nature portfolio

Peer Review File

Improved overall survival in an anti-PD-L1 treated cohort of newly diagnosed glioblastoma patients is associated with distinct immune, mutation, and gut microbiome features: a single arm prospective phase I/II trial

Corresponding Author: Dr Shiao-Pei Weathers

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)
This is a clinical trial for GBM with 60 patients with multi-modal therapy including chemo-radiation and atezo (PDL1 inhibitor). Then, multi-modal data was collected to further understand treatment response.

The study is clearly presented, and reports interesting findings in the context of mutation profiles (EGFR vs PTEN), immune cell infiltration and preliminary findings in the context of microbiome. In particular two biomarkers stand out: EGFR, and immune infiltration and only are predictive of overall survival in the context of PDL1 inhibition. Validation in two external data sets (TCGA & GLASS) shows that this difference is not there when not treated with PDL1 inhibitors. Overall this is a great study that has uncovered interesting new biomarkers for treatment response and a potential path for immunotherapy use in GBM, in particular in the mesenchymal subtype which the authors show are enriched in immune infiltration and have better OS.

Since the trial included a small set of IDH-mutant cases, to the best of my knowledge, these are now not defined as GBM anymore per updated WHO criteria? Anyhow, it would be great to better specify this in the introduction that some IDH mutant cases were included.
Where these “secondary GBMs” or what was the rationale for including these, if the trial was focused only on “molecular GBM”?

Reviewer #2

(Remarks to the Author)
There is a significant need to define biomarkers that may predict the response to checkpoint immunotherapy in patients with glioblastoma. In the manuscript by Weathers et al., the authors report findings from a phase 1 and phase 2 clinical trial of glioblastoma patients treated with anti-PD-L1 checkpoint immunotherapy in combination with standard care (radiation therapy and temozolomide). They used a multiomics approach such as whole exome sequencing (WES), transcriptomics, and multiplex immunofluorescence imaging on the pretreated biopsy tissue to define features that may predict response to anti PD-L1 checkpoint therapy. Analysis of the transcriptome, microbiome, exome sequencing, and multiplex imaging revealed distinct immune microenvironments, microbiome (absence of Bacteroides. thetaiotaomicron), and tumor genetics (PTEN mutation) in patients that responded to anti-PDL-1 checkpoint therapy in combination with standard care. The manuscript by Weathers et al., which is well-organized and clearly written, could be accepted for publication with the following revisions.

Comments:

1)"In Figure 4B and 4C, the immune features predictive of responders vs. non-responders could be described in greater depth. Could the authors elaborate on these features and validate them with other techniques such as multiplex imaging? It

appears the authors used only CD8 and CD3 markers for immunofluorescence to define cytotoxic T cells. Could the authors delve deeper into T cell subsets by examining Granzyme, Perforin, FOXP3, CD8, CD4, and cytotoxic markers? Similarly, the polarization, activation, and subsets of myeloid cells could be better defined at the transcriptome and imaging levels, as described in the paper by Lee et al., Nature Comm. 12, 6938 (2021).

2)It would be interesting if the study could illustrate how tumor molecular subtypes, such as PTEN and EGFR mutations, correlate with immune features. Do patients with PTEN versus EGFR mutations have particularly distinct tumor microenvironments evident in baseline tumor specimens?

3)What is the status of PD-L1 expression in responders compared to non-responders in anti-PD-L1 immunotherapy, as it isn't reported? Could PD-L1 expression be presented using IHC or multiplex imaging? Is there a correlation between anti-PDL1 therapy and PD-L1 expression in the tumor? Which cell types of the tumor microenvironment express PD-L1 in the brain tumor microenvironment? How do tumor mutations, like PTEN and EGFR, correlate with PD-L1 expression? Is PD-L1 expression upregulated after temozolomide treatment?

4)The correlation of the microbiome with the response to immunotherapy is very interesting. It would be intriguing to demonstrate how the microbiome correlates with immune features and molecular subtypes.

5)The Methods section should be revised to include a description of how the microbiome is analyzed in the paper. Additionally, in the Materials and Methods section, the treatment schedule, and the route of treatment for the therapy should be detailed.

6)There is no information about the sequencing data. Has the data already been deposited in a public server?

7)It would be interesting to see if any pretreatment blood parameters (cytokines, immune cell types) correlate with survival, molecular subtypes of the tumor, and the microbiome.

Reviewer #4

(Remarks to the Author)
In this manuscript, the authors investigate biomarkers of response to anti-PDL1 immunotherapy in glioblastoma patients and their results seem to indicate that several markers across different omics identify patients with longer survival after anti-PDL1 therapy. Glioblastoma is a devastating disease with very poor prognosis, and improving the detection of those patients with higher response to treatments is essential. It is a comprehensive, multiomics study that has a potential clinical interest. I have a handful of comments aiming at helping the authors improve their manuscript.

My first comment pertains to the conflicts of interest. In the disclosure of DOI the authors declare none, but two authors are affiliated with Genentech, which is also the support listed in the acknowledgements. This looks like a conflict of interest to me and should be clarified.

