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Peer Review File

Transcriptomic Classification of Diffuse Large B-Cell Lymphoma Identifies a High-Risk Activated B-cell-like Subpopulation with Targetable MYC Dysregulation

Corresponding Author: Dr Anita Gandhi

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors of the manuscript entitled "Transcriptomic Classification of Diffuse Large B-Cell Lymphoma Identifies a High-Risk Activated B-cell-like Subpopulation with Targetable MYC Dysregulation" start by proposing seven molecular subtypes of DLBCL obtained from an unsupervised clustering approach using transcriptomic data that recovers both tumor-intrinsic and tumor microenvironment features. The authors developped an algorithm (SubLymE) to validate the clusters in two additional cohorts which recapitulated the main features across clusters. This was followed by describing the clical impact of the clusters, the cluster-specific biological themes and their correlation with previously proposed DLBCL classifications like the Lymphoma Ecotypes (LEs) (Steen, et al., 2021), Lymphoma Microenvironment (LME) (Kotlov, et al., 2021), LymphGen (Wright, et al., 2020), HMRN (Lacy, et al., 2020), Dark Zone Signature (DZsig) (Alduaij W, 2023), TME26 (Risueño, et al., 2020), Reddy COO (Reddy, et al., 2017), and HRsig-Mayo classifiers (Wenzl K, 2023). Given the negative impact of the A7 subpopulation on survival outcome, the authors focused the remaining part of the article on characterizing this group, finding an upregulation of MYC/MYC signatures probably driven by a upregulation of TCF4 driven by a gain in 18q21 which was later validated using protein measurements, IHC, DLBCL cell lines protein expression, abd a TCF4 knockdown in DLBCL cell lines. The authors finished by indirectly evaluating the effect of using lenalidomide + R-CHOP regimen in the A7/non-A7 subpopulations versus conventional R-CHOP treatment. Overall, the bioinformatic methods are well explained and applied for finding and describing the clusters, the manuscript is easy to follow and has a clear clinical focus for the A7 subtype; however there are some points that could improve the manuscript.

Major comments:

- For the Figures 1A and 7F, it would be useful to provide within each KM a table with the log.rank test result of comparing each cluster to the other.

- In lines 544-546 the authors mention how the value of K was selected in the unsupervised clustering method, it would be useful to provide a supplementary figure with the measures of the silhouette metric, gap statistic, and co-cluster frequency distribution that arguments their initial choice of 8 clusters.

- In the section "Comparison among lymphoma subtypes identifies cluster-specific biological themes" and in lines 195-203, the authors described that the A7 subpopulation was associated with the S5 high-risk B-cell state, the Unclassified Ecotype, the High180 Risk HRsig-Mayo class, the MYD88 HMRN group, the Depleted LME, ABC COO, and TME26 negativity.
* Given that the supplementary table 2 has the presence of extranodal sites and that it presents MYD88 mutations, it could be useful if the authors can describe the percentage of extranodal lymphomas within the A7 group given that MYD88 mutation is a hallmark of PCNSL.
* Many of the characteristics of the A7 subpopolation are shared with a recently described molecular subtype of PCNSL (subtype CS1; Hernandez, et al., 2023 Annals of oncology) which includes a depleted tumor microenvironment, PI3K activity, glycolytic activity, high proliferation, IRF4/OCT2 upregulation, Germinal center B-cell related signatures upregulation, MYC activation, MYD88 mutations, amplifications in loci 9p21 (MTAP, CDKN2A), and 18q21 (involving TCF4). It would be interesting to see if transcriptionally the A7 subtype proposed in the present article is similar to the CS1 PCNSL subtype. If most of the most of the A7 tumors are NOT extranodal this could suggest that the A7 subpopulation could potentially travel to the CNS and produce a CS1 PCNSL tumor, which would be very interesting.

- Given that lenalidomide can improve immune visibility of DLBCL cells through increased MHC I/II expression and increase

trafficking of CD8+ T318 cells to the DLBCL tumor. The authors could perform the same experiments for A1 cluster or do a KM analysis similar to Figure 7F using the A1 cluster as this cluster present both lack of MHC-I/II expression and cold TME.

Minor comments:

- Text for the axis of the plots in Figure 1c, 5d-f, and 7b,c,e is hardly readable.

- The authors focus on A7 cluster but are there any recommendations of therapeutic targets for the other clusters at least on the RNA level?

I thank the authors for their work and hope they find my comments helpful.

Reviewer #2

(Remarks to the Author)
The research article titled "Transcriptomic Classification of Diffuse Large B-Cell Lymphoma Identifies a High-Risk Activated B-cell-like Subpopulation with Targetable MYC Dysregulation" was well-written. The authors have included both the tumor intrinsic and tumor microenvironment (TME) factors to develop Subtypes of Large B-cell Lymphoma Expression (SubLymE) classifier by unsupervised clustering of transcriptomic data (both microarray and bulk RNAseq) of a large cohort of Diffuse Large B-cell Lymphoma (DLBCL) patients. One of the newly identified clusters, A7 was found to be high-risk, and enriched for activated B cell (ABC) cell-of-origin (COO), along with low immune infiltration, high cMYC gene mRNA expression, and copy number gains in chromosomes 3 and 18.
The following comments should be addressed before it is ready for acceptance:
1. The rationale behind the selection of the discovery and replication cohorts is not clearly explained in the manuscript. Several other large cohorts of lymphoma studies are available [PMID: 32505213; PMID: 28985567 etc.] to further enhance the training as well as testing of the SubLymE classifier.
2. It is commendable that the authors have provided a piece of critical information in Supplementary Fig S2. The RNAseq normalization plots may enhance this data.
3. The authors mentioned that “We next examined the survival outcomes of the seven clusters under R-CHOP and RCHOP-like treatment regimens in the MER cohort.” However, the model is binarized between A7 and non-A7 clusters only. The authors may provide a complete estimation of the prognostic implications of all clusters. As “clusters A1, A2, and A7 show strong downregulation of tumor microenvironment signatures, in contrast to cluster A6 which shows strong upregulation of microenvironment signatures” it will be interesting to explore the clinical relevance of all the identified clusters.
4. The authors have suggested based on results generated from cell line models that TCF4 may be a “potential therapeutic target for the A7 population”. How elevated TCF4 expression is correlated with the A7 population of DLBCL patients only when compared to other clusters?
5. The authors may refer to the specific “computational immune deconvolution algorithms” that they have used to extract the TME information of the clusters.
6. The rationale behind the association of Lenalidomide treatment with the prognosis of A7 is unclear. The authors have not discussed how the treatment model of Lenalidomide in mice resembles the A7 population of DLBCLs. The authors may discuss the biological significance of the association of MYD88 mutation with the A7 cluster while exploring Lenalidomide treatment.
7. There is a typographical error in line 560-561 of the manuscript.

Reviewer #3

(Remarks to the Author)
In this manuscript by Stokes, Wenzl, Huang and col, the authors conducted unsupervised clustering of transcriptomes of 1208 nodal DLBCL cases and identified a high-risk cluster enriched for ABC-DLBCL subtypes, low immune infiltrated TME, high MYC expression, MYC signature and chr3 and 18 copy number gains. In a pre-clinical model, the use of lenalidomide improved the T cell infiltration of tumors. One of the major concerns is whether as this “A7” patients truly represent a biological and clinical distinct entity than the already described categories including ABC-DLBCL with low tumor microenvironment infiltration and/or negative for MHC I/II. Regarding clinical response to lenalidomide or ibrutinib + R-CHOP vs. R-CHOP, at least two reports (one prospective and one retrospective) showed that ABC-DLBCL patients with low immune infiltration benefit from lenalidomide + R-CHOP (Zhang et al) while ABC-DLBCL patients with CD8 (S1) immune infiltration benefit from ibrutinib + R-CHOP (Steen et al). Same models (cell lines and animal models) do not carry the key molecular mechanisms associated with the A7 phenotype.

