHC-I independent mechanism of action. Indeed, very little is known about how anti-PD1 blockade can influence CD4+ T cell states.
3) What is the underlying biological explanation for the naïve/memory CD4+ T cell diversity being related to response to anti-PD1? Is the effect independent of age? T cell frequencies and diversity are associated with age (https://pubmed.ncbi.nlm.nih.gov/24510963/), so it would be interesting to know if both the naïve CD4+ T cell diversity and B cell diversity are correlated with or independent of age.
4) As the authors are proposing that their IL1B+ monocyte population / state is unique to cancer patients, it is important to contextualize this cell subsets with regards to previously defined monocyte subsets in healthy individuals. Is there any relationship of this subset to previously described monocyte subsets such as those described by Villani et al Science 2017 (https://pubmed.ncbi.nlm.nih.gov/28428369/)?
5) The monocyte cluster 0 signature is associated with response to anti-PD1 in cHL and a second cohort of mUC that they authors use to validate their findings. However, it is unclear if this signature is simply prognostic for good outcome or if it is predictive of response to anti-PD1 or both. If data from other studies are available, it would be ideal to assess whether this signature is associated with better progression free survival or overall survival in patients that did not receive anti-PD1.

Minor Comments

1) Throughout Figure 1, the authors report results as a percent of CD3+ cells which by definition must include CD8+ T cells. However, no CD8+ T cell data are presented. The authors should, at a minimum, report the frequency of conventional and regulatory CD4+ T cells and CD8+ T cells, even if no analysis of CD8+ T cells is undertaken.
2) The authors are to be complimented for making their code publicly available and for having an accession number for their

scRNAseq data. A link to the accession was provided, but there is no reviewer token provided to access the data. The authors should ensure that raw and processed scRNAseq data are available privately during review and publicly upon acceptance.
3) Stylistically, this reviewer would recommend that the authors only include UMAPs in each primary figure for the cell type that is being evaluated. For example, the focus of Figure 3 is NK cells but B cells and monocytes are also introduced in Figure 3A and then shown independently in subsequent figures.
4) Why have the authors decided to use version 3.1.0 of Cellranger? There have been many subsequent releases since v3.1.0 with v8.0.0 available now.
5) Please include a more thorough methodological description of the bioinformatics pipeline for generation of the single-cell TCR and BCR data. Did the authors use different tools for TCR and BCR reconstruction and if so, why?

Recommendation: Major revisions to address key points about the patient cohort, changes in immune signatures from baseline to post treatment, and contextualization of findings with other studies.

(Remarks on code availability)
The code is publicly available and well organized, but a thorough evaluate of the code is not possible because the data are not available.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all my questions. Although the predictive power is less than expected, it is an important paper discussing blood tests for predicting the efficacy of immunotherapies. Now the scRNA-seq and scTCR-seq data are deposited in EGA. The processed gene expression matrix and TCR clone data should be deposited into the NCBI GEO database, which is applied in most scRNA-seq studies and will further increase the impacts.

(Remarks on code availability)

Reviewer #2

(Remarks to the Author)
In this revised manuscript, the authors described shared circulating and TME features of the immune response to PD1 blockade in HD. Main findings were a higher number of circulating CD4 cells and B cells as well as of CD4 TCD and BCR repertoires in responding patients. Conversely, presence of IL1B monocytes were associated with resistance to PD1 blockade, a finding validating previous studies in solid tumors. Most concerns raised by reviewers in the previous submission were satisfactorily addressed in this version.

(Remarks on code availability)

Reviewer #3

(Remarks to the Author)
In this revised manuscript by Paczkowska et al, the authors have built further upon their initially submitted work and have robustly addressed my comments. This manuscript is important for the field of cHL, but also for the broader field of immunotherapy due to the findings related to MHC-I independent mechanisms of tumor control following anti-PD1 therapy and IL1B+ myeloid cells as a potential driver of resistance. One final comment: the authors mention the potential of blood assays throughout the manuscript, and this reviewer assumes they are referring to measuring the frequency of the IL1B+ monocyte population by flow cytometry as a biomarker? However, there would also seemingly be the potential to measure inflammatory cytokines e.g. IL1B, IL6, IL8, etc as a surrogate for this population. This would be much easier to measure in a clinical setting versus a flow-based assay. This is a minor point, but perhaps worth mentioning since the authors findings could potentially translate to blood-based assays for prognosis.

(Remarks on code availability)
The code is appropriately presented and robust.

Reviewer #4

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature

Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

(Remarks on code availability)

REVIEWER COMMENTS

Reviewer #1 (scRNAseq, systems immunology) (Remarks to the Author):

In this manuscript, the authors conducted single-cell RNA-seq of peripheral blood samples from Hodgkin lymphoma (cHL), which had different responses to PD-1 blockade **treatment. They identify that CD4+ T cells and B cells are enriched in responders and IL-**1β+ monocytes are enriched in nonresponders. Overall the findings are interesting and expected from the immunological perspective. As the authors discussed, these features can be captured with a peripheral blood test. My major concern is how the blood test should be established.

We thank the reviewer for his/her interest in our findings and share the enthusiasm regarding translating this information into a peripheral blood test.

First, the authors separated CD3+ from CD3- cells before single-cell RNA-seq. Is such separation necessary to set up a blood test predicting the responses of PD-1 blockade?

We do not think that it will be necessary to initially separate $CD3^+$ and $CD3^-$ cells in a peripheral blood test to predict responses to PD-1 blockade. In the current single-cell RNA sequencing (scRNAseq) analyses, we first separated the $CD3^+$ and $CD3^-$ cells and processed 17,000 cells of each type ($CD3^+$ and $CD3^-$) to maximize our discovery efforts (Methods, lines 502-507). Now that the data have been analyzed and cell types of particular interest have been identified, we will use this information to develop a peripheral blood test that does not require initial separation of $CD3^+$ and $CD3^-$ cells.

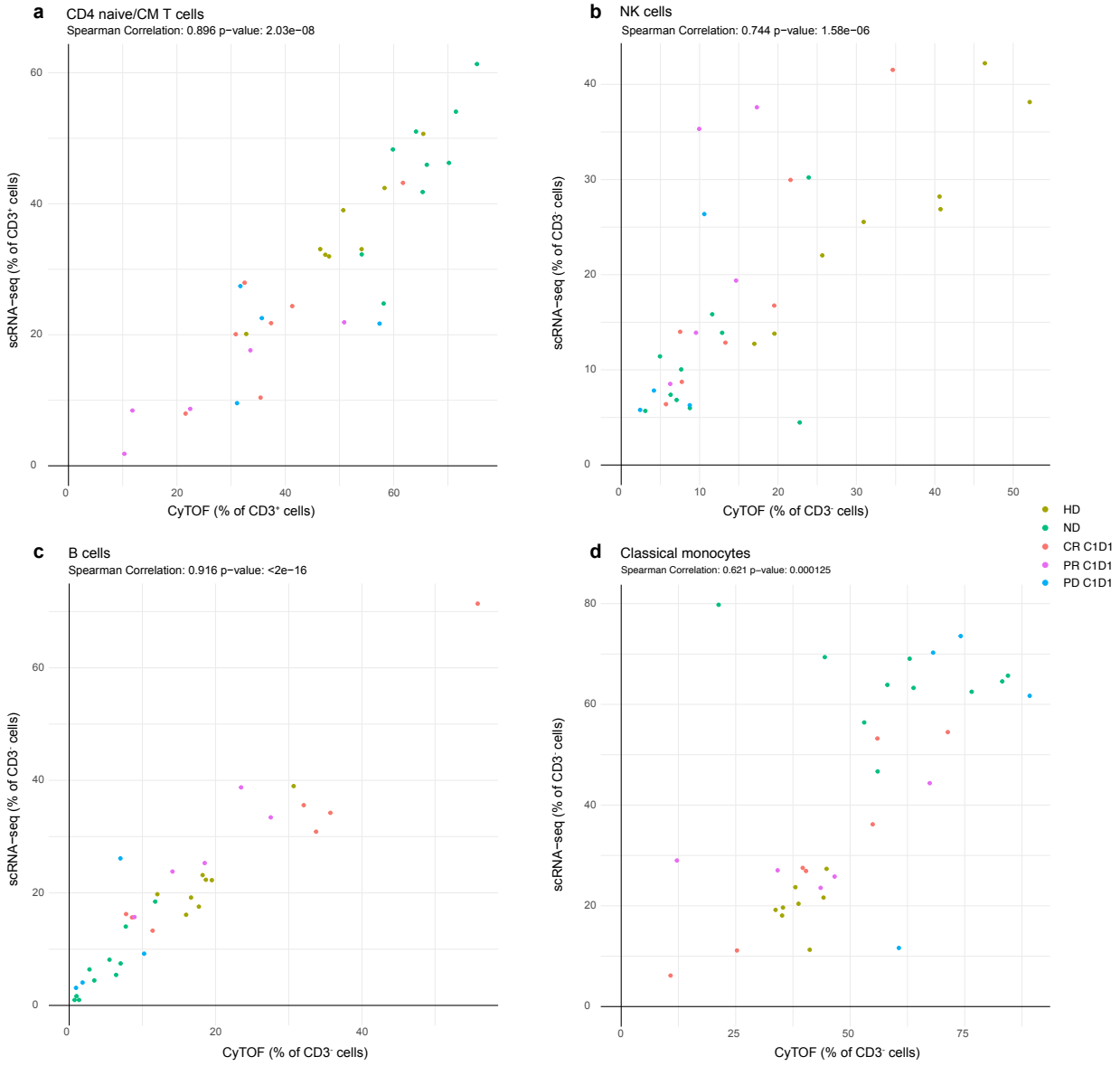
Second, protein-level analysis is absent in the current study.

We do perform protein-based multiplex immunofluorescence analysis to further characterize the newly identified $CD4^+$ Cluster 19 cycling ($CTLA4^+$) cells (Figure 2I and J).

We also utilize multiple RNA-based methods to further characterize the Cluster 0 monocytes/ macrophages in the intact Hodgkin TIME (Figure 5D) and tumor-infiltrating monocytes/ macrophages with Cluster 0 features in a compendium of solid tumors (Figure 5E). We also identify response-related transcriptional features of Cluster 0 monocytes that are negatively associated with response to PD-1 blockade in cHL and a solid tumor type (metastatic urothelial carcinoma, Figure 6A-E).

In the revised manuscript, we also take advantage of the previously performed CyTOF analyses[1] and current scRNAseq analyses (this manuscript) to assess the correlation between protein-based flow cytometric and scRNAseq analysis of identified circulating immune cell types. We have protein-based CyTOF data and scRNAseq analyses on serial aliquots of the same C1D1 peripheral blood mononuclear cell samples from 8 of 13 healthy donors, 10 of 11 patients with newly diagnosed cHL, and 7 of 9 complete responders, 5 of 5 partial responders and 4 of 6 patients with progressive disease from the cohort of patients

with relapsed/refractory Hodgkin lymphoma treated with PD-1 blockade. We enclose an assessment of the correlation between protein-based CyTOF and scRNAseq for all circulating CD4⁺ naïve/central memory (N/CM) T cells, NK cells, B cells, and classical monocytes (which include the Cluster 0 monocytes and additional classical monocytes Cluster 1 and 2) and provide these analyses as new supplemental Figure 5. As shown, **there are excellent correlations between the abundance of these cell types as determined by flow cytometry (CyTOF) and scRNAseq** (CD4⁺ N/CM T cells Spearman correlation 0.896 and p-value 2.03e-08, NK cells Spearman correlation 0.744 and p-value 1.58e-06, B-cell Spearman correlation 0.916 and, p-value<2e-16 and classical monocytes Spearman correlation 0.621 and p-value 0.000125).

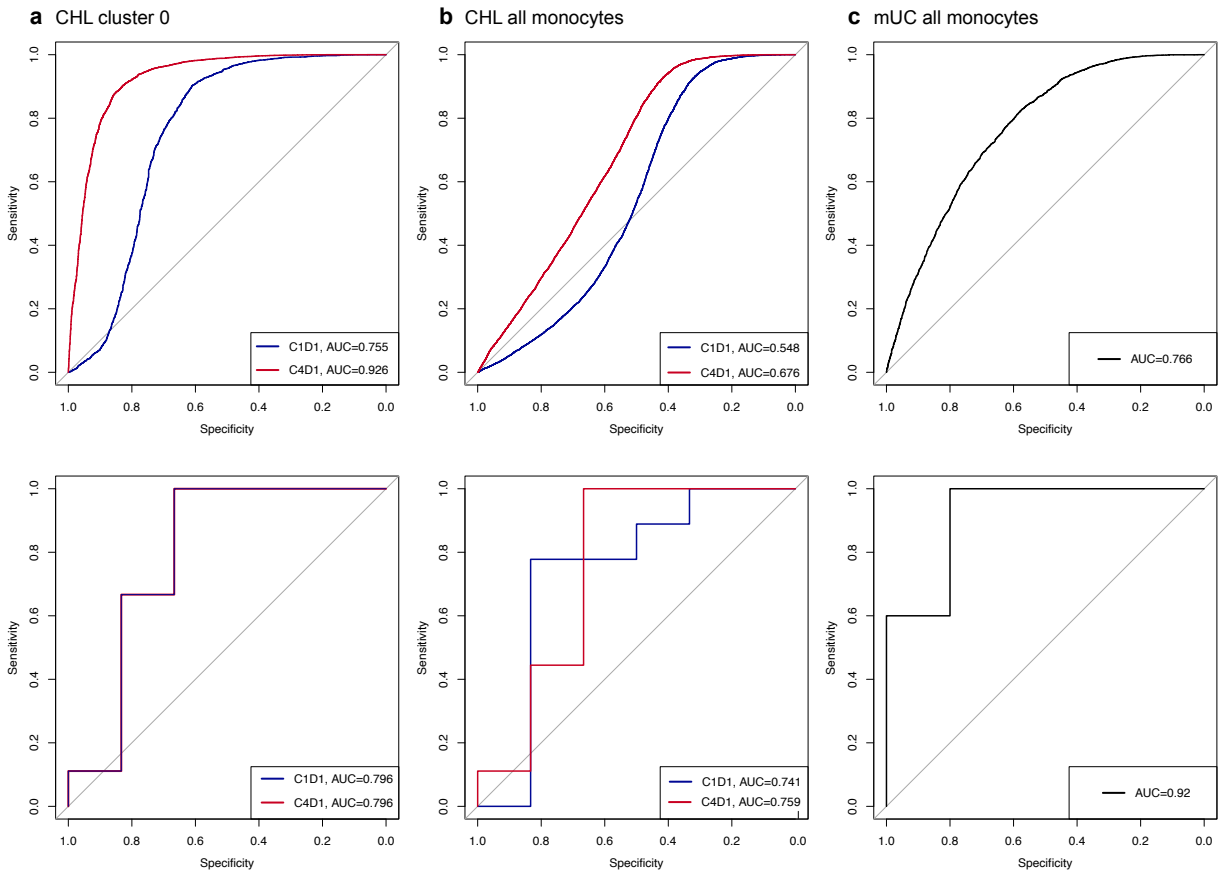


The authors should clarify whether the future blood test should be protein-based or RNA-based.

We are interested in developing a robust protein-based peripheral blood assay. However, we are also exploring the complementary predictive value of additional RNA-based components such as the Cluster 0 monocyte AUCell transcriptional signature. Although this is an active area of investigation for us, we believe that it would be premature to speculate on the exact components of the future blood test.

Third, the authors only demonstrated the statistical trends of the predictive values, without providing an evaluation of the real sensitivity, and specificity. Machine learning algorithms should be applied to demonstrate the predictive power of such testing.

We appreciate the reviewer's comment and now include previously generated ROC curves that assess the sensitivity and specificity of the Cluster 0 AUCell transcriptional signature below and in the new Supplementary Figure 10. These ROC curves align with the AUCell analyses in cHL and an independent solid tumor (metastatic urothelial cell carcinoma [MUC]) in the main manuscript (Figure 6, panels C, D and E).



We previously explored the utility of machine learning algorithms in conjunction with a local expert and colleague, Faisal Mahmood, Brigham and Women's Department of Pathology. However, the size of the current data set limited our implementation of current machine learning algorithms.

Overall, this manuscript provides important data on the predictive value of peripheral blood testing on PD-1 blockade responses. If this point is further enhanced, I am very glad to recommend its publication.

We thank the reviewer for his/her favorable recommendation.

Reviewer #2 (Cancer biomarker) (Remarks to the Author):

In this manuscript by Paczkowska et. al, the authors described immune cells features associated with response and lack or response to PD1 inhibition in cHL. By using SC RNA seq and spatial analysis they described circulating and TME features of the immune response to anti-PD1 That include more circulating CD4+ naïve/ TCM and B cells, that showed more diverse TCR and BCR repertoires. Also, IL1B+ monocytes were more abundant in non-responders, features previously described in other tumors. Overall, this a descriptive manuscript with little insight into mechanisms apart from what was extensively reported by this (e.g., Nat Med 2020) and other groups, decreasing the impact of the findings.

We appreciate the reviewer providing detailed feedback regarding our manuscript.

We believe that the current manuscript provides multiple important insights beyond our earlier more limited protein-based CyTOF analyses[1] as summarized below.

