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METHODS. We conducted a pilot study to evaluate the safety and immunological effects of vaccination with GBM6-AD, lysate of an allogeneic glioblastoma stem cell line, with poly-ICLC in patients with LGG. Patients were randomized to receive the vaccines before surgery (Arm 1) or not (Arm 2) and all patients received adjuvant vaccine. Co-primary outcomes were to evaluate the safety and immune response in the tumor.

RESULTS. A total of 17 eligible patients were enrolled – nine into Arm 1 and eight into Arm 2. This regimen was well-tolerated with no regimen-limiting toxicity. Neoadjuvant vaccination induced upregulation of type-1 cytokines and chemokines, and increased activated CD8+ T-cells in peripheral blood. Single-cell RNA/TCR-sequencing detected CD8+ T-cell clones that expanded with effector phenotype and migrated into tumor microenvironment (TME) in response to neoadjuvant vaccination. Mass cytometric analyses detected increased tissue resident-like CD8+ T-cells with effector memory phenotype in […]
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Conflict of interest
Michael R Olin and Christopher L Moertel received a portion of royalties as inventors of GBM6-AD which is exclusively licensed to the University of Minnesota. Andres M Salazar is an employee of Oncovir Inc. Andres M Salazar is Chairman, CEO, Scientific Director, and co-founder of Oncovir, Inc.
Abstract

BACKGROUND. Long-term prognosis of WHO grade II low-grade glioma (LGG) is poor secondary to risk of recurrence and malignant transformation into high-grade glioma. Given the relatively intact immune system of patients with LGG and the slow tumor growth rate, vaccines are an attractive treatment strategy.

METHODS. We conducted a pilot study to evaluate the safety and immunological effects of vaccination with GBM6-AD, lysate of an allogeneic glioblastoma stem cell line, with poly-ICLC in patients with LGG. Patients were randomized to receive the vaccines before surgery (Arm 1) or not (Arm 2) and all patients received adjuvant vaccine. Co-primary outcomes were to evaluate the safety and immune response in the tumor.

RESULTS: A total of 17 eligible patients were enrolled – nine into Arm 1 and eight into Arm 2. This regimen was well-tolerated with no regimen-limiting toxicity. Neoadjuvant vaccination induced upregulation of type-1 cytokines and chemokines, and increased activated CD8+ T-cells in peripheral blood. Single-cell RNA/TCR-sequencing detected CD8+ T-cell clones that expanded with effector phenotype and migrated into tumor microenvironment (TME) in response to neoadjuvant vaccination. Mass cytometric analyses detected increased tissue resident-like CD8+ T-cells with effector memory phenotype in TME following the neoadjuvant vaccination.

CONCLUSION. The current regimen induces effector CD8+ T-cell response in peripheral blood and
enables vaccine-reactive CD8+ T-cells to migrate into TME. Further refinements of the regimen may have to be integrated into future strategies.

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250 words
Introduction

Gliomas are the most common primary malignant central nervous system (CNS) tumors (1). As of 2016, they are classified according to histology and molecular characteristics as grade I-IV by the World Health Organization (WHO) (2). Among these clinically and molecularly diverse tumors, WHO grade II low-grade gliomas (LGGs), which include diffuse astrocytomas and oligodendrogliomas, are more common in young adults during the third and fourth decades of life. Secondary to their infiltrative nature, they are not curable with resection (3). Survival is shortened by the tendency for malignant transformation into more aggressive WHO grade III or IV high-grade glioma (HGG) (3, 4). Even with multimodal therapy (i.e., surgery, radiation therapy [RT], chemotherapy), their invasive growth and resistance to therapy result in recurrence and death in most patients within one to two decades of diagnosis (5-7). Furthermore, the majority of LGG harbor gain-of-function mutations in isocitrate dehydrogenase (IDH) 1 or 2 (8, 9). The oncometabolite D-2-hydroxyglutarate (2-HG) produced by mutant IDH is known to promote gliomagenesis and to induce immunosuppressive effects in the tumor microenvironment (TME) (10, 11).

Taken together, LGG can be considered a premalignant condition for HGG, such that novel interventions to prevent malignant transformation may improve outcomes. Immunotherapeutic modalities, such as vaccines, may offer a safe and effective option for these patients due to their tumors’ slower growth rate.
(in contrast with HGG), which should allow sufficient time for multiple immunizations and higher levels of anti-glioma immunity. The immune system of patients with LGGs may not be as compromised as that of patients with HGG because LGG patients demonstrated an excellent immunological response to the vaccines (12). Furthermore, the generally mild toxicity of vaccines may have advantages over chemotherapy or RT for long-term cognitive and quality of life impairments.

To implement an effective immunoprevention against recurrence with malignant transformation to HGG, we employed an allogeneic cell lysate-based vaccine from the glioma stem cell line GBM6-AD, which was isolated from a patient diagnosed with glioblastoma multiforme (GBM) (13). These cells express several glioma-associated immunogenic antigens (GAAs) – r.g., IL-13Rα2, EphA2, and Her-2 – that are frequently expressed at some levels in LGG and higher levels in HGG (12), suggesting that immune responses against GBM6-AD may target existing LGG tissues and provide protective immunity against HGG. In the initial study in patients with GBM, the autologous dendritic cell-based vaccine loaded with GBM6-AD was well-tolerated and associated with an immune response in a subset of patients (13).

Toll-like receptor (TLR) 3 ligands are known to serve as natural inducers of pro-inflammatory cytokines capable of promoting Type-1 adaptive immunity, and TLR3 is abundantly expressed by cells within CNS
We have previously reported that co-administration of a TLR3 ligand, polyinosinic-polycytidylic acid (poly-IC) stabilized with poly-lysine and carboxymethylcellulose (poly-ICLC), enhances CNS tumor- trafficking of vaccine-induced effector T-cells, resulting in a therapeutic effect in rodent CNS tumor models in CXCL10-dependent manners (14). Our pilot study evaluating the combination of poly-ICLC with a peptide-based vaccine in LGG patients demonstrated robust vaccine-specific response and was well tolerated (12).

Finally, the effect of immunotherapy on TME needs to be evaluated properly. A sampling of recurrent tumors after immunotherapy failure is not ideal due to the inconsistency in the timing of sampling among the patients and the potential for acquired resistance. As such, we conducted a pilot vaccine study to evaluate the safety and immunological effects of vaccination with GBM6-AD lysate and poly-ICLC with pre-surgical randomization and immunotherapy of patients, allowing for prospective procurement and evaluation of tumor samples.
Results

Patient characteristics

From September 2016 until November 2019, a total of 33 patients were screened with eight excluded for various reasons. Twenty-five patients were randomized – 13 into Arm 1 and 12 into Arm 2 – and underwent resection. Seventeen patients were available for analysis with four patients excluded after resection secondary to confirmation of malignant transformation; one excluded secondary to insufficient tissue, and three withdrawing consent (Supplemental Figure 1). Nine patients (median age, 43 years; 33.3% female, 66.6% male) were available for analysis from Arm 1 and eight patients (median age, 33 years; 62.5% female, 37.5% male) from Arm 2 (Table 1). Only one case in each arm was newly diagnosed, and all patients harbored an IDH1 mutation, though this was not required for eligibility, and one patient received neoadjuvant vaccine prior to glioma diagnosis. Oligodendrogliomas comprised 89% of patients randomized to Arm 1 and 38% in Arm 2. To help account for this bias and delineate the immunological characters of oligodendrogliomas and astrocytomas, we compared the immune cell compositions in these tumor types by CIBERSORTx deconvolution analyses of the RNA-seq gene expression data from The Cancer Genome Atlas (TCGA) (15) (Supplemental Figure 2). We found no remarkable differences between oligodendrogliomas and astrocytomas, while the differences between IDH-wild type and IDH-mutant gliomas were much more remarkable. Furthermore, we analyzed our clinical data in the current
study 1) with all eligible patients and 2) with oligodendroglioma patients only. There were no differences between Arms 1 and 2 regarding race, ethnicity, the median time between surgical resection and first adjuvant vaccination, and the median number of administered adjuvant vaccines.