Major comments:
1. Conceptually, the premise that existing classifications focus on either tumor intrinsic or TME features is not entirely correct as most later classifications integrate these features at some point. Furthermore, given the biological and functional crosstalk between cancer cells and TME cells, the question remain to what extend deep, multimodality analysis of cancer cells’

intrinsic features is informative on the TME.
2. Not clear whether DH-HGBL patients were excluded or considered as DLBCL patients.
3. Several of these classifications have been used in the clinic (even reporting a 20-gene genetic subtyping) and prospectively validated, even for the clinical efficacy of lenalidomide (and ibrutinib) as reported for the GUIDANCE R-CHOP-X trial for N1-like patients and patients with immune cold TME (Zhang et al, Genetic subtype-guided immunochemotherapy in diffuse large B cell lymphoma: The randomized GUIDANCE-01 trial, Cancer Cell 41, 1705-7116).
4. The proportion of lymphoma cells compared to TME cells for each sample (i.e., tumor "purity") should be explicitly indicated because the clustering could be biased by this proportion (critical for A6 and A7). This could be done, for example, by multiparametric imaging.
5. The cluster A7 appears to be unique in its composition, however, it is not clear how the other clusters differ from A7 and between them. Please provide any data that indicates that each cluster is biologically differently from each other (e.g., by conducting additional analysis form the "pathways" in Fig. 3a but in unbiased manner).
6. Number of patients (prevalence, Fig. 1c) in each cluster between MET and REMoDL-B replication appears inconsistent (A1 and A5 are bigger in REMoDL while A3 is bigger in MER). This is more notorious for the REMoDL-B replication cohort in which overall gene expression and clustering appears "noisier" (see for example A5). Again, A6 and A7 appear to behave differently from the other clusters that behave more inconsistently.
7. The differences in prognosis when comparing A7 vs. non-A7 appears to be due to the highest proportion of ABC-DLBCL in A7 vs. non-A7 (in fact this difference in prognosis is lost when comparing within ABC-DLBCL and the HR is lost when considering ABC-DLBCL as in Table 2).
8. It is not clear how the "lymphoma related processes" and respective categories were selected (and why others were excluded). In addition, Fig. 3a shows that is not clear cut between most of the pathways and the cluster making it difficult to understand whether these represent true biological differences in DLBCL. Statistical analysis must be provided. Validations must be provided (see points 3 and 4).
9. Additional signatures should be run against the clusters as in Fig. 3b, these include DHITsig and MGH signatures. These are MYC-driven signatures mostly seen in GCB-DLBCLs but a small proportion were described in ABC-DLBCL as well. This feature could be overrepresented here due to the method chosen for the COO classification, a mixed nature of the samples (samples containing both ABC-like and GCB-like DLBCL cells like recently described in lymphoma ecotypes) and a selection bias in the patient population.
10. The characteristics of A7 appear to overlap (and thus represent the similar populations of patients) with B-cell state S5 (Steen et al, Cancer Cell 2021). Majority of S5 are represented in Lymphoma EcoTyper LE1, however A7 could not be classified in a specific LE. This should be revisited. The S5 has strong correlate with ABC-DLBCL MCD LymphGen, however this appear not to be the case with A7, please explain.
11. There appear to be discrepancies in the copy number alterations, transcriptional signatures, and COO class for A7, most notoriously in the enrichment of BCL6 (3q) gain and BCL2 gain with no enrichment of BCL6 signature (Fig 3) and BCL2 HMRN signature (fig 3) and being ABC-DLBCL. This needs to be clarified by analyzing an independent dataset and with orthogonal methodology.
12. Full quantification of the MYC positive lymphoma cells (vs. MYC negative lymphoma cells) should be provided for all the patients in the dataset as shown with a few examples in Fig. 5c. This is important given the intra- and inter-tumor variability in MYC protein expression particularly in absence of genetic drivers like translocations and amplifications.
13. The mechanism driven MYC expression is A7 patients is unclear. In some cases, the author identified putative driver TCF4 but from the analysis provided is unclear how typical this mechanism could be, whether TCF4 expression directly correlate with MYC expression in A7 and other clusters, etc. Other known mechanisms leading to MYC expression (in absence of genetic drivers) including epigenetic and metabolic were not explored or considered.
14. It is unclear what methodology the authors used to identify the ABC-DLBCL cell lines RIVA and U2932 as being A7 cluster-like (apparently due to similar genomic alterations, although defined in a very broad manner). This is a very unlikely association given the TME, as the authors claimed, is a critical component for clustering and subsequent classification of DLBCLs. It could be much more meaningful and informative to develop a patient-derived model for a previously classified A7 patient.
15. The baseline (or shRNA treated) protein expression levels of MYC in cell lines with and without TCF4 amplification is similar (or probably even higher in SU-DHL2 harboring 2 copies of TCF4, Fig. 5g).
16. Please provide quantification for Figure 5g. Data must be shown for each of the time points as in Figure 5h (baseline to day 5). This is critical since MYC is a very short lived protein.
17. For the experiments shown in Figure 5h is unclear to determine if the effects on proliferation are due to MYC downregulation (not shown) or to TCF4 downregulation (not shown). The fact that RIVA and U2932 appear to be more responsive to TCF4 downregulation (than to the other cell lines) does not imply that this is due to MYC downregulation as RIVA and U2932 also have higher expression of TCF4 that is genetically driven.
18. The experiments in Figure 5h should be done in presence of TCF4 plasmid that rescue the TCF4 shRNA, at least for some time points. In addition, if MYC downregulation is claimed as the driver of the effect, it must be done with MYC rescue as well.
19. The is no indication that the level of MYC downregulation induced by TCF4 shRNA decreases MYC activity to a significant level. This could be done by RNA-sequencing, for example.
20. Experiments shown in Figure 5 must also be done with MYC shRNA in these conditions since TCF4 is a known driver of ABC-DLBCL.
21. As mentioned earlier, data shown in panels Fig 6 c and d must be shown for all the patients in the cohort.
22. No clear what non-A7 cases represent (as in Fig. 6d). This should be done comparing ABC-DLBCL patients with low TME infiltration that are non-A7.
23. The concept that A7 DLBCL tumors exhibit a less permissive tumor microenvironment is unclear and not supported by the data provided. Also, the "mechanism" described to explain the lack of recruitment to the TME (Fig. 6d, middle panel) lacks references and probably does not apply to DLBCL.

24. There is no clear mechanism by which A7 patients lack expression of MHC class I/II. Is this genetic? Epigenetic? How frequent is the single vs double loss? How these compare with non-A7 particularly other ABC-DLBCL with low tumor infiltration?
25. Effect of lena on A7 vs. non-A7 should be done only in ABC-DLBCL with low tumor infiltration since is a know fact (now supported by several publications) that low tumor infiltration patients benefit by adding lenalidomide (or ibrutinib) to R-CHOP. Alternatively, this data on R2-CHOP vs. R-CHOP benefit should be shown for ABC-DLBCL with low immune infiltration vs. ABC-DLBCL with immune infiltration (for example by applying the LME classification as in Zhang et al and/or the EcoTyper as in Steen et al).
26. Data in Fig 7A-B and 7C were not conducted in A7-like cell lines (as previously identified).
27. Eu-Myc mice do not represent DLBCL an certainly do not represent A7-like tumors. This experiment should be conducted using a cell line in which the TME as well as genetic constitution of the lymphoma cell (or at least the key mechanism leading to decrease class I and II expression and MYC expression as well as TCF4 expression) represent A7-like tumors.

Minor comments
1. Not clearly indicated what methodology/algorithm was implemented for TME cell deconvolution from bulk RNA-seq data.
2. Order of panel/data: Figure 7 F is described before Figure 7A.