- The current scRNAseq analysis includes 172,274 $CD3^+CD8^-$ and 229,670 $CD3^-$ peripheral blood mononuclear cells in 26 $CD3^+CD8^-$ and 24 $CD3^-$ extensively annotated clusters.
- Newly identified features of $CD3^+CD8^-$ peripheral blood mononuclear cells in the clinically annotated cohorts include **qualitative, in addition to quantitative, differences** in $CD4^+$ N/CM cells in healthy donors, patients with newly diagnosed cHL and those with relapsed/refractory cHL annotated for responses to PD1 blockade. These differences include the disease-associated relative decreased expression of CCR7, SELL, TCF7 and increased expression of S100A4 and CD69 in the $CD4^+$ N/CM compartment, potentially reflecting increased antigen exposure and activation (Figure 2C).
- Additional insights regarding the $CD4^+$ effector populations that would not have been possible without scRNAseq analysis (Figure 2D-H) include: 1) demonstration of shared TCR clonotypes in the $CD4^+$ CTL populations (Figure 2E); 2) identification of disease-associated deficits in circulating $\gamma\delta$VD2 cells; and 3) identification of a new $CD4^+CTLA4^+Ki67^+$ population with regulatory features (Figure 2G and H, Cluster 19) that was also detected in the intact cHL TIME in close proximity to malignant HRS cells (Figure 2I and J).
- Interferon-responsive NK cells like those described in chronic viral infections were identified and associated, for the first time, with cancer (Hodgkin lymphoma).
- The new B-cell scRNAseq data were used to reconstruct individual BCR sequences and directly associate BCR clonal diversity with response to PD-1 blockade in cancer (Hodgkin lymphoma) for the first time.
- Circulating monocyte heterogeneity in cHL was characterized in a way that was not previously possible because of the limited number of monocyte markers in our prior CyTOF analysis.

- ScRNAseq was required to identify the major circulating population of Cluster 0 monocytes in patients with cHL, but not healthy donors, and define the unique transcriptional signature of these cells (Figure 5C).
- After identifying these circulating Cluster 0 monocytes in scRNAseq analysis, cells with similar transcriptional features were detected in the intact cHL TIME, in close proximity to HRS cells (Figure 5D). Cells with similar transcriptional features were also identified in independent solid tumor datasets (Figure 5E and Figure 6E).
- Transcriptional features of circulating Cluster 0 monocytes associated with lack of response to checkpoint blockade were also defined (Figures 6A and B) and translated into an outcome-associated transcriptional signature in cHL and an additional solid tumor dataset (Figure 6C-E).

Most findings remain speculative leading to overstated conclusions. In general, there is not consideration of intra- and interpatient variability in the proportion of a particular cell population that question the strength and statistical validity of the reported associations with outcomes.

We are deeply committed to ensuring the strength and statistical validity of our findings. For this reason, we previously included the data points for each cHL patient (newly diagnosed and relapsed/refractory annotated by best response to PD-1 blockade) and healthy donor in the analyses and performed Benjamini-Hochberg corrections for multiple hypothesis testing as described in detail in Figure legends 2, 4, 5 and the online methods section (lines 662-668).

Specific comments:
1. Comparison with healthy donors and/or treatment naïve cHL are biased for the apparent lack of matching of sex and age with the PD1 treatment samples. Given that many of these cell populations and transcriptional profiles of cell subsets are affected by sex and, to a greater extend, by age, (and comorbidities, non-cancer treatments, etc.) corrections should be applied when comparing very small datasets.

We have now added a detailed demographic table that includes the ages and sexes of our healthy donors and patients with newly diagnosed cHL and relapsed/refractory cHL annotated for best response to PD-1 blockade (new Supplementary Table 1A (PPMCs) and B (FFPE samples). As cHL is largely a disease of young adults and our healthy donors are similar in age (and sex), age- (and/or sex) -related differences do not explain our described findings (new Supplementary Table 1C). For illustration, we enclose a new analysis that includes age as an additional data point in the comparative assessment of TCR and BCR diversity (below and new Extended Data Figure 3C [TCR diversity] and new Extended Data Figure 7E [BCR diversity). In the revised manuscript, we added the following sentences (lines 162-163, 292-294 and 302, respectively).

- “Of note, the identified differences in TCR diversity were not age-related (Extended Data Figure 3C).”

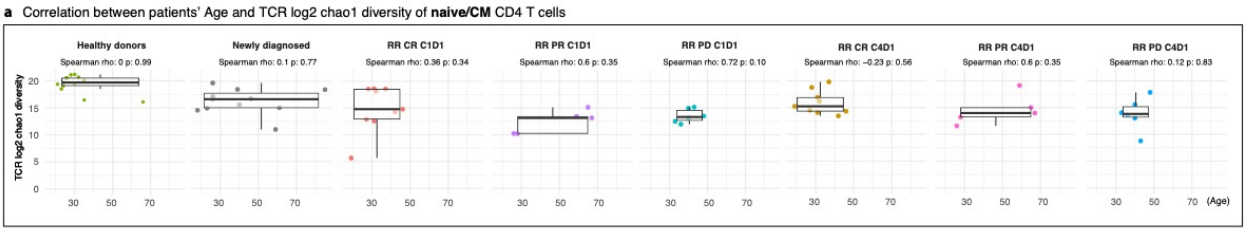
- “In the subset of patients with concurrent scRNAseq and CyTOF analyses there was an excellent correlation between circulating B-cell numbers (supplementary Figure 5C)
- The identified differences in BCR diversity were not age-related (Extended Data Figure 7E).

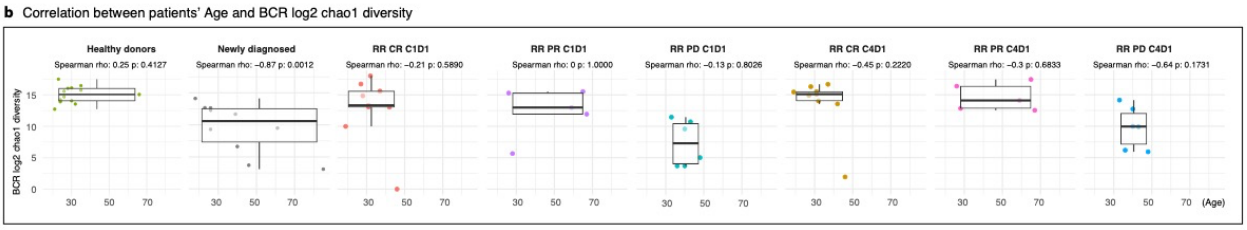
	Healthy donors (n=13)	Newly diagnosed (n=11)	CR (n=9)	PR (n=5)	PD (n=6)
Median Age	28.00	39.00	31.00	59.00	40.00
Mean Age	30.77	40.82	32.56	49.00	40.00
Min Age	21.00	18.00	19.00	26.00	33.00
Max Age	66.00	85.00	46.00	67.00	48.00

Sex - no. (%)	Healthy donors	Newly diagnosed	CR (n=9)	PR (n=5)	PD (n=6)
Male	7 (54%)	7 (64%)	4 (44%)	3 (60%)	2 (33%)
Female	6 (46%)	4 (36%)	5 (56%)	2 (40%)	4 (67%)

Age	Mann-Whitney U test
HD vs ND	0.3081
CR vs PR	0.3162
CR vs PD	0.0673
PR vs PD	0.6473

Age	Kruskal-Wallis test
CR, PR and PD	0.189





As noted above, we previously included appropriate corrections for multiple hypothesis testing as described in Figure legends 2, 4, 5 and the online methods section (lines 662-668).

2. In Figure 1, the authors describe several T cell populations with potential cytotoxic function (clusters 5, 22, 16, 7b and 23). Given their relevance for anti PD-1 therapy efficacy, authors should provide functional evidence of the cytotoxic capacity of these cells

Our manuscript highlights the information that can be obtained by analyzing the circulating immune signature in patients with cHL including identification of T-cell populations with likely cytotoxic function ($CD4^+$ CTLs and $CD4^+$ interferon-responsive CTLs [Clusters 5 and 22], γδVD2 cells [Cluster 7B and 23] and NK/NKT cells [Cluster 16]). Our analyses of circulating $CD4^+$ T-cell populations was driven, in part, by the challenges of obtaining viable cryopreserved single-cell suspensions from pretreatment and on-treatment tumor biopsies in patients with relapsed/refractory cHL on trials of PD-1 blockade. Cryopreserved tumor cell suspensions and additional peripheral mononuclear cell samples from study patients are not available, precluding further functional analyses of the cytotoxic capacity of the identified circulating CD4+ T-cell populations.

3. There are certain inconsistencies in several populations in relation with response, for example, cluster 19 T cells (Fig 2g): C1D1 cluster 19 cells are low in PR, high in PD and intermediate in CR. This could suggest that measurements are in fact very variable with minimal differences in percentages very likely falling within the interpatient variability. Moreover, cluster 19 T cells measurements at C1D1 are not consistent with C4D1 measurements. This is also seen for the data shown in Fig. 4e.

We opted to use the same type of statistical analyses, Cuzick's trend tests, for circulating immune cell subsets annotated by each patient's best response to PD-1 blockade (CR, PR or PD), in all of the analyses of circulating $CD4^+$ T cells and B cells (Figures 2 and 4). For certain immune cell populations, including $CD4^+$ Cluster 19 $CTLA4^+$ $Ki67^+$ cells (Figure 2G) and the B-cell subpopulations (Figure 4E), the response-related differences are most striking between responders (CRs and PRs) versus those with progressive disease (PD). Comparisons of the medians in the box and whiskers plots in each of these panels is consistent with this interpretation. For the reviewer's interest, we have also performed statistical comparisons of CR/PR versus PD in: 1) $CD4^+$ Cluster 19 $CLA4^+$ $Ki67^+$ cells (C1D1, Figure 2G, P=0.01) and 2) B-cell clusters (Cluster 3 Mature-naïve IgK: C1D1 P=0.0163, C4D1 P=0.01; Cluster 6 Mature-naïve IgL: C1D1 P=0.0077, C4D1 P=0.0204; Cluster 9 Memory: C1D1 P=0.01, C4D1 P=0.0059; Cluster 12 Mature-naïve/ memory IgK (V1-39) : C1D1 P=0.0084, C4D1 P=0.01) (from Figure 4E) using a one-sided Mann Whitney test for all comparisons.

A difference between circulating C1D1 and C4D1 Cluster 19 T cells and B cells is the intervening treatment.

4. What is the correlation between measurements in peripheral blood vs. TME, for example, for cluster 19 cells, in the same patients and sampling time? Are cells in circulation and TIME functionally equivalent?

Pretreatment biopsies were not available for the study patients, precluding a direct comparison between circulating and tumor-infiltrating $CD4^+$ Cluster 19 cells in the same patients at the same sampling times. We recognized the importance of characterizing tumor-infiltrating $CD4^+$ T cells with Cluster 19 features and performed multiplex

immunofluorescent analyses of the intact TIME in an additional cohort of patients with newly diagnosed cHL (Figure 2I and J). As indicated, we identified $CD4^+$ T cells with Cluster 19 immunophenotypic features in the intact cHL TIME in close proximity to malignant Hodgkin Reed Sternberg (HRS) cells.

5. In Figure 2I, the definition of "proximal" and "distal" is rather arbitrary. Instead, the authors should compare the distribution of distances separating HRS cells from CD4+ CTLA4+ Ki67+ and from 'other' cells.

Our definition of "proximal" and "distal" is based on our extensive prior multiplex immunofluorescent analyses of the intact cHL TIME and the definitions derived from these earlier publications[2,3]. We respectfully believe that the best way to characterize the proximity of $CD4^+$ CTLA4 $Ki67^+$ cells to malignant HRS cells is to use our previously described analytical framework (within or beyond 75 μm of HRS cells in the intact TIME). This is because the $CD4^+$ $CTLA4^+$ $Ki67^+$ cells are not the only immune cells in near proximity to HRS cells. As described in the *Nature Communications* manuscript introduction (lines 73-75), "In intact cHLs, malignant HRS cells are in close proximity to $CD4^+$ T cells and PD-$L1^+$ macrophages which form a localized immunoprotective niche". We build on our earlier identification of PD-$L1^+$ macrophages in proximity to HRS cells in the intact TIME[2] with the identification and characterization of Cluster 0 monocyte/macrophages in Figure 5.

6. Similarly, in Figure 3 the authors describe multiple CD3- cell populations, including B cells, NK and dendritic cells. Given the apparent association of some of these populations with anti-PD1 therapy response, the authors should provide evidence that circulating populations are equivalent to the ones found in the TIME.

In the $CD3^-$ space, the most striking associations with response to PD1 blockade were for the circulating B-cell populations (Figure 4) and the transcriptionally polarized Cluster 0 monocytes (Figures 5 and 6). We previously noted, in the manuscript discussion, that our findings regarding the associations between peripheral B-cell abundance and high BCR diversity and response to PD-1 blockade "extend prior observations regarding the prognostic significance of tumor-infiltrating B cells in cHL" (lines 414-415)[4,5]. Given these earlier observations[4,5] and the association across B-cell clusters, we did not think that it was necessary to directly evaluate tumor-infiltrating B-cells in the current manuscript.

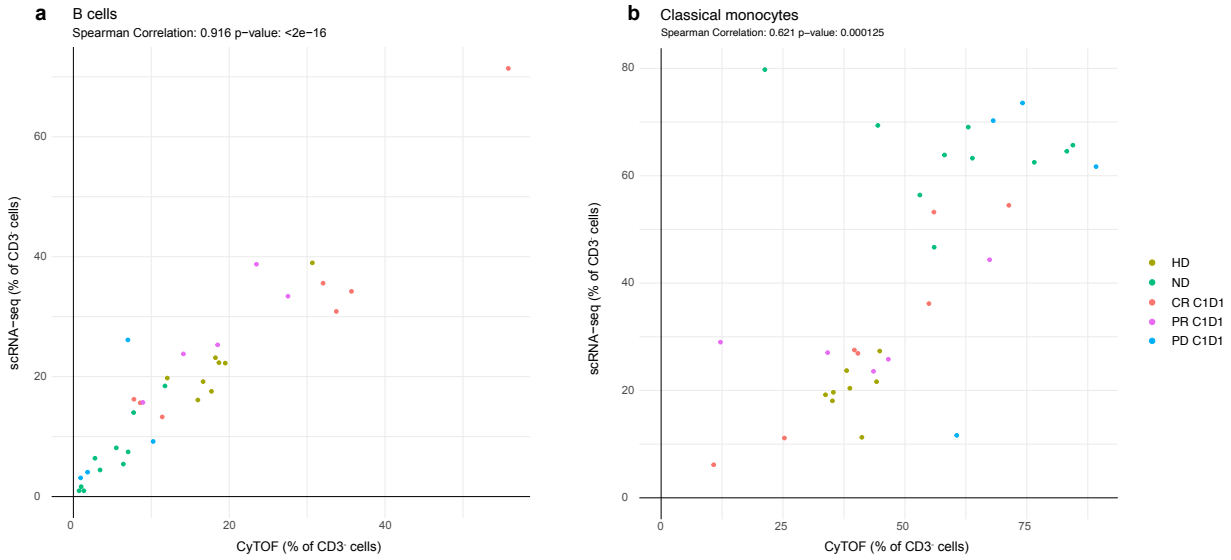
As described above, the likely biological importance of circulating Cluster 0 monocytes in patients with cHL prompted us to determine whether tumor-associated macrophages with Cluster 0 transcriptional features were present in the intact TIME of patients with newly diagnosed cHL. As noted, we used RNAscope analyses to both identify tumor-associated macrophages with Cluster 0 transcriptional features in the intact TIME and localize these cells in near proximity (within 75 μm) to malignant HRS cells (Figure 5D).

7. The proportion of circulating cells, like B cells shown in Fig. 4d and myeloid in Figs 5b (IL1B+ monocytes) and 6 should be validated by flow-cytometry that is a more reliable methodology to identify cell populations since processing of cells for single cell RNA-

seq usually affect the proportion of these cells. In addition, this should be done indicating individual patient contributions to the mean proportions. This is important to establish interpatient variability.

To address this important suggestion, we took advantage of the previously performed CyTOF analyses[1] and current scRNAseq analyses (this manuscript) of circulating B-cells and classical monocytes (which include Cluster 0 cells) in our study patients. We have protein-based CyTOF data and scRNAseq analyses on C1D1 serial cryopreserved peripheral blood mononuclear cell samples from 8 of 13 healthy donors, 10 of 11 patients with newly diagnosed cHL, and 7 of 9 complete responders, 5 of 5 partial responders and 4 of 6 patients with progressive disease from the cohort of patients with relapsed/refractory Hodgkin lymphoma treated with PD-1 blockade. We enclose an assessment of the correlation between protein-based CyTOF and scRNAseq for all circulating B cells and classical monocytes (which include the Cluster 0 monocytes and additional classical monocytes Clusters 1 and 2) and provide these analyses as new supplemental Figure 5. As shown, **there are excellent correlations between the abundance of circulating B cells and classical monocytes as determined by flow cytometry (CyTOF) and scRNAseq** (B-cell Spearman correlation 0.916 and, P<2E-16 and classical monocyte Spearman correlation 0.621 and P value 0.000125). Interpatient variability is addressed by showing the CyTOF and scRNAseq data for each individual patient.