One global comment pertains to the writing, that largely tacitly assume some significant background knowledge on the terminology and concepts. Nature Communication is a multidisciplinary journal, and it is anticipated that many readers are not in the oncology field. Examples include the acronyms that are not defined or not defined at first use (ex. MGMT, PDL-1, IDH). Definitions of acronyms should be associated with explanation of their biological importance (ex. The importance of MGMT status in drug resistance; the general reminder overview of a sentence on the principle of PDL-1 immunotherapy). Authors should also define what an ”exhausted T cell” is.

The authors should always use the term “statistically” alongside “significance”, owing to the double meaning of the concept of significance in plain English. This is a confusion that is present throughout the text, for example line 342 the authors claim that “Patients with cluster 1 tumors trended toward achieving a better mOS ($p = 0.076$, HR = 0.548 (0.279, 1.074)) than those in cluster 2”. Here the hazard ratio is biologically significant indicating that the patients did achieve a better mOS, but not statistically significant. Similarly, in Figure 6, GTR or STR/Bx:STR seems to have a biologically significant effect size (HR 0.84-16.76) but that fails to achieve statistical significance, which may indicate failure to statistically identify an important effect due to lack of statistical power. P-values do not indicate the probability of the null hypothesis and do not inform about the magnitude of the effect. Similar sentences should be re-written throughout the text.

Throughout the text, effect sizes (eg. HR, Cohen’s d) should always be disclosed together with the p-values. P-values are only (an incomplete) part of the

Again, I have several comments regarding the statistical aspect of the study. The value of the false discovery rate is not specified in the methods. In the “Estimate score and immune deconvolution” paragraph of the methods, which t-test was used? Two independent Student’s t test? Welch test? Were the parametric assumptions verified and how? (In Fig 3B-C it seems that parametric assumptions of normality and homoscedasticity might possibly be violated). The level of confidence should be disclosed (usually conventionally 5% but it should be disclosed).

In the “Unsupervised cluster-based analysis…” section of the results (Figure 2), the fact that the 2 clusters in Fig 2A-C and the two clusters in Fig 2D-E are all called cluster 1 and 2 is a little confusing. In this figure again, there is a discrepancy between the HR given in the text (0.548 (0.279-1.074)) and in the figure (1.826(0.931-3.583)), which are clearly linked by an inverse relationship and an opposite definition of the HR. This should be clarified.

There is an important mismatch between Fig 6A and the text related to it. The HR, CI and p-values disclosed in the text do not correspond to those in the figure. Just like the authors are reporting results from another experiment.

In figure 2 and 3, the colour selection is very confusing with sex classification ending up with the same colour code as mutation status. It would be helpful to change that.

The sources (providers, catalogue numbers, species) of antibodies used for multiplex immunofluorescence should be provided.

The authors should harmonise how they report confidence intervals throughout the text.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
All my comments have been addressed.

Reviewer #2

(Remarks to the Author)
In the revised manuscript, Weathers et al. present additional data that strengthen their findings. Their Phase I/II clinical trial suggests novel genetic, immune, and microbiome biomarkers that could help identify GBM patients who are more likely to respond to immunotherapy. Overall, the manuscript is well-written, and the revisions successfully address the reviewers' concerns. It can be accepted for publication in its current form.

Reviewer #4

(Remarks to the Author)
The authors made a clear and significant effort to change the manuscript according to the many reviewers’ comments, in particular mine. Acronyms are defined, statistical significance is used as one undivided idiom, effect sizes are reported, and disclosure of confidence intervals harmonised and statistics in Fig6A edited. They must be congratulated for that.

However, I sincerely think there is still an issue with the disclosure of conflicts of interests. The authors claim to have added a clear statement on the COI regarding the authors employed by Genentech. But I could not find such a statement. Nature Communications defines employment as a financial COI (see https://www.nature.com/ncomms/editorial-policies/competing-interests). Disclosure is mandatory.

Regarding comments made by the other reviewers, it seems that in the Data Availability section, the EGA reference number has not been really added (I guess the text highlighted in yellow is where the authors wanted to insert the number, but without actually doing it).

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

This is a clinical trial for GBM with 60 patients with multi-modal therapy including chemo-radiation and atezo (PDL1 inhibitor). Then, multi-modal data was collected to further understand treatment response.

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Where these “secondary GBMs” or what was the rationale for including these, if the trial was focused only on “molecular GBM”?

We thank the Reviewer for the opportunity to clarify in the manuscript the reason for the inclusion of a small number of IDH mutant-cases. At the time of trial conception in 2016, the WHO 2016 criteria was used in determination of patient eligibility with glioblastoma (GBM). The WHO 2016 criteria permitted IDH mutant GBM cases to be classified as GBM. This was described in the results in the initial manuscript and additional text has been added to make it clearer why the IDH mutant cases were initially included but ultimately excluded for the analyses.

Reviewer #2 (Remarks to the Author):

There is a significant need to define biomarkers that may predict the response to checkpoint immunotherapy in patients with glioblastoma. In the manuscript by Weathers et al., the authors report findings from a phase 1 and phase 2 clinical trial of glioblastoma patients treated with anti-PD-L1 checkpoint immunotherapy in combination with standard care (radiation therapy and temozolomide). They used a multiomics approach such as whole exome sequencing (WES), transcriptomics, and multiplex immunofluorescence imaging on the pretreated biopsy tissue to define features that may predict response to anti PD-L1 checkpoint therapy. Analysis of the transcriptome, microbiome, exome sequencing, and multiplex imaging revealed distinct immune microenvironments, microbiome (absence of Bacteroides. thetaiotaomicron), and tumor genetics (PTEN mutation) in patients that responded to anti-PDL-1 checkpoint therapy in combination with standard care. The manuscript by Weathers et al., which is well-organized and clearly written, could be accepted for publication with the following revisions.