Treatment and safety

The study design is shown in Figure 1. All Arm 1 patients received four doses of neoadjuvant vaccination, and most of the patients in both arms completed all scheduled adjuvant vaccinations (Table 1). Treatment was well-tolerated with only one grade 3 and no grade 4 or 5 treatment-related adverse events (TRAEs). The most common TRAE was injection site reaction (Table 2). No patients experienced any regimen-limiting toxicity (RLT).

Clinical outcomes

The median time of follow-up for all patients enrolled and who received the A1 vaccine was 20.81 months (95% CI 15.2 – 28.9 months) and all patients remain alive. Progression-free survival (PFS) was calculated from the time of post-operative A1 vaccine to time of centrally confirmed imaging progression per Response Assessment in Neuro-Oncology (RANO) for low-grade glioma (16). Median PFS was 11.0 months (95% CI 10.8 – 15.4 months) (Supplemental Figure 3A). Event-free survival (EFS) was
calculated from the time of the post-operative A1 vaccine to the time of new therapy. Median EFS was 23.7 months (95% CI 19.5 – not reached) (Supplemental Figure 3B). Of the six patients who went on to receive additional treatment, three had second surgery with one confirming malignant progression to anaplastic oligodendroglioma and two confirming recurrent low-grade gliomas. Of the three patients who went on to receive additional treatment without surgery, one had evidence of enhancement at the time of recurrence to suggest malignant transformation. There were no significant differences between PFS or EFS between Arms (Supplemental Figure 3C and D).

**Neoadjuvant vaccination with GBM6-AD lysate and poly-ICLC induces type-1 cytokines and chemokines in peripheral blood**

To evaluate the immune response induced by the study regimen in peripheral blood, we first performed the Luminex multiplex assay with serum samples to evaluate the induction of cytokines and chemokines. Serum samples were drawn at screening (for Arm 1 patients only), day of surgery, A1, and A16 (Figure 1). We previously demonstrated that tumor-specific type-1 T-cells, which predominantly secrete IFN-\(\gamma\), can efficiently traffic into CNS tumors and mediate effective therapeutic efficacy via type-1 chemokine CXCL10 (17, 18). We detected significantly elevated serum concentration levels of CXCL10, IFN-\(\gamma\), TNF-\(\alpha\), and IL-10 in Arm 1 patients on the day of surgery, which was within 48 hours after the last dose of the
neoadjuvant vaccinations, compared with Arm 1 samples at screening or Arm 2 samples on the day of surgery (Figure 2). On the other hand, in the serum samples at A16, which was three weeks after the final adjuvant vaccination, there was no upregulation in either group (Supplemental Figure 4A), suggesting a short window for cytokine response in peripheral blood, which is consistent with our previous results (17). We observed the same trends when we selectively analyzed the data from oligodendroglioma patients, though the differences in IFN-γ or TNF-α concentration between screening and day of surgery timepoints in Arm 1 patients only achieved borderline significance because of the smaller number of cases (p = 0.06, Supplemental Figure 4B).

**PD-1**

**GZMB**

**Tbet**

**effector memory and GZMB**

**Tbet**

**effector CD8**

**T-cells increase following neoadjuvant vaccination**

To evaluate the regimen-induced changes of phenotype in peripheral blood mononuclear cells (PBMC), we conducted mass cytometric analyses from samples collected at screening (in Arm 1 only), day of surgery, A1, and A16. We first extracted CD8+ T-cells using a conventional CD8+ gating strategy (Supplemental Figure 5). CD8+ T-cells were clustered on the t-distributed stochastic neighbor embedding (t-SNE) plot and grouped into 10 subpopulations and annotated based on the expression status of differentiation markers, such as CD62L, CD27, CD127, CCR7, CD45RO, CD45RA (Figure 3A
and B). By analyzing the proportions of each subpopulation among CD8⁺ T-cells, we found that PD-1⁺ Granzyme B (GZMB)hi Tbethi effector memory and GZMBhi Tbethi effector CD8⁺ T-cells were upregulated at surgery while naïve CD8⁺ T-cells were downregulated in Arm 1 patients (Figure 3C). We found that some activation markers, such as CD38, Tbet, and PD-1, were expressed on all cells in this effector memory cluster and upregulated following neoadjuvant vaccination (Figure 3D). However, we did not observe any significant differences when directly comparing the proportions of these subpopulations between Arm 1 and Arm 2 (Supplemental Figure 6). The same trend was observed in the oligodendroglioma patients (Supplemental Figure 7). These results support that the study regimen induces type-1 immune responses in peripheral blood.

We also evaluated associations between vaccine-induced immune responses and clinical outcomes. Among Arm 1 patients, we defined an immunological responder as a patient who demonstrated a 10% or higher increase in the proportion of either PD-1⁺ GZMBhi Tbethi effector memory or GZMBhi Tbethi effector population following neoadjuvant vaccination. This led to identification of four patients (with oligodendrogliomas) (103-018, -026, -29, -51) as immunological responders. There were no clear associations between the immunological response and PFS (Supplemental Figure 8).
Vaccine-reactive CD8+ T-cells with effector phenotype migrate into the tumor microenvironment

To characterize the gene expression, subset proportions, and T-cell receptor (TCR) profile of T-cells in PBMCs, we analyzed pre- and post-neoadjuvant vaccinated PBMCs from four immunological responders (103-018, -26, -29, -51), using droplet-based 5’ single-cell RNA-sequencing (scRNA-seq) and single-cell T-cell receptor-sequencing (scTCR-seq) with 10x GENOMICS platform. We obtained scRNA-seq profiles from a total of 154,929 PBMCs with paired TCR sequences in 63,932 out of 76,432 T-cells (83.6%). We identified 17 cell clusters based on scRNA-seq profiles (Figure 4A), and confirmed TCR-α and -β sequences in five T and NKT-cell clusters (Figure 4B). We then re-clustered these T and NKT-cells into nine populations based on their gene expression profiles (Figure 4C and Supplemental Figure 9A).

When comparing pre- versus post-vaccination cytotoxic T-cells, effector CD8+ T-cells and NKT-cells were enriched in post-vaccinated samples (Figure 4D and Supplemental Figure 9B). In post-vaccinated cells, there were an increased proportion of effector CD4 and CD8 cells and decreased proportion of naïve CD4 and CD8 populations, consistent with results from the mass cytometry analysis (Figure 4E and F).