In the revised manuscript, lines 292-294, we added the following sentence: "In the subset of patients with concurrent scRNAseq and CyTOF analysis, there was an excellent correlation between circulating B-cell numbers (Supplementary Figure 5)."



8. Data shown in Fig 5e is irrelevant for this manuscript. There are plenty manuscripts showing the relevance of IL1B monocytes/TAMs in solid tumors in prognosis and response to immunotherapies, including anti-PD1.

We respectfully believe that the analysis of monocyte/macrophages with Cluster 0 transcriptional features in an additional large solid tumor compendium (Figure 5E) underscores the likely importance of cells in the biology of multiple tumors. We note that Reviewer #3 commended our inclusion of this additional dataset.

Reviewer #3 (Checkpoint therapy, T anti-tumor) (Remarks to the Author):

Summary

In this manuscript by Paczkowska et al, the authors leverage PBMC from healthy donors and 20 cHL patients that received anti-PD1 therapy as part of Checkmate 205 plus additional FFPE tissues to evaluate MHC-I independent mechanisms underlying response or resistance to anti-PD1 blockade. The authors find several intriguing cell subsets including cytotoxic CD4+ T cells and B cell features that are associated with response to anti-PD1 and CD4+ Treg and IL1B+ monocytes that were associated with resistance to anti-PD1. The authors also leveraged several external datasets to corroborate their findings with respect to IL1B+ monocytes. Overall, the authors are to be commended for a thorough and clearly presented analysis of their scRNAseq cohort and for including external validation experiments and datasets. The findings are compelling, but several major points outlined below related to the biological interpretation should be addressed. MHC-I independent effects of PD1 blockade are an important topic with broad implications, but there are several important points that would help better explain the underlying mechanisms.

We appreciate Reviewer 3's very thoughtful consideration and detailed review of our manuscript.

Major Comments

1) The authors should include a table of patient demographics and relevant clinical characteristics for the Checkmate 205 cohort, the cohort of FFPE samples, and the cohort of healthy donors that they utilize for this study.

We now include a new Supplementary Table 1 of patient demographics and clinical characteristics for healthy donors, patients with newly diagnosed cHL, patients with relapsed/refractory cHL treated with PD-1 blockade (Checkmate 205 cohort) and the additional cohort of patients with newly diagnosed cHL whose FFPE biopsy specimens were used in the multiplex immunofluorescence and RNAscope analyses.

2) This paper reports several immunologic features from blood that are associated with response or resistance to therapy in cHL, which is to be commended. However, it seems that authors have missed the opportunity to address another key question: how do immunologic states change from prior to and following anti-PD1 therapy? It would be important to understand these changes especially in the context of the MHC-I independent mechanism of action. Indeed, very little is known about how anti-PD1 blockade can influence CD4+ T cell states.

In our manuscript, we found that **baseline differences** in the abundance of circulating $CD4^+$ naïve/central memory cells, circulating B-cells and transcriptionally polarized Cluster 0 monocytes were associated with subsequent responses to PD-1 blockade. We also found that there were significant response-related differences in $CD4^+$ N/CM cell numbers

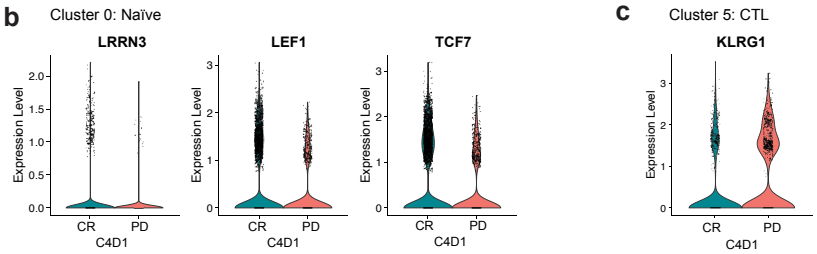
following PD-1 blockade (Extended Data Figure 4). Additionally, we identified response-related changes in the transcriptional signature of circulating Cluster 0 monocytes in patients following PD-1 blockade (shown in Figure 6A and further analyzed in Figure 6B and C). It is likely that there are additional qualitative changes in these circulating immune cell subsets, including maturation of CD4⁺ N/CM T-cells to effectors, when they migrate into the intact cHL TIME.

To address Reviewer 3's excellent suggestion to assess treatment-related CD4⁺ T cell changes in more detail, we now include the following analyses of differentially expressed transcripts in CD4⁺ T-cell clusters: CR vs PD at C1D1, CR vs PD at C4D1, CR C1D1 vs C4D1 and PD C1D1 vs C4D1 (new Supp. Table 5). We have included 2 of the most interesting findings from the CR vs PD at C4D1 analysis in the main manuscript):

1) In the main manuscript, lines 167-170 and new Extended Data Figure 4B, we note that responding patients' (CRs) C4D1 naïve Cluster 0 CD4⁺ T cells had increased expression of *TCF1, LEF1* and *LRNN3*, in comparison to Cluster 0 cells from patients with progressive disease (PD), potentially reflecting greater capacity for self-renewal.

2) In lines 194-198 of the main manuscript and new Extended Data Figure 4C, we describe "… response-related differences in Cluster 5 CD4⁺ CTLs from patients who achieved CRs or had progressive disease (PD) following treatment (C4D1). These included increased expression of the inhibitory Killer cell lectin-like receptor G1 (KLRG1) in patients with progressive disease (Extended Data Figure 4C and Supp. Table 5). In recent preclinical models, KLRG1 expression on CD4⁺ T-effector cells was associated with tumor progression and lack of response to PD-1 blockade[6] suggesting that the receptor may be an attractive and complementary treatment target."

For ease of review, we have included the new Extended Data Figure 4 panels B and C below:



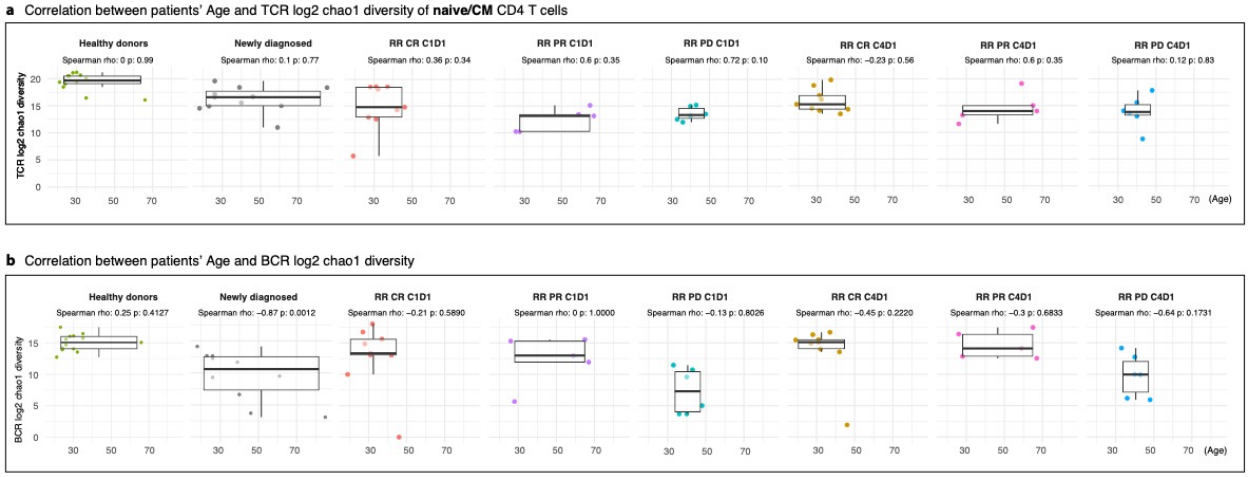
We appreciate Reviewer 3's suggestion to perform these additional analyses which reveal response-related differences in likely self-renewal capacity in CD4⁺ Cluster 0 naïve T cells and a targetable complementary exhaustion pathway in CD4⁺ Cluster 5 CTLs.

3) What is the underlying biological explanation for the naïve/memory CD4+ T cell diversity being related to response to anti-PD1? Is the effect independent of age? T cell frequencies and diversity are associated with age

(https://pubmed.ncbi.nlm.nih.gov/24510963/), so it would be interesting to know if both the naïve CD4+ T cell diversity and B cell diversity are correlated with or independent of age.

We believe that the underlying biological explanation for the association between naïve/memory CD4 T cell diversity and response to PD1 blockade relates to the need for ongoing CD4+ T-cell responses to new tumor neoantigens. In lines 395-398 of the discussion, we state that "In this largely MHC class I-negative tumor, quantitative differences in circulating CD4$^+$ N/CM T-cell abundance and TCR diversity were associated with the response to PD-1 blockade, highlighting the importance of a continued capacity to respond to new tumor neoantigens." In our analysis of the genomic signature of cHL, we found that EBV- cHLs (which constitute the majority of cHLs in patients in the US and Northern Europe), had an extraordinarily high molecular tumor burden and marked genomic instability[7]. In this context, the continued ability to respond effectively to new cHL tumor neoantigens is particularly important. Additionally, it is increasingly recognized that terminally differentiated, epigenetically modified effector T cells are not "reinvigorated" following PD-1 blockade. We note, in lines 400-402 of the manuscript discussion, that our findings build on "recent observations (from others) regarding the importance of *TCF7*$^+$ T-cell progenitors in the response to PD-1 blockade and extend these findings to the circulating CD4$^+$ T-cell compartment".

We performed a detailed demographic analysis and confirmed that disease-associated and response-related differences in CD4$^+$ N/CM T-cell diversity and B-cell diversity were independent of age. We have included these data below to facilitate review and added the information in new Extended Data Figures 3C and 7E.



4) As the authors are proposing that their IL1B+ monocyte population / state is unique to cancer patients, it is important to contextualize this cell subsets with regards to previously defined monocyte subsets in healthy individuals. Is there any relationship of this subset to previously described monocyte subsets such as those described by Villani et al Science 2017 (https://pubmed.ncbi.nlm.nih.gov/28428369/)?

In our analyses, we found that Cluster 0 monocytes were largely restricted to patients with cHL, in contrast to healthy donors. We previously reviewed the scRNAseq analyses of normal circulating monocyte subsets described by Villani et al in *Science* 2017[8]. None of the circulating monocyte subsets described by Villani et al have the Cluster 0-defining transcriptional signature.

5) The monocyte cluster 0 signature is associated with response to anti-PD1 in cHL and a second cohort of mUC that they authors use to validate their findings. However, it is unclear if this signature is simply prognostic for good outcome or if it is predictive of response to anti-PD1 or both. If data from other studies are available, it would be ideal to assess whether this signature is associated with better progression free survival or overall survival in patients that did not receive anti-PD1.

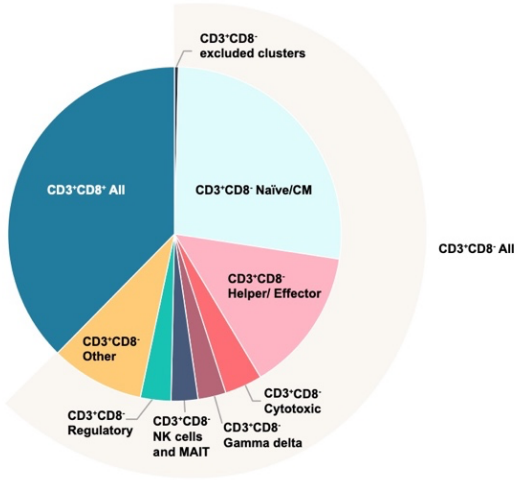
The reviewer raises an important point regarding the potential adverse association of Cluster 0 monocytes with additional treatments as well as PD-1 blockade. We do not yet have peripheral blood mononuclear cell scRNAseq data from patients with cHL who were treated with other therapies to address this important question. However, we are developing the appropriate clinical studies to evaluate this issue in the future.

In the manuscript discussion (lines 426-429), we do note the recent description of IL1β^+ tumor-associated macrophages that resemble Cluster 0 monocytes/macrophages in pancreatic ductal adenocarcinoma[9]. In the referenced paper, the authors find that proinflammatory IL1β^+ tumor-associated macrophages were associated with disease progression in patients who were not treated with PD-1 blockade. For this reason, we state that "the data suggests that IL1β^+ proinflammatory monocytes/macrophages negatively impact outcome in settings beyond PD-1 blockade" (Discussion, lines 432-433).

Minor Comments

1) Throughout Figure 1, the authors report results as a percent of CD3+ cells which by definition must include CD8+ T cells. However, no CD8+ T cell data are presented. The authors should, at a minimum, report the frequency of conventional and regulatory CD4+ T cells and CD8+ T cells, even if no analysis of CD8+ T cells is undertaken.

We have now included the requested analysis of the annotated CD4 T-cell subtypes and additional CD8$^+$ T cells in our scRNAseq dataset as a pie chart below and in Supplementary Figure 1 (new panel B).



2) The authors are to be complimented for making their code publicly available and for having an accession number for their scRNAseq data. A link to the accession was provided, but there is no reviewer token provided to access the data. The authors should ensure that raw and processed scRNAseq data are available privately during review and publicly upon acceptance.

We have contacted EGA regarding the process for assuring the availability of our scRNAseq data to Reviewer 3 during the review process. In brief, EGA will contact the editor who will provide Reviewer 3's email address and issue a token for time-limited review of the scRNAseq data. The EGA correspondence is included below.

"In order for you to give a reviewer access to download your data, we suggest the following steps:
1. Please add the contact from the journal to this email list (ticket #592501) and confirm that you agree to have your data downloaded by an anonymous person.
2. Once you have confirmed that you agree to the first step, I will create a new ticket with the journal contact person to confirm the download details. I will explain to them that I will set up a download account for the reviewer and we will need the reviewer's email for this purpose only (For security reasons). The journal needs to contact the reviewer to make them aware that:
    (i) The reviewer must inform us (EGA) as soon as the data is downloaded.
    (ii) That the data will be deleted once they have completed their review.
3. Once I have the reviewer's email address, I will generate the download account accordingly and pass on the login details (only to the reviewer via a brand-new RT ticket). I'll explain to the reviewer that they must confirm as soon as the data has been downloaded and that it must be deleted once the review is completed.
4. Once the review is complete, I will inform you that the review of the data is complete and that the reviewer has deleted the data."

3) Stylistically, this reviewer would recommend that the authors only include UMAPs in

each primary figure for the cell type that is being evaluated. For example, the focus of Figure 3 is NK cells but B cells and monocytes are also introduced in Figure 3A and then shown independently in subsequent figures.

We respectfully think that it is important to begin the description of the $CD3^-$ space with an overview of the major identified $CD3^-$ cell subsets, NK cells, B cell, and monocytes, as shown in Figure 3A and B. This is followed by the in-depth analyses of the NK cells (Figure 3C, D and E), B cells (Figure 4) and monocytes (Figures 5 and 6).

4) Why have the authors decided to use version 3.1.0 of Cellranger? There have been many subsequent releases since v3.1.0 with v8.0.0 available now.

Version 3.1.0 of Cell Ranger was the version available at the time of our initial scRNAseq analysis.

5) Please include a more thorough methodological description of the bioinformatics pipeline for generation of the single-cell TCR and BCR data. Did the authors use different tools for TCR and BCR reconstruction and if so, why?

In the online methods section (lines 515-518), we indicate that "for $CD3^+$ samples, 2 microliters of post-ctDNA amplification material was also used to prepare scTCRseq libraries. The sequencing libraries for scRNAseq and scTCRseq were normalized ... and sequenced on Illumina NovaSeq S4 300 cycle platform". Because we separately generated scTCRseq libraries, we did not do TCR reconstruction to analyze TCR diversity and clonality. In the online methods section entitled "TCR and B-cell receptor (BCR) analyses" (lines 566-574), we provide detailed descriptions of the TCR and BCR characterization. We indicate that the results from the TCR single-cell V (D) J sequencing were read into Immunarch to calculate TCR diversity. We note that the individual BCR sequences were reconstructed by TRUST4[10] from the single-cell RNAseq BAM files. The TRUST4 output for the reconstructed BCRs was also imported to Immunarch to calculate BCR Chao1 diversity.

Recommendation: Major revisions to address key points about the patient cohort, changes in immune signatures from baseline to post treatment, and contextualization of findings with other studies.

Reviewer #3 (Remarks on code availability):

The code is publicly available and well organized, but a thorough evaluate of the code is not possible because the data are not available.

References

1. Cader, F.Z., *et al*. A peripheral immune signature of responsiveness to PD-1 blockade in patients with classical Hodgkin lymphoma. *Nat Med* **26**, 1468-1479 (2020).