Comments:

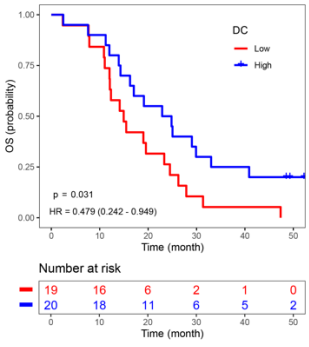
1)"In Figure 4B and 4C, the immune features predictive of responders vs. non-responders could be described in greater depth. Could the authors elaborate on these features and validate them with other

techniques such as multiplex imaging? It appears the authors used only CD8 and CD3 markers for immunofluorescence to define cytotoxic T cells. Could the authors delve deeper into T cell subsets by examining Granzyme, Perforin, FOXP3, CD8, CD4, and cytotoxic markers? Similarly, the polarization, activation, and subsets of myeloid cells could be better defined at the transcriptome and imaging levels, as described in the paper by Lee et al., Nature Comm. 12, 6938 (2021).

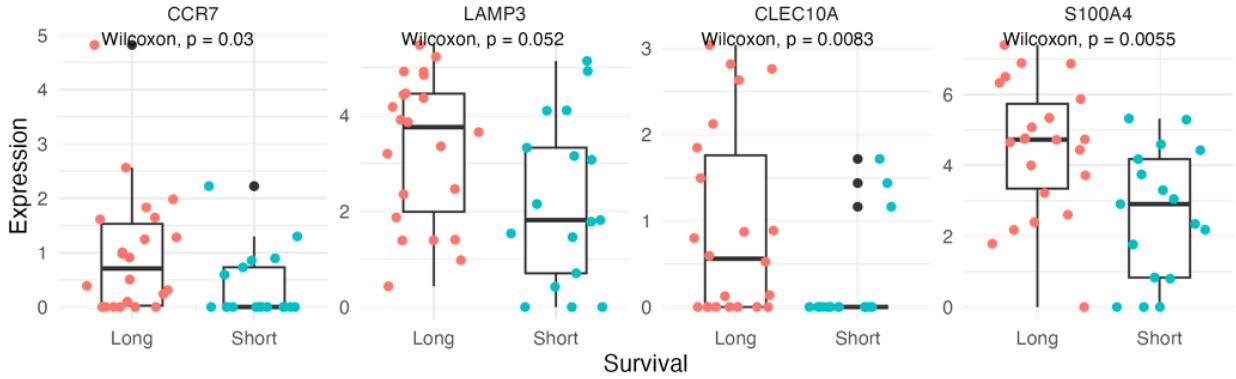
We thank the Reviewer for suggesting a more in-depth description of the immune features predictive of clinical outcome. The examination of T cell subsets using immunofluorescence markers for CD45RO+, FOXP3+, Granzyme B+ did not reveal levels of subsets expressing these markers to be statistically associated with differences in OS following treatment. Likewise, CD68+ cell density by immunofluorescence did not trend with either low or higher OS. Further marker exploration of clinically predictive markers by immunofluorescence was not possible due to limited tissue availability.

We used the transcriptomic data to further analyze myeloid cells, as described in the paper by Lee et al., Nature Comm. 12, 6938 (2021). Using the 11 scRNAseq subclusters of myeloid cells described in Lee, et al. we performed gene set enrichment analysis to derive a score for each myeloid subcluster type for each patient tumor sample. We then employed Kaplan-Meier analysis for each subcluster type separating patients by low vs. high myeloid subcluster values. Upon assessment, only the dendritic cell "DC" cluster demonstrated a statistically significant OS difference (p=0.031, HR=0.479, CI= 0.242 – 0.949). Patients with tumors possessing a high DC score displayed an OS benefit compared to those with a low DC score (see Figure below). These data are consistent with our determination that activated myeloid dendritic cells make up a statistically significant fraction of more highly immune infiltrated GBM tumors, which corresponds with better OS (Figure 4b). Lee et al.'s other macrophage and monocyte subclusters did not show a statistically significant difference in low vs. high OS in our cohort. It is certainly possible that the higher degree of heterogeneity inherent in the bulk tumor samples upon which we performed RNAseq did not expose differences that a scRNAseq approach would reveal.