Data from scTCR-seq identified T-cell clones that expanded in post-vaccination PBMC. When we focused on the top 15 frequent clonotypes in post-vaccinated samples, we found them to be enriched in post-vaccine samples compared to screening (Figure 5A). By extracting the TCR clonotypes enriched in the
post-vaccine samples with adjusted p-value < 0.15, we identified 26, 5, 13, and 32 enriched TCR-β sequences in patient 103-018, -26, -29, and -51, respectively (Figure 5B). We also performed bulk TCR-seq using genomic DNA from paired resected tumor specimen using ImmunoSEQ and found that some TCR-β clonotypes enriched in post-vaccinated peripheral blood were also identified in the corresponding tumor tissue (Figure 5B). The T-cell clones from these shared clonotypes were from the effector CD8 cluster in the PBMCs (Figure 5C) and GZMB expression was upregulated in these post-vaccine samples (Figure 5D). Moreover, within the effector CD8 cluster, the T-cells with shared clonotypes expressed a higher level of GZMB than other T-cells (Supplemental Figure 10) suggesting that some of the vaccine-reactive T-cells with cytotoxic CD8+ phenotype migrated into the TME.

To address whether the patients’ T-cell responses were directed against the vaccine-derived antigens, we conducted further evaluations of the CD8+ T-cells derived from these immunological responders at the surgery timepoint (post-neoadjuvant vaccinations). GBM6-AD cells express some of the well-characterized GAAs, such as EphA2 and IL-13Rα2 (Supplemental Figure 11A). Per RNA-seq, both GBM6-AD and patient-derived LGG tumors express these antigens, albeit at different levels (Supplemental Figure 11B). We then stimulated those T-cells with autologous dendritic cells pulsed with either GBM6-AD lysate or recombinant protein for EphA2 and IL-13Rα2 and evaluated whether the TCR
clones that had expanded following the vaccines in PBMC could further expand in response to the \textit{in vitro} stimulations. In one immunological responder (103-018), four of the top 15 most abundant CD8$^+$ T-cell TCR clonotypes (\textit{Supplemental Figure 12A}; clones 1, 3, 7, and 9) enriched more than 20\% in response to the GAA-stimulation when compared with the control group with no stimulation. However, we were not able to demonstrate a robust response of these TCR clones to GBM6-AD lysate (\textit{Supplemental Figure 12A}). Nevertheless, scTCR-seq analyses showed that the frequencies of these four CD8$^+$ T-cell clones among total T-cells in PBMC increased following the vaccination with GBM6-AD in this patient (\textit{Supplemental Figure 12B}). We also confirmed that none of these TCR clonotypes matched the known viral antigen-specific TCRs that are listed in VDJdb database (https://vdjdb.cdr3.net/). These results suggest that at least some of the TCR clones responded to the vaccine-derived antigens.

\textbf{The proportion of PD-1$^+$ CXCR3$^{hi}$ effector memory CD8$^+$ T-cells was significantly higher in the vaccinated tumor microenvironment}

We analyzed the immune profile of tumor-infiltrating lymphocytes (TILs) by mass cytometry. Four samples from Arm 1 and six from Arm 2 were available for this analysis. Because of the low frequency of leukocytes in the resected tumor samples, we extracted CD3$^+$ T-cells instead of CD8$^+$ T-cells (\textit{Supplemental Figure 13} and sub-grouped these cells into 15 clusters (\textit{Figure 6A and B}). The
proportion of CD103+ CD8+ T-cells with an effector memory phenotype (19) was significantly higher in Arm 1 tumors (Figure 6C). These T-cells were also highly positive for the CXCL10 receptor CXCR3. The proportion of regulatory T-cell (Treg) in Arm 1 trended higher but was not statistically significant (Figure 6C). Among the tissue resident-like CD8+ T-cell cluster, TILs in Arm 1 tumors demonstrated significantly higher expression levels for CXCR3, GZMB, and Tbet (Figure 6D). When we evaluated only oligodendrogliomas (three cases in each arm), TILs in Arm 1 trended a higher proportion of CD103+ CXCR3hi tissue resident-like CD8+ T-cells and effector CD8+ T-cell populations, but without statistical significance (Supplemental Figure 14). These results suggest a possibility that the CXCR3+ CD8+ T-cells were recruited into the TME by CXCL10/CXCR3 axis, and differentiated to tissue resident-like cells by the neoadjuvant vaccine. However, we were unable to detect any significant difference in expression of immune-related genes in the tumors derived from Arm 1 versus 2 patients using bulk RNA-seq analyses (Supplemental Figure 15).
Discussion

Despite the unmet need for developing effective and safe therapy for WHO grade II LGG, immunotherapy has not been extensively investigated in this population (12). By implementing a neoadjuvant design, we prospectively evaluated the impact of the GBM6-AD lysate vaccine with poly-ICLC in the peripheral blood and TME. Although the neoadjuvant vaccination-induced systemic immune response was detectable in peripheral blood, we did not observe an increase of TILs by mass cytometry (Supplemental Figure 13) or remarkable immune responses, such as effector molecule or chemokine productions, based on bulk RNA-seq analysis of the resected tumor tissues (Supplemental Figure 15). Divergence of the response between the peripheral blood and the TME suggests a substantial barrier for the systemic immune response to adequately manifest in the TME. In contrast to the current study, our prior dendritic cell-based vaccine study in combination with poly-ICLC detected induction of CXCL10 in GBM samples that recurred following vaccination (17). Gliomas, especially LGG, have a low degree of T-cell infiltration when compared with a variety of other cancers (20), and the blood-brain barrier appears to be more intact in LGG than in HGG (21).

However, we were able to detect the presence of vaccine-reactive CD8\(^+\) T-cell clones in the TME (Figure 5B, C) and increased frequency of activated CD103\(^+\) CXCR3\(^{hi}\) CD8\(^+\) T-cells in TME by single cell-based
analyses following the neoadjuvant vaccination (Figure 6C). These high-resolution analyses allowed us to detect some impact of the peripherally administered vaccination on the LGG TME. Taken together, future studies will have to integrate more effective strategies to render the LGG TME more permissive to the immune response. As tumor cells produce a variety of immunoregulatory factors (22), such as TGF-β, combination regimens with blockade of immunosuppressive pathways should be considered.

In the peripheral blood analysis, we detected a clear immune response only on the day of surgery (post neoadjuvant vaccination) in Arm 1, but not on A16 (post adjuvant vaccination) in either arm (Figure 2, 3, and Supplemental Figure 4). This may partially be secondary to differences in frequency of vaccine administration (neoadjuvant and adjuvant vaccines were administered weekly and every three weeks, respectively). It may also be due to the interval between the most recent vaccine administration and blood collection (two days in neoadjuvant vaccine while three weeks in the adjuvant vaccine). We previously reported that a dendritic cell-based peptide vaccine with poly-ICLC induced a robust upregulation of CXCL10 that peaked at 24 hours after the first vaccination in peripheral blood (17). Therefore, it is not straightforward to compare the immunological effects of neoadjuvant and adjuvant vaccines directly in the current analyses.
While we did not observe upregulated IFN-γ signatures in the post-vaccine LGG tumors compared to the control LGG tumors (Supplemental Figure 15), recent neoadjuvant immunotherapy trials evaluating anti-PD-1 antibodies in HGG patients have reported upregulated IFN-γ-related genes (23, 24). Cloughesy, et al. showed that neoadjuvant immunotherapy treatment in recurrent GBM was associated with prolonged overall survival (23). The lack of the upregulated IFN-γ signatures in the current study may reflect the difference of the immunotherapy regimen and lesser immune-permissive characteristics of the IDH-mutant LGG compared with HGG (25, 26).