2. Carey, C.D.*, et al*. Topological analysis reveals a PD-L1-associated microenvironmental niche for Reed-Sternberg cells in Hodgkin lymphoma. *Blood* **130**, 2420-2430 (2017).
3. Patel, S.S.*, et al*. The microenvironmental niche in classic Hodgkin lymphoma is enriched for CTLA-4-positive T cells that are PD-1-negative. *Blood* **134**, 2059-2069 (2019).
4. Grund, J.*, et al*. Low B-cell content is associated with a CD73-low tumour microenvironment and unfavourable prognosis in classic Hodgkin lymphoma. *Br J Haematol* **201**, 1097-1102 (2023).
5. Tudor, C.S.*, et al*. B cells in classical Hodgkin lymphoma are important actors rather than bystanders in the local immune reaction. *Hum Pathol* **44**, 2475-2486 (2013).
6. Ager, C.R.*, et al*. KLRG1 marks tumor-infiltrating CD4 T cell subsets associated with tumor progression and immunotherapy response. *Journal for immunotherapy of cancer* **11**(2023).
7. Wienand, K.*, et al*. Genomic analyses of flow-sorted Hodgkin Reed-Sternberg cells reveal complementary mechanisms of immune evasion. *Blood Adv* **3**, 4065-4080 (2019).
8. Villani, A.C.*, et al*. Single-cell RNA-seq reveals new types of human blood dendritic cells, monocytes, and progenitors. *Science* **356**(2017).
9. Caronni, N.*, et al*. IL-1beta(+) macrophages fuel pathogenic inflammation in pancreatic cancer. *Nature* (2023).
10. Song, L.*, et al*. TRUST4: immune repertoire reconstruction from bulk and single-cell RNA-seq data. *Nat Methods* **18**, 627-630 (2021).

REVIEWER COMMENTS

Reviewer #1 (scRNAseq, systems immunology) (Remarks to the Author):

In this manuscript, the authors conducted single-cell RNA-seq of peripheral blood samples from Hodgkin lymphoma (cHL), which had different responses to PD-1 blockade **treatment. They identify that CD4+ T cells and B cells are enriched in responders and IL-**1β+ monocytes are enriched in nonresponders. Overall the findings are interesting and expected from the immunological perspective. As the authors discussed, these features can be captured with a peripheral blood test. My major concern is how the blood test should be established.

We thank the reviewer for his/her interest in our findings and share the enthusiasm regarding translating this information into a peripheral blood test.

First, the authors separated CD3+ from CD3- cells before single-cell RNA-seq. Is such separation necessary to set up a blood test predicting the responses of PD-1 blockade?

We do not think that it will be necessary to initially separate $CD3^+$ and $CD3^-$ cells in a peripheral blood test to predict responses to PD-1 blockade. In the current single-cell RNA sequencing (scRNAseq) analyses, we first separated the $CD3^+$ and $CD3^-$ cells and processed 17,000 cells of each type ($CD3^+$ and $CD3^-$) to maximize our discovery efforts (Methods, lines 525-541). Now that the data have been analyzed and cell types of particular interest have been identified, we will use this information to develop a peripheral blood test that does not require initial separation of $CD3^+$ and $CD3^-$ cells.

Second, protein-level analysis is absent in the current study.

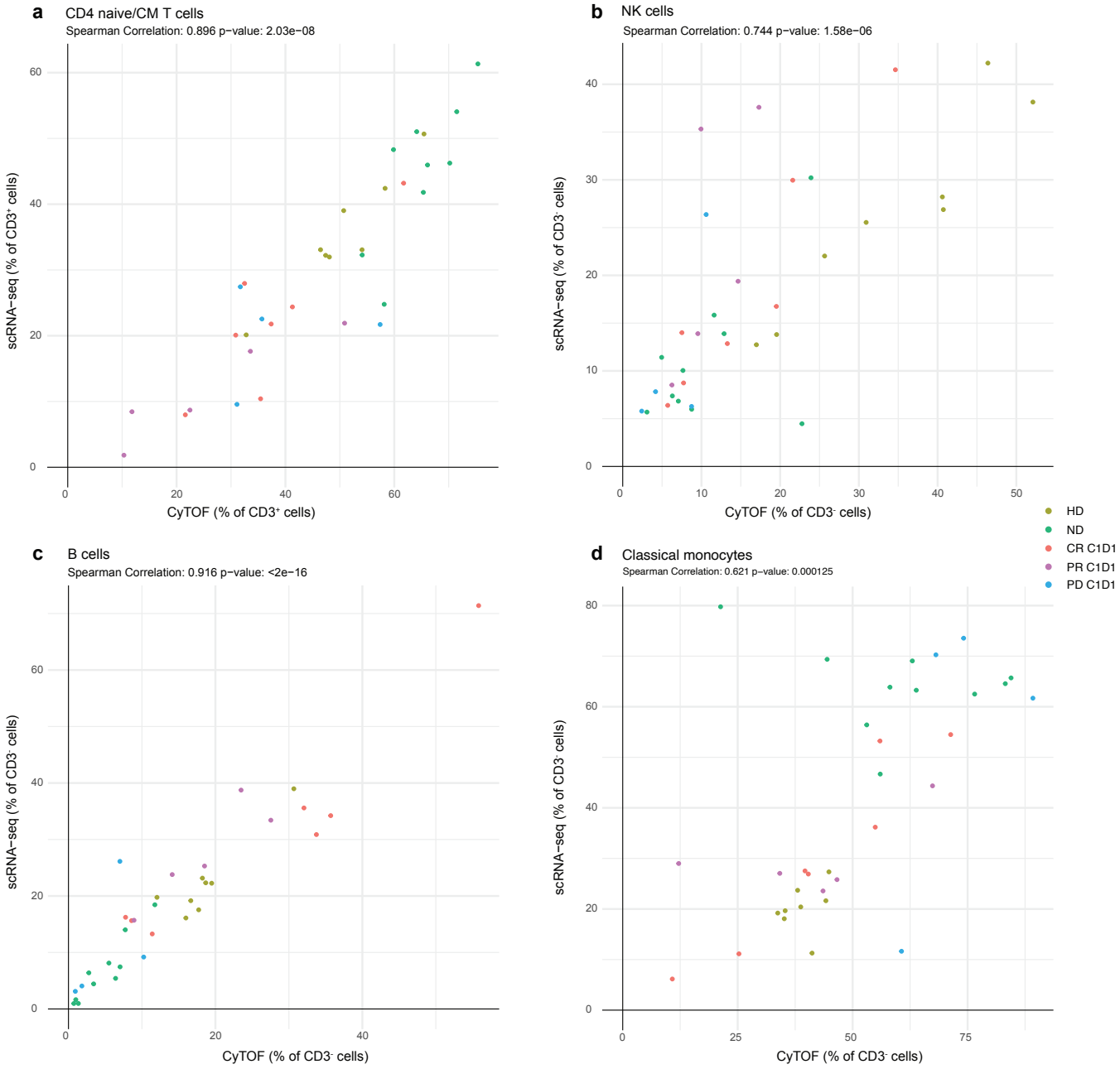
In the original manuscript, we did perform protein-based multiplex immunofluorescence analysis to further characterize the newly identified $CD4^+$ Cluster 19 cycling ($CTLA4^+$) cells (**Figure 2i and j**).

In the original manuscript, we also utilized multiple RNA-based methods to further characterize the Cluster 0 monocytes/ macrophages in the intact classic Hodgkin lymphoma (cHL) TIME (**Figure 5d**) and tumor-infiltrating monocytes/ macrophages with Cluster 0 features in a compendium of solid tumors (**Figure 5e**). We also identified response-related transcriptional features of Cluster 0 monocytes that were negatively associated with response to PD-1 blockade in cHL and a solid tumor type (metastatic urothelial carcinoma, **Figure 6a-e**).

In the revised manuscript, we also take advantage of the previously performed CyTOF analyses (Cader et al. Nat. Med. 26:1468-1479 [2020]) and current scRNAseq analyses (this manuscript) to assess the correlation between protein-based flow cytometric and scRNAseq analyses of identified circulating immune cell types. We have protein-based CyTOF data and scRNAseq analyses on serial aliquots of the same C1D1 peripheral blood mononuclear cell samples from 8 of 13 healthy donors, 10 of 11 patients with newly diagnosed cHL, and 7 of 9 complete responders, 5 of 5 partial responders and 4 of 6 patients with progressive disease from the cohort of patients with relapsed/refractory Hodgkin lymphoma treated with PD-1 blockade.

We enclose an assessment of the correlation between protein-based CyTOF and scRNAseq for all circulating $CD4^+$ naïve/central memory (N/CM) T cells, NK cells, B cells, and classical monocytes (which include the Cluster 0 monocytes and additional classical monocytes Cluster 1 and 2) and provide these analyses as **new Supplementary Figure 6** in the revised manuscript.

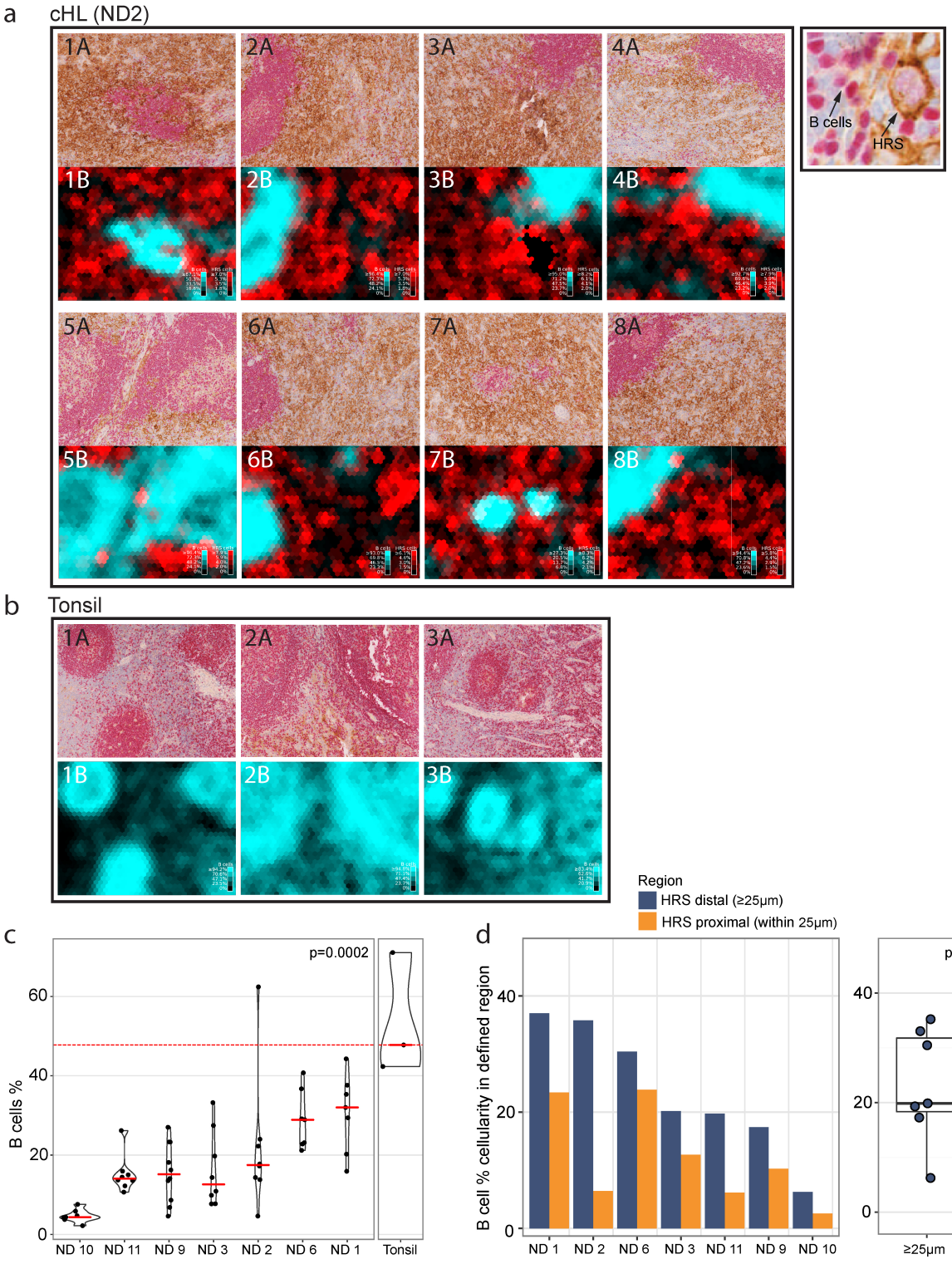
As shown, **there are excellent correlations between the abundance of these cell types as determined by flow cytometry (CyTOF) and scRNAseq** ($CD4^+$ N/CM T cells Spearman correlation 0.896 and p-value 2.03e-08, NK cells Spearman correlation 0.744 and p-value 1.58e-06, B-cell Spearman correlation 0.916 and, p-value<2e-16 and classical monocytes Spearman correlation 0.621 and p-value 0.000125).



Supplementary Figure 6. Correlation between the abundance of (a) CD4 naive/CM T cells, (b) NK cells, (c) B cells, and (d) classical monocytes in serial peripheral blood aliquots from healthy donors and patients with cHL determined by CyTOF (reported in Cader et al Nat. Med. 26:1468-1479 [2020]) and scRNAseq (this study). Protein-based CyTOF data and scRNAseq analyses on serial aliquots of the same C1D1 peripheral blood mononuclear cell samples from 8 of 13 healthy donors, 10 of 11 patients with newly diagnosed cHL, and 7 of 9 complete responders, 5 of 5 partial responders and 4 of 6 patients with progressive disease from the cohort of patients with relapsed/refractory Hodgkin lymphoma treated with PD-1 blockade are compared. Correlations between the abundance of these cell types as determined by flow cytometry (CyTOF) and scRNA seq assessed with Spearman correlations and p values.

In the revised manuscript, we also perform multiplex immunostaining to characterize the abundance and locations (HRS cell-proximal or -distal) of tumor-infiltrating B cells in diagnostic biopsies from the subset of patients with newly diagnosed cHL for whom we have circulating B-cell scRNA sequencing data (**Figure 4**) and available biopsy specimens (7 of 11 study patients). These new results are described in lines 299-308 of the revised manuscript and included in **new Extended Data Figure 7**, both of which are included below for ease of review.

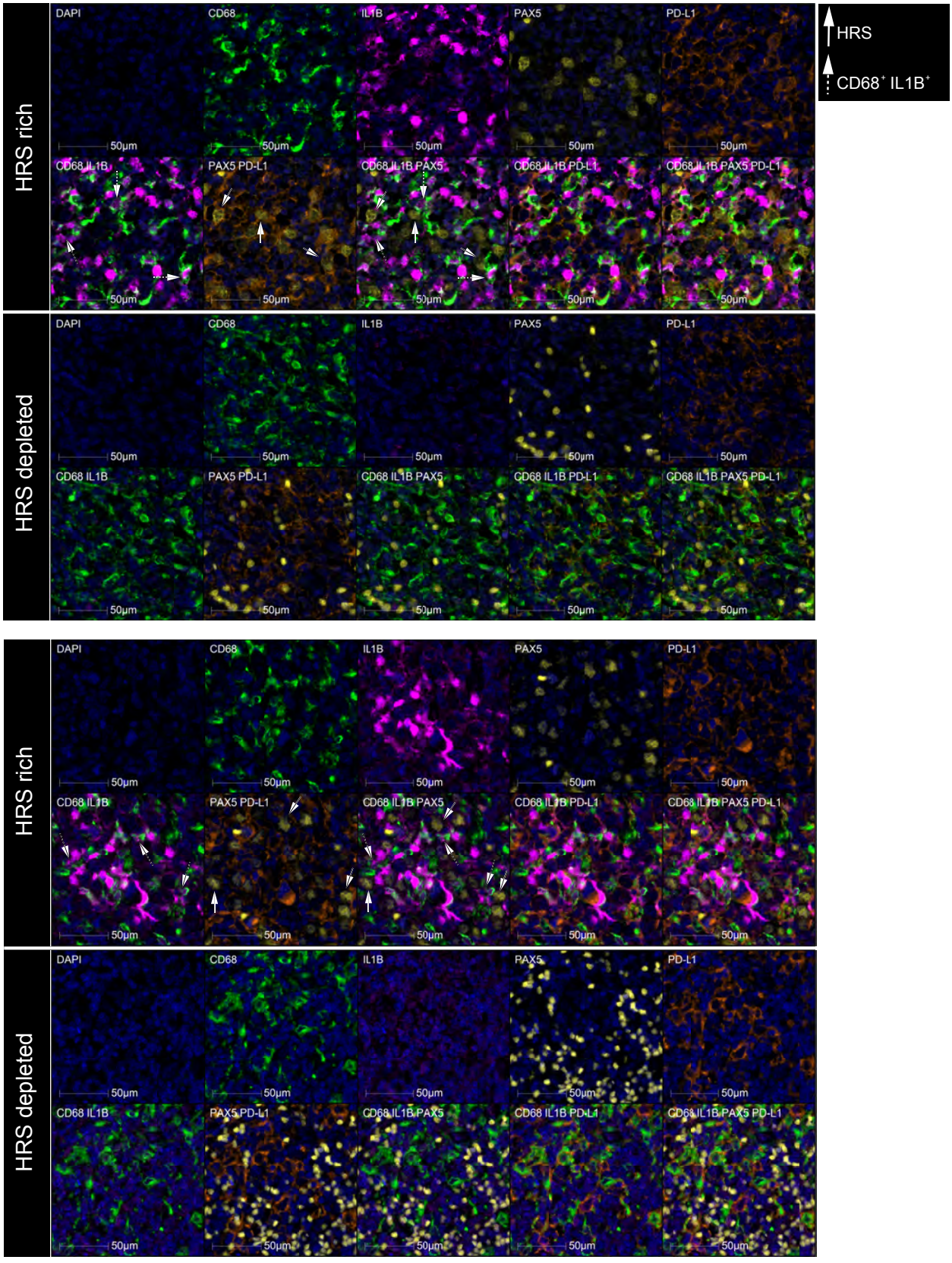
> "We also evaluated B-cell abundance and proximity to HRS cells in available diagnostic biopsies from 7 of the 11 patients with newly diagnosed cHL and scRNA-seq analyses of circulating B-cells (**Figure 4d, Extended Data Figure 7**). As all of the circulating B-cell subsets were significantly less abundant in patients with newly diagnosed cHL than in healthy donors (**Figure 4d**), we utilized a pan B-cell marker, PAX5, and PD-L1 to identify small $PAX5^{bright}$ normal infiltrating B-cells and $PAX5^{dim}$/$PD\text{-}L1^{+}$ HRS cells by dual immunohistochemistry and digital imaging (**Extended Data Figure 7**). Normal B cells were significantly less abundant in all evaluated newly diagnosed cHL biopsies than in control lymphoid tissue (**Extended Data Figure 7c**). Additionally, normal B cells were relatively excluded from the immediate HRS cell ($PAX5^{dim}$/ $PD\text{-}L1^{+}$) niche, defined as within 25 μm of the tumor cells (**Extended Data Figure 7d**)."