Myeloid Cluster: Dendritic Cell "DC"



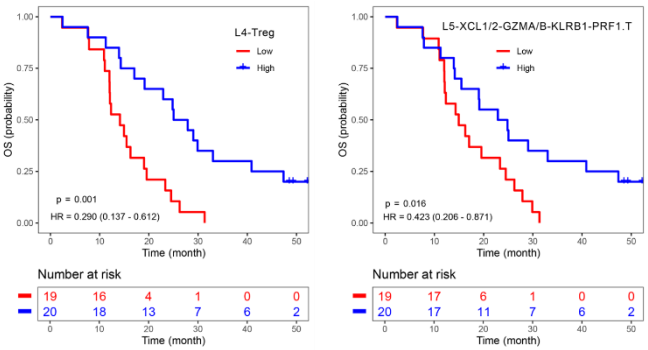
Lee, et al. further clustered the scRNAseq DC population, resulting in 9 subclusters, and performed pseudotime trajectory analysis resulting in 9 distinct cell fates. Notable from this analysis was the identification of an activated and/or migratory DC cluster characterized by elevated CCR7 and LAMP3. Further, the cell fates in their study following neoadjuvant anti-PD1, showed migratory CCR7+ DC populations to be increased following neoadjuvant treatment. Thus, we examined levels of activated/migratory DC markers and identified CCR7 and LAMP3, as well as, other markers (e.g., CLEC10A and S100A4) to be more highly expressed in the tumors of patients experiencing higher OS in our cohort.



These data are consistent with our identification of activated/migratory myeloid dendritic cells accounting for a statistically significant fraction of more highly immune infiltrated GBM tumors, which corresponds with better OS after anti-PD-L1 regimen treatment in our study. These observations may also support the inference that a subset of patients with primary GBM tumors more likely to benefit from anti-PD-L1 treatment have specific dendritic cell features akin to those identified after neoadjuvant anti-PD-L1 treatment by Lee et al., and therefore may be "primed" for anti-PD-L1 treatment in the primary GBM setting.

Provided the robust insights gleaned from the myeloid subclustering approach, we also used Lee at al.'s clustering outputs for lymphoid cells, and the further subclustering results for CD4 and CD8 lymphocytes to characterize the lymphoid profile in our cohort. Like the myeloid analysis, we used the 11 scRNAseq subclusters of lymphoid cells described in Lee, et al. to perform gene set enrichment analysis to derive a score for each lymphoid subcluster type for each patient tumor sample. We then employed Kaplan-Meier analysis for each subcluster type separating patients by low vs. high lymphoid subcluster values. Upon assessment, only the L4 and L5 lymphoid clusters demonstrated a statistically significant OS difference (L4, p=0.001, HR=0.290, CI= 0.137 – 0.612; L5, p=0.016, HR=0.423, CI= 0.206 – 0.871). Patients with tumors possessing a high L4 or L5 score displayed an OS benefit compared to those with a low scores (see Figure below).

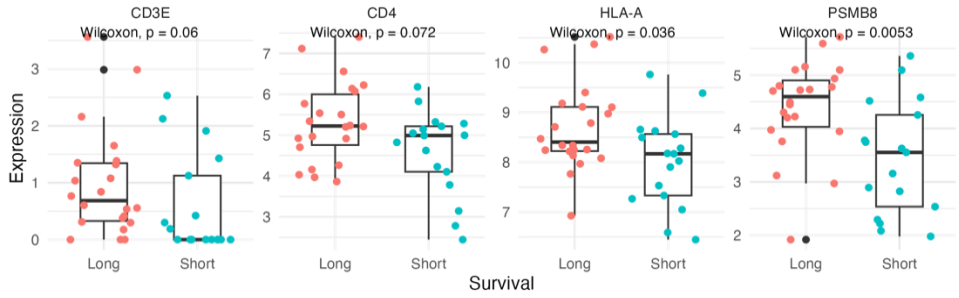
Lymphoid Clusters: L4 and L5



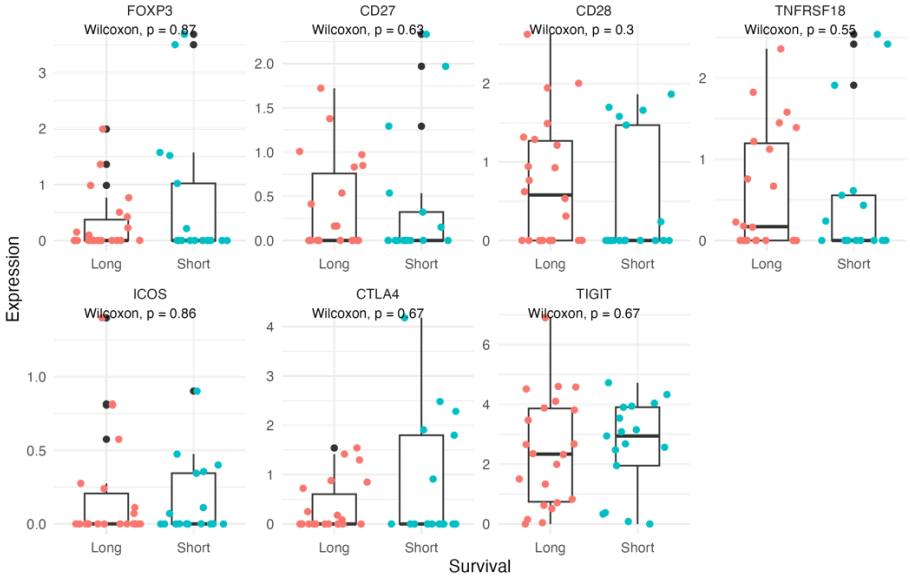
Although the L4 cluster is designated "Treg," a more granular analysis of the genes within this set reveals the expression of lymphocyte markers and antigen presenting genes (e.g., CD3E, CD4, HLA-A, PSMB8) to be more elevated in our cohort of GBM tumors associated with a higher OS, while the expression of multiple inhibitory genes within the L4 cluster gene set (e.g., FOXP3, CD27, CD28,

TNFRSF18, ICOS, CTLA4, TIGIT) show no statistically significant difference between GBM tumors associated with a lower vs. higher OS in our cohort.