Author Rebuttal letter:

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors of the manuscript entitled "Transcriptomic Classification of Diffuse Large B-Cell Lymphoma Identifies a High-Risk Activated B-cell-like Subpopulation with Targetable MYC Dysregulation" start by proposing seven molecular subtypes of DLBCL obtained from an unsupervised clustering approach using transcriptomic data that recovers both tumor-intrinsic and tumor microenvironment features. The authors developped an algorithm (SubLymE) to validate the clusters in two additional cohorts which recapitulated the main features across clusters. This was followed by describing the clical impact of the clusters, the cluster-specific biological themes and their correlation with previously proposed DLBCL classifications like the Lymphoma Ecotypes (LEs) (Steen, et al., 2021), Lymphoma Microenvironment (LME) (Kotlov, et al., 2021), LymphGen (Wright, et al., 2020), HMRN (Lacy, et al., 2020), Dark Zone Signature (DZsig) (Alduaij W, 2023), TME26 (RisueÃ±o, et al., 2020), Reddy COO (Reddy, et al., 2017), and HRsig-Mayo classifiers (Wenzl K, 2023). Given the negative impact of the A7 subpopulation on survival outcome, the authors focused the remaining part of the article on characterizing this group, finding an upregulation of MYC/MYC signatures probably driven by a upregulation of TCF4 driven by a gain in 18q21 which was later validated using protein measurements, IHC, DLBCL cell lines protein expression, abd a TCF4 knockdown in DLBCL cell lines. The authors finished by indirectly evaluating the effect of using lenalidomide + R-CHOP regimen in the A7/non-A7 subpopulations versus conventional R-CHOP treatment. Overall, the bioinformatic methods are well explained and applied for finding and describing the clusters, the manuscript is easy to follow and has a clear clinical focus for the A7 subtype; however there are some points that could improve the manuscript.

Major comments:

- For the Figures 1A and 7F, it would be useful to provide within each KM a table with the log.rank test result of comparing each cluster to the other.

We thank the reviewer for this suggestion. We believe the Figure 1A comment refers to the KM plots in Figure 2A. For the KM analysis in each cohort, we tested each cluster versus all others combined. The figure below shows the one-vs-other hazard ratio for each cluster in each dataset with the 95% confidence interval, and the following table shows the log-rank p-values, with $p<0.05$ highlighted in red. Only A7 consistently showed significantly different survival in each cohort. A5 showed better survival in ROBUST and A6 better survival REMoDL-B, but were not replicable in other cohorts. Text has been added to lines 149-150, âNo other cluster consistently showed a significantly different hazard ratio when compared to all other clusters combined,â and the hazard ratio figure has been included in the Supplemental Fig 3.

	A1	A2	A3	A4	A5	A6	A7
ROBUST	0.25	0.21	0.75	0.44	0.03	0.88	0.02
MER	0.90	0.95	0.31	0.25	0.24	0.91	0.05

REMoDL-B 0.20 0.49 0.78 0.83 0.30 0.04 0.002

GOYA 0.41 0.43 0.75 0.87 0.09 0.39 0.01

Reddy 0.22 0.09 0.26 0.39 0.71 0.71 0.003

We acknowledge the full set of cluster-pairwise tests would be illustrative if appropriately powered, however we did not conduct the tests given the lack of meaningful effect sizes using aggregated groups in the whole cohort, and the lack of statistical power that such tests would have when conducted in only a fraction of the cohort. Comparing the smallest clusters comprising only about 20% of the data, for example, would yield less than 30% power to detect even a fairly large HR above 2.

Using data in Figure 7A, a group-pairwise log-rank p-value table has been added (new table is added to Fig. 7F) and indicates that the only significant comparisons are between A7 R-CHOP and the other three groups (non-A7 R-CHOP, A7 R2-CHOP, non-A7 R2-CHOP).

Log-rank non-A7 A7 non-A7
p-value RCHOP R2CHOP R2CHOP
A7 RCHOP 0.02 0.01 0.04
non-A7 - 0.42 0.82
RCHOP
A7 R2CHOP - - 0.35

- In lines 544-546 the authors mention how the value of K was selected in the unsupervised clustering method, it would be useful to provide a supplementary figure with the measures of the silhouette metric, gap statistic, and co-cluster frequency distribution that arguments their initial choice of 8 clusters.

We thank the reviewer for this suggestion. A silhouette metric plot showing a peak at K=8 has been added as Supplemental Fig 17A, as well as the co-cluster frequency distribution supporting the elimination of the low-quality A8 cluster (Supplemental Fig 17B). We have removed the mention of the gap statistic as it was evaluated but did not show a compelling trend.

In the section "Comparison among lymphoma subtypes identifies cluster-specific biological themes" and in lines 195-203, the authors described that the A7 subpopulation was associated with the S5 high-risk B-cell state, the Unclassified Ecotype, the High180 Risk HRsig-Mayo class, the MYD88 HMRN group, the Depleted LME, ABC COO, and TME26 - negativity.

* Given that the supplementary table 2 has the presence of extranodal sites and that it presents MYD88 mutations, it could be useful if the authors can describe the percentage of extranodal lymphomas within the A7 group given that MYD88 mutation is a hallmark of PCNSL.

We thank the reviewer for this suggestion. We acknowledge that Myd88, PIM1 and ETV6 mutations are all significantly associated with C5 and the MYD88 HMRN cluster. While PIM1 and ETV6 are significantly enriched in A7, Myd88 mutations are not enriched in A7 over other clusters (Figure 8A). We speculate that the association between A7 and Myd88 enriched clusters stems from the enrichment of PIM1 and ETV6 in the A7 cluster.

A7 showed no significant association with extranodal involvement, defined as having 2 or more extranodal sites. The forest plot in Supplemental Fig 3 shows no significant association between A7 and extranodal sites in MER, where 17% (7/42) of A7 cases were extranodal and 18% (54/292) of non-A7 cases were extranodal (p=1).

We have also examined extranodal involvement in the two new validation cohorts. A similar non-significant trend was observed in GOYA, with 17% (13/75) of A7 being extranodal and 13% (25/196) of non-A7 being extranodal (p=0.33). The overall extranodal rate was somewhat higher in the Reddy dataset, but again showed no significant difference by cluster, with 29% (27/92) of A7 being extranodal, and 22% (71/327) of non-A7 being extranodal (p=0.13).

The forest plots in Supplemental Fig 3 have been updated to show the lack of significant association between A7 and extranodal involvement in GOYA and Reddy, in addition to MER where it was already shown.

* Many of the characteristics of the A7 subpopolation are shared with a recently described molecular subtype of PCNSL (subtype CS1; Hernandez, et al., 2023 Annals of oncology) which includes a depleted tumor microenvironment, PI3K activity, glycolytic activity, high proliferation, IRF4/OCT2 upregulation, Germinal center B-cell related signatures upregulation, MYC activation, MYD88 mutations, amplifications in loci 9p21 (MTAP, CDKN2A), and 18q21 (involving TCF4). It would be interesting to see if transcriptionally the A7 subtype proposed in the present article is similar to the CS1 PCNSL subtype. If most of the most of the A7 tumors are NOT extranodal this could suggest that the A7 subpopulation could potentially travel to the CNS and produce a CS1 PCNSL tumor, which would be very interesting.
We thank the reviewer for raising this interesting point. In fact, the majority of A7 cases are not extranodal (70-80%), and furthermore are not extranodal at higher rates than non-A7 cases. We applied the PCNSL subtype signatures from Hernandez et al. to compute a GSVA score for each subtype for each sample in the MER dataset. The results are shown below, organized to allow comparison of SubLymE clusters within PCNSL subtypes (top), or comparison of PCNSL subtypes within SubLymE clusters (bottom).

There is no strong unique association of A7 with CS1 PCNSL or any PCNSL cluster. This is due to 1) PCNSL CS1-4 are strongly driven by multiple mutations while A7 is mainly driven by few CNVs 2) There are only a few genetics abnormalities in common between CS1 and A7 (e.g., 18qamp, del9p, PIM1 mt, ETV6 mt) however there are many more differences (e.g. myd88 mt in CS1) leading to no association 3) The TME in C1,2 and 3 is neutral to mixed with even the âimmune depleteâ CS2 cluster having memory CD4 T cells and monocytes while A7 is immune cold. The most striking association is the distinction between A7 and CS4 (A7 is immune cold while CS4 is immune hot).