GBM6-AD cells express stem cell markers and some of the GAAs, such as EphA2 and IL-13Rα2 (Supplemental Figure 11) which have defined HLA-binding T-cell epitopes in the context of HLA-A*02:01 (27-29). However, because our eligibility criteria did not require HLA typing of individual patients, it was not feasible to identify T-cell epitopes included in the GBM6-AD lysate for each patient’s unique HLA type. Nevertheless, our scRNA/TCR seq analysis enabled us to identify TCR clonotypes that expanded following the vaccinations and existed in the LGG TME (Figure 5B, C). None of the expanded TCR clonotypes matched the known viral antigen-specific TCRs. Furthermore, some of the TCR clones that had expanded following the GBM6-AD vaccine in vivo further expanded in response to stimulations with recombinant protein for EphA2 and IL-13Rα2 in vitro (Supplemental Figure 12). These results suggest
that at least some of the TCR clones responded to the vaccine-derived GAAs and the immune response elicited by the GBM6-AD is relevant to the immunogenicity of the patient-derived gliomas. However, we observed the increase of TCR clones in response to GAAs in only one patient among the four immunological responders. The absence of detectable responses in the other patients could be due to the assay sensitivity and different levels of GAA epitope-presentation on a variety of HLA types in those patients. Also, immunosuppressive factors that are expressed by glioma cells, such as CD200 (30), may have suppressed the T-cell responses against the GBM6-AD lysate. Our co-authors’ group recently implemented a phase I clinical trial to evaluate the effects of CD200-blockade in the GBM6-AD lysate vaccine in patients with recurrent high-grade gliomas (NCT04642937). To improve the design of our future vaccine studies, the use of novel algorithms developed for the identification of target epitopes recognized by the TCR repertoire (31, 32) may allow us to identify the antigenic targets.

We recognize several limitations of the current study. First, this is a pilot study with small sample size, and the follow-up periods have been insufficient to evaluate clinical benefits in LGG patients, even preliminarily. Second, the amount of resected tumor available was limited to allow a more extensive analysis, such as scRNA/TCR-seq of TIL. Third, the histological types (oligodendroglioma vs. astrocytoma) were not adequately balanced in patient assignments between Arms 1 and 2. Due to the
small sample size, our randomization was based solely on whether the patient was newly diagnosed or recurrent, but not any other factors such as histological types, prior treatment, or time from diagnosis. Oligodendroglioma has a better prognosis than astrocytoma (33). However, to the best of our knowledge, no reports have shown significant immunological differences between these two histological types. Our analyses demonstrated that there were no remarkable differences in the immune cell composition between oligodendrogliomas and astrocytomas although the the densities of some immune cells, such as CD4 memory resting cells and follicular helper T-cells, showed slight differences (Supplemental Figure 2). On the other hand, the differences between IDH-wild type and IDH-mutant gliomas were more remarkable. Further, to evaluate the impact of the vaccine regimen within the same histology, we analyzed data from oligodendroglioma cases alone and observed overall consistent results, suggesting our findings may not be heavily biased on the histology. Fourth, we analyzed the TCR repertoire profiles by scRNA/TCR-seq only in immunological responders. Additional analyses in non-responders might help us to better understand the differences in response. Fifth, we treated all patients with the combination of GBM6-AD lysate and poly-ICLC but did not include a group with monotherapy with GBM6-AD vaccine alone or poly-ICLC alone due to the small sample size, although our preclinical data demonstrating the effects of the combination (14) provided a rationale for the design of the current study.
Because the majority of LGG patients experience malignant transformation to HGG (3), we aimed for patients to mount preemptive immunity against antigens expressed in HGG by vaccinating with an allogeneic glioblastoma-derived GBM6-AD lysate. Cancer immunoprevention is based on the hypothesis that a functioning immune system controls tumor onset and development. Prophylactic vaccines against virally-caused cancers have been in the clinic (34). For tumors without viral etiologies, vaccines targeting cancer-associated mucin 1 (MUC1) have been evaluated in patients with colon adenoma as a premalignant disease or resected non-small cell lung cancer (35, 36). Additional analyses will be required to determine whether vaccination with GBM6-AD cell lysate to LGG patients would have a potential of immunoprevention for malignant transformation to HGG. Further, collaborative approaches among investigators who are committed to developing immunoprevention approaches for patients with premalignant diseases would facilitate the advancement of the field.

In conclusion, the current pilot neoadjuvant vaccine study demonstrates that some of the vaccine-reactive CD8+ T-cells can traffic to the LGG TME, although further refinements of the regimen and more active disruption of the blood-brain barrier may have to be integrated into future immunotherapeutic strategies to achieve a better clinical outcome.
Materials and Methods

Study design and patients. This study (NCT02549833) is a pilot trial assessing the safety and immunoreactivity of subcutaneous administration of GBM6-AD lysate in combination with poly-ICLC (Hiltonol, Oncovir) in patients ≥ 18 years of age with newly diagnosed or recurrent WHO grade II gliomas (defined as an astrocytoma or oligodendroglioma). Presence of IDH mutation was not a predefined eligibility criteria as the trial was designed prior to the WHO 2016 classification system. Key eligibility criteria included Karnofsky performance status ≥ 70; the presence of a supratentorial, non-enhancing T2-FLAIR lesions; anticipation of at least 500 mg tumor tissue at resection; and no history or clinical suspicion of immune system abnormalities. Prior radiation therapy, chemotherapy, or molecularly targeted therapy was allowed. Patients must have been off corticosteroid for at least two weeks before the first neoadjuvant vaccine or adjuvant vaccine.

The study design and flow diagram are summarized in Figure 1 and Supplemental Figure 1, respectively. In brief, after the consent, patients were randomized to Arm 1 or 2. Arm 1 (neoadjuvant vaccination group) patients received GBM6-AD lysate and poly-ICLC subcutaneously on days -23±2, -16±2, -9±2, and -2 relative to the scheduled surgery. At least two weeks after the post-op steroid was tapered, but within ten weeks post-surgery, the GBM6-AD/poly-ICLC vaccines were given and repeated
every three weeks for five doses (Weeks A1, A4, A7, A10, and A13; defined as the weeks from first adjuvant vaccine dose) followed by booster vaccines at Weeks A32 and A48. 2) Arm 2 (control group) patients received no vaccine prior to surgery and only received adjuvant vaccination in the same way as Arm 1 patients. The randomization took place in a 1:1 ratio between the two arms, stratified by newly diagnosed vs. recurrent.

Blood samples were obtained on days -23±2 (only Arm 1 patients), on the day of surgery, A1, A10, A16, and A32/48 if applicable. MRI was taken at screening (within 28 days prior to study enrollment and randomization), pre-operatively (24 – 48 hours prior to surgery), post-operatively (within 14 days of surgery and 28 days prior to A1 vaccine), A16, and A32/48 if applicable.

The co-primary endpoints of this study are 1) safety (the incidence and severity of adverse events associated with the treatment regimen, with an early stopping rule based on the frequency of RLT); and 2) detection of the vaccine-induced immune response in the resected tumor.

