Neoadjuvant anti-PD-1 immunotherapy promotes a survival benefit with intratumoral and systemic immune responses in recurrent glioblastoma

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Glioblastoma, with an incidence of 3.2 per 100,000 population, is the most common primary malignant central nervous system tumor and carries an abysmal 3-year survival rate of just 10.1% (ref. 1). The median progression-free survival in primary glioblastoma is 6.9 months, and median overall survival is 14.6 months with standard care surgery, radiation therapy and temozolomide 2. In recurrent glioblastoma, median overall survival is an estimated 24–44 weeks 3–5. New therapies are needed for patients diagnosed with this type of cancer.

Among the array of cancer immunotherapeutics available, PD-1 monoclonal antibody blockade has yielded promising results in patients with metastatic cancer 6–12. PD-1 inhibition is thought to disrupt the engagement of PD-1 with its inhibitory ligands, spurring cytotoxic T cell–mediated tumor elimination 11,13. Pembrolizumab, an anti-PD-1 monoclonal antibody, has demonstrated benefit as monotherapy in multiple cancer types 14–17, but primarily in the adjuvant setting 17. However, a preclinical metastatic breast cancer study suggested that neoadjuvant immune checkpoint inhibition could generate enhanced and sustained antitumor immune responses, resulting in a survival benefit over adjuvant therapy alone 17. Such concepts were recently validated by a small single-arm clinical study in resectable lung cancer 18, a small randomized trial in melanoma 19 as well as a phase 2 trial in melanoma comparing neoadjuvant nivolumab to neoadjuvant nivolumab with ipilimumab 20. All studies demonstrated enhanced T cell responses and a clinical benefit with neoadjuvant checkpoint inhibition.

To date, PD-1 blockade has demonstrated limited efficacy in patients with glioblastoma, except in isolated case reports associated

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with mismatch repair deficiency. Preclinical studies, however, have suggested that the PD-1–programmed death-ligand 1 (PD-L1) axis is immunologically relevant and that a therapeutic window exists. To address the question of whether neoadjuvant PD-1 blockade would alter the functional immune landscape and extend survival in patients, the Ivy Consortium initiated a multi-institution, randomized, open-label pilot study of pembrolizumab in patients with recurrent, surgically resectable glioblastoma. We used T cell receptor sequencing, gene expression profiling, mass cytometry and quantitative multiplex immunofluorescence to explore the intratumoral immune consequences of PD-1 monoclonal antibody administration and identify potential biomarkers of response.

Results

Trial patient characteristics. A total of 35 patients were enrolled and randomized between October 2016 and September 2017 at seven institutions and comprise the intention-to-treat population (Extended Data Fig. 1). Sixteen patients were randomized into the neoadjuvant pembrolizumab group and 19 into the adjuvant-only group. Three patients in the adjuvant-only group withdrew consent before surgery, and two patients (one in the neoadjuvant group and one in the adjuvant-only group) were replaced following surgery according to the study protocol on the basis of insufficient histological evidence of glioblastoma. These five patients were excluded from tissue studies but were included in the intention-to-treat efficacy analysis. The baseline patient characteristics are dichotomized by treatment group in Table 1. There were no statistically significant differences in age, sex, Karnofsky performance status, isocitrate dehydrogenase (IDH) mutation status, O6-methylguanine DNA methyltransferase (MGMT) methylation status, pre- or post-surgery tumor volume or steroid administration at registration. Furthermore, the extent of resection or the fraction of patients who received a gross total resection was not different between groups. As of the cutoff date of 2 July 2018, 31 of 32 patients had discontinued pembrolizumab (two for unacceptable toxicity; one by investigator decision, one by withdrawal of consent and 27 owing to progressive disease). Of the 27 patients with progressive disease, 24 went on to receive a bevacizumab-containing regimen. Dexamethasone was also administered to individual patients at varying times at the site investigator’s discretion (Fig. 1b). The Kaplan–Meier estimated median followup time was 476d (15.6 months; interquartile range 414–522 d (13.6–17.2 months)).

Safety. Pembrolizumab was generally well tolerated (Supplementary Table 1); the neoadjuvant administration of the antibody was not associated with any new, unreported toxicities in this patient population. As of the analysis cutoff date, ten patients (67%) in the neoadjuvant group experienced grade 3–4 adverse events that were deemed unlikely, possible, probable or likely attributable to the study drug; this occurred in seven patients (47%; two-sided P = 0.46, Fisher exact test, 95% confidence interval 0.42–12.95) in the adjuvant cohort. No patients in the neoadjuvant group had surgery delayed because of adverse events before tumor resection. Two patients, both in the neoadjuvant group, experienced toxicities leading to discontinuation of pembrolizumab; one patient developed grade 3 pneumonia and the other a grade 4 elevation in alanine aminotransferase. The most common treatment-related toxicities were muscle weakness (50%), headache (47%) and hyperglycemia (37%). Such treatment-related adverse events are commonly seen in patients with central nervous system tumors and/or those who have received corticosteroids; indeed, several of these adverse events were deemed unlikely to be related to the study drug by the site investigator but were included for completeness of data reporting.

Neoadjuvant pembrolizumab confers significant improvement in overall and progression-free survival. As of the analysis cutoff date, there have been nine deaths to date in the neoadjuvant arm and 12 in the adjuvant-only arm. In the intention-to-treat analysis, patients in the neoadjuvant arm demonstrated a statistically significant increase in overall survival, with a hazard ratio of 0.39 compared to the adjuvant-only group (95% confidence interval 0.17–0.94; P = 0.04, log-rank test). Patients in the adjuvant-only group had a median overall survival of 228d (7.5 months), whereas those in the neoadjuvant arm had a median overall survival of 417d (13.7 months; Fig. 1a). Median progression-free survival was 72.5d (2.4 months) in the adjuvant-only group and 99.5d (3.3 months) in the neoadjuvant group (hazard ratio 0.43; 95% confidence interval 0.20–0.90; P = 0.03, log-rank test; Fig. 1b and Extended Data Fig. 2). However, the establishment of progression-free survival may be complicated by pseudoprogression (Fig. 1c), a finding previously observed in malignant glioma patients treated with immunotherapy. In patients that received surgery and had histologic evidence of tumor (n = 15 patients per group), the median overall survival of the neoadjuvant treatment cohort was 400d (13.2 months) from registration date, while that of the adjuvant treatment cohort was 192d (6.3 months; hazard ratio 0.35, 95% confidence interval 0.14–0.88; P = 0.03, log-rank test).

Neoadjuvant PD-1 blockade induces distinct tumoral gene expression changes. To evaluate whether neoadjuvant PD-1 blockade would alter the gene expression profile of recurrent glioblastoma, we first analyzed transcriptional changes within patients tumors utilizing direct multiplexed messenger RNA (mRNA) quantification. We analyzed the differential gene expression between treatment groups, which revealed several statistically significant differences in tumor samples (Supplementary Table 2). Kyoto Encyclopedia of Genes and Genomes gene set

| Table 1 | Patient characteristics by group |
|---------|---------------------------------|
| Patient characteristics | Neoadjuvant (n = 16) | Adjuvant (n = 16) | Total (n = 32) |
| Demographics | | | |
| Age at enrollment, years, mean ± s.d. | 55.4 ± 13.5 | 59.3 ± 12.3 | 57.4 ± 12.8 |
| Gender, n (%) | | | |
| Male | 7 (44) | 5 (31) | 12 (38) |
| Female | 9 (56) | 11 (69) | 19 (63) |
| Karnofsky performance status, mean ± s.d. | 80 ±8.9 | 86 ± 6.2 | 83 ± 8.2 |
| Steroid use at registration | | | |
| Patients receiving steroids (n) | 7 | 7 | |
| Daily dosage, median, mg day⁻¹ (range) | 0 (0–4) | 0 (0–6) | |
| MGMT status, n (%) | | | |
| Methylated | 6 (38) | 11 (69) | 17 (53) |
| Unmethylated | 7 (44) | 4 (26) | 11 (34) |
| Unknown | 3 (19) | 1 (6) | 4 (13) |
| IDH status, n (%) | | | |
| WT | 12 (75) | 13 (81) | 25 (78) |
| Mutant | 3 (19) | 2 (13) | 5 (16) |
| Unknown | 1 (6) | 1 (6) | 2 (6) |
| Preoperative tumor volume (cm³), mean ± s.e.m. | 21.8 ± 6.2 | 18.9 ± 3.2 | |