Overall, none of the clusters showed a high average score for the CS1 PCNSL subtype. Although clusters A4, A5 and A7 are among the highest-scoring clusters for the CS1 subtype, each of those clusters on average exhibits the CS2 expression pattern even more strongly. Roughly 2/3 of A7 cases score highest for CS2, the highest enrichment of CS2 in any cluster. We find several biological themes in common between A7 and CS2, including increased MYC signaling, immunological depletion, p53 activity, and hypothesized lenalidomide sensitivity.

Cluster A2 had the highest proportion of samples with CS1 as the top-scoring subtype, but still only 14% of A2 scored highest for CS1. One of the clearest associations between clusters and subtypes is the strong association of A6 with CS4, consistent with the âimmune-hotâ nature of both groups.

Given the lack of clear associations with A7, the above explanation and figures are not included in the manuscript.

- Given that lenalidomide can improve immune visibility of DLBCL cells through increased MHC I/II expression and increase trafficking of CD8+ T cells to the DLBCL tumor. The authors could perform the same experiments for A1 cluster or do a KM analysis similar to Figure 7F using the A1 cluster as this cluster present both lack of MHC-I/II expression and cold TME.

We again thank the reviewer for this suggestion. We repeated the 4-way KM analysis in Figure 7F stratifying by A1 instead of A7, but found no significant differences by cluster or by treatment arm (see KM plot below). While A1 tends to have low MHCI/II like A7, it is a mix of ABC and GCB. Since lenalidomide has known preferential activity in ABC tumors (Nowakowski JCO 2014), it is not surprising that R2-CHOP does not perform well in cluster A1. Given this lack of association, this explanation and figure are not included in the manuscript.

Minor comments:

- Text for the axis of the plots in Figure 1c, 5d-f, and 7b,c,e is hardly readable.
We thank the reviewers for pointing this out and for this suggestion. The axis text has been improved.

- The authors focus on A7 cluster but are there any recommendations of therapeutic targets for the other clusters at least on the RNA level?

We have made an effort to identify potential therapies for clusters A1-A6 based on transcriptomic/genetic features of each cluster (see table below). However, as these are based on speculation and not experimental data, we have not included this in the manuscript.

Gene expression Potential therapy
Cluster Mutations Genomic Lesion pathways approach

A1 NID1, PTGIR, NLPR2 2p11 deletion NFKB downregulation Unknown
1q36 deletion, 18q12
deletion, 18q21 PI3K/AKT/MTOR
deletion and 8p23 signaling, E2F targets, EZH2 inhibition MTOR
A2 EZH2, DAB1, IL15 deletion IRF4 downregulation inhibitor

Notch signaling,
Stromal-1 signature
A3 CDC42, DSP 11q25 deletion upregulation Notch inhibitor

PI3K upregulation,
2q21 gain, 2q23 gain Stromal-1 signature
A4 TP63, INO80, ZNF717 and 8q21 gain upregulation PI3K inhibitors
18p11 gain, 3q25
gain, 3q27-28 gain, Lenalidomide, BCL6
2p21 deletion and NFKB upregulation, inhibitor, MYD88
A5 MYD88, CSMD1 2q32 deletion OxPhos upregulation inhibitor

B2M, TNFAIP3, CD24, 18q21 gain and 9p21 TME upregulation, JAK
A6 NEAT1 deletion upregulation JAK inhibitor

Reviewer #2 (Remarks to the Author):

The following comments should be addressed before it is ready for acceptance:
1. The rationale behind the selection of the discovery and replication cohorts is not clearly explained in the manuscript. Several other large cohorts of lymphoma studies are available [PMID: 32505213; PMID: 28985567 etc.] to further enhance the training as well as testing of the SubLymE classifier.

We thank the reviewer for raising this point. While there are currently many large publicly available front-line DLBCL genomic datasets, at the time our research started in 2018 there were limited datasets available. We started this project with NGS data generation for ROBUST and a commercial cohort because we had tissue for sequencing and clinical data. Therefore, we used them as our discovery cohort. Text has been added to lines 96-99 to explain this as âWhile there are currently many publicly available datasets available, this discovery cohort was selected based upon its large size, consistent profiling methodology, and availability of genomic and clinical data at the inception of this research.â. As datasets have become available over time, we have incorporated them into our work as replication cohorts and this latest revised version has now added GOYA and Reddy et al. cohorts as suggested. Our new analysis (Figure 1) shows replication of relevant cluster-specific patterns in terms of both outcome and biology.

2. It is commendable that the authors have provided a piece of critical information in Supplementary Fig S2. The RNAseq normalization plots may enhance this data.

We thank the reviewer for this suggestion and have added PCA plots of all 5 cohorts (Supplemental Figure 16) together in the space of all genes, COO genes, and SubLymE classifier genes. This RNAseq normalization analysis indicates that all datasets are well-normalized to a common gene expression space.
3. The authors mentioned that âWe next examined the survival outcomes of the seven clusters under R-CHOP and RCHOP-like treatment regimens in the MER cohort.â However, the model is binarized between A7 and non-A7 clusters only. The authors may provide a complete estimation of the prognostic implications of all clusters. As âclusters A1, A2, and A7 show strong downregulation of tumor microenvironment signatures, in contrast to cluster A6 which shows strong upregulation of microenvironment signaturesâ it will be interesting to explore

the clinical relevance of all the identified clusters.

We thank the reviewer for this suggestion and agree that based on biology some of the non-A7 clusters may be prognostic. However, we tested the prognostic power of each cluster in binarized one-vs-other model and found that only A7 consistently had a significantly different outcome from all others. The figure below of the one-vs-other HRs for each cluster in each dataset has been included in the supplement as Figure 3, illustrating the consistency of both A7âs prognostic power, as well as the lack of prognostic power in other clusters. We have added text to this effect motivating the focus on A7 specifically (lines 144-146, âNo other cluster consistently showed a significantly different hazard rate when compared to all other clusters combined (Supplemental Fig 3).

4. The authors have suggested based on results generated from cell line models that TCF4 may be a âpotential therapeutic target for the A7 populationâ. How elevated TCF4 expression is correlated with the A7 population of DLBCL patients only when compared to other clusters?

The thank the reviewer for this question and our new analysis shows TCF4 gene expression is significantly and reproducibly upregulated in A7 patients compared to non-A7 in all five cohorts. A new representative figure (below) from all cohorts has been added as Supplemental Figure 18.

5. The authors may refer to the specific âcomputational immune deconvolution algorithmsâ that they have used to extract the TME information of the clusters.

A pointer to the Methods section has been included following the quoted text, where the deconvolution approach is described and referenced â line 541.

6. The rationale behind the association of Lenalidomide treatment with the prognosis of A7 is unclear. The authors have not discussed how the treatment model of Lenalidomide in mice resembles the A7 population of DLBCLs. The authors may discuss the biological significance of the association of MYD88 mutation with the A7 cluster while exploring Lenalidomide treatment.

We thank the reviewer for these comments. The rationale behind the association of Lenalidomide treatment with the prognosis of A7 is based on the known mechanism of action by Lenalidomide. Lenalidomide is a known T cell activator (Gandhi CCDT 2010, Gandhi BJH 2014, Kronke Science 2013, Hagner BJH 2015) and based on this rationale, was hypothesized to overcome the low T cell phenotype of A7 tumors and perhaps overcome itâs poor prognostic behavior. In addition, another Aiolos/Ikaros degrader Avadomide has been shown to enhance T cell trafficking into DLBCL tumor tissue in a clinical trial (Hagner, Blood 2015). This has been addressed in Lines 306-311.

We acknowledge that Myd88, PIM1 and ETV6 mutation are all significantly associated with C5 and MYD88 HMRN cluster. While PIM1 and ETV6 are significantly enriched in A7, Myd88 mutations are not enriched in A7 over other clusters (Supplemental Fig 8A). We speculate that the association between A7 and Myd88 enriched clusters stems from the enrichment of PIM1 and ETV6 in the A7 cluster.

In our discovery cohort, 35% of A7 patients (compared to 20% of non-A7 patients) have Myd88 mutations, however based on our research, there is no mechanistic rationale to suggest that Lenalidomide would have greater activity in Myd88 mutated tumors as Aiolos and Ikaros degradation resulting in apoptosis would occur in both Myd88 wild-type and mutant settings.