L4: Lymphocyte and Antigen Presentation Genes:

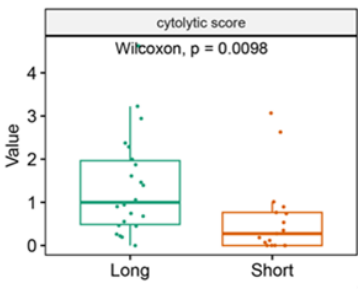


L4 Cluster: Immune Inhibitory Genes

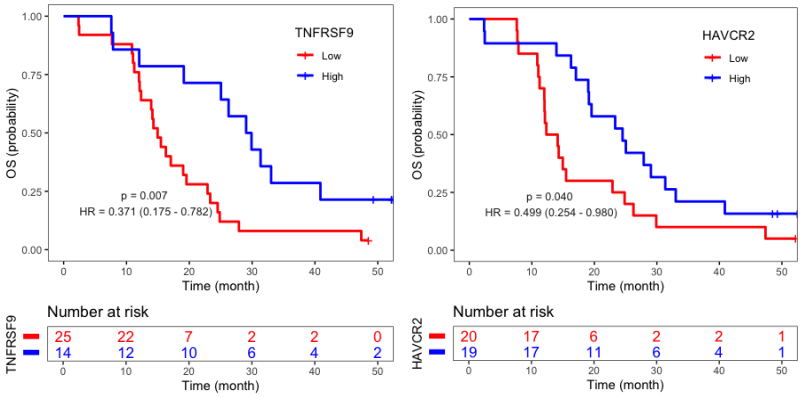


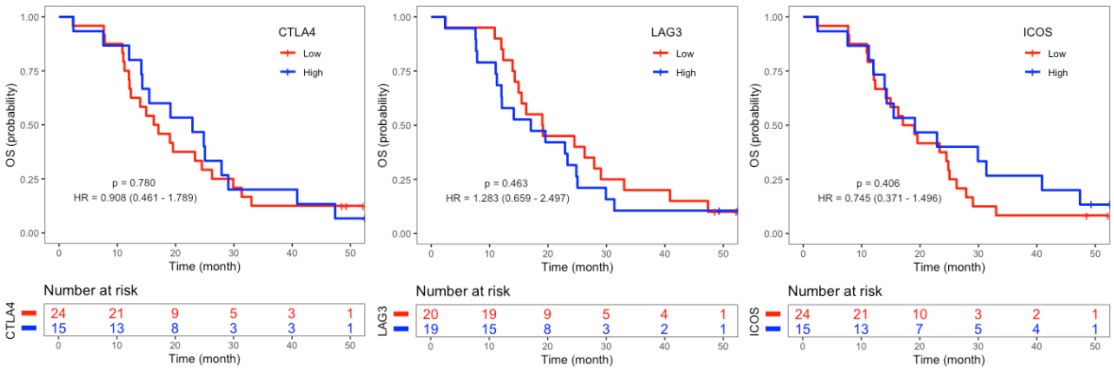
The L5 cluster is designated “cytolytic.” Patients in our cohort with tumors enriched in the expression of L5 cluster genes exhibited a longer survival benefit. This is consistent with our observation of a higher cytolytic score (geometric mean of GZMA and PRF1 gene expression, p=0.0098) being statistically significantly higher in the tumors of patients with greater OS, as shown in Figure 3c of the main text

Manuscript Figure 3C:



Finally, we observed that the CD4+ effector memory T cell profile accounted for a statistically significant fraction of more highly immune infiltrated GBM tumors, which corresponds with better OS after treatment with the anti-PD-L1 regimen in our study (Figure 4a). Lee et al.'s subclustering of the CD4+ T cell compartment, also contained multiple genes associated with the immune inhibitory system [e.g., CTLA4, PDCD1, LAG3, ICOS, TNFRSF9 (aka, 4-1BB), HAVCR2 (aka, TIM-3)]. Of these, only patients with pre-treatment tumors having either higher expression of TNFRSF9 or HAVCR2 exhibited a statistically significant difference in OS (see Figures below). In the context of CD4+ T cells, TNFRSF9 propagates a signal that enhances cell proliferation, survival and anti-tumor cytokine release. HAVCR2, however, may serve as a target in addition to PD-L1. This possibility is surfaced in the Discussion section of the manuscript.