*Follow-up.* All patients were followed for response and toxicity assessments until disease progression, the start of a new therapy, or for a maximum of 18 months from study registration (whichever occurs
earlier). Toxicity was determined using the revised NCI Common Toxicity Criteria version 5.0 for Toxicity and Adverse Event Reporting (CTCAE). RLTs were defined as grade 2 or more bronchospasm or generalized urticarial; grade 2 or more allergic reaction; grade 2 or more autoimmune disease; any grade 3 toxicity related to the vaccine such as grade 3 injection site reaction, hematological or hepatic toxicity, or neurotoxicity.

Vaccine formulation with GBM6-AD lysate and poly-ICLC. GBM6-AD lysate was prepared in batches by the University of Minnesota Molecular and Cellular Therapeutics Facility using the established an allogeneic glioblastoma stem cell line GBM6-AD as the antigen source, as previously described (13). Dose vials were made under Good Manufacturing Practice (GMP) conditions for administration under IND #16,794, and provided by Dr. David McKenna Jr MD at University of Minnesota. The lysate was supplied in vials each containing 0.5 mL solution with a concentration of 2 mg/mL and stored in liquid nitrogen. Poly-ICLC, a synthetic complex of polyinosinic and polycytidylic acid, stabilized with polylysine and carboxymethyl cellulose, was available from Oncovir, Inc. It was supplied in vials each containing 1 mL of translucent solution with a concentration of 2 mg/mL and stored in a refrigerator. On the day of the scheduled vaccine, GBM6-AD lysate (1 mg protein in 0.5 ml) was mixed with 0.7 ml (1.4 mg) poly-ICLC to formulate a dose for subcutaneous administration.
Processing of human samples. Patients peripheral blood mononuclear cells (PBMC) and serum were isolated by density gradient centrifugation with Ficoll-Paque (GE Healthcare) and cryopreserved for further analysis. The freshly resected tumor tissue was minced using scalpels and digested (3 mg/mL Collagenase IV, 1 mg/mL DNase, and 2 mg/mL Trypsin Inhibitor Soybean in phosphate-buffered saline (PBS)) at 37°C for 30 minutes using a shaking heater. The samples were then filtered through the 70 mm cell strainer and washed twice with PBS. The cells were then cryopreserved for further analysis.

Luminex multiplex assay. Cytokine and chemokine analyses by multiplex assay were performed by the Immune Assessment Core at University of California, Los Angeles (UCLA). A Milliplex Human magnetic bead kit with a panel of 38 analytes (EMD Millipore, Cat #HCYTMAG-60K-PX38) was used per the manufacturer’s instructions on a DropArray 96-well plate (Curiox). Briefly, 5 mL undiluted human serum samples were mixed with 5 mL magnetic beads, and allowed to incubate overnight at 4°C while shaking. After washing the plates three times with wash buffer (PBS with 0.1% BSA and 0.05% Tween-20) in a DropArray LT Washing Station MX96 (Curiox), 5 mL of detection antibody was added and incubated for 1 hour at room temperature. 5 mL streptavidin-phycoerythrin conjugate was then added to the reaction mixture and incubated for another 30 minutes at room temperature. Following three washes, beads were
resuspended in sheath fluid, and fluorescence was quantified using a Luminex 200 instrument. Data were analyzed using MILLIPLEX Analyst 5.1 software.

Mass cytometry data acquisition. Cryopreserved patient-derived PBMCs or tumor dissociated cells were thawed 1:10 in thawing media (2% Human-AB serum-containing X-VIVO + 25 U/mL Benzonase). Cells were incubated in 5 mM of cisplatin (Cell-ID Cisplatin; Fluidigm), allowing for the distinguishing of live cells. PBMCs, but not tumor dissociated cells, were then fixed with 1.6% PFA and barcoded with Cell-ID 20-Plex Pd Barcoding Kit (Fluidigm). After Fc blocking (Human TruStain FcX; Biolegend), cells were stained with metal-conjugated surface antibodies cocktail (Supplemental Table 1). Cells were then permeabilized with Perm-S Buffer (Fluidigm) and stained with intracellular antibodies cocktail (Supplemental Table 1), followed by resuspension in Iridium intercalator (Cell-ID Intercalator; Fluidigm) solution overnight. Cells were then washed and resuspended in running buffer consisting of a 1:10 dilution of normalization beads (EQ Four Element Calibration Beads; Fluidigm) in deionized water. Samples were then acquired on the Fluidigm Helios Mass Cytometer and resultant data was exported to FCS files for further processing. In the PBMC analyses, eight samples (samples from four time points from two patients) were barcoded, stained, and acquired on Mass Cytometer simultaneously in each experiment.
Processing of mass cytometry data. To control for sensitivity variability of the Helios Mass Cytometer both within and across samples, raw FCS files were processed by the normalizer function provided by the Parker Institute of Cancer Immunotherapy premessa package on R Studio. Normalization beads were removed on the same platform. The processed files were uploaded to the Cytobank platform and de-barcoded manually. Each immune subpopulation such as CD8$^+$ T-cells was gated and exported as shown in Supplemental Figures 5 and 13. These exported files were then uploaded to the cytofkit package (37), where immune cells were subjected to dimension reductional algorithm t-Distributed Stochastic Neighboring Embedding (tSNE) for visualization in 2D space, and clustered using FlowSOM. The cells in each cluster were then phenotyped and analyzed using z-score normalized marker expression and population data respectively. All analytic outputs were generated on R Studio unless noted otherwise.

Single-cell RNA-sequencing and single-cell TCR-sequencing. Preparation of scRNA-seq and scTCR-seq libraries and sequencing were performed by CoLabs at University of California San Francisco (UCSF). Chromium Next GEM Single Cell V(D)J Reagent Kits v1.1 (10x GENOMICS) was used per the manufacturer's instructions. In brief, cryopreserved patient-derived PBMCs were washed with PBS containing 0.04% bovine serum albumin (BSA) and resuspended in PBS containing 0.04% BSA to a final concentration of 1,000 cells per mL. Cells were captured in droplets and nanoliter-scale Gel Beads-in-
emulsion (GEMs) were generated. Following reverse transcription and cell barcoding, GEMs were broken, and cDNA was purified using Silane magnetic beads followed by amplified via PCR (98°C for 45 s; 13-18 cycles of 98°C for 20 s, 67°C for 30 s, 72°C for 1 min; 72°C for 1 min). Amplified cDNA was then used for both 5' gene expression library construction and TCR enrichment. For gene expression library construction, 50 ng of amplified cDNA was fragmented and end-repaired, double-sided size-selected with SPRIselect beads, PCR-amplified with sample indexing primers (98°C for 45 s; 14-16 cycles of 98°C for 20 s, 54°C for 30 s, 72°C for 20 s; 72°C for 1 min), and double-sided size-selected with SPRIselect beads. For TCR library construction, TCR transcripts were enriched from 2 mL of amplified cDNA by PCR (primer sets 1 and 2: 98°C for 45 s; 10 cycles of 98°C for 20 s, 67°C for 30 s, 72°C for 1 min; 72°C for 1 min). Following TCR enrichment, 50 ng of enriched PCR product was fragmented and end-repaired, size-selected with SPRIselect beads, PCR-amplified with sample-indexing primers (98°C for 45 s; 9 cycles of 98°C for 20 s, 54°C for 30 s, 72°C for 20 s; 72°C for 1 min), and size-selected with SPRIselect beads. The scRNA-seq and scTCR-seq libraries were sequenced on an Illumina NovaSeq 6000 to sequencing depth of 500 million reads and 60 million reads per sample, respectively. The sequencing data are available in the Gene Expression Omnibus (GEO) database (GEO GSE188620).