Regarding the connection to Lenalidomide reducing the negative prognostic value of A7 in 1L DLBCL patients treated with R-CHOP, it has been demonstrated in multiple clinical trials that lenalidomide has preferential activity in ABC-DLBCL (Nowakowski JCO 2021, Hernandez-Ilizaliturri Cancer 2011, Czuczman CCR 2017) either as single agent or in combination with R-CHOP. The dual mechanism of action of lenalidomide has been demonstrated to have direct anti-DLBCL proliferative activity as well as immunomodulatory activity through enhancement of T-cell and NK-cell mediated activities (Gandhi CCDT 2010, Gandhi BJH 2014, Kronke Science 2013, Hagner BJH 2015). We hypothesize that in addition to direct effects of lenalidomide in this ABC-DLBCL malignant clone, that lenalidomide would enhance immune cell recruitment and activity to the tumor microenvironment which is beneficial as the A7 DLBCL patients have a clear phenotype of an immune depleted tumor. We utilize the mouse model, engineered to overexpress myc which is strongly overexpressed in A7 DLBCL cells and also has a depleted immune constituency in the spleen post-transfer of the eu-myc cells, to demonstrate this angle of immune recruitment of T-cell populations to the tumor microenvironment.

7. There is a typographical error in line 560-561 of the manuscript.

We thank the reviewer for pointing this out and it has been corrected.

Reviewer #3 (Remarks to the Author): expertise in diffuse large B cell lymphoma immunology

In this manuscript by Stokes, Wenzl, Huang and col, the authors conducted unsupervised clustering of transcriptomes of 1208 nodal DLBCL cases and identified a high-risk cluster enriched for ABC-DLBCL subtypes, low immune infiltrated TME, high MYC expression, MYC signature and chr3 and 18 copy number gains. In a pre-clinical model, the use of lenalidomide improved the T cell infiltration of tumors. One of the major concerns is whether as this âA7â patients truly represent a biological and clinical distinct entity than the already described categories including ABC-DLBCL with low tumor microenvironment infiltration and/or negative for MHC I/II. Regarding clinical response to lenalidomide or ibrutinib + R-CHOP vs. R-CHOP, at least two reports (one prospective and one retrospective) showed that ABC-DLBCL patients with low immune infiltration benefit from lenalidomide + R-CHOP (Zhang et al) while ABC-DLBCL patients with CD8 (S1) immune infiltration benefit from ibrutinib. Same models (cell lines and animal models) do not carry the key molecular mechanisms associated with the A7 phenotype.

Major comments:
1. Conceptually, the premise that existing classifications focus on either tumor intrinsic or TME features is not entirely correct as most later classifications integrate these features at some point. Furthermore, given the biological and functional crosstalk between cancer cells and TME cells, the question remain to what extend deep, multimodality analysis of cancer cellsâ intrinsic features is informative on the TME.

We thank the reviewer for this comment and agree this is a very important perspective as the science evolves on how the DLBCL genetics interacts/trains the surrounding microenvironment. The majority of historical classifications (Lenz, Chapuy, Schmitz, Reddy) have primarily focused on the DLBCL genetics and only recently with seminal work from Steen and Kotlov has the integration of the malignant tumor DNA and the immune system come into focus. We believe our study further adds to the field by describing not only a high-risk segment of the disease which has elements of both the tumor and the immune system, but a therapeutic intervention that has a mechanism which acts on both angles and was shown to overcome the poor prognosis of these patients when added to R-CHOP.

This statement has been modified in the text lines 67-70, as âOnly recently with seminal work on Lymphoma Microenvironment ((the tumor microenvironment into classification efforts such as LME (Kotlov, et al., 2021)) and EcoTyper (Steen, et al., 2021) has the crosstalk between tumor cells and their microenvironment come into focus.â

2. Not clear whether DH-HGBL patients were excluded or considered as DLBCL patients.

We thank the reviewer for this comment. The table below summarizes the DH-HGBL status, as well as HGBL NOS status for the clinical cohorts in our study.

	HGBL assessed by morphology	HGBL-NOS included	DH-HGBL included
MER	yes	yes (6/343)	no
ROBUST	yes	no	no
Commercia	yes	no	no
GOYA	not specified in publication	not specified in publication	not specified in publication
REMoDL-B	not specified in publication	not specified in publication	not specified in publication
Reddy	not specified in publication	not specified in publication	not specified in publication

The above table is for explanation only and not included in the manuscript. The following sentence was added to manuscript (lines 468): âSubLymE was trained entirely on DLBCL NOS patients without HGBLâ A small number of HGBL patients were included in the MER cohort, with no clear enrichment of SubLymE clusters (1 was A1, 2 were A2, 2 were A3, and 1 was A7). We would generally expect a lower prevalence of A7 patients among DH patients given A7 patients are mostly ABC and double hit patients are mostly GCB .

3. Several of these classifications have been used in the clinic (even reporting a 20-gene genetic subtyping) and prospectively validated, even for the clinical efficacy of lenalidomide (and ibrutinib) as reported for the GUIDANCE R-CHOP-X trial for N1-like patients and patients with immune cold TME (Zhang et al, Genetic subtype-guided immunochemotherapy in diffuse large B cell lymphoma: The randomized GUIDANCE-01 trial, Cancer Cell 41, 1705-7116).

We thank the reviewer for pointing us to a recent Cancer Cell article which reports findings from the

GUIDANCE-01 trial. We have revised language to capture recent progress towards applying molecular classifications in clinical studies (lines 71 -76) as âThese molecular classifications may eventually take DLBCL into the era of tailored targeted therapies, exemplified by a recent report in a R-CHOP + X trial, x being an agent that could specifically target the biology of a predefined patient segment (Zhang et al., 2023).â

While the study was limited by small numbers and was not powered to detect a difference in each genetic subtype, the authors do report that DP-LME patients characterized by low microenvironmental cells, do benefit from R-CHOP plus Lenalidomide. Interestingly, almost all A7 patients fall into the DP-LME category supporting our hypothesis that R2-CHOP can overcome a low TME tumor type. It is also important to note however that only one-third of DP-LME patients are A7 therefore these two classifications are distinct.

4. The proportion of lymphoma cells compared to TME cells for each sample (i.e., tumor âpurityâ) should be explicitly indicated because the clustering could be biased by this proportion (critical for A6 and A7). This could be done, for example, by multiparametric imaging.

We have added statistics describing tumor purity estimates by cluster. Lines 113 â115 âMeantumor purity (74% overall) varied by cluster with A1 and A7 showing higher (80% and 83%) and A4 and A6 showing lower purity (70% and 68%). â

We have also added a description of the tumor purity to the Methods-Datasets section. Lines 468 - 470 âSamples from the MER cohort generally had high tumor cellularity by pathology review, with 83% of samples having >50% purity, and 57% having >70% purity.â)

We interpret the varying tumor abundance among generally high-purity samples to be a real source of biological variability across DLBCL patients, rather than a source of confounding bias. The fact that patient-level outcomes vary significantly between the SubLymE classes suggests that they represent true differences in patient biology, instead of uninformative bias which would be expected to have no association with outcome. We have also provided patient-level multiparameter imaging data (Supplemental Fig. 15).

5. The cluster A7 appears to be unique in its composition, however, it is not clear how the other clusters differ from A7 and between them. Please provide any data that indicates that each cluster is biologically differently from each other (e.g., by conducting additional analysis form the âpathwaysâ in Fig. 3a but in unbiased manner).

We thank the reviewer for this request. We have included a Supplemental Figure 9 showing the significant dysregulated Hallmark pathways in each cluster.

The data showed that each cluster has a unique set of differentially expressed biological pathways. For example: A1 shows downregulation of most of the pathways tested, with the exception of spermatogenesis; A2 shows a mix of up- and down-regulated pathways, with E2F targets, G2M checkpoint, OxPhos, and MYC targets all upregulated, while immune and inflammatory pathways were downregulated; A3 showed downregulation of most pathways similar to A1, but also showed dysregulation of KRAS signaling; A5 showed upregulation of most pathways with the exception of KRAS signaling; A6 showed strong upregulation of immune and inflammatory pathways, but was notably downregulated in E2F targets, G2M checkpoint, and MYC target signals. A4 was the least distinct clusters, showing relatively few significant pathways compared to other clusters.