Given the use of Lee et al.'s more granular subsetting of the myeloid and lymphoid cells on tumor data from our cohort, we have now added the following statement under the section heading **Highly immune infiltrated GBM tumors are associated with specific immune cell types, the mesenchymal GBM subtype, and fecal microbiome profiles**, "A recent study by Lee et al., using scRNAseq to interrogate immune cell subsets in GBM identified 11 subclusters of myeloid cells. We leveraged these subcluster transcription profiles to analyze our cohort samples. Notably, the dendritic cell "DC" subcluster profile demonstrated a statistically significant mOS difference (Supplemental Figure 3A), consistent with the immune deconvolution we performed (Figure 4B) identifying activated myeloid dendritic cells to make up a statistically significant fraction of more highly immune infiltrated tumors, which corresponded with a better OS. Further subset analysis by Lee et al., identified a CCR7+ LAMP3+ "activated and/or

migratory" DC subpopulation to be upregulated following neoadjuvant anti-PD-L1. We identified these two markers to be higher in tumors associated with increased OS in our cohort (Supplemental Figure 3B). These data suggest that the dendritic cell profile of primary GBM pretreatment tumors associated with a improved clinical outcome may be better primed for effective anti-PD-L1 treatment."

"We also leveraged the 11 scRNAseq derived subclusters composing the lymphoid cell compartment of GBM to assess pretreatment tumors in our cohort. Among these lymphoid subclusters, only L4 and L5 were associated with better OS in our cohort (Supplemental Figure 3C). A granular analysis of the genes within the L4 set revealed the expression of lymphocyte markers and antigen presenting genes (e.g., CD3E, CD4, HLA-A, PSMB8) to be more elevated in our cohort of GBM tumors associated with a higher OS, while the expression of multiple inhibitory genes (e.g., FOXP3, CD27, CD28, TNFRSF18, ICOS, CTLA4, TIGIT) showed no statistically significant difference. A more detailed examination of the genes within the L5 set revealed the expression of cytolytic enzyme genes, consistent with the cytolytic score (geometric mean of GZMA and PRF1 gene expression)(Figure 3C), to be statistically higher in the tumors of patients in our study with greater OS. The CD4 compartment markers have multiple genes associated with the immune inhibitory system (e.g., CTLA4, PDCD1, LAG3, ICOS, TNFRSF9 (aka, 4-1BB), HAVCR2 (aka, TIM-3)). Of these, TNFRSF9 and HAVCR2 were more elevated in the tumors of patients who demonstrated a longer OS, whilst no statistically significant expression difference was observed in any of the other immune checkpoint genes (Supplemental Figure 3D)."

2) It would be interesting if the study could illustrate how tumor molecular subtypes, such as PTEN and EGFR mutations, correlate with immune features. Do patients with PTEN versus EGFR mutations have particularly distinct tumor microenvironments evident in baseline tumor specimens?

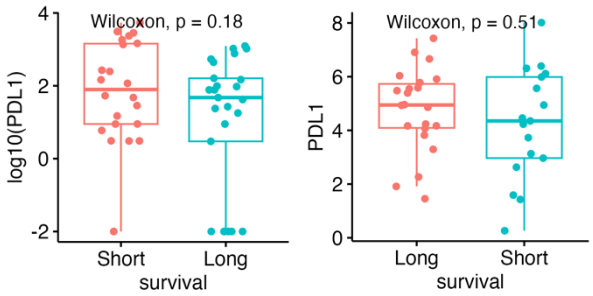
We thank the Reviewer for surfacing this important question regarding a potential linkage between the distinct tumor microenvironments and PTEN versus EGFR mutation in the baseline tumor specimens of our cohort. An evaluation of the multiplex immunofluorescence data failed to reveal any statistically significant associations between the density measures of any immune features and PTEN versus EGFR mutations across the tumor samples of the cohort.

We further evaluated the transcriptional immune features we highlighted in the submitted manuscript (EIS and the deconvoluted cell types), as well as, the immune profiles from Lee et al. (myeloid – dendritic cell; dendritic cell activated – CCR7 and LAMP3; and lymphoid – L4, L5) for association with either PTEN versus EGFR mutation in the tumors of patients in our cohort. Neither the immune profiles we generated (EIS, deconvolution), nor the immune profiles from the Lee et al. paper, were significantly associated with PTEN versus EGFR mutation.

3) What is the status of PD-L1 expression in responders compared to non-responders in anti-PD-L1 immunotherapy, as it isn't reported? Could PD-L1 expression be presented using IHC or multiplex imaging? Is there a correlation between anti-PDL1 therapy and PD-L1 expression in the tumor? Which cell types of the tumor microenvironment express PD-L1 in the brain tumor microenvironment? How do tumor mutations, like PTEN and EGFR, correlate with PD-L1 expression? Is PD-L1 expression upregulated after temozolomide treatment?

We examined the PD-L1 expression in tumors of patients achieving longer versus shorter OS. PD-L1 expression by IHC (VENTANA PD-L1 (SP263) Assay) at thresholds of >1% or >5%, was not associated with

clinical outcome. Neither PD-L1 protein expression by multiplex immunofluorescence nor transcript level by RNAseq were associated with overall survival outcome (See Figure below).



A more granular assessment of the cell types within tumors showed that PD-L1 is expressed on GFAP+, CD3+ and CD68+ cells. However, we observed that among the major cell types, patients whose tumors harbored a higher percentage of cytotoxic T cells (CD3+CD8+) expressing PD-L1 had a greater OS (p=0.030, HR=0.439 (0.205-0.940)). This was not the case for either GFAP+, CD68+ or CD3+ (non-CD8+) cells. We have added a statement to this effect under the section heading **Highly immune infiltrated GBM tumors are associated with specific immune cell types, the mesenchymal GBM subtype, and fecal microbiome profiles** and the associated Kaplan-Meier graph as Figure 4D, replacing the prior supplemental Figure 3A: "Patients whose tumors had a higher percentage of cytotoxic (CD3+CD8+) T cells that expressed PD-L1 achieved a longer mOS (p=0.030, HR=0.439 (0.205-0.940)) (Figure 4D), not observed in PD-L1 expressing GFAP+, CD68 + or CD3+CD8- cells."