s.d.: standard deviation; s.e.m.: standard error of the mean.
enrichment analysis identified gene products associated with cytokine–cytokine interaction, the chemokine signaling pathway and the Toll-like receptor signaling pathway. To quantify the T cell–inflamed microenvironment, we compared an immune-related gene expression signature score (Supplementary Table 3) between treatment groups. There was a significant transcriptional increase in genes related to interferon-γ responsiveness (P = 0.03, U = 49; Mann–Whitney U-test; Fig. 2a and Extended Data Fig. 3). We also sought to determine whether neoadjuvant PD-1 blockade would induce other transcriptional changes in these tumors. As such, we performed RNA sequencing on patient tumor samples and applied an unbiased screen for differentially expressed gene signatures with an interquartile range > 1. Our data demonstrated interferon- and T cell–pathway induction in 9 of 14 tumors in the neoadjuvant group and 5 of 15 in the adjuvant-only group, corroborating our mRNA quantification data. In addition, 3 out of 14 tumors in the neoadjuvant group demonstrated positive enrichment of cell cycle/cancer proliferation signatures, whereas this occurred in 11 of 15 tumors in the adjuvant group (Fig. 2b; two-sided \( P < 0.01 \), Fisher exact test, 95% confidence interval 0.01–0.70). We found no statistically significant correlation between steroid dose at registration and signature scores for representative interferon, T cell and cell cycle gene expression. The proportion of cell cycle signatures in our study as a whole was similar to that of publicly available RNA sequencing datasets from patients with recurrent glioblastoma (11 of 20 pre-treatment tumors; Extended Data Fig. 4; two-sided \( P = 0.8 \), Fisher exact test, 95% confidence interval 0.21–2.77) and The Cancer Genome Atlas (73 of 166 samples; two-sided \( P = 0.7 \), Fisher exact test, 95% confidence interval 0.50–2.83; Extended Data Fig. 5). Notably, only one of the samples with interferon pathway and T cell gene set enrichment in the neoadjuvant group demonstrated cell cycle pathway activation (Fig. 2b), suggesting that neoadjuvant PD-1 blockade induced relevant immune cell activation within the tumor microenvironment that subsequently repressed the cell-cycle-related transcriptional activity of tumor cells.

Fig. 1 | Neoadjuvant pembrolizumab confers significant improvement in overall and progression-free survival in patients with recurrent glioblastoma. Patients in the neoadjuvant arm (red) received 200 mg pembrolizumab 14 ± 5 d before surgical resection; patients in the adjuvant-only arm (blue) did not; both groups received 200 mg adjuvant pembrolizumab every 3 weeks. a, Kaplan–Meier plot of overall survival. Median overall survival for patients receiving adjuvant treatment only was 228.5 d, whereas median survival in the neoadjuvant group was 417 d (hazard ratio 0.39 neoadjuvant/adjuvant; 95% confidence interval 0.21–2.77) and The Cancer Genome Atlas (36). The proportion of cell cycle signatures in our study as a whole was similar to that of publicly available RNA sequencing datasets from patients with recurrent glioblastoma (11 of 20 pre-treatment tumors; Extended Data Fig. 4; two-sided \( P = 0.8 \), Fisher exact test, 95% confidence interval 0.21–2.77) and The Cancer Genome Atlas (73 of 166 samples; two-sided \( P = 0.7 \), Fisher exact test, 95% confidence interval 0.50–2.83; Extended Data Fig. 5). Notably, only one of the samples with interferon pathway and T cell gene set enrichment in the neoadjuvant group demonstrated cell cycle pathway activation (Fig. 2b), suggesting that neoadjuvant PD-1 blockade induced relevant immune cell activation within the tumor microenvironment that subsequently repressed the cell-cycle-related transcriptional activity of tumor cells.
Neoadjuvant PD-1 antibody blockade is associated with focal upregulation of PD-L1 and CD8+ T cell infiltrate in recurrent glioblastoma. To address whether neoadjuvant PD-1 blockade would increase the infiltration of T cells, we quantified the immune cell infiltrate in each patient’s tumor tissue via multiplex immuno-fluorescence. The density of tumor-infiltrating CD8+ T cells was not different between groups but demonstrated significant variability in the neoadjuvant cohort. Because PD-L1 is known to be upregulated in response to interferon-γ produced by infiltrating T cells, we evaluated whether PD-L1 would be increased with neoadjuvant PD-1 blockade. Samples were classified as having either a constitutive, focal or negative PD-L1 expression pattern with varying degrees of CD8+ T cell infiltration. Seven neoadjuvant patients and three adjuvant patients exhibited a focal phenotype with a high CD8 infiltrate (Fig. 3 and Supplementary Table 4), suggesting that neoadjuvant treatment is associated with focal induction of PD-L1 expression. Qualitatively, the focal upregulation of PD-L1 in the adjuvant cases was not as striking, suggesting that the endogenous functional T cell response was not as dramatic.

**Fig. 2 | Tumor gene expression profile altered by neoadjuvant PD-1 blockade.**

*a*, Heat map of tumor mRNA expression of interferon-γ-related gene panel for individual patients. Within this panel, ‘A’ denotes a patient in the neoadjuvant group, and ‘B’ denotes a patient in the adjuvant-only group. Dendrograms represent unsupervised hierarchical clustering by Ward’s minimum distance. Green coloration represents decreased expression; red coloration represents increased expression. **b**, Top, heat map showing the gene set variation analysis enrichment scores of gene sets with interquartile range ≥1. The gene sets can be grouped into the three categories: (i) interferon pathway induction, (ii) T cell activity and (iii) cell cycle/proliferation. Middle, heat map of mRNA expression of the representative genes corresponding to the gene set enrichments above. Bottom, heat map of progression-free and overall survival of each patient (in log2 scale). MHC, major histocompatibility complex; OS, overall survival; PFS, progression-free survival.
that the clinical effectiveness of neoadjuvant PD-1 blockade might occur because this specific timing resulted in a detectable local and systemic T cell response. To test this, we performed αβ T cell receptor sequencing on all patients’ peripheral blood mononuclear cells at baseline, at the time of surgery and at approximately 6 weeks after surgery, as well as on all evaluable tumor samples. We statistically identified T cell receptor rearrangements that were differentially abundant in each patient’s peripheral blood mononuclear cells at on-treatment time points relative to baseline and quantified the number of clones that significantly increased in frequency (Fig. 4a) as well as the proportion of T cell receptors shared between tumor and blood (Fig. 4b). There was a trend toward an increased number of expanded clones between baseline and surgery in the neoadjuvant compared with the adjuvant group (two-sided P = 0.07, unpaired t-test). We observed no significant differences in tumor-infiltrating lymphocyte content, T cell receptor diversity or proportion of T cell receptors shared between tumor and blood by treatment group or clinical time point. However, in neoadjuvant patients, we noted commensurate increases in the fraction of expanded T cell clones following the post-surgery PD-1 blockade cycle and T cell receptor overlap between tumor and blood across all three collected time points (r = 0.86, 0.84, 0.89; P = 0.0002, 0.0003, 0.00004, respectively, asymptotic t approximation, n = 25 patients). Similarly, the amount of T cell receptor overlap at the time of surgery was also significantly associated with the tumor-infiltrating lymphocyte fraction, but only in the neoadjuvant treatment group (r = 0.83; P = 0.0005, asymptotic t approximation, n = 25 patients; Fig. 4c). These correlations were not found in the adjuvant-only group, suggesting that neoadjuvant PD-1 blockade uniquely initiated a coordinated local and systemic T cell response.