Extended Data Figure 7. Immunohistochemical analyses of normal infiltrating B cells in the intact cHL tumor immune microenvironment. a, (Left, top panels, 1A-8A) Eight selected regions of interest (ROIs) of double PAX5 (red) and PD-L1 (brown) IHC images (17x magnification) from a representative newly diagnosed cHL (ND2). (**Right upper panel**). High magnification (57x) image of a larger PAX5^{dim} PD-L1^{+} HRS cell and additional smaller PAX5^{bright} normal B cells. **A (Left, bottom panels, 1B-8B)** Composite heatmaps for PAX5^{dim} PD-L1^{+} HRS cells (red – high density of HRS cells) and PAX5^{bright} B cells (cyan – high density of B cells) overlaid on the original IHC images (from **1A-8A**). The color intensities for each heatmap were determined by calculating the percentage of cells with the given phenotype (HRS: PAX5^{dim} PD-L1^{+}, B cells: PAX5^{bright}) among all cells within a 37µm radius distance of each hexagon's centroid. Each hexagon has a horizontal width (short diagonal) of 25 µm. The color scale for each ROI is shown in the lower right corner. **b, (Top panels, 1A-3A)** Selected ROIs of double PAX5 (red) and PD-L1 (brown) IHC images (17x magnification) from a control tonsil. **b, (Bottom panels, 1B-3B)** Heatmaps for normal B cells (cyan – high density of PAX5^{bright} B cells) overlaid on the original IHC images (from **1A-3A**). **c,** Fraction of PAX5^{bright} B cells in the intact microenvironment of 7 newly diagnosed cHLs and a control tonsil. Each dot is a separately evaluated ROI. P value (p 0.0002) reflects the difference between the median normal B-cell percentage in the control tonsil and the 7 evaluated newly diagnosed cHLs. **d,** B-cell cellularity in defined regions represented as % of normal B cells among all cells proximal to HRS cells (within 25µm, orange) or distal to HRS cells (>25µm, blue), paired for each individual case (**left panel**) and summarized for all cases together (**right panel**).

In the revised manuscript, we also perform multiplex immunostaining to characterize, at a protein level, the numbers and locations (HRS-cell proximal or distal) of tumor-infiltrating Cluster 0 IL1β^+ monocytes/macrophages in biopsies of newly diagnosed cHL. We previously used slides from the same series of diagnostic cHL biopsies to analyze Cluster 0 transcripts by RNAscope in **Figure 5c and d** of the original manuscript. We initially thought that it was preferable to characterize the Cluster 0 signature in tumor-infiltrating monocytes/macrophages at a transcriptional level because the defining Cluster 0 features are soluble chemokines and cytokines that might be less well defined with protein-based assays. In the revised manuscript, we now use a 4-plex antibody panel (CD68, IL1β, PAX5 and PD-L1) and multiplex immunofluorescence (mIF) of the above-mentioned cHL biopsy specimens to document the presence of IL1β^+ monocytes/macrophages in the intact cHL TIME, primarily in HRS-cell proximal regions. The new text describing the additional studies is included in lines 355-361 and **Extended Data Figure 10** and is enclosed below for ease of review.

> "To further validate our findings, we performed multiplex immunofluorescence (mIF) with a 4-plex panel (CD68, IL1β, PAX5 and PD-L1) on the same cHL cases and confirmed the presence of IL1β^+ CD68$^+$ monocytes/ macrophages in the intact TIME at the protein level. IL1β^+ monocytes/macrophages were primarily detected in HRS-rich regions of the intact TIME whereas monocytes/macrophages in HRS-poor regions were largely IL1β^- (**Extended Data Figure 10**). These mIF analyses reinforce our RNAscope results (**Figure 5d**) and highlight the likely significance of tumor-infiltrating IL1β^+ monocytes/macrophages in cHL."



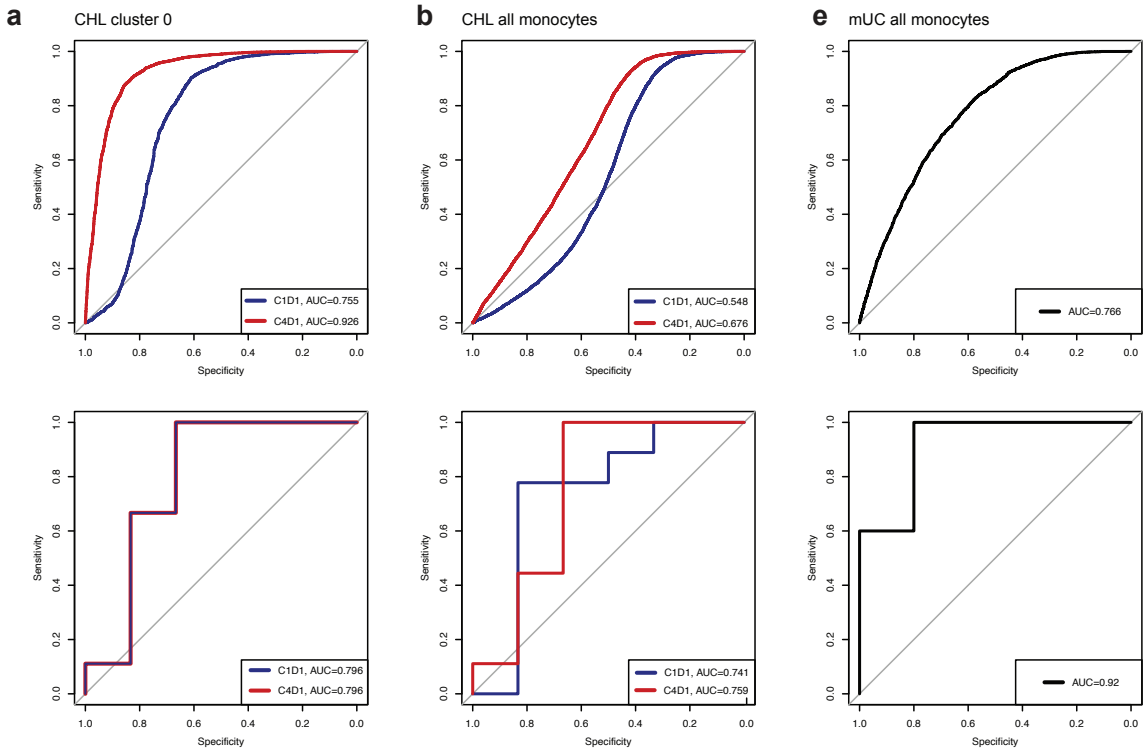
Extended Data Figure 10. Multiplex immunofluorescence imaging of IL1β+ monocyte/macrophages in the intact cHL tumor immune microenvironment. Two representative newly diagnosed cHLs (**a** and **b**) with regions including abundant PAX5^{dim} PD-L1^{+} HRS cells (upper panel) and rare HRS cells (lower panel) are shown. Arrows identify PAX5^{dim} PD-L1^{+} HRS cells ↑ and IL1β^{+} CD68^{+} monocytes/macrophages ↑ which are co-localized in HRS-rich regions (upper panel). Regions with rare PAX5^{dim} PD-L1^{+} HRS cells primarily include smaller PAX5^{bright} normal B cells and IL1β^{-} CD68^{+} macrophages (bottom panel). CD68 green, IL1β magenta, PAX5 yellow and PD-L1 orange.

The authors should clarify whether the future blood test should be protein-based or RNA-based.

We are interested in developing a robust protein-based peripheral blood assay. However, we are also exploring the complementary predictive value of additional RNA-based components such as the Cluster 0 monocyte AUCell transcriptional signature. Although this is an active area of investigation for us, we believe that it would be premature to speculate on the exact components of the future blood test.

Third, the authors only demonstrated the statistical trends of the predictive values, without providing an evaluation of the real sensitivity, and specificity. Machine learning algorithms should be applied to demonstrate the predictive power of such testing.

We appreciate the reviewer's comment and now include previously generated ROC curves that assess the sensitivity and specificity of the Cluster 0 AUCell transcriptional signature below and in the **new Supplementary Figure 9, panels a, b, e**. These ROC curves align with the AUCell analyses in cHL and an independent solid tumor (metastatic urothelial cell carcinoma [MUC]) in the main manuscript (**Figure 6, panels c, d and e**).



Supplementary Figure 9. ROC curves showing the sensitivity and specificity of the Cluster 0 AUCell transcriptional signature in cHL Cluster 0 monocytes at the single-cell (a, upper panel) and patient levels (a, lower panel), cHL all monocytes at the single-cell (b, upper panel) and patient levels (b, lower panel), and metastatic urothelial carcinoma all monocytes at the single-cell (e, upper panel) and patient levels (e, lower panel), (Figure 6c-e, main manuscript).

We previously explored the utility of machine learning algorithms in conjunction with a local expert and colleague, Faisal Mahmood, Brigham and Women's Department of Pathology. However, the size of the current data set limited our implementation of current machine learning algorithms.

Overall, this manuscript provides important data on the predictive value of peripheral blood testing on PD-1 blockade responses. If this point is further enhanced, I am very glad to recommend its publication.

We thank the reviewer for his/her favorable recommendation.

Reviewer #2 (Cancer biomarker) (Remarks to the Author):

In this manuscript by Paczkowska et. al, the authors described immune cells features associated with response and lack or response to PD1 inhibition in cHL. By using SC RNA seq and spatial analysis they described circulating and TME features of the immune response to anti-PD1 That include more circulating CD4+ naïve/ TCM and B cells, that showed more diverse TCR and BCR repertoires. Also, IL1B+ monocytes were more abundant in non-responders, features previously described in other tumors. Overall, this a descriptive manuscript with little insight into mechanisms apart from what was extensively reported by this (e.g., Nat Med 2020) and other groups, decreasing the impact of the findings.

We appreciate the reviewer providing detailed feedback regarding our manuscript.

We believe that the current manuscript provides multiple important insights beyond our earlier more limited protein-based CyTOF analyses (Cader et al. Nat. Med. 26:1468-1479 [2020]) as summarized below.

- The current scRNAseq analysis includes 172,274 $CD3^+CD8^-$ and 229,670 $CD3^-$ peripheral blood mononuclear cells in 26 $CD3^+CD8^-$ and 24 $CD3^-$ extensively annotated clusters.
- Newly identified features of $CD3^+CD8^-$ peripheral blood mononuclear cells in the clinically annotated cohorts include **qualitative, in addition to quantitative, differences** in $CD4^+$ N/CM cells in healthy donors, patients with newly diagnosed cHL and those with relapsed/refractory cHL annotated for responses to PD1 blockade. These differences include the disease-associated relative decreased expression of CCR7, SELL, TCF7 and increased expression of S100A4 and CD69 in the $CD4^+$ N/CM compartment, potentially reflecting increased antigen exposure and activation (**Figure 2c**).
- Additional insights regarding the $CD4^+$ effector populations that would not have been possible without scRNAseq analysis (**Figure 2d-h**) include: 1) demonstration of shared TCR clonotypes in the $CD4^+$ CTL populations (**Figure 2e**); 2) identification of disease-associated deficits in circulating gdVD2 cells; and 3) identification of a new $CD4^+CTLA4^+Ki67^+$ population with regulatory features (**Figure 2g and h**, Cluster 19) that was also detected in the intact cHL TIME in close proximity to malignant HRS cells (**Figure 2i and j**).
- Interferon-responsive NK cells like those described in chronic viral infections were identified and associated, for the first time, with cancer (Hodgkin lymphoma).
- The new B-cell scRNAseq data were used to reconstruct individual BCR sequences and directly associate BCR clonal diversity with response to PD-1 blockade in cancer (Hodgkin lymphoma) for the first time.
- Circulating monocyte heterogeneity in cHL was characterized in a way that was not previously possible because of the limited number of monocyte markers in our prior CyTOF analysis.
- ScRNAseq was required to identify the major circulating population of Cluster 0 monocytes in patients with cHL, but not healthy donors, and define the unique transcriptional signature of these cells (**Figure 5c**).
- After identifying these circulating Cluster 0 monocytes in scRNAseq analysis, cells with similar transcriptional features were detected in the intact cHL TIME, in close proximity to HRS cells (**Figure 5d**). Cells with similar transcriptional features were also identified in independent solid tumor datasets (**Figure 5e and Figure 6e**).
- Transcriptional features of circulating Cluster 0 monocytes associated with lack of response to checkpoint blockade were also defined (**Figures 6a and b**) and translated

into an outcome-associated transcriptional signature in cHL and an additional solid tumor dataset (**Figure 6c-e**).

Most findings remain speculative leading to overstated conclusions. In general, there is not consideration of intra- and interpatient variability in the proportion of a particular cell population that question the strength and statistical validity of the reported associations with outcomes.

We are deeply committed to ensuring the strength and statistical validity of our findings. For this reason, we previously included the data points for each cHL patient (newly diagnosed and relapsed/refractory annotated by best response to PD-1 blockade) and healthy donor in the analyses and performed Benjamini-Hochberg corrections for multiple hypothesis testing as described in detail in **Figure legends 2, 4, 5** and the online methods section (lines 706-712).

Specific comments:
1. Comparison with healthy donors and/or treatment naïve cHL are biased for the apparent lack of matching of sex and age with the PD1 treatment samples. Given that many of these cell populations and transcriptional profiles of cell subsets are affected by sex and, to a greater extend, by age, (and comorbidities, non-cancer treatments, etc.) corrections should be applied when comparing very small datasets.

We have now added a detailed demographic table that includes the ages and sexes of our healthy donors and patients with newly diagnosed cHL and relapsed/refractory cHL annotated for best response to PD-1 blockade (**new Supplementary Table 1a** (PPMCs) **and b** (FFPE samples). As cHL is largely a disease of young adults and our healthy donors are similar in age (and sex), age- (and/or sex) -related differences do not explain our described findings (**new Supplementary Table 1c**). For illustration, we enclose a new analysis that includes age as an additional data point in the comparative assessment of TCR and BCR diversity (below and **new Extended Data Figure 2c** [TCR diversity] and **new Extended Data Figure 6e** [BCR diversity). In the revised manuscript, we added the following sentences (lines 163-164 and 316-317, respectively).

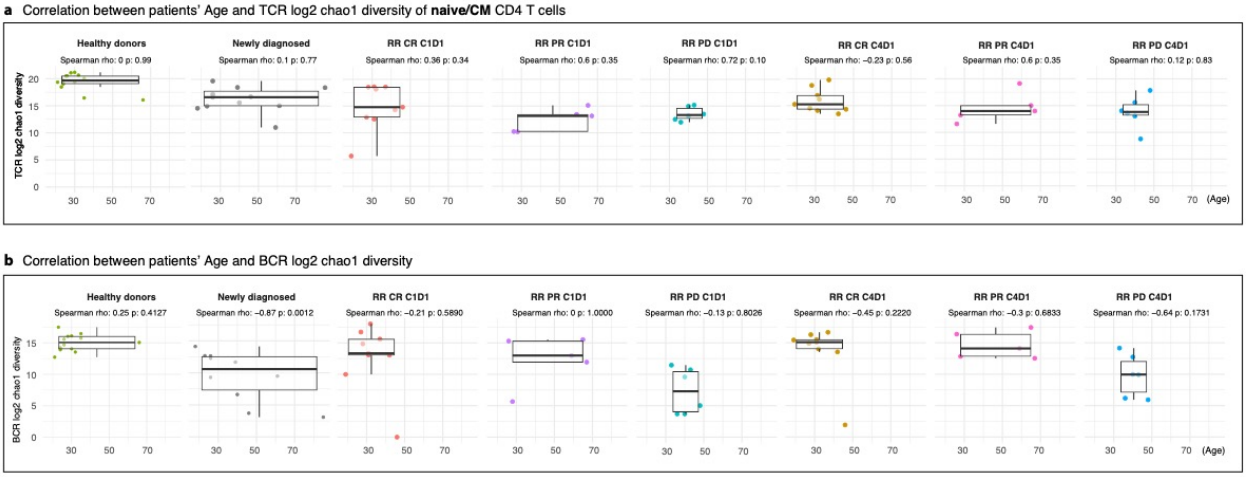
- "Of note, the identified differences in TCR diversity were not age-related (**Extended Data Figure 2c).**"
- "The identified differences in BCR diversity were not age-related (**Extended Data Figure 6e**)."