The fraction of PD-L1 expressing cells, assessed by individual cell type or in total, were not statistically correlated with PTEN or EGFR mutation status.

Although derived from a small number of patients, there was no clear trend in the change of PD-L1 abundance by either IHC or multiplex immunofluorescence following temozolomide treatment (n=10, n=11, patients with on- vs. pre-treatment samples, respectively)

4) The correlation of the microbiome with the response to immunotherapy is very interesting. It would be intriguing to demonstrate how the microbiome correlates with immune features and molecular subtypes.

We recognize that the relatively low number of microbiome samples compared to tumor samples available in this study cohort makes observations gleaned from an analysis only hypothesis generating. However, carrying forward the Lee et al. myeloid and lymphoid scRNAseq subcluster profiles from GBM tumors, we tested for any correlative relationships between the fecal microbiome bacterial species and specific tumor immune profiles. Bacteroides thetaiotaomicron, the only species whose prevalence was associated with either a low tumor immune score or low overall survival, was significantly anti-correlated with the lymphoid L4 and L5 subclusters, as well as, the myeloid dendritic cell subcluster (ρ = -0.77, -0.88 and -0.56, respectively). An inverse relationship was observed with the fecal bacterial species with the highest prevalence in patients with high tumor immune scores or greater overall survival (viz, Agathobaculum butyriciproducens, Dorea formicigenerans, Roseburia inulinivorans, Roseburia intestinalis). We are pursuing further microbiome studies in patients with GBM tumors to better characterize and understand the relationships between host, tumor and microbiome features.

	Bacteroides thetaiotaomicron		Agathobaculum butyriciproducens		Dorea formicigenerans		Roseburia inulinivorans		Roseburia intestinalis	
	p	ρ	p	ρ	p	ρ	p	ρ	p	ρ
Lymphoid cells: L4 cluster	0.0025	-0.77	0.03	0.67	0.039	0.68	<0.001	0.87	0.0013	0.8
Lymphoid cells: L5 cluster	<0.001	-0.88	0.092	0.56	ns	ns	0.004	0.74	<0.001	0.86
Myeloid cells: DC cluster	0.05	-0.56	0.016	0.74	0.039	0.65	0.0014	0.81	<0.001	0.89

p = p-value; ρ = Spearman's rho

5) The Methods section should be revised to include a description of how the microbiome is analyzed in the paper. Additionally, in the Materials and Methods section, the treatment schedule, and the route of treatment for the therapy should be detailed.

We thank the Reviewer for this comment. The Methods section has been revised to include a description of the microbiome analysis as well as details surrounding the treatment the patients received.

6) There is no information about the sequencing data. Has the data already been deposited in a public server?

In accordance with the policy of Nature Communications, we are uploading the sequencing data to the European Genome-phenome Archive (EGA)

7) It would be interesting to see if any pretreatment blood parameters (cytokines, immune cell types) correlate with survival, molecular subtypes of the tumor, and the microbiome.

We thank the Reviewer for this comment. We fully agree that analysis of the blood parameters would be very interesting. This is currently an ongoing separate project that has been undertaken in collaboration with another academic institution. This subproject work is planned to be reported out as a separate manuscript.

Reviewer #4 (Remarks to the Author):

In this manuscript, the authors investigate biomarkers of response to anti-PDL1 immunotherapy in glioblastoma patients and their results seem to indicate that several markers across different omics identify patients with longer survival after anti-PDL1 therapy. Glioblastoma is a devastating disease with very poor prognosis, and improving the detection of those patients with higher response to treatments is essential. It is a comprehensive, multiomics study that has a potential clinical interest. I have a handful of comments aiming at helping the authors improve their manuscript.

My first comment pertains to the conflicts of interest. In the disclosure of DOI the authors declare none, but two authors are affiliated with Genentech, which is also the support listed in the acknowledgements. This looks like a conflict of interest to me and should be clarified.

We thank the Reviewer for improving our manuscript and pointing out this very important disclosure. A disclosure of DOI for Katja Schulze and Walter C. Darbonne, both from Genentech, has now been made clear. The trial was supported by the MD Anderson and Genentech Rare Tumor Alliance. Katja and Walter from the Genentech team participated in our scientific discussions regarding the correlative work that was performed and reviewed the final manuscript.

One global comment pertains to the writing, that largely tacitly assume some significant background knowledge on the terminology and concepts. Nature Communication is a multidisciplinary journal, and it is anticipated that many readers are not in the oncology field. Examples include the acronyms that are not defined or not defined at first use (ex. MGMT, PDL-1, IDH). Definitions of acronyms should be associated with explanation of their biological importance (ex. The importance of MGMT status in drug resistance; the general reminder overview of a sentence on the principle of PDL-1 immunotherapy). Authors should also define what an ”exhausted T cell” is.