Neoadjuvant PD-1 blockade is associated with monocyteic and T cell phenotypic changes. To evaluate whether neoadjuvant PD-1 blockade would alter the phenotype of systemic immune cell populations, we performed time-of-flight mass cytometry on peripheral blood samples collected from study subjects at baseline, before surgery and after receiving at least one adjuvant treatment cycle, enabling use of the adjuvant group as a concurrent control. After staining and sample acquisition, we concatenated a total of 84 samples. We utilized unsupervised clustering to conduct cell
Fig. 4 | Neoadjuvant PD-1 blockade alters correlative relationships between blood and tumor repertoire features and alters circulating immune cell phenotypes. a, Box-and-whisker plots comparing the number of expanded T cell receptor clones between baseline and surgery (left, \( P = 0.07 \), two-tailed \( t \)-test, \( t = 1.98, \) \( df = 14.33, \) 95% confidence interval \(-1.94 \) to \( 50.7, n = 26 \) patients), and between surgery and 1–2 cycles of pembrolizumab (right, \( P = 0.85 \), two-tailed \( t \)-test, \( t = 0.19, \) \( df = 26.0, \) 95% confidence interval \(-30.2 \) to \( 36.3, n = 28 \) patients). The y axis denotes the number of expanded clones. b, Box-and-whisker plot comparing T cell receptor overlap between peripheral blood and tumor at the time of surgery \( (P = 0.59, \) two-tailed \( t \)-test, \( t = 0.54, \) \( df = 24.8, \) 95% confidence interval \(-0.01 \) to \( 0.008, n = 27 \) patients). On the y axis, 0 indicates no clonal overlap and 1 indicates complete overlap. For a and b, whiskers represent minima and maxima, boxes extend from 25th to 75th percentiles, horizontal line represents median; red coloration denotes neoadjuvant+adjuvant group; blue coloration denotes adjuvant-only group. c, Hierarchically ordered Spearman correlation plots of the T cell receptor sequencing data. Numbers on the color key indicate the Spearman correlation coefficient; boxes marked with an X had Benjamini–Hochberg-adjusted, two-tailed \( P \)-values of \( < 0.01 \) and \( < 0.001 \), respectively. d, Scatter plot of the proportions of a peripheral cluster of intermediate monocytes (CD11b+CD11c+CD14+CD16+HLA-DR+) at baseline and after one or two cycles of adjuvant therapy. The y axis indicates percentage of live mononuclear cells; \( n = 28 \) patients; the neoadjuvant group is represented by the plot on the left, the adjuvant group on the right; Benjamini–Hochberg-corrected, two-sided \( P = 0.007 \) by general linear hypothesis test. e, Scatter plot of selected cell surface markers on CD4+ T cells before and after the first dose of pembrolizumab. Note the decreased PD-1 expression and increased CD152 (Benjamini–Hochberg-corrected, two-sided \( P = 0.025 \) and 0.0015, respectively, general linear hypothesis test, \( n = 28 \) patients) in the neoadjuvant group (left). * \( P < 0.05 \); ** \( P < 0.01 \); NS, non-significant; N+A, neoadjuvant+adjuvant; A, adjuvant-only.
Population identification (Extended Data Fig. 6) and evaluated the characteristics of the identified clusters. There was a statistically significant decrease in an intermediate monocyte population in the neoadjuvant group between baseline and after at least one cycle of adjuvant PD-1 blockade, which was not observed in the adjuvant-only group (baseline mean proportion 1.35 ± 0.31% versus on-treatment mean proportion 0.56 ± 0.12%; Benjamini–Hochberg-corrected, two-sided $P = 0.007$, general linear hypothesis test, $n = 28$; Fig. 4d). We then compared the expression of functional markers within clustered cell populations and noted statistically significant decreases in PD-1 and increases in CTLA-4 (Benjamini–Hochberg-corrected, two-sided $P = 0.025$ and 0.0015, respectively, general linear hypothesis test, $n = 28$) on CD127+ T cell clusters (Fig. 4c) solely in the neoadjuvant group between baseline and post-surgery. After correcting for multiple comparisons, no significant changes were observed in peripheral blood mononuclear cell populations from the adjuvant-only treatment group.

Cell cycle gene signature, baseline T cell receptor clonality and tumor-infiltrating lymphocyte density may be associated with clinical response to neoadjuvant PD-1 blockade. Finally, we sought to determine which clinical and laboratory factors were directly associated with survival, utilizing an elastic net-regularized Cox regression for variable selection. In the 30 patients with evaluable tumor, neoadjuvant PD-1 blockade therapy, IDH mutation status, lower baseline peripheral T cell receptor clonality and increased tumor-infiltrating lymphocyte density were associated with increased overall survival. We then fit these variables, combined with age and sex, into a Cox proportional hazards regression and confirmed that neoadjuvant treatment was associated with improved survival (hazard ratio 0.33, $P = 0.045$; Supplementary Table 5). Standardized baseline peripheral T cell receptor clonality trended toward a survival association, with a hazard ratio of 1.48 for each standard deviation increase of 1 ($P = 0.12$). Given the significant treatment effect on the cell cycle signature in neoadjuvant patients, we sought to determine whether a representative gene set could be utilized as a biomarker for overall survival, calculating the $R^2$ statistic to evaluate the potential predictive power. We found that the cell-cycle-related gene set variation analysis enrichment score explained 57% of the variance in overall survival ($R^2 = 0.57$). When added to the previous Cox model, the $R^2$ value increased from 0.36 to 0.62, suggesting that the cell cycle gene set variation analysis enrichment score may be one of the most significant predictors of overall survival.

**Discussion**

In this study, PD-1 monoclonal antibody blockade was associated with statistically significant improvements in overall survival and progression-free survival when administered in the neoadjuvant setting to patients with recurrent glioblastoma. We leveraged T cell receptor sequencing, mRNA expression profiling, quantitative multiplex immunofluorescence and mass cytometry on patient blood and tumor samples to understand the distinct biological effects induced by this neoadjuvant timing. Our data suggest that neoadjuvant PD-1 monoclonal antibody blockade induces functional activation of tumor-infiltrating lymphocytes, producing an interferon response within the tumor microenvironment. Tumor infiltration with interferon-γ-producing, PD-1/PD-L1-suppressed
T cells is probably essential for the systemic priming of tumor-specific T lymphocytes after neoadjuvant pembrolizumab. Indeed, tumor-infiltrating lymphocyte density was one of the variables selected in our elastic net regression as being possibly related to survival; conversely, presurgical tumor volume, postsurgical tumor volume, percentage resection, gross total resection (as a dichotomous variable) and dexamethasone dosage at time of registration were not. This suggests that the survival benefit derived from neoadjuvant PD-1 blockade is largely driven by the immune response. After neoadjuvant PD-1 blockade and resection, tumor-specific T cell clones maintained functionality with repeated anti-PD-1 monoclonal antibody administration in the adjuvant setting (Fig. 5). We believe that the T cell–mediated interferon response induces downregulation of cell-cycle-related gene expression within tumor cells, enabling a therapeutic window and resulting in a survival benefit. It is already known that many interferon-regulated gene products block cell cycle activities and tumor cell proliferation, suggesting that the transcriptional suppression of such cell-cycle-related genes is a key feature of the efficacy for PD-1 blockade. Of note, a single-arm phase 2 clinical trial by Melero and colleagues in this same issue, utilizing neoadjuvant nivolumab in newly diagnosed or relapsed glioblastoma (NCT02550249), demonstrated intratumoral and systemic immune changes similar to those found by us.