	Healthy donors (n=13)	Newly diagnosed (n=11)	CR (n=9)	PR (n=5)	PD (n=6)
Median Age	28.00	39.00	31.00	59.00	40.00
Mean Age	30.77	40.82	32.56	49.00	40.00
Min Age	21.00	18.00	19.00	26.00	33.00
Max Age	66.00	85.00	46.00	67.00	48.00

Sex - no. (%)	Healthy donors	Newly diagnosed	CR (n=9)	PR (n=5)	PD (n=6)
Male	7 (54%)	7 (64%)	4 (44%)	3 (60%)	2 (33%)
Female	6 (46%)	4 (36%)	5 (56%)	2 (40%)	4 (67%)

Age	Mann-Whitney U test
HD vs ND	0.3081
CR vs PR	0.3162
CR vs PD	0.0673
PR vs PD	0.6473

Age	Kruskal-Wallis test
CR, PR and PD	0.189



As noted above, we previously included appropriate corrections for multiple hypothesis testing as described in **Figure legends 2, 4, 5** and the online methods section (lines 706-712).

2. In Figure 1, the authors describe several T cell populations with potential cytotoxic function (clusters 5, 22, 16, 7b and 23). Given their relevance for anti PD-1 therapy efficacy, authors should provide functional evidence of the cytotoxic capacity of these cells

Our manuscript highlights the information that can be obtained by analyzing the circulating immune signature in patients with cHL including identification of T-cell populations with likely cytotoxic function ($CD4^+$ CTLs and $CD4^+$ interferon-responsive CTLs [Clusters 5 and 22], gdVD2 cells [Cluster 7B and 23] and NK/NKT cells [Cluster 16]). Our analyses of circulating $CD4^+$ T-cell populations was driven, in part, by the challenges of obtaining viable cryopreserved single-cell suspensions from pretreatment and on-treatment tumor biopsies in patients with relapsed/refractory cHL on trials of PD-1 blockade. Cryopreserved tumor cell suspensions and additional peripheral mononuclear cell samples from study patients are not available, precluding further functional analyses of the cytotoxic capacity of the identified circulating CD4+ T-cell populations.

3. There are certain inconsistencies in several populations in relation with response, for example, cluster 19 T cells (Fig 2g): C1D1 cluster 19 cells are low in PR, high in PD and intermediate in CR. This could suggest that measurements are in fact very variable with minimal differences in percentages very likely falling within the interpatient variability. Moreover, cluster 19 T cells measurements at C1D1 are not consistent with C4D1 measurements. This is also seen for the data shown in Fig. 4e.

We opted to use the same type of statistical analyses, Cuzick's trend tests, for circulating immune cell subsets annotated by each patient's best response to PD-1 blockade (CR, PR or PD), in all of the analyses of circulating $CD4^+$ T cells and B cells (**Figures 2 and 4**). For certain immune cell populations, including $CD4^+$ Cluster 19 $CTLA4^+$ $Ki67^+$ cells (**Figure 2g**) and the B-cell subpopulations (**Figure 4e**), the response-related differences are most striking between responders (CRs and PRs) versus those with progressive disease (PD). Comparisons of the medians in the box and whiskers plots in each of these panels is consistent with this interpretation. For the reviewer's interest, we have also performed statistical comparisons of CR/PR versus PD in: 1) $CD4^+$ Cluster 19 $CLA4^+$ $Ki67^+$ cells (C1D1, **Figure 2g**, P=0.01) and 2) B-cell clusters (Cluster 3 Mature-naïve IgK: C1D1 P=0.0163, C4D1 P=0.01; Cluster 6 Mature-naïve IgL: C1D1 P=0.0077,

C4D1 P=0.0204; Cluster 9 Memory: C1D1 P=0.01, C4D1 P=0.0059; Cluster 12 Mature-naïve/ memory IgK (V1-39) : C1D1 P=0.0084, C4D1 P=0.01) (from **Figure 4e**) using a one-sided Mann Whitney test for all comparisons.

A difference between circulating C1D1 and C4D1 Cluster 19 T cells and B cells is the intervening treatment.

4. What is the correlation between measurements in peripheral blood vs. TME, for example, for cluster 19 cells, in the same patients and sampling time? Are cells in circulation and TIME functionally equivalent?

Pretreatment biopsies were not available for the study patients, precluding a direct comparison *between* circulating and tumor-infiltrating $CD4^+$ Cluster 19 cells in the same patients at the same sampling times. We recognized the importance of characterizing tumor-infiltrating $CD4^+$ T cells with Cluster 19 features and performed multiplex immunofluorescent analyses of the intact TIME in an additional cohort of patients with newly diagnosed cHL (**Figure 2i and j**). As indicated, we identified $CD4^+$ T cells with Cluster 19 immunophenotypic features in the intact cHL TIME in close proximity to malignant Hodgkin Reed Sternberg (HRS) cells.

5. In Figure 2I, the definition of “proximal” and “distal” is rather arbitrary. Instead, the authors should compare the distribution of distances separating HRS cells from CD4+ CTLA4+ Ki67+ and from ‘other’ cells.

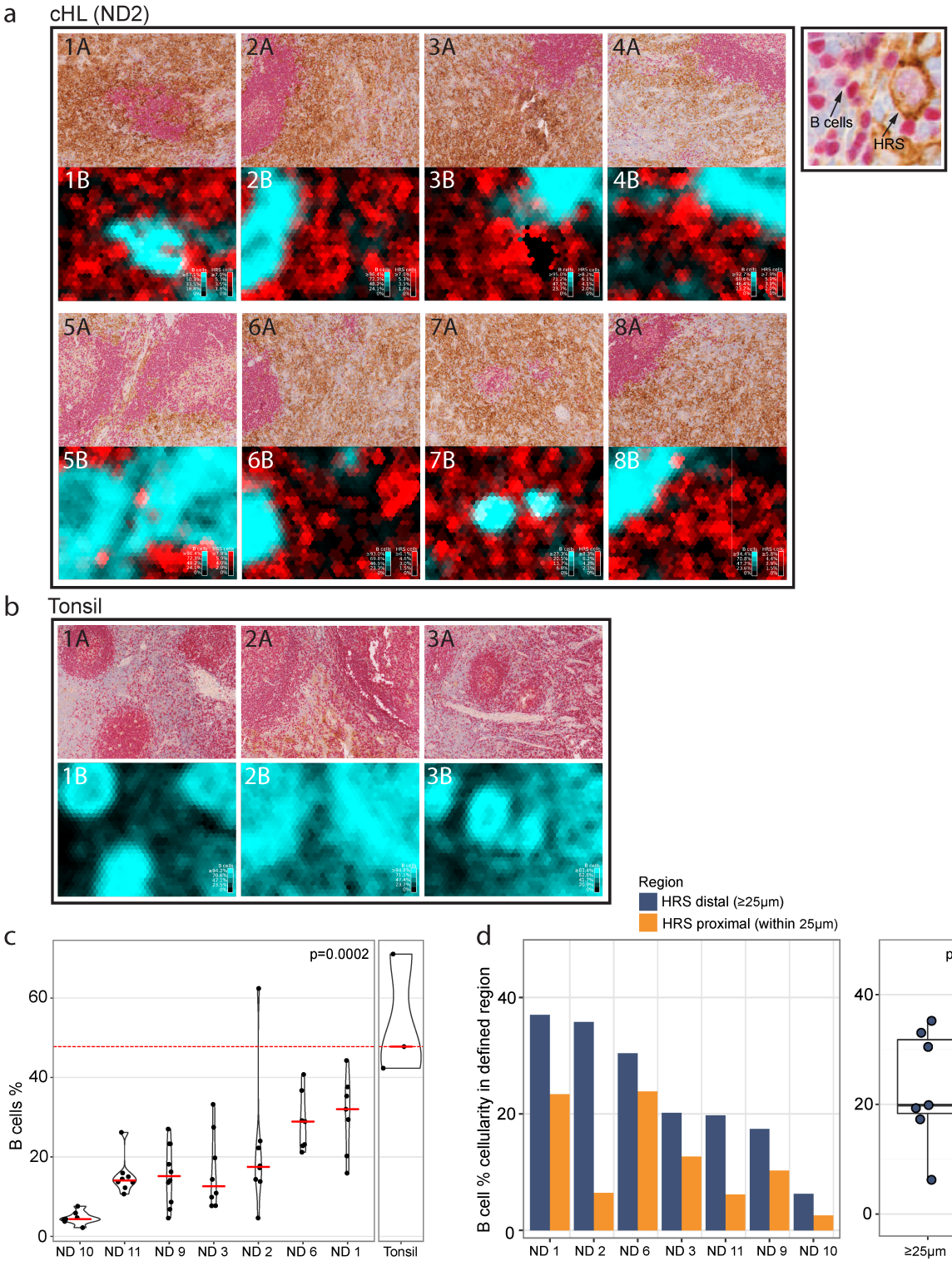
Our definition of “proximal” and “distal” is based on our extensive prior multiplex immunofluorescent analyses of the intact cHL TIME and the definitions derived from these earlier publications (Carey et al. Blood 130:2420-2430 [2017] and Patel et al. Blood 134:2059-2069 [2019]). We respectfully believe that the best way to characterize the proximity of $CD4^+$ CTLA4 $Ki67^+$ cells to malignant HRS cells is to use our previously described analytical framework (within or beyond 75 µm of HRS cells in the intact TIME). This is because the $CD4^+$ $CTLA4^+$ $Ki67^+$ cells are not the only immune cells in near proximity to HRS cells. As described in the *Nature Communications* manuscript introduction (lines 73-75), “In intact cHLs, the malignant HRS cells are in close proximity to PD-1^+ $CD4^+$ T cells and PD-$L1^+$ macrophages which form a localized immunoprotective niche (Carey et al. Blood 130:2420-2430 [2017]).” We build on our earlier identification of PD-$L1^+$ macrophages in proximity to HRS cells in the intact TIME(Carey et al. Blood 130:2420-2430 [2017]) with the identification and characterization of Cluster 0 monocyte/macrophages in **Figure 5.**

6. Similarly, in Figure 3 the authors describe multiple CD3- cell populations, including B cells, NK and dendritic cells. Given the apparent association of some of these populations with anti-PD1 therapy response, the authors should provide evidence that circulating populations are equivalent to the ones found in the TIME.

In the $CD3^-$ space, the most striking associations with response to PD1 blockade were for the circulating B-cell populations (**Figure 4**) and the transcriptionally polarized Cluster 0 monocytes (**Figures 5 and 6**). We previously noted, in the manuscript discussion, that our findings regarding the associations between peripheral B-cell abundance and high BCR diversity and response to PD-1 blockade “extend prior observations regarding the prognostic significance of tumor-infiltrating B cells in cHL” (lines 436-437) (Grund, et al. Br J Haematol. 201:1097-1102 [2023] and Tudor et al. Hum. Pathol. 44:2475-2486 [2013]).

In the revised manuscript, we also performed multiplex immunostaining to characterize the abundance and locations (HRS cell-proximal or -distal) of tumor-infiltrating B cells in diagnostic biopsies from the subset of patients with newly diagnosed cHL for whom we have circulating B-cell scRNA sequencing data (**Figure 4**) and available biopsy specimens (7 of 11 study patients). These new results are described in lines 299-308 of the revised manuscript and included in **new Extended Data Figure 7**, both of which are included below for ease of review.

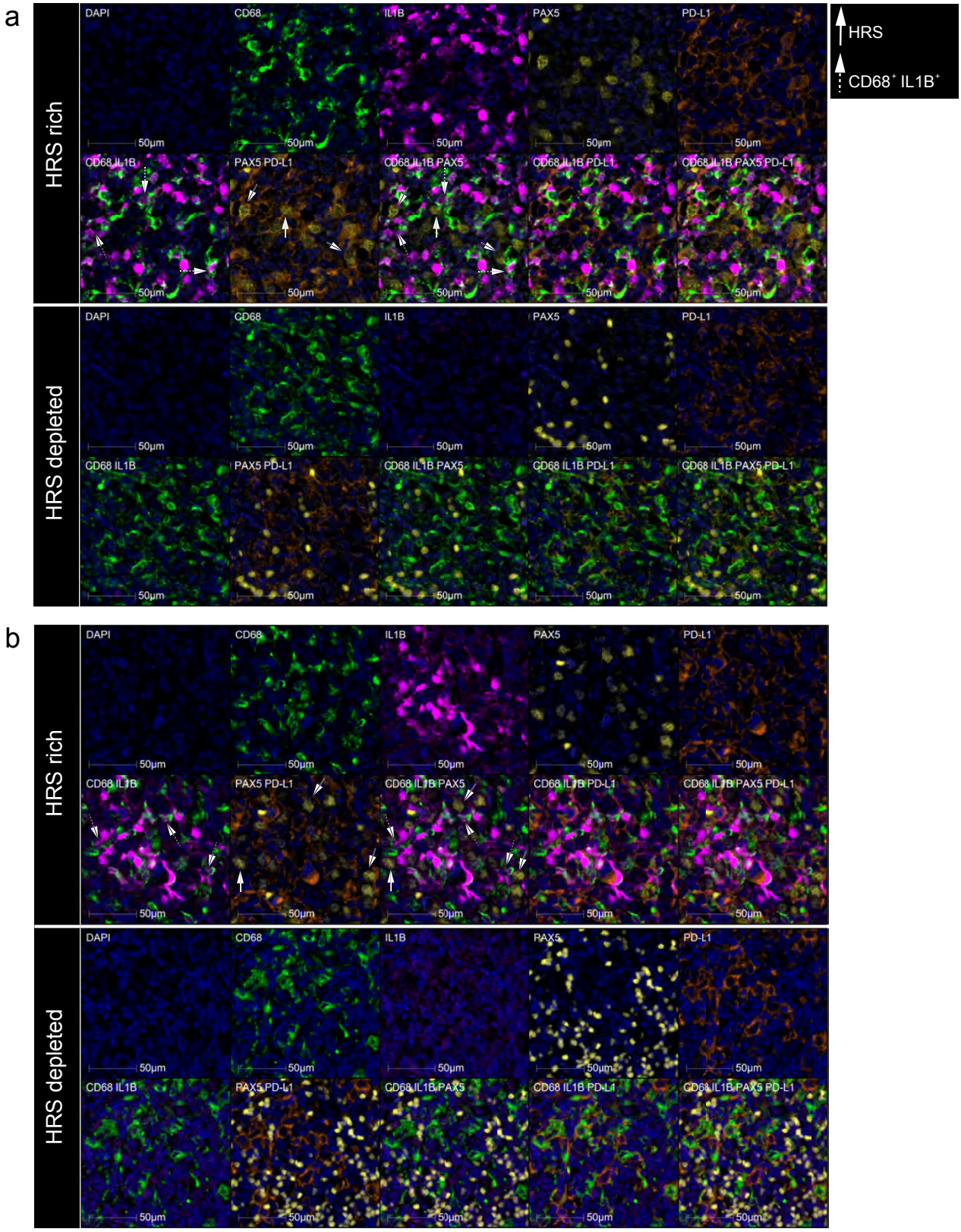
> "We also evaluated B-cell abundance and proximity to HRS cells in available diagnostic biopsies from 7 of the 11 patients with newly diagnosed cHL and scRNA-seq analyses of circulating B-cells (**Figure 4d, Extended Data Figure 7**). As all of the circulating B-cell subsets were significantly less abundant in patients with newly diagnosed cHL than in healthy donors (**Figure 4d**), we utilized a pan B-cell marker, PAX5, and PD-L1 to identify small $PAX5^{bright}$ normal infiltrating B-cells and $PAX5^{dim}$/$PD\text{-}L1^{+}$ HRS cells by dual immunohistochemistry and digital imaging (**Extended Data Figure 7**). Normal B cells were significantly less abundant in all evaluated newly diagnosed cHL biopsies than in control lymphoid tissue (**Extended Data Figure 7c**). Additionally, normal B cells were relatively excluded from the immediate HRS cell ($PAX5^{dim}$/ $PD\text{-}L1^{+}$) niche, defined as within 25 μm of the tumor cells (**Extended Data Figure 7d**)."