The Reviewer makes an excellent point. The definitions of acronyms and terms, as well as, an explanation of their biological importance has now been incorporated into the manuscript.

The authors should always use the term “statistically” alongside “**significance**”, owing to the double meaning of the concept of significance in plain English. This is a confusion that is present throughout the text, for example line 342 the authors claim that “Patients with cluster 1 tumors trended toward achieving a better mOS (p = 0.076, HR = 0.548 (0.279, 1.074)) than those in cluster 2”. Here the hazard ratio is biologically significant indicating that the patients did achieve a better mOS, but not statistically significant. Similarly, in Figure 6, GTR or STR/Bx:STR seems to have a biologically significant effect size (HR 0.84-16.76) but that fails to achieve statistical significance, which may indicate failure to statistically identify an important effect due to lack of statistical power. P-values do not indicate the probability of the null hypothesis and do not inform about the magnitude of the effect. Similar sentences should be re-written throughout the text.

We have changed the wording of ‘significant’ to ‘statistically significant’ where significance was determined by a statistical test and removed the terms ‘significant’ or ‘significance’ when not associated with a statistical test..

Throughout the text, **effect sizes (eg. HR, Cohen’s d)** should always be disclosed together with the p-values. P-values are only (an incomplete) part of the

The effect size has now been added with the p-values throughout the text.

Again, I have several comments regarding the statistical aspect of the study. The value of the **false discovery rate** is not specified in the methods. In the “Estimate score and immune deconvolution” paragraph of the methods, **which t-test** was used? Two independent Student’s t test? Welch test? Were the parametric assumptions verified and how? (In Fig 3B-C it seems that parametric assumptions of normality and homoscedasticity might possibly be violated). The level of confidence should be disclosed (usually conventionally 5% but it should be disclosed).

The FDR-corrected p-value significance threshold of 0.05 is now specified, the use of the non-parametric Wilcoxon t-test in the “Estimate score and immune deconvolution” paragraph and the level of confidence is made clear in the manuscript.

In the “Unsupervised cluster-based analysis…” section of the results (Figure 2), the fact that the 2 clusters in Fig 2A-C and the two clusters in Fig 2D-E are all called cluster 1 and 2 is a little confusing. In this figure again, there is a discrepancy between the HR given in the text (0.548 (0.279-1.074)) and in the figure (1.826(0.931-3.583)), which are clearly linked by an inverse relationship and an opposite definition of the HR. This should be clarified.

We changed the unsupervised mutation cluster names to cluster A and cluster B and corrected the discrepancy between the HR in the text and figure.

There is an important mismatch between Fig 6A and the text related to it. The HR, CI and p-values disclosed in the text do not correspond to those in the figure. Just like the authors are reporting results from another experiment.

We appreciate the Reviewer pointing out this mismatch. The HR, Cl and p-values of the text and related Figure 6A are now corrected.

In figure 2 and 3, the **colour selection** is very confusing with sex classification ending up with the same colour code as mutation status. It would be helpful to change that.

The color selections in Figures 2 and 3 have been changed to avoid any confusion between sex classification and mutation status.

The sources (providers, catalogue numbers, species) of antibodies used for multiplex immunofluorescence should be provided.

The providers, catalogue numbers and species of the antibodies used for the multiplex immunofluorescence are now provided in Supplemental Table 2.

The authors should harmonise how they report confidence intervals throughout the text. Confidence intervals.

The reporting of confidence intervals is now harmonized throughout the manuscript.

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

All my comments have been addressed.

Reviewer #2 (Remarks to the Author):

In the revised manuscript, Weathers et al. present additional data that strengthen their findings. Their Phase I/II clinical trial suggests novel genetic, immune, and microbiome biomarkers that could help identify GBM patients who are more likely to respond to immunotherapy. Overall, the manuscript is well-written, and the revisions successfully address the reviewers' concerns. It can be accepted for publication in its current form.

Reviewer #4 (Remarks to the Author):

The authors made a clear and significant effort to change the manuscript according to the many reviewers' comments, in particular mine. Acronyms are defined, statistical significance is used as one undivided idiom, effect sizes are reported, and disclosure of confidence intervals harmonised and statistics in Fig6A edited. They must be congratulated for that.

However, I sincerely think there is still an issue with the disclosure of conflicts of interests. The authors claim to have added a clear statement on the COI regarding the authors employed by Genentech. But I could not find such a statement. Nature Communications defines employment as a financial COI (see https://www.nature.com/ncomms/editorial-policies/competing-interests). Disclosure is mandatory.

Thank you for the comment. We have added the following language to the disclosures in the manuscript for clarification. KS and WCD are members of the MD Anderson Genentech Joint Steering Committee and provided input on the design of the translational research as well as review of data and their scientific interpretation. KS and WCD participated in funding decisions for the translational research and manuscript review.

Regarding comments made by the other reviewers, it seems that in the Data Availability section, the EGA reference number has not been really added (I guess the text highlighted in yellow is where the authors wanted to insert the number, but without actually doing it).

Thank you for this comment. An unexpected delay occurred with acquisition of the EGA reference number which has since been resolved. The EGA dataset accession ID is EGAD50000001154 has been added to the manuscript. The expected release date is 12/26/24.