6. Number of patients (prevalence, Fig. 1c) in each cluster between MET and REMoDL-B replication appears inconsistent (A1 and A5 are bigger in REMoDL while A3 is bigger in MER). This is more notorious for the REMoDL-B replication cohort in which overall gene expression and clustering appears ânoisierâ (see for example A5). Again, A6 and A7 appear to behave differently from the other clusters that behave more inconsistently.

We thank the reviewer for the comment and acknowledge variability of the prevalence of each cluster in different patient cohorts.

Due to the nature of SubLymE clusters being driven by underlying biology, we do expect the prevalence of each cluster to vary from cohort to cohort, as different cohorts do not recruit patients with exact same characteristics. To better address this, we added two more cohorts (Reddy and GOYA) which provide a more comprehensive view of cohort-to-cohort variability (Fig. 1C). We see a consistent prevalence for A7 between 10-20% in all 5 cohorts, within the range of expected statistical variability. Similar cross-cohort prevalence is also seen for A2 and A6. The distinctness and consistency of the

clusters can also be inferred from the number of significant hallmark pathways shown in Supplemental Figure 9, and the correlation of enrichment scores between datasets shown in Supplemental Fig 2B. We find that cluster A7 is indeed a particularly reproducible segment of DLBCL, showing good consistency of its size, molecular characteristics, and survival outcomes.

7. The differences in prognosis when comparing A7 vs. non-A7 appears to be due to the highest proportion of ABC-DLBCL in A7 vs. non-A7 (in fact this difference in prognosis is lost when comparing within ABC-DLBCL and the HR is lost when considering ABC-DLBCL as in Table 2).

While the log-rank comparison of A7 vs. non-A7 among ABC-only is not significant in 3 of the 5 datasets, each of the non-significant datasets has an ABC subpopulation size too small to yield even 50% power with the expected effect size. Even with identical behavior between cohorts, we should not expect to find significance in each, but we do find consistency of the effect size, with the hazard ratio estimates varying in a small range from 1.5 to 1.7. The Cox models shown in Table 2 indicate that A7 a significant prognostic factor on its own (HR=2.00, p=0.006), and that its HR is only slightly diminished but still significant when accounting for COO (HR=1.91, p=0.02). In fact, none of the clinical features shown in Table 2 account for more than a fraction of A7âs prognostic power; the A7 p-value is significant in all multivariable models tested.

8. It is not clear how the âlymphoma related processesâ and respective categories were selected (and why others were excluded). In addition, Fig. 3a shows that is not clear cut between most of the pathways and the cluster making it difficult to understand whether these represent true biological differences in DLBCL. Statistical analysis must be provided. Validations must be provided (see points 3 and 4).

We thank the reviewer for these comments. While we acknowledge the biological categories in Figure 3 are not comprehensive, however, they broadly reflect known molecular aspects of lymphoma, and have been used by others (e.g. Wright et al. Cancer Cell 2020, HernÃ¡ndez-Verdin 2022). We have provided additional heatmaps showing the selected signatures in MER, REMoDL-B, GOYA and Reddy as well as heatmaps indicating statistical significance of differences aggregated by cluster (Supplemental Figure 5). The directionality and significance of many cluster-defining signals are observed across datasets, notably, the immune depletion of A2 and A7, the immune upregulation of A6, MYC upregulation in A7 and downregulation in A3, and opposite dysregulation of B-cell transcription factors in A2 and A7, among others.

9. Additional signatures should be run against the clusters as in Fig. 3b, these include DHITsig and MGH signatures. These are MYC-driven signatures mostly seen in GCB-DLBCLs but a small proportion were described in ABC-DLBCL as well. This feature could be overrepresented here due to the method chosen for the COO classification, a mixed nature of the samples (samples containing both ABC-like and GCB-like DLBCL cells like recently described in lymphoma ecotypes) and a selection bias in the patient population.

We thank the reviewer for this suggestion and agree these known myc-driven signatures are important to explore in our study and indeed DHITsig has already been included under its updated name DZsig. We also included two additional classifiers LymphProg (Ren 2024) and MHG (Sha 2019) as additional points of comparison in Figure 3B.

We find that DZsig positivity is associated with A2, owing to the GCB-enriched nature of both groups, while DZsig negativity is associated with clusters A5 and A6. The LymphProg High Risk group is associated with A7, as might be expected of two significantly prognostic classifiers, while the LymphProg Low Risk group is associated with A6. In the MHG classification, we find expected association between the MHG-COO groups and the COO-enriched clusters, namely MHG-ABC with A7, and MHG-GCB with A2 and A3. The MHG group itself is associated with both A1 and A2.

The proportion of cases classified as DHITsig/DZsig positive was consistent across cohorts, ranging from 13% to 17%, suggesting no particular bias in patient population. We additionally tested the Hans and Nanostring COO calls where available in addition to the Reddy calls used throughout the manuscript, and found consistent association of DHITsig positivity with the GCB COO group regardless of method.

10. The characteristics of A7 appear to overlap (and thus represent the similar populations of patients) with B-cell state S5 (Steen et al, Cancer Cell 2021). Majority of S5 are represented in Lymphoma EcoTyper LE1, however A7 could not be classified in a specific LE. This should be revisited. The S5 has strong correlate with ABC-DLBCL MCD LymphGen, however this appear not to be the case with A7, please explain.

We thank the reviewer for making these points and agree that similarities in biologies could certainly lead to some expected overlaps in classifications. Indeed B cell state S5 is largely ABC and associated with poor prognosis (Steen et al). While we applied the published algorithms for Ecotyper and B cell state (Steen et al), LE1 and MCD (Wright 2020) were both called at unexpectedly low rates in our data, making statistical analysis challenging. We do find non-significant enrichment of A7 above background prevalence in both of these classes - among 4 MCD patients in MER, 2 were A7 (p=0.07), and among 8 LE1 patients, 2 were A7 (p=0.22). We also find expected associations between LE1 and S5 (p=0.01), but not between MCD and LE1 (p=1) or between MCD and S5 (p=0.4). Ultimately in our data, A7 is spread between S4, S5 and unclassified B cell state, and mostly is assigned to an unclassified Ecotyper LE class. While this may be a technical issue, we would like to point out that A7 is not enriched in Myd88 mutations, and therefore it is not unexpected that A7 is somewhat distinct from S5 and MCD classes.

11. There appear to be discrepancies in the copy number alterations, transcriptional signatures, and COO class for A7, most notoriously in the enrichment of BCL6 (3q) gain and BCL2 gain with no enrichment of BCL6 signature (Fig 3) and BCL2 HMRN signature (fig 3) and being ABC-DLBCL. This needs to be clarified by analyzing an independent dataset and with orthogonal methodology.

We find distinct and consistent relationships between BCL2/BCL6 copy number, expression, and mutation in both MER and ROBUST. Both the A7 and ABC subtypes are enriched for both BCL2 and BCL6 copy number gain, but only show increased expression of BCL2, with no association between BCL6 copy gains and increased BCL6 expression. The BCL2 HMRN group is a mutation-based, GCB-associated group which is associated with the GCB-enriched A2 subtype, and anti-associated with the ABC-enriched A7 subtype. Overall, A7âs BCL2 copy number gain is consistent with ABC COO, its BCL6 copy number gain is consistent with no enrichment of BCL6 signature, and lack of GCB-associated BCL2 mutation consistent with lack of association with GCB-associated BCL2 HMRN signatures.

Below is a table summarizing the copy number, mutation, and expression patterns of BCL2 and BCL6 in ROBUST and MER, stratified to show associations with the A7 and ABC subtypes. While orthogonal data to confirm these associations would be beneficial, we do not have such data available at this time.