In our gene expression analysis of tumor samples, we noted an upregulation of interferon-γ-related gene products in patients who received neoadjuvant PD-1 blockade, whereas patients without this upregulation demonstrated enrichment of cell cycle pathway gene sets. In fact, in a murine neoadjuvant immunotherapy model of breast cancer, the neutralization of interferon-γ nullified the efficacy of neoadjuvant anti-PD-1 plus anti-CD137 antibody therapy, indicating a vital role in promoting antitumor activity. This, together with our data, strongly suggests that increased interferon-γ signaling is a consequence of anti-PD-1 therapy and mediator of the functional immunologic response. Our study indicates that, although increased interferon signaling occasionally occurs in the presence of recurrent glioblastoma (as demonstrated by patients in the adjuvant-only group with upregulated interferon-γ-related gene expression), checkpoint release in the presence of tumor (i.e., in the neoadjuvant setting) may be a crucial component in the enhancement of tumor-specific T cell effector function, a finding consistent with that observed by Liu et al. in a murine model. In addition, mice that received no treatment and no surgery demonstrated increased tumor-specific T cells, but poor effector function compared to groups that received neoadjuvant therapy and tumor resection, suggesting that both timing and surgery may play a role in tumor-specific T cell expansion. A similar mechanism may account for the failure of anti-PD-1 therapy in recurrent, unresected glioblastoma.

Quantitative multiplex immunohistochemistry revealed an increased proportion of patients in the neoadjuvant group that exhibited focal upregulation of PD-L1. However, three patients in the adjuvant-only group also demonstrated this finding, albeit with qualitatively limited PD-L1 intensity. Two of these three patients also demonstrated upregulation of cell cycle-related genes, suggesting that their endogenous T cell response was insufficient to disrupt tumor cell proliferation. The third patient, with focal PD-L1 upregulation on multiplex immunofluorescence, exhibited some CD8+ T cell infiltration but did not have an elevated cell cycle signature. The T cell– and interferon-related genes also were not increased, suggesting an aberrant mixture of low-level activity. The overall survival of these three patients was below the median for all patients.

After analyzing the T cell receptor data, we found that expansion of tumor-associated T cell receptor clones in the neoadjuvant group after treatment was correlated with high overlap between tumor and blood. While the overall number of expanded clones did increase after the initial pembrolizumab dose in the adjuvant-only group, these additional correlations were not seen. This suggests that PD-1 blockade can lead to increases in T cell clones regardless of timing; however, pre-surgical checkpoint inhibition enables a selective, primary tumor-specific T cell clonal modulation, driving systemic expansion of tumor-specific T lymphocytes.

Neoadjuvant pembrolizumab was not associated with a significant change in T cell receptor diversity, supporting previous findings that diversification of the T cell repertoire is not mediated via the PD-1 axis. However, as our data indicate that increased baseline T cell receptor clonality may be associated with reduced survival, it appears that greater initial T cell diversity may portend improved responsiveness to PD-1 blockade. Our data suggest a role for combination immunotherapy with CTLA-4 blockade, which was shown to increase the number and complexity of T cell receptor variants in patients with melanoma and has been demonstrated to be efficacious in other tumor types. Although this combination was evaluated in a small cohort of recurrent glioblastoma patients lacking an obvious survival signal, it has yet to be explored in a neoadjuvant investigation.

In our single-cell peripheral blood analysis, no specific cell population appeared to consistently predict survival, contrasting with previous studies in other cancer types. In our evaluation of differentially expressed functional markers on various cell populations, CD4+ T cells demonstrated statistically significant increases in CD152 and CD127 and a decrease in PD-1, suggesting that the degree of phenotypic shift toward activation and memory may play a significant role in the post-surgical antitumor immune response.

Because of the established role of corticosteroid usage in the treatment of both central nervous system malignancies and immune-related adverse events, we evaluated the correlation between dexamethasone dosage at the time of registration as continuous and dichotomous variables and expression scores of interferon-γ, T cell– and cell-cycle-related gene sets, as well as overall survival. We found no statistically significant correlations, differing from published findings in patients with non-small-cell lung cancer that received PD-1/PD-L1 blockade on high-dose baseline corticosteroids. This may be due to the fact that the protocol for this study excluded patients receiving high-dose dexamethasone at baseline >4 mg/day. Many patients continued to receive, or were started on, low doses of dexamethasone during their treatment course. Our analyses corroborate previous findings that systemic corticosteroids can be utilized as management for adverse events without affecting objective response rates in patients receiving nivolumab for advanced melanoma.

Given the noted improvement in survival, we intend to expand the current study and pursue further clinical trials with neoadjuvant combination immunotherapeutics. Although this was a randomized clinical study, it was powered for tissue analyses. As such, interpretation of efficacy outcomes is limited by small sample size. Similarly, the observed immune effects may be influenced by an imbalance in the number of pembrolizumab doses received by patients in each group; the nature of this clinical trial design as a multicenter study also limited tissue availability for in-depth biologic analyses. We also did not find statistically significant correlations between patients that exhibited large numbers of expanded T cell clones, focal upregulation of PD-L1 expression and the above gene expression signature score. This may be due to the small sample size and/or the heterogeneity in tumor sampling for quantitative multiplex immunofluorescence analysis. Notably, almost all patients in this study went on to receive bevacizumab. The precise role of bevacizumab is unclear, given the inter-patient heterogeneity in post-progression therapies and timing. Data suggest that bevacizumab in this setting is not associated with poorer survival outcomes. Overall, the consistency of the data from this study indicates that there is an observable tissue-based and clinical treatment effect.
As such, the neoadjuvant approach to immunotherapy and brain cancer may provide a unique clinical development path while also providing a therapeutic window to study the immunobiology of malignant brain tumors.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41591-018-0337-7.

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**Author contributions**
The conceptualization, methodology and supervision were carried out by R.M.P., P.Y.W. and T.F.C.; the investigation was carried out by R.M.P., T.F.C., P.Y.W., J.R.O., A.H.L., A.Y.M., A.C.W., T.B.D., W.H.Y., J.L.C., I.C.A.-R., H.C., T.I.K., J.F.d.G., D.A.R., I.K.M., A.L.C., E.Q.L., P.L.N., B.J.O. and N.A.B.; writing of the original draft was handled by A.Y.M.; and the draft was reviewed and edited by all the authors; funding was acquired by L.M.L., R.M.P., T.F.C. and P.Y.W.; the data were curated by J.R.O. and S.C.G.; formal analysis was carried out by J.R.O., B.M.E., A.Y.M., G.L., L.D., E.S.K., W.H., C.M.S. and J.A.R.; and project administration was carried out by J.R.O. and S.C.G.

**Competing interests**
J.A.R. and C.M.S. have a financial interest in Adaptive Biotechnologies. T.F.C. and D.A.R. have received compensation from Merck as consultants on advisory boards. P.Y.W. and H.C. have received honoraria from Merck. J.F.d.G. has done consulting and/or received honoraria with Merck and Bristol-Myers Squibb. I.K.M. reports research funding from General Electric, Amgen and Lilly; advisory roles with Agios, Puma Biotechnology and Debiopharm Group; and honoraria from Roche for a presentation.

**Additional information**
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Methods

Study design and patients. Patients were ≥18 years of age, had recurrent glioblastoma and were candidates for surgical debulking. Key eligibility criteria included Karnofsky performance status ≥70, previous first-line therapy with at least radiation therapy, first or second relapse with unequivocal evidence of tumor progression, adequate organ function, no high-dose systemic corticosteroids (defined as >4 mg day⁻¹ of dexamethasone or bio-equivalent for at least three consecutive days within two weeks of registration) and absence of previous antiangiogenic or antivascular endothelial growth factor agents. All patients provided written informed consent; the study was approved by institutional review boards at all sites (Dana-Farber Cancer Institute; Huntsman Cancer Institute; MD Anderson Cancer Center; Massachusetts General Hospital; Memorial Sloan Kettering Cancer Center; University of California, Los Angeles; University of California, San Francisco) and was conducted according to the Declaration of Helsinki.