Extended Data Figure 7. Immunohistochemical analyses of normal infiltrating B cells in the intact cHL tumor immune microenvironment. a, (Left, top panels, 1A-8A) Eight selected regions of interest (ROIs) of double PAX5 (red) and PD-L1 (brown) IHC images (17x magnification) from a representative newly diagnosed cHL (ND2). (**Right upper panel**). High magnification (57x) image of a larger PAX5^{dim} PD-L1^{+} HRS cell and additional smaller PAX5^{bright} normal B cells. **A (Left, bottom panels, 1B-8B)** Composite heatmaps for PAX5^{dim} PD-L1^{+} HRS cells (red – high density of HRS cells) and PAX5^{bright} B cells (cyan – high density of B cells) overlaid on the original IHC images (from **1A-8A**). The color intensities for each heatmap were determined by calculating the percentage of cells with the given phenotype (HRS: PAX5^{dim} PD-L1^{+}, B cells: PAX5^{bright}) among all cells within a 37µm radius distance of each hexagon's centroid. Each hexagon has a horizontal width (short diagonal) of 25 µm. The color scale for each ROI is shown in the lower right corner. **b, (Top panels, 1A-3A)** Selected ROIs of double PAX5 (red) and PD-L1 (brown) IHC images (17x magnification) from a control tonsil. **b, (Bottom panels, 1B-3B)** Heatmaps for normal B cells (cyan – high density of PAX5^{bright} B cells) overlaid on the original IHC images (from **1A-3A**). **c,** Fraction of PAX5^{bright} B cells in the intact microenvironment of 7 newly diagnosed cHLs and a control tonsil. Each dot is a separately evaluated ROI. P value (p 0.0002) reflects the difference between the median normal B-cell percentage in the control tonsil and the 7 evaluated newly diagnosed cHLs. **d,** B-cell cellularity in defined regions represented as % of normal B cells among all cells proximal to HRS cells (within 25µm, orange) or distal to HRS cells (>25µm, blue), paired for each individual case (**left panel**) and summarized for all cases together (**right panel**).

In the original manuscript, the likely biological importance of circulating Cluster 0 monocytes in patients with cHL prompted us to determine whether tumor-associated macrophages with Cluster 0 transcriptional features were present in the intact TIME of patients with newly diagnosed cHL. As noted, we initially used RNAscope analyses to both identify tumor-associated macrophages with Cluster 0 transcriptional features in the intact TIME and localize these cells in near proximity (within 75 μm) to malignant HRS cells (**Figure 5d**). We thought that it was preferable to characterize the Cluster 0 signature in tumor-infiltrating monocytes/macrophages at a transcriptional level because the defining Cluster 0 features were soluble chemokines and cytokines that might be less well defined with protein-based assays. In the revised manuscript, we also perform multiplex immunostaining to characterize, at a protein level, the numbers and locations (HRS-cell proximal or distal) of tumor-infiltrating Cluster 0 IL1β^+ monocytes/macrophages in biopsies of newly diagnosed cHL. For these new studies, we used slides from the same series of diagnostic cHL biopsies analyzed for Cluster 0 transcripts by RNAscope in **Figure 5c and d** of the original manuscript. The new text describing the additional studies is included in lines 355-361 and **new Extended Data Figure 10** and is enclosed below for ease of review.

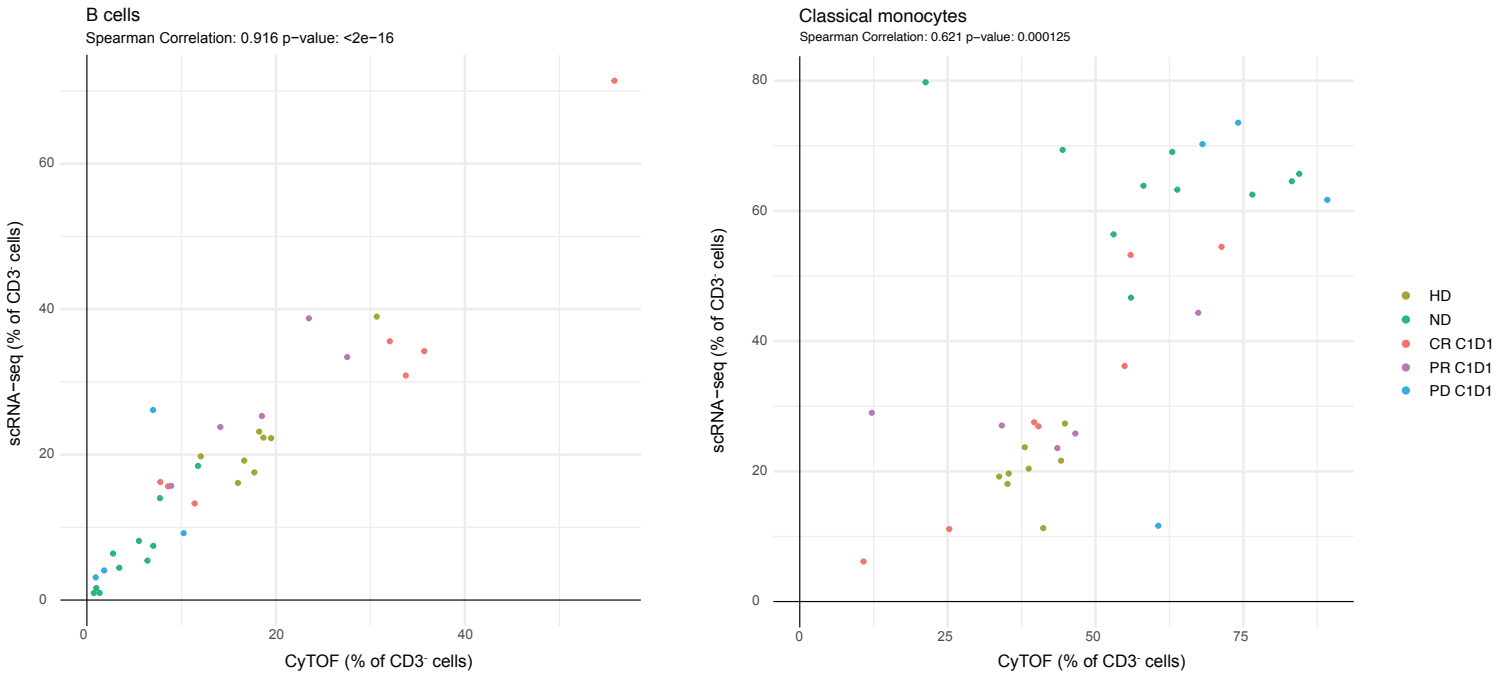
> "To further validate our findings, we performed multiplex immunofluorescence (mIF) with a 4-plex panel (CD68, IL1β, PAX5 and PD-L1) on the same cHL cases and confirmed the presence of IL1β^+ CD68$^+$ monocytes/ macrophages in the intact TIME at the protein level. IL1β^+ monocytes/macrophages were primarily detected in HRS-rich regions of the intact TIME whereas monocytes/macrophages in HRS-poor regions were largely IL1β^- (**Extended Data Figure 10**). These mIF analyses reinforce our RNAscope results (**Figure 5d**) and highlight the likely significance of tumor-infiltrating IL1β^+ monocytes/macrophages in cHL."



Extended Data Figure 10. Multiplex immunofluorescence imaging of IL1β+ monocyte/macrophages in the intact cHL tumor immune microenvironment. Two representative newly diagnosed cHLs (**a** and **b**) with regions including abundant PAX5^{dim} PD-L1^{+} HRS cells (upper panel) and rare HRS cells (lower panel) are shown. Arrows identify PAX5^{dim} PD-L1^{+} HRS cells ↑ and IL1β^{+} CD68^{+} monocytes/macrophages ⇡ which are co-localized in HRS-rich regions (upper panel). Regions with rare PAX5^{dim} PD-L1^{+} HRS cells primarily include smaller PAX5^{bright} normal B cells and IL1β^{-} CD68^{+} macrophages (bottom panel). CD68 green, IL1β magenta, PAX5 yellow and PD-L1 orange.

7. The proportion of circulating cells, like B cells shown in Fig. 4d and myeloid in Figs 5b (IL1B+ monocytes) and 6 should be validated by flow-cytometry that is a more reliable methodology to identify cell populations since processing of cells for single cell RNA-seq usually affect the proportion of these cells. In addition, this should be done indicating individual patient contributions to the mean proportions. This is important to establish interpatient variability.

To address this important suggestion, we took advantage of the previously performed CyTOF analyses (Cader et al. Nat. Med. 26:1468-1479 [2020]) and current scRNAseq analyses (this manuscript) of circulating B-cells and classical monocytes (which include Cluster 0 cells) in our study patients. We have protein-based CyTOF data and scRNAseq analyses on C1D1 serial cryopreserved peripheral blood mononuclear cell samples from 8 of 13 healthy donors, 10 of 11 patients with newly diagnosed cHL, and 7 of 9 complete responders, 5 of 5 partial responders and 4 of 6 patients with progressive disease from the cohort of patients with relapsed/refractory Hodgkin lymphoma treated with PD-1 blockade. We enclose an assessment of the correlation between protein-based CyTOF and scRNAseq for all circulating B cells and classical monocytes (which include the Cluster 0 monocytes and additional classical monocytes Clusters 1 and 2) and provide these analyses as **new Supplementary Figure 6**. As shown, **there are excellent correlations between the abundance of circulating B cells (left panel) and classical monocytes (right panel) as determined by flow cytometry (CyTOF) and scRNAseq** (B-cell Spearman correlation 0.916 and, P<2e-16 and classical monocyte Spearman correlation 0.621 and P value 0.000125). Interpatient variability is addressed by showing the CyTOF and scRNAseq data for each individual patient.



In the revised manuscript, lines 295-297, we added the following sentence:

"In the subset of patients with current scRNA-seq and prior CyTOF analyses, there was an excellent correlation between circulating B-cell numbers (**Supplementary Figure 6c**)."

8. Data shown in Fig 5e is irrelevant for this manuscript. There are plenty manuscripts showing the relevance of IL1B monocytes/TAMs in solid tumors in prognosis and response to immunotherapies, including anti-PD1.

We respectfully believe that the analysis of monocyte/macrophages with Cluster 0 transcriptional features in an additional large solid tumor compendium (**Figure 5e**) underscores the likely importance of cells in the biology of multiple tumors. We note that Reviewer #3 commended our inclusion of this additional dataset.

Reviewer #3 (Checkpoint therapy, T anti-tumor) (Remarks to the Author):

Summary

In this manuscript by Paczkowska et al, the authors leverage PBMC from healthy donors and 20 cHL patients that received anti-PD1 therapy as part of Checkmate 205 plus additional FFPE tissues to evaluate MHC-I independent mechanisms underlying response or resistance to anti-PD1 blockade. The authors find several intriguing cell subsets including cytotoxic CD4+ T cells and B cell features that are associated with response to anti-PD1 and CD4+ Treg and IL1B+ monocytes that were associated with resistance to anti-PD1. The authors also leveraged several external datasets to corroborate their findings with respect to IL1B+ monocytes. Overall, the authors are to be commended for a thorough and clearly presented analysis of their scRNAseq cohort and for including external validation experiments and datasets. The findings are compelling, but several major points outlined below related to the biological interpretation should be addressed. MHC-I independent effects of PD1 blockade are an important topic with broad implications, but there are several important points that would help better explain the underlying mechanisms.

We appreciate Reviewer 3's very thoughtful consideration and detailed review of our manuscript.

Major Comments

1) The authors should include a table of patient demographics and relevant clinical characteristics for the Checkmate 205 cohort, the cohort of FFPE samples, and the cohort of healthy donors that they utilize for this study.

We now include a **new Supplementary Table 1** of patient demographics and clinical characteristics for healthy donors, patients with newly diagnosed cHL, patients with relapsed/refractory cHL treated with PD-1 blockade (Checkmate 205 cohort) and the additional cohort of patients with newly diagnosed cHL whose FFPE biopsy specimens were used in the multiplex immunofluorescence and RNAscope analyses.

2) This paper reports several immunologic features from blood that are associated with response or resistance to therapy in cHL, which is to be commended. However, it seems that authors have missed the opportunity to address another key question: how do immunologic states change from prior to and following anti-PD1 therapy? It would be important to understand these changes especially in the context of the MHC-I independent mechanism of action. Indeed, very little is known about how anti-PD1 blockade can influence CD4+ T cell states.

In our manuscript, we found that **baseline differences** in the abundance of circulating $CD4^+$ naïve/central memory cells, circulating B-cells and transcriptionally polarized Cluster 0 monocytes were associated with subsequent responses to PD-1 blockade. We also found that there were significant response-related differences in $CD4^+$ N/CM cell numbers following PD-1 blockade (**Extended Data Figure 3**). Additionally, we identified response-related changes in the transcriptional signature of circulating Cluster 0 monocytes in patients following PD-1 blockade (shown in **Figure 6a** and further analyzed in **Figure 6b and c**). It is likely that there are additional qualitative changes in these circulating immune cell subsets, including maturation of $CD4^+$ N/CM T-cells to effectors, when they migrate into the intact cHL TIME.

To address Reviewer 3's excellent suggestion to assess treatment-related CD4$^+$ T cell changes in more detail, we now include the following analyses of differentially expressed transcripts in CD4$^+$ T-cell clusters: CR vs PD at C1D1, CR vs PD at C4D1, CR C1D1 vs C4D1 and PD C1D1 vs C4D1, all individual CD4$^+$ T-cell clusters (**new Supplementary Table 5a**), and the combined naïve/CM clusters (Clusters 0, 1, 9, 11, 12, 15, 18, 21 and 24) (**new Supplementary Table 5b**). We found that the greatest response-related differences were between CR and PD at C4D1 as visually depicted in the enclosed graphic (below).

		CR vs. PD at C1D1		**CR vs. PD at C4D1**		**CR C1D1 vs. C4D1**		**PD C1D1 vs. C4D1**	
		Number of transcripts more abundant in CR (vs. PD) at C1D1	Number of transcripts less abundant in CR (vs. PD) at C1D1	Number of transcripts more abundant in CR (vs. PD) at C4D1	Number of transcripts less abundant in CR (vs. PD) at C4D1	Number of transcripts more abundant at C1D1	Number of transcripts more abundant at C4D1	Number of transcripts more abundant at C1D1	Number of transcripts more abundantat C4D1
0	Naïve	8	0	21	129	5	0	1	4
1	Naïve/CM	16	32	15	71	2	0	1	4
9	Naïve/CM	6	4	0	78	3	0	0	3
11	Naïve/CM (TRBV30)	0	6	0	6	0	0	0	0
12	Naïve/CM (TRAV8-2)	4	0	0	18	2	0	0	0
15	Naïve/CM	1	6	0	23	8	0	2	0
18	Naïve/CM (SOX4+)	0	16	0	33	2	1	0	0
21	Naïve/CM	0	0	0	4	0	0	0	0
24	Naïve/CM	0	1	0	0	0	0	0	0
2	CXCL13+	22	30	17	111	6	0	0	5
3	Th2	11	20	1	22	3	0	1	1
6	Th17-like	6	7	0	12	3	0	0	1
5	CTL	5	8	11	18	18	10	9	0
22	IFN-responsive CTL	6	0	0	3	0	5	0	2
10	Treg	5	10	10	40	3	2	2	1
17	Tr1	1	5	1	36	36	0	1	0
4	Other	18	27	6	32	11	0	2	7
8	IFNg stimulated	6	8	11	69	4	0	0	1
14	Other	3	4	3	20	3	0	2	3
19	Cycling (CTLA4+)	6	9	2	3	9	4	0	0
20	Other	1	15	0	10	8	1	1	0
7b	γδ VD2	0	0	2	7	5	3	0	0
23	γδ non-VD2	0	0	0	0	2	0	0	0
13	γδ VD2	0	0	0	17	9	1	0	0
16	NK NKT	4	2	0	0	48	4	0	0
7a	MAIT	4	4	9	43	17	1	0	0

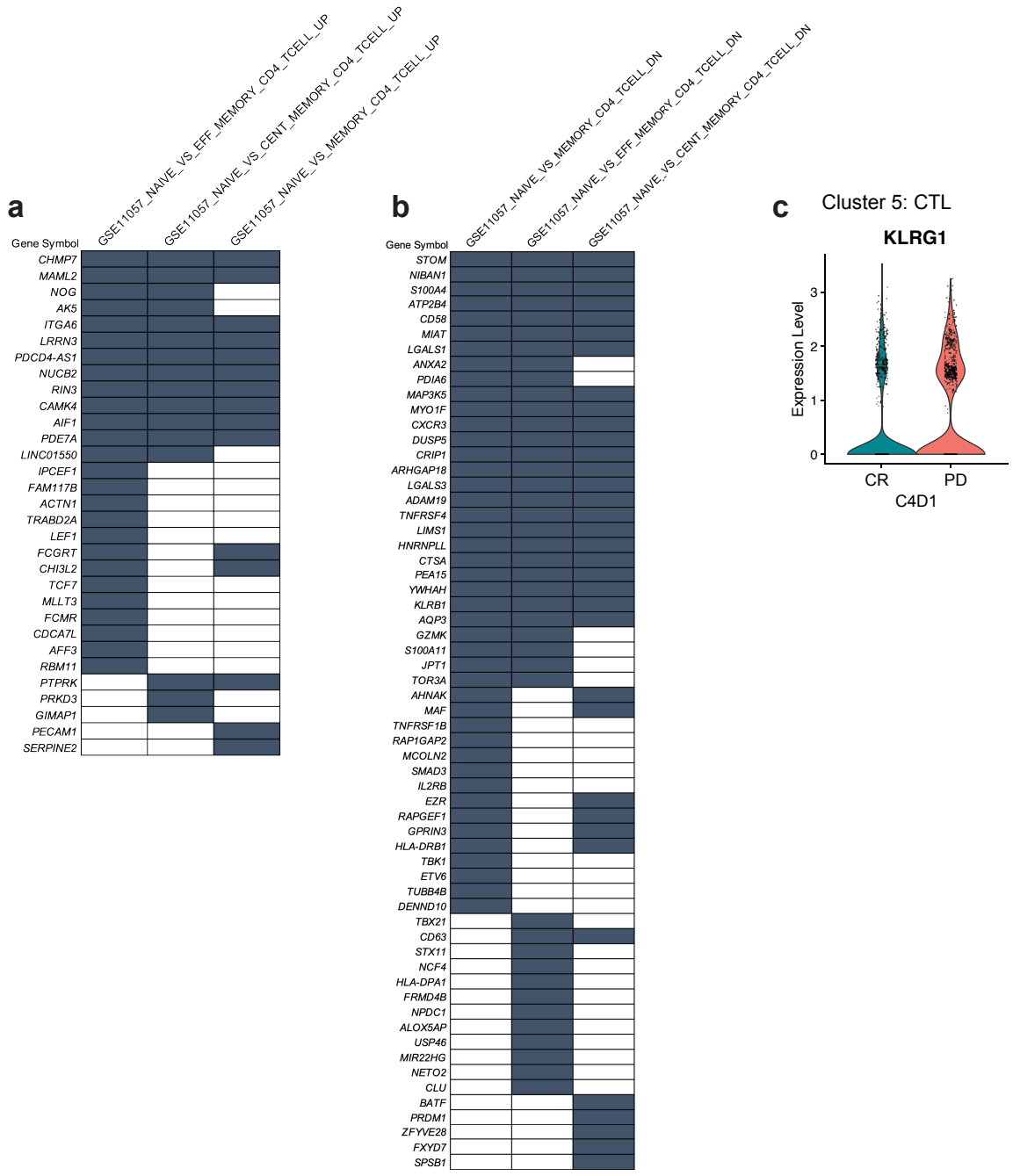
We have included 2 of the most interesting findings from the CR vs PD at C4D1 analysis in the revised manuscript. The associated text and data items are enclosed for ease of review.