	Non-A7 (MER)	ABC (MER)	Non-ABC (MER)	A7 (ROBUST)	Non-A7 (ROBUST)	ABC (ROBUST)	Non-ABC (ROBUST)
BCL2 copy number gain	56%	38%	53%	30%	65%	36%	58%	27%
BCL2 mutation	3%	13%	2%	17%	2%	8%	5%	13%
BCL2 expression (mean)	0.60	0.16	0.22	0.21	0.57	0.11	0.41	0.01
BCL6 copy number gain	59%	30%	41%	29%	58%	37%	52%	25%
BCL6 mutation	8%	7%	6%	9%	10%	8%	7%	10%
BCL6 expression (mean)	-0.15	0.15	-0.47	0.57	-0.49	-0.36	-0.75	-0.03

12. Full quantification of the MYC positive lymphoma cells (vs. MYC negative lymphoma cells) should be provided for all the patients in the dataset as shown with a few examples in Fig. 5c. This is important given the intra- and inter-tumor variability in MYC protein expression particularly in absence of genetic drivers like translocations and amplifications.

We thank the reviewer for the comment. In defining myc positive vs negative status of patients, we rely on the standard binary 40% cutoff as defined by Johnson and Gascoyne (JCO 2012) to classify patients as myc positive vs negative. In the effort within this manuscript, we randomly selected a handful of samples for IHC evaluation as representative cases of myc expression via immunohistochemistry in A7

vs non-A7. This effort was to buttress the computational analysis demonstrating myc mRNA expression is higher in A7 vs non-A7 clusters of DLBCL patients, which has now been expanded to include 5 trial cohorts (Figure 5B).

13. The mechanism driven MYC expression is A7 patients is unclear. In some cases, the author identified putative driver TCF4 but from the analysis provided is unclear how typical this mechanism could be, whether TCF4 expression directly correlate with MYC expression in A7 and other clusters, etc. Other known mechanisms leading to MYC expression (in absence of genetic drivers) including epigenetic and metabolic were not explored or considered.

We thank the reviewer for highlighting this point within the manuscript. Given the central role of myc in B-cell malignancies, especially DLBCL, there are many paths for the malignant cell to exploit leading to enhancement of myc activity. In this specific manuscript, our analysis of the patient data has highlighted the overactivation of myc at a transcriptional pathway level (HALLMARK myc v1/v2 gene sets) along with very few genomic insults (mutation, copy number alterations, etc) being statistically associated with A7 tumors. One of the very strong signals we observe associated with A7 is the arm level amplification of 18q. Within 18q21.2 resides the TCF4 gene which has previously been shown to modulate the transcription of c-myc in ABC-DLBCL cells (Jain Sci Trans Med 2019). Our work in this manuscript strove to further enhance that association between TCF4 gene amplification and dysregulation of c-myc with respect to the high-risk clinical nature of A7.

Specifically, when examining correlations in the ROBUST dataset, we see TCF4 copy number strongly correlates with TCF4 expression. And within that dataset, there is a significant association between TCF4 copy number and myc mRNA expression. However, given myc is a driver of DLBCL and there are many avenues leading to its overactivation as the reviewer highlights, we find low Spearman correlation between TCF4 mRNA and myc mRNA expression in any of the patient segments including A7 (no higher than 0.27). We wished to examine this specific TCF4/myc connection in the laboratory utilizing models that had the 18q amplification. In those experiments, we demonstrated in DLBCL cell line models with TCF4 amplification that shRNA targeting TCF4 specifically downregulated myc protein/mRNA expression, gene expression of myc target genes, and proliferative decrease compared to ABC-DLBCL cells without TCF4 (Figure 5F-I, Supplemental Figure S13A-E).

14. It is unclear what methodology the authors used to identify the ABC-DLBCL cell lines RIVA and U2932 as being A7 cluster-like (apparently due to similar genomic alterations, although defined in a very broad manner). This is a very unlikely association given the TME, as the authors claimed, is a critical component for clustering and subsequent classification of DLBCLs. It could be much more meaningful and informative to develop a patient-derived model for a previously classified A7 patient.

We agree with the reviewer that it is a difficult challenge to find models that represent all aspects of the tumor, especially as the SubLymE classifier relies on signals from both the malignant clone biology and the immune constituents to accurately define tumors. In our preclinical work, given the strong association of Chr 18q and 3 amplifications with A7 DLBCL tumors, we selected cell lines based on the presence or absence of these events. Through this selection criteria, we characterized Riva and U2932 as more like A7 DLBCL clones than SUDHL-2 and TMD8. This was the basis for further comparative experiments exploring the connection between TCF4 amplification/expression and c-myc expression/transcriptional activity.

15. The baseline (or shRNA treated) protein expression levels of MYC in cell lines with and without TCF4 amplification is similar (or probably even higher in SU-DHL2 harboring 2 copies of TCF4, Fig. 5g).

We agree with the reviewer that DLBCL cells will exhibit high levels of c-myc and achieve this through many routes (translocation, amplification, epigenetic dysregulation, etc). We have added additional data demonstrating the importance of c-myc to DLBCL cell lines models independent of the TCF4 amplification (Supplemental Figure S12F,G). These data demonstrate that knockdown of c-myc in 4 ABC-DLBCL cell lines results in decreased proliferative capacity of all models (independent of TCF4 status). This in conjunction with the shTCF4 data supports the idea that in models with 18q amplification, TCF4 is an important axis in regulating myc expression, however in models lacking TCF4 amplification, the DLBCL cells have harnessed other methods to elevate c-myc expression/activity.

16. Please provide quantification for Figure 5g. Data must be shown for each of the time points as in Figure 5h (baseline to day 5). This is critical since MYC is a very short lived protein.

These data have been added as Figure 5H. We have also provided western blot data for TCF4 shRNA in the 4 DLBCL cell lines at two additional time points (Supplemental Figure S12A,B).

17. For the experiments shown in Figure 5h is unclear to determine if the effects on proliferation are due to MYC downregulation (not shown) or to TCF4 downregulation (not shown). The fact that RIVA and U2932 appear to be more responsive to TCF4 downregulation (than to the other cell lines) does not imply that this is due to MYC downregulation as RIVA and U2932 also have higher expression of TCF4 that is genetically driven.

We have added qPCR data for myc target genes to Supplemental Figure S12C to demonstrate that the anti-proliferative activity of shTCF4 correlates with downregulation of c-myc protein and mRNA expression and downstream myc transcriptional activity.

18. The experiments in Figure 5h should be done in presence of TCF4 plasmid that rescue the TCF4 shRNA, at least for some time points. In addition, if MYC downregulation is claimed as the driver of the effect, it must be done with MYC rescue as well.

We thank the reviewer for the comment and agree this is a valuable experiment. We believe that the additional data including measurements of myc target gene expression and the new shMYC data add orthogonal evidence that TCF4 regulates c-myc expression/activity in models with Chr18q amplification. While the rescue experiment is a good suggestion, we have not performed this specific task as we had to balance the preclinical efforts to complete all suggested experiments for this revision.

19. The is no indication that the level of MYC downregulation induced by TCF4 shRNA decreases MYC activity to a significant level. This could be done by RNA-sequencing, for example.

We agree with the reviewer and have provided qPCR data for several myc target genes to demonstrate the impact on myc transcriptional activity post-TCF4 shRNA induction (Supplemental Figure S12C).

20. Experiments shown in Figure 5 must also be done with MYC shRNA in these conditions since TCF4 is a known driver of ABC-DLBCL.

We agree with the reviewer and have generated these new cell line models and included the western blot and proliferation data in Supplemental Figure S12F,G. These data support the TCF4 specific regulation of c-myc in 18q amplified cell lines through the demonstration that inhibiting c-myc levels directly with a shRNA has broad activity in DLBCL models.

21. As mentioned earlier, data shown in panels Fig 6 c and d must be shown for all the patients in the cohort.

We thank the reviewer for the comments and have provided the data for the remainder of the patients in the cohort within Supplemental Figure 13-14.
22. No clear what non-A7 cases represent (as in Fig. 6d). This should be done comparing ABC-DLBCL patients with low TME infiltration that are non-A7.