After consent, patients randomized to the neoadjuvant group received pembrolizumab 200 mg by intravenous infusion every 3 weeks for 5 before scheduled surgical resection. Tumor resection was performed according to institutional standards. After recovery from surgery, patients received pembrolizumab 200 mg by intravenous infusion every 3 weeks until either tumor progression or an adverse event requiring study drug discontinuation. Blood samples were obtained every second cycle (6 weeks). Patients were followed for MRI changes, clinical exams and steroid doses until death or second progression. After second progression, patients were followed every 3 months for vital status until death.

RNA isolation and quantification. RNA was isolated from tumor sections stored in AllProtect tissue reagent (QIAGEN) at the time of surgery; peripheral blood mononuclear cells were isolated at baseline, time of surgery and at cycle 2 of therapy and lyzed to obtain RNA and protein. The nCounter GX analysis system (NanoString) was utilized to quantify RNA and protein expression according to the manufacturer's directions. The 770-gene nCounter PanCancer immune profiling panel was utilized (Nanostring; list of genes available from manufacturer).

RNA sequencing and gene set enrichment. Paired-end, 2 × 150 base pair (bp) transcriptome reads were mapped to the Genome Reference Consortium Human Build 38 (GRCh38) reference genome using HISAT2 (ref. 29). These raw sequencing files have been uploaded to the Gene Expression Omnibus (No. GSE121810). The gene level counts are generated by the HTSeq-count program; we took log2 counts per million (CPM) as normalized gene expression values. To calculate single-sample gene set enrichment, we used the Gene Set Variation Analysis (GSVA) package60 to derive the absolute enrichment scores of the following perturbation gene sets from the Broad Institute's Molecular Signatures Database:7

1. c2-egg.v6.0
2. c6.all.v6.0
3. c7.all.v6.0
4. hallmark.v6.0

To ensure robustness, we chose only gene sets with at least 15 genes. We also included curated immune/stromal cell signatures from the CIBERSORT program71 and from single-cell RNA sequencing studies on patient tumors.72 To run GSVA, we first performed one of our own normalization (logCPM normalization is less than twofold (log, CPM interquartile range ≥ 1)). The shortlisted gene raw HTSeq-computed counts were then supplied to the GSVA program using the kdist='Poisson' mode. Once the GSVA scores of all gene sets were computed, we selected those with GSVA score interquartile range ≥1 for further analysis.

Tumors with GSVA score ≥0.2 in both the T ‘generic’ (reflecting general T cell activation markers) and Averys et al. ‘IFN’ genes (interferon activation signatures reported by Avers et al.,31) and ≥0.2 in the ‘FARMER_BREAST_CANCER_CLUSTER_2’ (reflecting cell-cycle-related genes), are defined as having high T cell infiltration and interferon pathway activation and decreased cell cycle. To evaluate for potential correlations between steroid administration and immune function, we calculated Spearman’s rank correlation coefficient for steroid dose in milligrams at registration and the above representative gene sets for interferon – T cell – and cell-cycle-related signature scores.

Finally, to compare the enrichments of the shortlisted gene sets within our datasets and that of other glioblastomas, we combined the log, CPM-normalized expression of The Cancer Genome Atlas (TCGA) glioblastomas (based on HTSeq-counts data generated from the TCGA RNA-Seq V2 data at the Cellranger alliance) and the pre-treatment RNA sequencing dataset GSE79671 (a study of patients with recurrent glioblastoma) with our own logCPM and CPM removed potential batch effects across the two datasets using the RemoveBatchEffect function in the limma R package. The combined CPM is input into the GSVA program using the default kdist='Poisson' option. We compared the statistical difference in the fraction of tumors with positive enrichment of each gene set, defined as having a GSVA score ≥0.2 for that gene set, in the neoadjuvant (n = 14), adjuvant (n = 15), GSE79671 (n = 20) and TCGA patient groups (n = 166) by one-sided Fisher exact test.

Immunosequencing of the T cell receptor β-chain. Genomic DNA was extracted from patient tumors and peripheral blood mononuclear cells (collected at baseline, before surgery, at the time of the first postsurgical pembrolizumab dose, every 6 weeks thereafter or at the end of treatment). Genomic DNA was extracted using the Qiagen DNeasy blood extraction kit. Utilizing the immunoSEQ Assay (Adaptive Biotechnologies) for peripheral blood mononuclear cell (PBMC) samples and the immunoSEQ Assay for formalin-fixed, paraffin-embedded tumor samples, T cell receptor (TCR) complementarity-determining region 3 (CDR3) regions were amplified and sequenced from 2 μg of genomic DNA (or all available extracted DNA if <2 μg was available). Regions were amplified using a bias-controlled multiplexed polymerase chain reaction method, followed by high-throughput sequencing. Sequences were collapsed and filtered to identify and quantify the absolute abundance of each unique TCRβ CDR3 for further analysis as previously described32–34.

To measure tumor-infiltrating lymphocyte density, the immunoSEQ Assay for formalin-fixed, paraffin-embedded tumor samples amplifies both the TCRβ CDR3 and selected reference genes, which quantitate the total number of T cells and nucleated cells, respectively. Tumor-infiltrating lymphocyte density is calculated by dividing the total number of T cells by the total number of nucleated cells.

We assessed T cell receptor overlap between tumor-infiltrating lymphocytes and PBMCs as previously described35. To summarize, T cell receptor overlap between tumor and PBMC samples from each patient was assessed by identifying the shared TCRβ CDR3 amino acid sequences found between two samples. The sum of shared sequences between two samples was then divided by the total number of unique TCRβ CDR3 sequences present across both samples. To evaluate T cell receptor diversity, we utilized the Daley–Smith Richness estimator, a Bayesian approach that enables characterization of the complexity of DNA sequencing data; this methodology provides an estimate of repertoire diversity that is more informative than differences in input DNA between samples.

No significant difference was observed in the number of expanded clones between treatment groups from baseline to surgery (two-sided P = 0.40, Wilcoxon rank sum test with continuity correction, W = 101, 95% confidence interval –7.0 to 44.0, n = 26 independent biological samples) or from surgery to the second adjuvant cycle (two-sided P = 0.89, Wilcoxon rank sum test with continuity correction, W = 101.5, 95% confidence interval –0.05 to 0.06, n = 30 independent biological samples) or peripheral blood (two-sided P = 0.35, Wilcoxon rank sum test, W = 83, 95% confidence interval 0.15 to 0.05, n = 30 independent biological samples) or proportion of T cell receptors shared between tumor and blood at baseline (two-sided P = 0.62, Wilcoxon rank sum test, W = 117, 95% confidence interval –0.03 to 0.007, n = 29 independent biological samples), the time of surgery was not independent biological samples).

Mass cytometry. Peripheral blood mononuclear cells were collected at baseline, at the time of surgery and after one or two cycles of adjuvant therapy, and prepared for mass cytometry analysis according to the Maxpar protocol. Briefly, 0.5 × 10⁶ to 3.5 × 10⁶ cells were washed with phosphate buffered saline then resuspended in 5 μM Cell-Id isosfatin (Fluidigm) as a live/dead marker for 5 min at room temperature. After quenching with cell-staining buffer, the cells were incubated with a 24-marker panel (Supplementary Table 6) for 30 min at room temperature. After washing with cell-staining buffer, cells were incubated overnight in 125 mM iridium intercalation solution (×10,000 dilution of 125 μM Cell-ID Intercaler-Ir in Maxpar Fix and Perm Buffer (Fluidigm)) to label intracellular DNA. Cells were then washed with cell-staining buffer and distilled water.