Processing of scRNA-seq and TCR-seq data. The scRNA-seq reads were aligned to the GRCh38
reference genome and quantified using cellranger count (10x GENOMICS, version 4.0.0). Filtered gene-barcode matrices that contained only barcodes with unique molecular identifier (UMI) counts that passed the threshold for cell detection were used for further analysis. The scTCR-seq reads were aligned to the GRCh38 reference genome and consensus TCR annotation was performed using cellranger vdj (10x GENOMICS, version 4.0.0). To identify the TCR clonotypes which were enriched in post vaccinated samples, the frequencies of each TCR clonotype (TRA and TRB combination) in pre- and post-vaccinated samples in each patient were compared, and the clonotypes which frequencies in the post-vaccinated sample were higher than the pre-vaccinated sample with adjusted p-value <0.15 (calculated by Benjamini-Hochberg procedure) were defined as “enriched clonotype”.

All additional analyses were performed using Seurat (version 4.0.0) (38). Cells with less than 200 or greater than 3500 genes detected or greater than 10% mitochondrial RNA content were excluded from the analysis. Seurat objects were generated from raw UMI counts in each sample and counts data were log-normalized independently. The TCR information was added to the corresponding Seurat object using the AddMetaData function. For clustering of all cell types in PBMC, variable genes and anchors were called on using FindVariableFeatures and FindIntegrationAnchors function, respectively, resulted in generating the integrated Seurat object. Scaled z-scores for each gene were calculated using the
ScaleData function and user input into a principal component analysis (PCA) based on variable genes. Clusters were identified using shared nearest neighbor (SNN)-based clustering based on the first 10 principal components with $k = 30$ and resolution $= 0.3$, and the same principal components were used to generate the Uniform manifold approximation and projection (UMAP). Clusters were then annotated based on the expression of known marker genes (39). The cells in five clusters that represent T- and NKT-cell clusters were extracted and re-clustered using SNN-based clustering based on the first 10 principal components with $k = 30$ and resolution $= 0.2$.

**Bulk TCR-seq.** For bulk TCR-seq in tumor-infiltrating lymphocytes (TILs), genomic DNAs were extracted from frozen resected tumor specimens using AllPrep DNA/RNA Mini Kit (Qiagen) per manufacturer’s instructions. TCR-β complementarity-determining region 3 (CDR3) regions were amplified and sequenced from 2.5-3 mg of genomic DNA utilizing the immunoSEQ Assay (Adaptive Biotechnologies). Sequences were collapsed and filtered to identify and quantitate the absolute abundance of each unique TCR-β CDR3. To evaluate whether the T-cells with enriched clonotypes in peripheral blood migrated into the TME, we assessed TCR-β overlap between TILs determined by bulk TCR-seq and PBMCs determined by scTCR-seq and visualized Venn diagram using VennDiagram package on R Studio.
Coculture of CD8+ T-cells with dendritic cells (DCs). Human CD14+ cells and T-cells were isolated from cryopreserved PBMCs using CD14 MicroBeads (Miltenyi Biotec) and EasySep Human T Cell Isolation Kit (STEMCELL Technologies), respectively. Monocyte-DCs were generated from human CD14+ cells using CellXVivo Human Monocyte-derived DC Differentiation Kit (R&D Systems). Immature DCs were incubated with or without either GBM6-AD lysate (10 µg/mL) or recombinant human EphA2 and IL-13Rα2 (10 µg/mL, Sino Biological) for 8h. DCs and T-cells were cocultured at the ratio of 1:4 in X-vivo supplemented with 2% human AB serum and 5 ng/ml of human IL-7 (PeproTech) for 5 days. CD8+ T-cells were isolated from the cocultured cells using CD8+ T-Cell Isolation Kit (Miltenyi Biotec). Genomic DNA was extracted from CD8+ T-cells using NucleoSpin Tissue (Takara Bio) for bulk TCR-seq.

Tumor bulk RNA-seq library preparation. Total RNA was extracted from frozen resected tumor specimens using AllPrep DNA RNA Mini Kit (Qiagen) following the manufacturer's protocol. RNA integrity was evaluated using Agilent Bioanalyzer 2100 (Agilent) and the samples with RIN ≥ 7 were used for the following analyses. The following library preparation and sequencing were performed by DNA Technologies and Expression Analysis Core at University of California Davis (UC Davis) Genome Center. Strand-specific and barcode indexed RNA-seq libraries were generated from 300 ng total RNA each after poly-A enrichment using the Kapa mRNA-seq Hyper kit (Kapa Biosystems) following the instructions of
the manufacturer. The fragment size distribution of the libraries was verified via micro-capillary gel electrophoresis on a Bioanalyzer 2100. The libraries were quantified by fluorometry on a Qubit fluorometer (LifeTechnologies) and pooled in equimolar ratios. The pool was quantified by qPCR with a Kapa Library Quant kit (Kapa Biosystems) and sequenced on an Illumina NovaSeq 6000 (Illumina) with paired-end 150 bp reads. The sequencing data are available in the Gene Expression Omnibus (GEO) database (GEO GSE188620).

**Tumor bulk RNA-seq data analyses.** Quality check (QC) and adapter-trimming were performed on the generated FASTQ sequencing data using fastq (v0.21.0) with default settings (40). An average of 180.8 million pass-QC sequencing read was obtained per tumor sample. Pass-QC read were then mapped to the human reference genome hg19 (GRCh37.p13) using STAR (v2.5.4b) with the guidance of transcriptome annotation Homo_sapiens.GRCh37.87.chr.gtf. The subsequent sorting and indexing were carried out using samtools (v1.10). Gene-level expression abundance was calculated using stringtie (v2.1.4) as count and transcript-per-million reads (TPM) values (41).

**The Cancer Genome Atlas Data Analysis.** RNA-seq gene expression data was downloaded through the UCSC Xena Toil web portal (dataset ID: TcgTargetGtex_rsem_gene_tpm; version: 2016-09-03;
From the whole dataset, 656 cases were extracted as subject to analysis (151 cases from TCGA-GBM and 447 cases from TCGA-LGG). We classified the cases into IDH-wildtype glioma (IDHwt), IDH-mutant, 1p19q-non-codeleted astrocytoma (IDH-A), and IDH-mutant, 1p19q-codeleted oligodendroglioma (IDH-O), based on their previously defined molecular diagnoses (43). The values in the downloaded data were then converted back to transcript-per-million (TPM) values for subsequent analyses.

**Immune Cell Deconvolution Analysis.** For immune cell composition prediction deconvolution, the TPM-summarized gene expression data was uploaded to and analyzed by CIBERSORTx with ‘absolute’ mode and with quantile normalization disabled (15) (https://cibersortx.stanford.edu/). The analysis estimated the score of each of the samples as to the 22 distinct immune cell compositions (‘LM22’), which can be compared among samples as well as cell types, but does not represent the cell fraction. The scores were compared among the three molecular categories with the Kruskal-Wallis test with Holm’s multiple testing corrections followed by the Dunn post hoc test.