1) In lines 179-184 of the revised manuscript and **new Extended Data Figure 4,**

"We further assessed qualitative response-related differences in circulating naïve/CM CD4$^+$ T cells in patients who achieved CRs or progressed following PD-1 blockade. Pathway enrichment analyses revealed that naïve/CM CD4+ T-cell subsets (Cluster 0, 1, 9, 11, 12, 15, 18, 21 and 24) from complete responders had increased expression of TCF7, LEF1 and LRNN3 and a more naïve cell phenotype, potentially reflecting a greater capacity for self renewal (Supplementary Table 5 and **Extended Data Figure 4a-b**) (Cano-Gamez et al. Nat. Commun. 11:1801 [2020] and Wang et al. Clin Immunol. 241:109078 [2022])."

2) In lines 198-204 of the revised manuscript and **new Extended Data Figure 4c**, we describe

"… response-related differences in Cluster 5 CD4$^+$ CTLs from patients who achieved CRs or had progressive disease (PD) following treatment (C4D1) (**Supplementary Table 5)**. These included increased expression of the inhibitory Killer cell lectin-like receptor G1 (KLRG1) in patients with progressive disease (**Extended Data Figure 4c and Supplementary Table 5**). In recent preclinical models, KLRG1 expression on CD4$^+$ T-effector cells was associated with tumor progression and lack of response to PD-1 blockade (Ager et al. J Immunother Cancer. 11(9) [2023])."



Extended Data Figure 4. Differential expression of response-related genes in CD4⁺ T cells. a-b, Pathway enrichment analysis of response- related differentially expressed genes in the naïve/central memory (CM) CD4 T-cell clusters (0, 1, 9, 11, 12, 15, 18, 21 and 24) using Molecular Signatures Database (MSigDB) and C7: Immunologic gene sets. Results are based on a differential analysis of naive/CM CD4 T-cell transcripts in patients who achieved a CR or progressed (PD) on PD-1 blockade at C4D1. **a,** Transcripts that are more abundant in CRs than in PDs. **b,** Transcripts that are less abundant in CRs than in PDs. **c,** Violin plots showing the relative expression of KLRG1 in Cluster 5: CTL in CRs versus PDs.

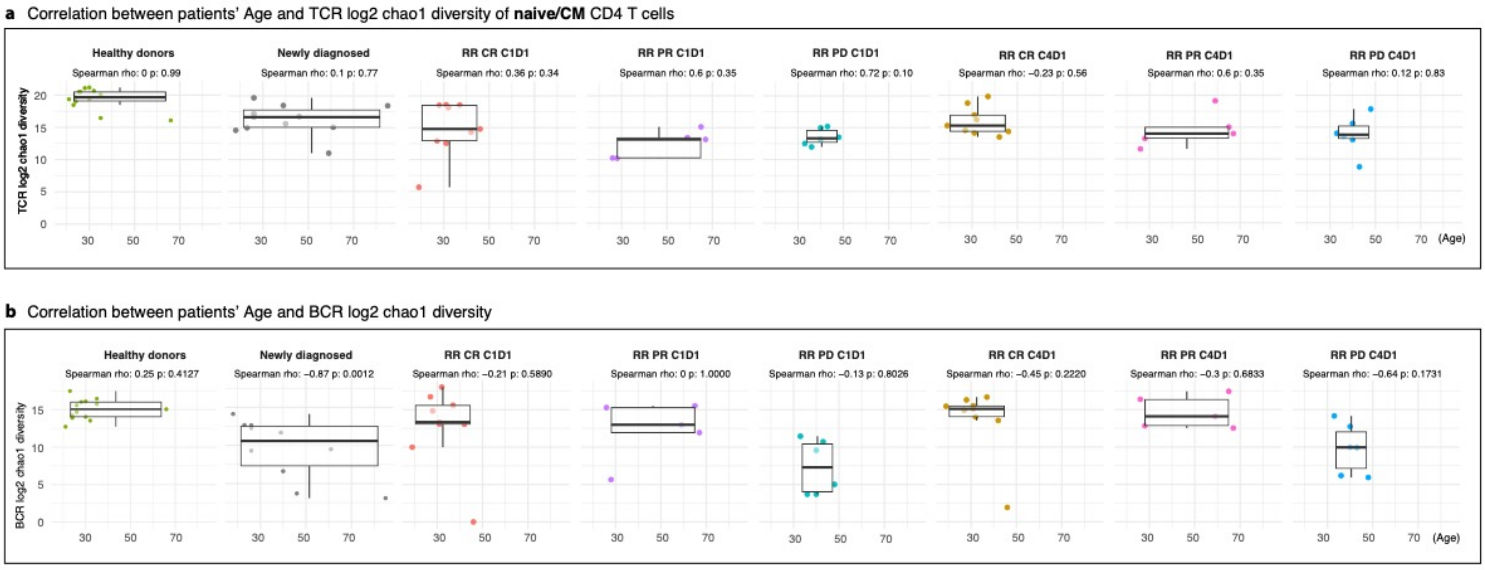
We appreciate Reviewer 3's suggestion to perform these additional analyses which reveal response-related differences in likely self-renewal capacity in CD4⁺ naïve/CM T cells and identify a potentially targetable complementary exhaustion pathway in CD4⁺ Cluster 5 CTLs.

3) What is the underlying biological explanation for the naïve/memory CD4+ T cell diversity being related to response to anti-PD1? Is the effect independent of age? T cell frequencies and diversity are associated with age (https://pubmed.ncbi.nlm.nih.gov/24510963/), so it would be interesting to know if both the naïve CD4+ T cell diversity and B cell diversity are correlated with or independent of age.

We believe that the underlying biological explanation for the association between naïve/memory CD4 T cell diversity and response to PD1 blockade relates to the need for ongoing CD4+ T-cell responses to new tumor neoantigens. In lines 417-420 of the discussion, we state that "In this largely MHC class I-negative tumor, quantitative differences in circulating $CD4^+$ N/CM T-cell abundance and TCR diversity were associated with the response to PD-1 blockade, highlighting the importance of a continued capacity to respond to new tumor neoantigens."

In our analysis of the genomic signature of cHL, we found that EBV- cHLs (which constitute the majority of cHLs in patients in the US and Northern Europe), had an extraordinarily high molecular tumor burden and marked genomic instability (Wienand et al. Blood Adv. 3:4065-4080 [2019]). In this context, the continued ability to respond effectively to new cHL tumor neoantigens is particularly important. Additionally, it is increasingly recognized that terminally differentiated, epigenetically modified effector T cells are not "reinvigorated" following PD-1 blockade. We note in lines 422-424, of the manuscript discussion, that our findings build on "recent observations (from others) regarding the importance of *TCF7*$^+$ T-cell progenitors in the response to PD-1 blockade and extend these findings to the circulating $CD4^+$ T-cell compartment".

We performed a detailed demographic analysis and confirmed that disease-associated and response-related differences in $CD4^+$ N/CM T-cell diversity and B-cell diversity were independent of age. We have included these data below to facilitate review and added the information in **new Extended Data Figures 2c and 6e.**



4) As the authors are proposing that their IL1B+ monocyte population / state is unique to cancer patients, it is important to contextualize this cell subsets with regards to previously defined monocyte subsets in healthy individuals. Is there any relationship of this subset to previously described monocyte subsets such as those described by Villani et al Science 2017 (https://pubmed.ncbi.nlm.nih.gov/28428369/)?

In our analyses, we found that Cluster 0 monocytes were largely restricted to patients with cHL, in contrast to healthy donors. We previously reviewed the scRNAseq analyses of normal circulating monocyte subsets described by Villani et al in *Science* 2017. None of the circulating monocyte subsets described by Villani et al have the Cluster 0-defining transcriptional signature.

5) The monocyte cluster 0 signature is associated with response to anti-PD1 in cHL and a second cohort of mUC that they authors use to validate their findings. However, it is unclear if this signature is simply prognostic for good outcome or if it is predictive of response to anti-PD1 or both. If data from other studies are available, it would be ideal to assess whether this signature is associated with better progression free survival or overall survival in patients that did not receive anti-PD1.

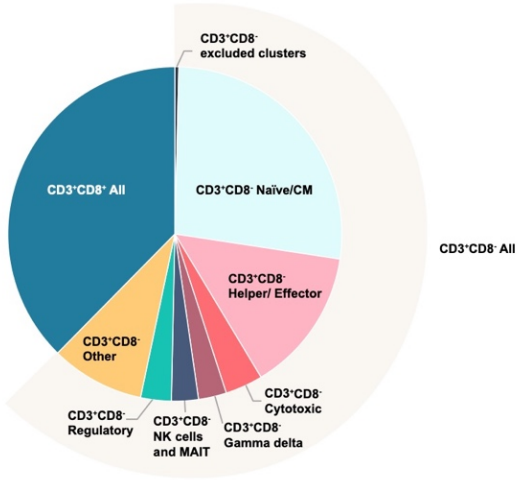
The reviewer raises an important point regarding the potential adverse association of Cluster 0 monocytes with additional treatments as well as PD-1 blockade. We do not yet have peripheral blood mononuclear cell scRNAseq data from patients with cHL who were treated with other therapies to address this important question. However, we are developing the appropriate clinical studies to evaluate this issue in the future.

In the manuscript discussion (lines 448-451), we do note the recent description of IL1β^+ tumor-associated macrophages that resemble Cluster 0 monocytes/macrophages in pancreatic ductal adenocarcinoma (Caronni et al. Nature 623:415–422 [2023]). In the referenced paper, the authors find that proinflammatory IL1β^+ tumor-associated macrophages were associated with disease progression in patients who were not treated with PD-1 blockade. For this reason, we state that "the data suggests that IL1β^+ proinflammatory monocytes/macrophages negatively impact outcome in settings beyond PD-1 blockade" (Discussion, lines 454-455).

Minor Comments

1) Throughout Figure 1, the authors report results as a percent of CD3+ cells which by definition must include CD8+ T cells. However, no CD8+ T cell data are presented. The authors should, at a minimum, report the frequency of conventional and regulatory CD4+ T cells and CD8+ T cells, even if no analysis of CD8+ T cells is undertaken.

We have now included the requested analysis of the annotated CD4 T-cell subtypes and additional CD8$^+$ T cells in our scRNAseq dataset as a pie chart below and in **Supplementary Figure 1** (**new panel b**).



2) The authors are to be complimented for making their code publicly available and for having an accession number for their scRNAseq data. A link to the accession was provided, but there is no reviewer token provided to access the data. The authors should ensure that raw and processed scRNAseq data are available privately during review and publicly upon acceptance.

We have contacted EGA regarding the process for assuring the availability of our scRNAseq data to Reviewer 3 during the review process. In brief, EGA will contact the editor who will provide Reviewer 3's email address and issue a token for time-limited review of the scRNAseq data. The EGA correspondence is included below.

"In order for you to give a reviewer access to download your data, we suggest the following steps:
1. Please add the contact from the journal to this email list (ticket #592501) and confirm that you agree to have your data downloaded by an anonymous person.
2. Once you have confirmed that you agree to the first step, I will create a new ticket with the journal contact person to confirm the download details. I will explain to them that I will set up a download account for the reviewer and we will need the reviewer's email for this purpose only (For security reasons). The journal needs to contact the reviewer to make them aware that:
    (i) The reviewer must inform us (EGA) as soon as the data is downloaded.
    (ii) That the data will be deleted once they have completed their review.
3. Once I have the reviewer's email address, I will generate the download account accordingly and pass on the login details (only to the reviewer via a brand-new RT ticket). I'll explain to the reviewer that they must confirm as soon as the data has been downloaded and that it must be deleted once the review is completed.
4. Once the review is complete, I will inform you that the review of the data is complete and that the reviewer has deleted the data."

3) Stylistically, this reviewer would recommend that the authors only include UMAPs in each primary figure for the cell type that is being evaluated. For example, the focus of Figure 3 is NK cells but B cells and monocytes are also introduced in Figure 3A and then shown independently in subsequent figures.

We respectfully think that it is important to begin the description of the $CD3^-$ space with an overview of the major identified $CD3^-$ cell subsets, NK cells, B cell, and monocytes, as shown in **Figure 3a and b.** This is followed by the in-depth analyses of the NK cells (**Figure 3c, d and e**), B cells (**Figure 4**) and monocytes (**Figures 5 and 6**).

4) Why have the authors decided to use version 3.1.0 of Cellranger? There have been many subsequent releases since v3.1.0 with v8.0.0 available now.

Version 3.1.0 of Cell Ranger was the version available at the time of our initial scRNAseq analysis.

5) Please include a more thorough methodological description of the bioinformatics pipeline for generation of the single-cell TCR and BCR data. Did the authors use different tools for TCR and BCR reconstruction and if so, why?

In the online methods section (lines 538-541), we now indicate that:

> "For $CD3^+$ samples, 2 µL of post ctDNA amplification material was also used to prepare scTCRseq libraries. The sequencing libraries for scRNA-seq and scTCR-seq were normalized to 4nM concentration, pooled using a volume ratio of 4:1 and sequenced on Illumina NovaSeq S4 300 cycle platform."

Because we separately generated scTCRseq libraries, we did not do TCR reconstruction to analyze TCR diversity and clonality. In the online methods section entitled "TCR and B-cell receptor (BCR) analyses" (lines 590-598), we provide detailed descriptions of the TCR and BCR characterization. We indicate that the results from the TCR single-cell V (D) J sequencing were read into Immunarch to calculate TCR diversity. We note that the individual BCR sequences were reconstructed by TRUST4 (Song et al. Nat Methods 18, 627-630 [2021]) from the single-cell RNAseq BAM files. The TRUST4 output for the reconstructed BCRs was also imported to Immunarch to calculate BCR Chao1 diversity.

Recommendation: Major revisions to address key points about the patient cohort, changes in immune signatures from baseline to post treatment, and contextualization of findings with other studies.

Reviewer #3 (Remarks on code availability):

The code is publicly available and well organized, but a thorough evaluate of the code is not possible because the data are not available.

Reviewer 1. Now the scRNA-seq and scTCR-seq data are deposited in EGA. The processed gene expression matrix and TCR clone data should be deposited into the NCBI GEO database.

As noted by Reviewer 1, we previously deposited our scRNA-seq and scTCR-seq data in EGA. In order to keep the data from our manuscript in a single repository, we have now deposited the processed gene expression matrices in EGA. As indicated in the revised manuscript, the TCR data is included in Supplementary Data 3 and 4. BCR clone data is included in Supplementary Data 6. We believe that this is the easiest way for readers to access these additional data.

Reviewer 3. One final comment: the authors mention the potential of blood assays throughout the manuscript, and this reviewer assumes they are referring to measuring the frequency of the IL1B+ monocyte population by flow cytometry as a biomarker? However, there would also seemingly be the potential to measure inflammatory cytokines e.g. IL1B, IL6, IL8, etc as a surrogate for this population. This would be much easier to measure in a clinical setting versus a flow-based assay. This is a minor point, but perhaps worth mentioning since the authors findings could potentially translate to blood-based assays for prognosis.

The reviewer comments on a potential correlation between circulating IL1β monocyte numbers and the abundance of circulating inflammatory cytokines such as IL1β, IL6 and IL8. Our previous experience suggests that it may not be possible to precisely correlate the abundance of the above-mentioned circulating cytokines with a single cell population, such as IL1β^{+} monocytes, in the peripheral blood. For this reason, we would prefer not to comment on this point in the absence of definitive data.