Events were subsequently acquired on a Helios mass cytometer (Fluidigm) in the University of California, Los Angeles Jonsson Comprehensive Cancer Center flow cytometry core and the data were analyzed as described previously36. After acquisition, data were normalized utilizing EQ four-element calibration beads (Fluidigm) and preprocessed to remove dead cells. Each dataset was then loaded into R with the flowCore package. The raw marker intensities were transformed utilizing hyperbolic inverse sine (arcsinh) with cofactor of 5. We calculated Spearman’s rank correlation coefficient for TCRβ CDR3 amino acid sequences found between two samples. The sum of shared sequences between two samples was then divided by the total number of unique TCRβ CDR3 sequences present across both samples. To evaluate T cell receptor diversity, we utilized the Daley–Smith Richness estimator, a Bayesian approach that enables characterization of the complexity of DNA sequencing data; this methodology provides an estimate of repertoire diversity that is more informative than differences in input DNA between samples.
and PD-L1, using the Opal 4-Color Manual IHC Kit and protocol (PerkinElmer). Slides were first de-paraflinized with xylene and rehydrated with an ethanol gradient. Heat-induced antigen retrieval was then performed on slides before each antibody application using pH9 antigen retrieval buffer (AR9, PerkinElmer) for CD8, CD45, PD-1 and PD-L1 antibodies; pH6 antigen retrieval buffer (AR6, PerkinElmer) was used for GFAP. The following antibody clones and dilutions paired with Opal tyramide signal amplification reagent were used and applied in the following order for each panel: CD8 (C8/144B, 1:5,000, Dako), with Opal 520; PD-1 (P0414, 1:400, PerkinElmer) with Opal 570, and for PD-L1 (OPD77, 1:50,000, Abcam) and Opal 650; CD45 (2B11+PD727, 1:1,000, Dako) and Opal 520, PD-L1 (SP142, 1:4,000). All slides were nuclear counterstained with Spectral DAPI (PerkinElmer), mounted (ProLong Diamond Antifade Mountant, Life Tech) and whole sections were imaged at ≥20 resolution (0.3 μm pixel⁻¹) using a Leica Aperio Versa 200 Slide Scanning Microscope equipped with a 16-bit Andor iXon 887 × 1,080 with a 5.5-megapixel camera (Translation Pathology Core Laboratory). Eight-bit images were captured using the equipped filters: DAPI 350/460 excitation/emission (ex/em), green 495/537 ex/em, red 580/625 ex/em and Cy5 640/690 ex/em. Positive stain quantification and spatial cell analysis was performed using HELA image analysis software (Indica Labs). Positivity thresholds were determined using primary antibody-negative control slides for exclusion of auto-fluorescence and nonspecific background staining.

MRI acquisition and analysis. Anatomic magnetic resonance images were acquired for all patients in the current study using a 1.5T or 3T clinical MRI scanner, and the MR images were transferred to the image folder corresponding to their local standard of care protocols. Standard anatomic images were obtained with the axial T1-weighted fast spin-echo sequence or magnetization-prepared rapid acquisition gradient-echo (MPRAGE) sequence (repetition time [ms]/echo time [ms] inversion time [ms] = 400–3209/3.6–21.9/0–1238; slice thickness = 1.0–6.5 mm; intersection gap = 0–2.5 mm; number of averages = 1–2; matrix size = 176–512 × 256–512; and field of view = 24.0–25.6 cm). Additionally, T2-weighted fast spin-echo and fluid-attenuated inversion-recovery sequences were also obtained, and parameter-matched T1-weighted images enhanced with gadopentetate dimeglumine (Magnevist; Berlex; 0.1 mmol/kg) were acquired shortly after contrast material injection. All trial scans were compliant with the consensus recommendations for the international standardized brain tumor imaging protocol70.

Linear registration was performed between all images (T2, fluid-attenuated inversion-recovery, pre-contrast T1, post-contrast T1) and post-contrast T1-weighted images at screening using a 12-degree-of-freedom linear transformation approach with the software FreeSurfer (FSL; FMRI Software Library). Estimates of tumor volume were performed using contrast-enhanced T1-weighted digital subtraction maps to exclude areas of post-surgical blood products or other sources of T1 shortening, as described previously71,72. Initial segmentation was performed automatically using previously defined methodology72, and final segmented volumes were edited by an experienced investigator with more than 10 years of experience to include large vessels and any obvious non-tumor regions.

Statistics and reproducibility. For mRNA quantification data, expression values were normalized using positive and negative controls and housekeeping genes and analyzed using the nSolver analysis software 4.0 (NanoString). Differential expression based on treatment group was assessed via a mixed negative binomial model for each gene with correction for multiple comparisons using the Benjamini–Hochberg adjustment. Gene set enrichment analysis was performed using the Kyoto Encyclopedia of Genes and Genomes gene collection in the Molecular Signatures Database73,74, with P values determined by hypergeometric test and corrected using a false discovery rate of 0.05 with n = 28 independent biological samples. The interferon-γ-associated gene expression profile score was calculated as previously described75.

To compute the differential abundance of T cell receptor clones, TRCβ rearrangements were detected by the immunoSEQ Assay for each patient’s peripheral blood mononuclear cell samples at baseline (n = 5 independent biological samples), the time of surgery (n = 27 independent biological samples) and cycle 2 (n = 29 independent biological samples) and compared to the previous time point. Rearrangements with a count of under 10 between the two compared time points were excluded. For a TRCβ rearrangement to be considered significantly expanded or contracted, a two-sided binomial test with Benjamini–Hochberg adjustment was performed using a false discovery rate of 0.01. To determine whether there was a difference between the two groups, the number of expanded clones was compared using the Wilcoxon signed-rank test.

To detect differences between the neoadjuvant, steroid dose in milligrams and adjoint-only groups in mass cytometry data, we employed the generalized linear hypothesis function with the R function glht to test for the difference in marker expression, fitting fixed and mixed models using the stats and lme4 packages in R. To compare cell population proportions between groups, we employed a generalized linear mixed model using the sample identification and anonymized patient identifiers as random effects76. For differential and marker-specific expression function data, Bioinformatics, 34, 166–169 (2015).