**Statistical analysis.** The statistical differences in the concentration of chemokines/cytokines in serum
(Luminex), the proportion of each cluster among each sample (CyTOF and scRNA-seq) were calculated using paired Wilcoxon test for longitudinal analysis (among Arm 1 samples) while non-paired Wilcoxon test was used for the direct comparison between Arm 1 and 2 samples. The differences of the several markers expression on every single cell in CyTOF data were analyzed by a non-paired t-test. PFS (time from A1 date which the date of the first adjuvant vaccine to disease progression per RANO criteria) was estimated using Kaplan Meier survival curves and statistical differences were analyzed by Log-Lank test.

Statistical analysis and data visualization were performed with R version 4.0.2 or GraphPad Prism version 6.01, and p < 0.05 was regarded as statistically significant.

**Study approval.** This study (NCT02549833) was approved by institutional review boards at University of California, San Francisco, and was conducted according to the Declaration of Helsinki. All patients provided written informed consent.
Author contributions

JWT and H. Okada conceptualized and designed the study. H. Ogino, TN, PBW, KO, A. Saijo analyzed PBMC and tumor tissue samples. JWT, NAB, JLC, NAO, SHJ, PT, SMC, and MSB recruited patients and managed clinical care. A. Shai, CMW, and JJP processed the tumor specimens and performed the pathological analyses. DG, MT, and JER coordinated the trial and summarized the clinical data. AMM supervised the statistical and analytical methods used. MRO, CLM developed the GBM6-AD vaccine. AMS provided poly-ICLC. ALL authors participated in writing and revising the manuscript.
Acknowledgments

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References


Table 1. Patients' baseline characteristics

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Arm 1 (n = 9)</th>
<th>Arm 2 (n = 8)</th>
<th>Total</th>
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<tbody>
<tr>
<td>Median age, yr (range)</td>
<td>43 (24-62)</td>
<td>33 (31-69)</td>
<td>33 (24-69)</td>
</tr>
<tr>
<td>Female, no. (%)</td>
<td>3 (33.3%)</td>
<td>5 (62.5%)</td>
<td>8 (47.1%)</td>
</tr>
<tr>
<td>Recurrent, no. (%)</td>
<td>8 (88.8%)</td>
<td>7 (87.5%)</td>
<td>15 (88.2%)</td>
</tr>
<tr>
<td>Histology, no. (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Astrocytoma</td>
<td>1 (11.1%)</td>
<td>5 (62.5%)</td>
<td>6 (35.3%)</td>
</tr>
<tr>
<td>Oligodendroglioma</td>
<td>8 (88.8%)</td>
<td>3 (37.5%)</td>
<td>11 (64.7%)</td>
</tr>
<tr>
<td>IDH1 mutation, no. (%)</td>
<td>9 (100%)</td>
<td>8 (100%)</td>
<td>17 (100%)</td>
</tr>
<tr>
<td>Race</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>White</td>
<td>8 (88.8%)</td>
<td>6 (75.0%)</td>
<td>14 (82.3%)</td>
</tr>
<tr>
<td>African American</td>
<td>1 (11.1%)</td>
<td>0 (0%)</td>
<td>1 (5.9%)</td>
</tr>
<tr>
<td>Asian</td>
<td>0 (0%)</td>
<td>1 (12.5%)</td>
<td>1 (5.9%)</td>
</tr>
<tr>
<td>Other (not available)</td>
<td>0 (0%)</td>
<td>1 (12.5%)</td>
<td>1 (5.9%)</td>
</tr>
<tr>
<td>Ethnicity</td>
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<tr>
<td>Non-Hispanic</td>
<td>9 (100%)</td>
<td>7 (87.5%)</td>
<td>16 (94.1%)</td>
</tr>
<tr>
<td>Median no. of days from first pre-surgical</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>vaccine to surgery (range)</td>
<td>23 (21-25)</td>
<td></td>
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<tr>
<td>Median no. of days from surgery to first</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>post-surgical vaccine (range)</td>
<td>35 (26-48)</td>
<td>32 (27-36)</td>
<td>34 (26-48)</td>
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<tr>
<td>Median no. of adjuvant vaccines (range)</td>
<td>7 (5-7)</td>
<td>7 (6-7)</td>
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</tr>
<tr>
<td>No. of patients analyzed for immune profiling</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Luminex</td>
<td>8 (88.8%)</td>
<td>8 (100%)</td>
<td>16 (94.1%)</td>
</tr>
<tr>
<td>CyTOF (PBMC)</td>
<td>8 (88.8%)</td>
<td>8 (100%)</td>
<td>16 (94.1%)</td>
</tr>
<tr>
<td>CyTOF (tumor)</td>
<td>4 (44.4%)</td>
<td>6 (75.0%)</td>
<td>10 (58.8%)</td>
</tr>
<tr>
<td>scRNA-seq</td>
<td>4 (44.4%)</td>
<td>0 (0%)</td>
<td>4 (23.5%)</td>
</tr>
<tr>
<td>Median time from diagnosis to surgery on</td>
<td>6.3 (0-20)</td>
<td>4.7 (0-12)</td>
<td>4.7 (0-20)</td>
</tr>
<tr>
<td>trial, years</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prior radiotherapy</td>
<td>1 (11.2%)</td>
<td>1 (12.5%)</td>
<td>2 (11.8%)</td>
</tr>
<tr>
<td>Prior systemic therapy</td>
<td>3 (33.3%)</td>
<td>4 (50.0%)</td>
<td>7 (41.2%)</td>
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Table 2: Adverse Events

<table>
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<th>Arm 1 (n = 13)</th>
<th>Arm 2 (n = 8)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>(290 AE’s)</td>
<td>(155 AE’s)</td>
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<tr>
<td><strong>General Disorders</strong></td>
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<tr>
<td>Injection site reaction</td>
<td>137 (47.2%)</td>
<td>80 (51.6%)</td>
</tr>
<tr>
<td>Fatigue</td>
<td>29 (10.0%)</td>
<td>11 (7.1%)</td>
</tr>
<tr>
<td>Fever</td>
<td>1 (0.3%)</td>
<td>11 (7.1%)</td>
</tr>
<tr>
<td>Flu like symptoms</td>
<td>33 (11.4%)</td>
<td>9 (5.8%)</td>
</tr>
<tr>
<td><strong>Nervous System Related</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Headache</td>
<td>16 (5.5%)</td>
<td>9 (5.8%)</td>
</tr>
<tr>
<td>Seizure</td>
<td>1† (0.3%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Dizziness</td>
<td>3 (1.0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Myalgia</td>
<td>5 (1.7%)</td>
<td>1 (0.6%)</td>
</tr>
<tr>
<td><strong>Gastrointestinal Disorders</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td>8 (2.8%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Constipation</td>
<td>1 (0.3%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>0 (0%)</td>
<td>1 (0.6%)</td>
</tr>
<tr>
<td><strong>Skin Disorder</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pruritus</td>
<td>10 (3.4%)</td>
<td>2 (1.3%)</td>
</tr>
<tr>
<td><strong>Blood Disorders</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anemia</td>
<td>5 (1.7%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Leukopenia</td>
<td>2 (0.7%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Neutropenia</td>
<td>3 (1.0%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Lymphopenia</td>
<td>4 (1.4%)</td>
<td>1 (0.6%)</td>
</tr>
<tr>
<td><strong>Laboratory Results</strong></td>
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<tr>
<td>ALT increase</td>
<td>6 (2.1%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>AST increase</td>
<td>1 (0.3%)</td>
<td>0 (0%)</td>
</tr>
<tr>
<td>Bilirubin increase</td>
<td>1 (0.3%)</td>
<td>0 (0%)</td>
</tr>
</tbody>
</table>