We thank the reviewer for the suggestion. We have now included the remainder of the data from patients within the cohort. We were attempting to demonstrate a visual representation of the differences in tumor infiltration of immune cells between A7 and non-A7 clusters. In response to this specific question of how A7 tumors compare to other non-A7 ABC-DLBCL tumors with low immune infiltration, we have provided the data below. These data demonstrate from a visual inspection that there is equivalent lack of enrichment of immune cells in low TME infiltrated ABC-DLBCL patients that are not A7 defined as compared to tumors defined as A7. We have also included an ABC-DLBCL patient with high levels of immune cell infiltration (CD4+ T-cells in this case). These data were generated to further support the computational analysis provided in Figure 6B demonstrating there do exist ABC-DLBCL tumors with low immune infiltration, however this is only one angle of what defines a tumor as A7 as different genomic states will govern the malignant clones transcriptional output, providing another signal for differentiation between patient/tumor cluster calls.

23. The concept that A7 DLBCL tumors exhibit a less permissive tumor microenvironment is unclear and not supported by the data provided. Also, the âmechanismâ described to explain the lack of recruitment to the TME (Fig. 6d, middle panel) lacks references and probably does not apply to DLBCL.

We replaced âpermissiveâ with âimmune infiltratedâ in lines 271-272 to better reflect the situation in DLBCL as opposed to solid tumor. Multiple lines of evidence supported this statement including GSVA analysis showing reduced T cells, macrophages, NK cell populations in A7 (Fig 3A, Supplementary Fig 5), and orthogonal MIBI data corroborated this finding (Fig 6C, D and Supplementary Fig 13, 14).

We suspect that more than one mechanism may have contributed to the lack of recruitment to the TME and cited 3 references (Elpek 2007, God 2015; Han 2021). We also added the following to the manuscript (lines 304-306) âWe also hypothesize that chromosome 9p21 loss, which is known to be associated with cold tumors including B-cell neoplasms (Han, et al., 2021), may be one of the genetic drivers for low TME in A7. â

24. There is no clear mechanism by which A7 patients lack expression of MHC class I/II. Is this genetic? Epigenetic? How frequent is the single vs double loss? How these compare with non-A7 particularly other ABC-DLBCL with low tumor infiltration?

In A7-DLBCL patients within the MER cohort, there are 3 out of 34 patients (9%) with LOH MHC loss as measured by 6p loss and homozygous loss, which is not significantly different from the 19 out of 224 (8%) observed in non-A7. The majority of cases exhibiting loss of MHC-I genes also exhibit loss of MHC-II genes. As shown in Figure 3A, mRNA expression of class I/II molecules is lower in A7 patients compared to other clusters. The low expression of MHC I/II in A7 even without genomic loss of MHC genes suggests other mechanisms such as transcriptional regulation or epigenetic dysregulation.

Given this, it does not appear that lack of MHC I/II is primarily due to genomic loss of these genes. Ennishi et al have published that MHC loss is associated with EZH2 mutations, however A7 is predominantly ABC-DLBCL, in which EZH2 mutations are rare (3% of A7 is EZH2-mutated in MER, compared to 11% of non-A7).

25. Effect of lena on A7 vs. non-A7 should be done only in ABC-DLBCL with low tumor infiltration since is a know fact (now supported by several publications) that low tumor infiltration patients benefit by adding lenalidomide (or ibrutinib) to R-CHOP. Alternatively, this data on R2-CHOP vs. R-CHOP benefit should be shown for ABC-DLBCL with low immune infiltration vs. ABC-DLBCL with immune infiltration (for example by applying the LME classification as in Zhang et al and/or the EcoTyper as in Steen et al).

We thank the reviewer for these suggestions and have added some plots below. Our data corroborates the notion that patients with low tumor infiltration benefit from the addition of lenalidomide, showing that R2CHOP outperforms RCHOP in the TME26- group, but not in the TME26+ group.

We additionally stratified the TME26- subset of the ROBUST cohort (ABC-only) by A7 status and treatment arm and found similar results to the full population. A7 RCHOP still had worse outcome than any other group. There was a non-significant trend toward better survival in A7 R2CHOP over non-A7 R2CHOP, and the largest hazard ratio was between A7 R2CHOP and A7 RCHOP.

We do find that R2CHOP trends toward outperforming RCHOP in the general class of TME26- cases, but the effect is even stronger in the A7 subset of TME26- cases. Below are KM plots of treatment arm + A7 status in the TME26- population as well as the treatment effect alone in TME26-. There is no overall treatment effect in the full population regardless of TME status (HR=1.27), while the hazard ratio improves in the TME26- subpopulation (HR=1.45), and improves even further in A7 TME26- (HR=2.01) (we compare effect size point estimates rather than p-values due to varying sample size and power in the subpopulation comparisons).

26. Data in Fig 7A-B and 7C were not conducted in A7-like cell lines (as previously identified).

We agree with the reviewer and have re-performed the ChIP-seq in U2932 cells. The updated data are now available in Figure 7A. For Figure 7B-C, the antigen specificity of the T-cells required a cell line with HLA-A2 restriction. This constraint limited us to utilizing the RL cell line which had low MHC expression and were responsive to Len in modulation of expression for MHC class I/II genes. Again, this is to dissect a portion of the overall hypothesis that Len can mediate MHC upregulation through degradation of Aiolos/Ikaros.

27. Eu-Myc mice do not represent DLBCL an certainly do not represent A7-like tumors. This experiment should be conducted using a cell line in which the TME as well as genetic constitution of the lymphoma cell (or at least the key mechanism leading to decrease class I and II expression and MYC expression as well as TCF4 expression) represent A7-like tumors.

We agree with the reviewer that eu-myc do not represent A7 biology. We utilized this model for a variety

of reasons; overexpression of c-myc, lack of immune infiltrate once the tumor has been established (two important points highlighted within A7 tumors) and availability within our group for experimentation. We do not currently have access to additional models at this time, however, as this biology is an active research area for our group, we are exploring collaborations with academic investigators where we could evaluate additional aspects of the biology of new models being developed including PDX models. We hope the publication of this work will generate further interest in the community to collaboratively explore the intersection of disease biology with novel therapies in an effort to bring these forward to the benefit of patients.

Minor comments
1. Not clearly indicated what methodology/algorithm was implemented for TME cell deconvolution from bulk RNA-seq data.

We have provided references for the TME26 and ADAPTS deconvolution methodologies used for estimating TME (Danziger, S. A., Gibbs, D. L., Shmulevich, I., McConnell, M., Trotter, M. W., Schmitz, F., & ...Ratushny, A. V. (2019). ADAPTS: Automated deconvolution augmentation of profiles for tissue specific cells. PLoS One, 14(11), e0224693).

2. Order of panel/data: Figure 7 F is described before Figure 7A.

We have changed the text to reflect the figure ordering.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The authors have addressed all my comments

Reviewer #2

(Remarks to the Author)
My concerns have been addressed...

Reviewer #3

(Remarks to the Author)
This is a revised manuscript by Stokes, Wenzl, Huang, and collaborators on the identification of a transcriptional subgroup of DLBCL, termed A7, that exhibit up-regulation of MYC signatures (and TCF4 and 18q21) and low immune TME infiltration. Several other groups previously described transcriptional and functional DLBCL subgroups with highly overlapping features with A7, some of which have use the same subset of patients. The authors responded most of the issues raised by reviewers with additional bioinfomatic and statistical associations rather than biological and therapeutic mechanistic data. For most, the biological and therapeutic characterization of the A7 subgroup has been conducted using a variety of cell lines, human and mouse, and mouse models, each adding a piece of information (some models had TME, other do not, some are MCD but others not, some had MYC overexpression, others TCF4, others HLA expression, etc, etc) that together with the overlapping transcriptional characterization of DLBCL patient subsets (from several small datasets) question the notion that A7 is a distinct biological and clinical DLBCL subgroup. Whether the A7 identification could be applied prospectively in patients beyond datasets and whether it truly represents a subset of lymphomas amenable of, for example, preclinical modeling remains to be elucidated.