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Extended Data Fig. 1 | CONSORT diagram. Flow diagram of disposition of patients enrolled in the study.
Extended Data Fig. 2 | Kaplan–Meier plot of progression-free survival. Median progression-free survival (PFS) for patients who received pembrolizumab only in the adjuvant setting was 72.5 d; patients who received neoadjuvant and adjuvant pembrolizumab had a median PFS of 99.5 d (hazard ratio 0.43, 95% confidence interval 0.20–0.90; two-sided \( P = 0.03 \) by log-rank test).
Extended Data Fig. 3 | Eighteen gene interferon-γ-related signature scores in neoadjuvant versus adjuvant-only groups. Line in middle of box represents the median; box extends from the 25th to 75th percentiles; whiskers represent minimum and maximum values; n = 28 independent biological samples; P = 0.025, U = 49 by two-sided Mann-Whitney U-test. *: P < 0.05.
*Extended Data Fig. 4 | RNA sequencing comparison to other recurrent glioblastoma samples.* We combined our RNA sequencing dataset to that of GSE79671 (an RNA sequencing dataset of recurrent glioblastoma pre- and post-bevacizumab treatment; only pre-treatment (Pre-Tx) samples were used, and The Cancer Genome Atlas (TCGA) glioblastoma samples. We applied appropriate batch correction on log-transformed, normalized mRNA expression values using the removeBatchEffect function in the R package *limma* to estimate the fraction of glioblastoma patients with positive enrichment of cell cycle/cancer proliferation signatures (GSVA score ≥ 0.2). The proportion of positive enrichment of cell cycle/cancer proliferation signatures in our dataset as a whole is similar to GSE79671 (14 out of 29 (48%) versus 11 out of 20 (55%)). The number of samples with positive enrichment in the TCGA GBM is lower, at 41%. We observed that the neoadjuvant PD-1 monoclonal antibody therapy group is associated with a lower fraction of tumors with cell cycle signatures. Only 3 out of 14 tumors in the neoadjuvant group demonstrated positive enrichment, with 11 of 15 tumors in the adjuvant group and 11 of 20 tumors in the GSE79671 set (one-sided Fisher exact test, *P* = 0.01 and *P* = 0.05, respectively). GSVA, gene set variation analysis.
Extended Data Fig. 5 | RNA sequencing comparison to TCGA. We combined our RNA sequencing dataset to the TCGA glioblastoma dataset, with appropriate batch correction, to estimate the fraction of glioblastoma patients with positive enrichment of cell cycle/cancer proliferation signatures (GSVA score $\geq 0.2$). Three out of 14 tumors in the neoadjuvant group demonstrated positive enrichment, with 11 of 15 tumors in the adjuvant group and 73 of 166 tumors in The Cancer Genome Atlas set. TCGA: The Cancer Genome Atlas. GSVA: gene set variation analysis.
Extended Data Fig. 6 | Mass cytometry dimension reduction. a, Diffusion map of peripheral blood mononuclear cells (PBMCs) sampled from n = 28 patients at baseline, the time of surgery and on-treatment. Phenotypically similar cells are depicted in an unsupervised manner along the same continuous axes in a pseudotemporal progression. b, t-distributed stochastic neighbor-embedding (tSNE) plot of PBMCs from n = 28 patients at all three time points. Phenotypically similar cells are clustered in an unsupervised manner. All represented cells in both panels are colored by algorithmically assigned cluster numbers using the FlowSOM package. CD3+CD4+, CD3+CD8+, CD3–CD19+ and CD3–CD14+CD16+CD11b+CD11c+ cells are labeled to demonstrate how clustered cells in close proximity to one another are plotted.
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| n/a | Confirmed |
|-----|-----------|
| ☑   | The exact sample size \((n)\) for each experimental group/condition, given as a discrete number and unit of measurement |
| ☑   | An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly |
| ☑   | The statistical test(s) used AND whether they are one- or two-sided |
| ☑   | Only common tests should be described solely by name; describe more complex techniques in the Methods section. |
| ☑   | A description of all covariates tested |
| ☑   | A description of any assumptions or corrections, such as tests of normality and adjustment for multiple comparisons |
| ☑   | A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals) |
| ☑   | For null hypothesis testing, the test statistic (e.g. \(F\), \(t\), \(r\)) with confidence intervals, effect sizes, degrees of freedom and \(P\) value noted |
| ☑   | Give \(P\) values as exact values whenever suitable. |
| ☑   | For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings |
| ☑   | For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes |
| ☑   | Estimates of effect sizes (e.g. Cohen's \(d\), Pearson's \(r\)), indicating how they were calculated |
| ☑   | Clearly defined error bars |
| ☑   | State explicitly what error bars represent (e.g. SD, SE, CI) |

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection | Data was collected with Microsoft Excel or R on a secure server. |
| Data analysis | Statistical analysis was performed with SAS version 9.2, GraphPad Prism version 6.01, R version 3.4.3 or higher, HISAT2 version 2.1.0, CIBERSORT version 1.06. HALO image analysis platform version 2.1. R packages: ConsensusClusterPlus version 1.46.0, corrplot version 0.84, destiny version 2.12.0, flowCore version 1.48.0, FlowSOM version 1.14.0, ggplot2 version 3.1.0, glmnet version 2.0-16, GSVA version 1.30.0, limma version 3.38.2, lime4 version 1.1-19, mice version 3.3.0, multcomp version 1.4-8, premessa version 0.1.8, RColorBrewer version 1.1-2, readxl version 1.1.0, survival version 2.43-1, survminer version 0.4.3, Rtsne version 0.15. |

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data Policy information about availability of data
All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

RNA sequencing data is available in the Gene Expression Omnibus (GEO) under the accession code GSE121810. Source data for Figure 2B and Extended Data Figures 3, 4 and 5 are provided with the paper. The remainder of data that support the findings of this study are available from the corresponding authors upon reasonable request.

Field-specific reporting
Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

☐ Life sciences ☐ Behavioural & social sciences ☐ Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design
All studies must disclose on these points even when the disclosure is negative.

| Sample size | Based on our preliminary data, the mean of tumor infiltrating lymphocyte density was estimated to be 0.4 T cells per nucleated cell (standard deviation = 0.5) in the control group. Fifteen patients per group was deemed sufficient to achieve 85% power to detect an increase of 0.5 in tumor infiltrating lymphocyte density comparing the neoadjuvant group against the adjuvant-only group at an alpha of 0.05 (one sided) using a two-sample t test. |
| Data exclusions | Two patients were replaced according to the study protocol based on insufficient histological evidence of glioblastoma. Three other patients withdrew consent prior to receiving study intervention, but all were included in the intention-to-treat efficacy analysis. |
| Replication | For elastic net Cox regression, the value of lambda was tuned using 5-fold cross-validation; this was repeated by utilizing different starting seeds to ensure that identified nonzero coefficients were reproducible. For multiple imputation of missing variables, different starting seeds were also utilized and checked using density plots. Multiplex staining was performed in one standardized run per patient. For each staining run, two sequential slides were used as duplicates for each patient. Computational analysis was performed two or more times per sample. Mass cytometry, T cell receptor sequencing, Nanostring and bulk tumor RNA sequencing were performed once due to clinical sample availability. |
| Randomization | Subjects were randomized at enrollment into either the neoadjuvant group or the adjuvant-only group. |
| Blinding | Neither investigators nor patients were blinded to allocated treatment arm in this multi-institutional randomized study. As an investigator initiated pilot study, there was inadequate funding available for blinding. However, at no time before or during the study did the investigators or the patients consider an advantage to either arm as the study was principally designed to require a control for the immune monitoring performed in the perioperative and post operative period. Clinical equipoise was presumed maintained as both treatment arms received adjuvant study drug post operatively. The study evaluations were not analyzed until 4 months after the last patient was registered. Efficacy results were shared with the clinical investigators in June 2018 which is the time of the clinical data lock. |

Reporting for specific materials, systems and methods

Materials & experimental systems
n/a Involved in the study
☐ Unique biological materials
☐ Antibodies
☐ Eukaryotic cell lines
☐ Palaeontology
☐ Animals and other organisms
☐ Human research participants

Methods
n/a Involved in the study
☐ ChIP-seq
☐ Flow cytometry
☐ MRI-based neuroimaging
### Antibodies

Antibodies used for mass cytometry are as follows:

| Target (clone) | Vendor | Catalog number |
|----------------|--------|----------------|
| CD45RA (HI100) | Fluidigm | 3169008B |
| CD69 (FN50) | Fluidigm | 3144018B |
| CD4 (RPA-T4) | Fluidigm | 3145001B |
| CD25 (2A3) | Fluidigm | 3146001B |
| CD19 (HI90) | Fluidigm | 3142001B |
| CD274 (29E.2A3) | Fluidigm | 3148017B |
| CD14 (M5E2) | Fluidigm | 3151009B |
| CD279 (EH12.2H7) | Fluidigm | 3155009B |
| CD8a (RPA-T8) | Fluidigm | 3158001B |
| CD15 (W6D3) | Fluidigm | 3164001B |
| CD16 (3G8) | Fluidigm | 3165001B |
| CD3 (UCHT1) | Fluidigm | 3172007B |
| CD11b (ICRF44) | Fluidigm | 3173008B |
| HLA-DR (L243) | Fluidigm | 3174001B |
| CD127 (A019D5) | Fluidigm | 3176004B |
| CD38 (HIT2) | Fluidigm | 3172007B |
| CD206 (15-2) | Fluidigm | 3168008B |
| TIM-3 (F38-2E2) | Fluidigm | 3153008B |
| CD223 (LAG-3) | Fluidigm | 3154003B |
| CD152 (14D3) | Fluidigm | 3161004B |
| CD11c (Bu15) | Fluidigm | 3159001B |
| CD27 (O323) | Fluidigm | 3167002B |
| CD56 (NCAM 16.2) | Fluidigm | 3163007B |

### Validation

According to the manufacturer's website, each lot of conjugated antibodies is quality control tested by mass cytometry analysis of stained cells using the appropriate positive and negative cell staining and/or activation controls. Specifically, CD45RA (HI100) was validated by Fluidigm on human PBMCs; CD56 (NCAM 16.2) was validated by Fluidigm on human PBMCs - according to the manufacturer's website, human PBMCs were incubated for 6 hours in media alone or with PMA and ionomycin in the presence of monensin and brefeldin A. Cells were then fixed, permeabilized and stained with 154Sm-anti-CD45 (HI30) and 144Nd-anti-CD69 (FN50). Anti CD4-145Nd (RPA-T4), CD14 (M5E2), CD279 (EH12.2H7), CD33 (WM53), CD8a (RPA-T8), CD15 (W6D3), CD16 (3G8), CD3 (UCHT1), CD11b (ICRF44), HLA-DR (L243), CD127 (A019D5), CD38 (HIT2), CD206 (15-2), TIM-3 (F38-2E2), CD152 (14D3), CD11c (Bu15), CD27 (O323) were also validated by the manufacturer on human PBMCs; Custom conjugated antibodies CD25 (2A3) and CD223 (LAG-3) were validated on human PBMCs at the UCLA Jonsson Comprehensive Cancer Center Flow Cytometry Core.