†Grade 3 Adverse Event
Day 0: The day of surgery

Week A1: 1st post-op vaccine

Arm 1: Neoadjuvant Treatment

Arm 2: Control

Vaccination (GBM6 lysate + poly-ICLC)

Patients with newly diagnosed or recurrent WHO grade II glioma

Steroid

*Booster Vaccines (if applicable)

PBMC / Serum

PBMC / Serum

PBMC / Serum

PBMC / Serum

PBMC / Serum

Figure 1. The schema of this study.
Figure 2. Neoadjuvant vaccinations with GBM6-AD lysate and poly-ICLC induced the upregulation of type-1 chemokines and cytokines in peripheral blood. Serum concentrations of multiple chemokines and cytokines were measured by Luminex multiplex assay. The type-1 chemokine CXCL10 was elevated in Arm 1 samples on the day of surgery, within 48 hours of the last neoadjuvant vaccination. Effector cytokines, such as IFN-γ, TNF-α, and IL-10, also demonstrated significant upregulations following the neoadjuvant vaccines. *p<0.05 (calculated by paired Wilcoxon test) and **p<0.05 (calculated by non-paired Wilcoxon test).
Figure 3. Mass cytometric analyses detected increases of PD-1\(^+\) GZMB\(^{hi}\) Tbet\(^{hi}\) effector memory and GZMB\(^{hi}\) Tbet\(^{hi}\) effector CD8\(^+\) T-cells following the neoadjuvant vaccines. (A) T-distributed stochastic neighbor embedding (t-SNE) plot of CD8\(^+\) T-cells. To evaluate the vaccine-induced changes of phenotype in peripheral blood, mass cytometric analyses were conducted. CD8\(^+\) T-cells were subjected to dimension reductional algorithm t-SNE for visualization in 2D space and clustered by FlowSOM based on the expression status of seven differentiation markers (CD62L, CCR7, CD127, CD45RO, CD45RA, and PD-1). (B) Heatmap visualizing the relative expression (Z score) of T-cell-relevant markers in each subpopulation. Each cluster was annotated based on the expression status of differentiation markers as listed above. (C) The longitudinal analyses of proportions of each subpopulation in Arm 1 patients. Neoadjuvant vaccination with GBM6-AD and poly-ICLC increased PD-1\(^+\) GZMB\(^{hi}\) Tbet\(^{hi}\) effector memory and GZMB\(^{hi}\) Tbet\(^{hi}\) effector CD8\(^+\) T-cells while decreasing naive CD8\(^+\) T-cells. \(*p<0.05\) (paired Wilcoxon test). (D) The expression levels of activation markers, such as CD38, Tbet, and PD-1, on the PD-1\(^+\) GZMB\(^{hi}\) Tbet\(^{hi}\) effector memory cells were enhanced in the samples obtained following the neoadjuvant vaccines. \(*p<0.05\) (non-paired t-test).
Figure 4. Single-cell RNA-sequencing (scRNA-seq) analyses revealed the increases of effector CD4+ and CD8+, and decreases of naïve CD4+ and CD8+ T-cell populations following the neoadjuvant vaccinations. ScRNA-seq and scTCR-seq analyses on 10x GENOMICS platform were conducted in PBMCs obtained from the four immunological responders (103-018, -26, -29, -51) at the baseline and post-neoadjuvant vaccines. (A) UMAP of pooled PBMCs from all four patients at both baseline and post-neoadjuvant vaccines. Clusters were annotated based on the expression of known marker genes. Mono; monocyte, DC; dendritic cells, pDC; plasmacytoid dendritic cells, MK; megakaryocytes, B; B cells, NK; natural killer cells, NKT; natural killer T-cells. (B) UMAP was colored by TCR detection. TCRs were mainly detected in five clusters which represent T- and NKT-cell populations (pink). (C) UMAP of T- and NKT-cells. T- and NKT-cell populations were re-clustered and grouped into nine subpopulations. EM; effector memory, Treg; regulatory T-cells. (D) UMAP of T- and NKT-cells was colored by treatment status (either pre- or post-vaccination). Cytotoxic T-cells, such as effector CD8+ T-cells and NKT-cells, were enriched in post-vaccinated samples (light blue). (E) The bar plot showing the proportion of each cell cluster in each sample. (F) Quantification of each cell cluster in pre- and post-vaccinated samples. The proportion of effector T-cells showed a trend toward an increase in post-vaccinated samples while that of naïve T-cells showed a trend toward a decrease. P-values were calculated by paired Wilcoxon test.
Figure 5. Vaccine-reactive CD8+ T-cell clones with an effector phenotype migrated into the tumor microenvironment. (A) Top 15 frequent clonotypes in post-vaccinated samples were extracted, and their frequencies were compared. Most of these clonotypes showed higher frequencies in post-vaccinated samples than in the baseline. (B) The TCR clonotypes which were enriched in post-vaccinated PBMC were extracted with an adjusted p-value < 0.15. Patient 103-018, -26, -29, and -51 were found to have 26, 5, 13, and 32 enriched TCR-β clonotypes, respectively, in their PBMCs. Some of these clonotypes were also found in TCR repertoire of corresponding tumors (determined by bulk TCR-seq). (C) The T-cell clones that have these overlapped clonotypes mostly belonged to the effector CD8 cluster in PBMC in all cases. (D, E) The expression of GZMB was upregulated by neoadjuvant vaccinations in these T-cell clones. Log-normalized (count) on x-axis was calculated as log (count / [total count of the cell] x 10,000 + 1). *p<0.05 (non-paired t-test).
Figure 6. The proportion of tissue resident-like CD8+ T-cells with effector memory phenotype was significantly higher in the vaccinated tumor microenvironment. Single-cell suspensions dissociated from tumor samples from Arm 1 (4 cases) and Arm 2 (6 cases) were analyzed by mass cytometry. (A) CD3+ T-cells were subjected to dimension reductional algorithm t-SNE and clustered by FlowSOM based on the expression status of 10 differentiation markers (CD4, CD8a, CD62L, CD27, CD127, CCR7, CD45RO, CD45RA, CD25, and PD-1). (B) Heatmap visualizing the relative expression (Z score) of T-cell-relevant markers in each subpopulation. Each cluster was annotated based on the expression status of differentiation markers as listed above. (C) The proportion of tissue resident-like CD8+ T-cells with effector memory phenotype (CD103+, PD-1+, CXCR3hi, CCR7-, CD45RO+, GZMBhi) was significantly higher in Arm 1 samples. *p<0.05 (non-paired Wilcoxon test). The proportion of regulatory T-cells (Tregs) in Arm 1 showed a trend toward a higher percentage than Arm 2 but without statistical significance. (D) TILs in this tissue resident-like CD8+ T-cells cluster in Arm 1 tumors demonstrated significantly higher expression levels for the CXCL10 receptor CXCR3, GZMB, and Tbet than those in Arm 2 tumors.