### Human research participants

**Policy information about studies involving human research participants**

**Population characteristics** Patients were aged ≥ 18 years, male and female, of all ethnicities, with recurrent World Health Organization grade IV malignant glioma that were candidates for surgical debulking. Key eligibility criteria included Karnofsky performance status ≥ 70, previous first line therapy with at least radiotherapy, first or second relapse with unequivocal evidence of tumor progression, adequate organ function and absence of previous anti-angiogenic or anti-vascular endothelial growth factor agents.

**Recruitment** Participants were recruited by site-specific co-investigators at their respective institutions based on protocol eligibility criteria and verified by the study coordinator. Self-selection bias may be present and may affect survival; however, patients randomized to the control arm of this study exhibited overall survival similar to that of other patients with recurrent GM.

### Magnetic resonance imaging

**Experimental design**

**Design type** N/A - The current study did not involve “functional” MRI, only anatomic MRI for visualizing and quantifying treatment response.

**Design specifications** N/A - The current study involved acquisition of structural (anatomic) MRI at screening, prior to surgery, after surgery, and every treatment cycle until tumor progression, plus subsequent “off treatment” MRI scans until patient death.

**Behavioral performance measures** N/A
### Acquisition

| Imaging type(s) | Structural/Anatomic |
|-----------------|---------------------|
| Field strength  | 1.5T & 3T           |
| Sequence & imaging parameters | Parameter matched pre- and post-contrast (gadopentate dimegumine, 0.1 mmol/kg BW) T1-weighted images were acquired using either a 2D fast spin-echo or 3D gradient echo (MPRAGE, SPGR, or IR-SPGR) sequence (repetition time (msec)/echo time (msec)/inversion time (msec) = 400–3209/3.6–21.9/0–1238; slice thickness = 1–6.5 mm; intersection gap = 0–2.5 mm; number of averages = 1–2; matrix size = 176–512 x 256–512; and field of view = 24–25.6 cm). 2D T2-weighted fast spin-echo and fluid-attenuated inversion-recovery (FLAIR) images were also acquired but not used in the current study. All on trial scans were compliant with the consensus recommendations for the international standardized brain tumor imaging protocol (Ellingson et al., Neuro Oncol 2015; 17(9): 1188-98.) |
| Area of acquisition | Whole brain |
| Diffusion MRI | Not used |

### Preprocessing

| Preprocessing software | Linear registration was performed between all images (T2, FLAIR, pre-contrast T1, post-contrast T1) to post-contrast T1-weighted images at screening using a 12-degree-of-freedom linear transformation and a correlation coefficient cost function in FSL (FLIRT; FMRIB Software Library, Oxford, England; http://www.fmrib.ox.ac.uk/fsl/). Estimates of tumor volume were performed using contrast-enhanced T1-weighted digital subtraction maps to exclude areas of post-surgical blood products or other sources of T1 shortening. T1 subtraction maps were created by first performing linear registration as described above. Next, Gaussian normalization of image intensity was performed for both nonenhanced and contrast enhanced T1-weighted images using custom c-code courtesy of the National Institutes of Health Magnetoencephalography Core Facility (3dNormalize; NIMH MEG Core, Bethesda, MD; kurage.nimh.nih.gov/meglab/Med/3dNormalize), which normalizes image intensity by dividing each voxel by the standard deviation of the image intensity from the whole brain \[SNor(x,y,z) = S(x,y,z)/stdWB\], where S is raw image signal intensity, Nor is normalized, x,y,z are voxel coordinates, and stdWB is whole brain standard deviation. Next, voxel-by-voxel subtraction between normalized nonenhanced and contrast-enhanced T1-weighted images was performed using the Analysis of Functional NeuroImages software package (AFNI; 3dcalc; https://afni.nimh.nih.gov/). Image voxels with a positive (greater than zero) before-to-after change in normalized contrast enhancement signal intensity (i.e., voxels increasing in MR signal after contrast agent administration) within T2-weighted FLAIR hyperintense regions were isolated to create the final T1 subtraction maps in order to exclude large vessels and other hyperintense regions outside the primary tumor area. Estimates of tumor volume included areas of contrast enhancement on T1 subtraction maps. Initial segmentation was performed automatically and final segmented volumes were edited by an experienced independent observer with more than 10 years of experience to exclude large vessels and any obvious non-tumor regions. |
| Normalization | Intensity normalization was performed using custom c-code courtesy of the National Institutes of Health Magnetoencephalography Core Facility (3dNormalize; NIMH MEG Core, Bethesda, MD; kurage.nimh.nih.gov/meglab/Med/3dNormalize), which normalizes image intensity by dividing each voxel by the standard deviation of the image intensity from the whole brain \[SNor(x,y,z) = S(x,y,z)/stdWB\], where S is raw image signal intensity, Nor is normalized, x,y,z are voxel coordinates, and stdWB is whole brain standard deviation. |
| Normalization template | Images were not normalized/registered to a standard template space. All images were registered to the patient-specific screening MRI exam as mentioned above. |
| Noise and artifact removal | No noise or artifact removal was performed. |
| Volume censoring | N/A |

### Statistical modeling & inference

| Model type and settings | N/A |
| Effect(s) tested | N/A |
| Specify type of analysis | Whole brain | ROI-based | Both |
| Anatomical location(s) | Areas of contrast enhancing tumor burden |
| Statistic type for inference | N/A |
| Correction | N/A |
Models & analysis

n/a | Involved in the study
- [ ] Functional and/or effective connectivity
- [X] Graph analysis
- [X] Multivariate modeling or predictive analysis

Multivariate modeling and predictive analysis

Given the relatively low number of patients and large number of possible parameters with high correlation, we used an elastic net regularized regression for variable selection. Both clinical (including IDH mutation status, age, sex, MGMT methylation status, number of prior relapses, Karnofsky performance status, steroid dose in milligrams at time of registration) and laboratory data (including mass cytometry cluster percentages, T cell receptor overlap, tumor T cell density, expanded tumor-associated T cell clones, interferon-γ-related signature scores, presence or absence of inducible PD-L1 expression on multiplex immunofluorescence) were considered potential covariates. We used 5-fold cross-validation to obtain the value of λ that gave the minimum mean cross-validated error and determined the corresponding coefficients for each covariate. Variables with nonzero coefficients were then checked for collinearity and subsequently fitted into a Cox proportional hazards model, forcing in age and sex. Peripheral blood T cell receptor clonality and tumor infiltrating lymphocyte fractions were standardized before fitting into the Cox regression due to their wide ranges. As the tumor infiltrating T cell fraction was a measurement taken at the time of surgery, we ran both a two-sample t-test (P = 0.52, t = 0.65, df = 27.6) and Wilcoxon rank sum test (P = 0.41, W = 133) to ensure that there was no significant imbalance between the two groups at alpha = 0.05 that could potentially introduce confounding into the model. We performed a Cox-Snell residual plot, which did not suggest any lack-of-fit for the multivariate Cox model.