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Lessons learned from phase 3 trials of immunotherapy for glioblastoma: Time for longitudinal sampling?

Ethan Chen, Alexander L. Ling, David A. Reardon, and E. Antonio Chiocca

All author affiliations are listed at the end of the article

Corresponding Author: E. Antonio Chiocca, MD, PhD, Department of Neurosurgery, Brigham and Women's Hospital, Boston, MA 02115, USA (eachiocca@bwh.harvard.edu)

Abstract

Glioblastoma (GBM)'s median overall survival is almost 21 months. Six phase 3 immunotherapy clinical trials have recently been published, yet 5/6 did not meet approval by regulatory bodies. For the sixth, approval is uncertain. Trial failures result from multiple factors, ranging from intrinsic tumor biology to clinical trial design. Understanding the clinical and basic science of these 6 trials is compelled by other immunotherapies reaching the point of advanced phase 3 clinical trial testing. We need to understand more of the science in human GBMs in early trials: the "window of opportunity" design may not be best to understand complex changes brought about by immunotherapeutic perturbations of the GBM microenvironment. The convergence of increased safety of image-guided biopsies with "multi-omics" of small cell numbers now permits longitudinal sampling of tumor and biofluids to dissect the complex temporal changes in the GBM microenvironment as a function of the immunotherapy.

Key Points

There have been multiple failures of clinical trials in immunotherapies for glioblastoma (GBMs). The reasons for these failures are manifold. There is a need to improve the science of understanding human GBM clinical trial data. For complex therapies such as immunotherapies, longitudinal and spatial approaches to the science acquired from clinical trial specimens are required to understand the therapeutic perturbations on human tumor and biofluids.

The Current Treatment Landscape of GBM

Despite extensive knowledge related to the genetics of glioblastomas (GBMs), there have been no breakthrough therapies leading to extensive and durable survival of patients. Instead, there has been incremental improvement of reported median overall survival (mOS) of GBM patients since the early 2000s, first with the approval of temozolomide (TMZ) and then of tumor-treatment fields (TTFs).[1,2] Extensive surgical cytoreduction also leads to improved survival, based on evidence from retrospective analyses[3] and from post hoc analyses of subjects in randomized controlled trials (RCTs).[4,5] However, survivorship in clinical trials and/or surgery is based on strict trial eligibility criteria which routinely exclude subjects with less favorable demographic, performance, or imaging features. Clinical trial results can overestimate outcome as reflected by the discord of a median OS of only 8 months based on recent US registry data[6] relative to the 20+-month OS reported from the TTF trial.[2]

Several reasons are responsible for the lack of effective treatments. One is the significant intratumoral heterogeneity in the mutations and signal aberrancies within each individual tumor, as shown by single-cell genetics classifying the molecular and transcriptional programs of GBMs.[7] A second is that each tumor is composed of an extensive microenvironment characterized by endothelial cells and pericytes involved in neo-angiogenesis, reactive astrocytes, neurons and microglia, and several types of immune cells.[8] Such diversity could likely underlie why targeting one cancer-promoting vulnerability via targeted therapy or cell type within a tumor via anti-angiogenic therapy has led to tumor escape. Third, the blood–brain barrier (BBB) has been cited as a reason for failure of passage of antitumor agents into GBMs.[9] Fourth, there are now multiple preclinical and clinical data showing that the commonly utilized steroid, dexamethasone, leads to profound immunosuppression.[10–15] In fact, several immunotherapy trials now require that no or minimal steroid be used during treatment.[16,17] Lastly, the relative perceived inaccessibility of

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GBMs has limited the availability of biospecimens during trials. When biospecimens are harvested, they are usually a small fraction from all patients on the trial. There is a lack of systematic prospective and statistically valid analyses to determine the number of biospecimens required to support meaningful conclusions from the collected data.

One additional factor to consider in the interpretation of data from past clinical trials is that the latest World Health Organization (WHO) 2021 changed the classification of GBM such that it is now incorporates genetic markers with traditional histopathologic features.[18] Since several clinical trials (including immunotherapy trials) were completed before this new classification, some clinical trial data now need to be reanalyzed to distinguish therapy effects on GBMs versus other gliomas, such as Isocitrate Dehydrogenase mutant (IDHmut) astrocytoma. In fact, recent clinical data have questioned the clinical benefit of the Stupp regimen once GBMs are reclassified based on the new WHO 2021 criteria.[19]

Rationale Behind Immunotherapy for GBM

Three biologic features of GBM render immunotherapy attractive as a treatment. First, within each GBM there is significant clonal heterogeneity in terms of mutations and signaling anomalies[20]: several immunotherapies (such as immune checkpoint inhibitors) are agnostic to a priori knowledge of these GBM targets. Second, the GBM tumor microenvironment (TME) is highly immunosuppressive[21] and several immunotherapies aim to flip the suppressive phenotype into one that can help engender adaptive durable immunity against the GBM. Lastly, most systemic therapies must penetrate the BBB. However, the presentation of GBM antigens is thought to occur outside the confines of the BBB, primarily in head and neck lymph nodes.[22] This obviates the limitations posed for systemic immunotherapy to have to penetrate the BBB. Recently, immunotherapies have seen success with several solid cancers including CNS metastases, prompting renewed interest in applications to GBM as discussed below.

Phase 3 Clinical Trials of Immunotherapy for GBM

In recent times, after testing in early phase 1 and 2 trials, a total of 6 GBM immunotherapy phase 3 clinical trial studies have been performed and published (Table 1).

ACT IV: Epidermal Growth Factor Receptor Variant III Peptide Vaccine

Epidermal growth factor receptor variant III (EGFRvIII) is a truncated peptide of EGFR that is constitutively active.[23] It was first described in GBM and is a true tumor-specific antigen (TSA). It is estimated that approximately one-third of GBMs express it. EGFRvIII has been targeted preclinically by using chimeric antigen receptor (CAR)T cells,[24] dendritic cell vaccines pulsed with it,[25] and/or peptide vaccinations.[26] Phase 1[27] and 2 phase 2[28,29] clinical trials targeting EGFRvIII-expressing newly diagnosed GBMs (nGBM) were carried out by vaccinations with EGFRvIII linked to keyhole limpet hemocyanin (KLH) adjuvant (Rindopepimut) via subcutaneous injections: these showed the relative safety and encouraging survival data of 20+ months when compared to contemporaneous historical controls of less than 14 months. In fact, in a small phase 2 trial (ACTIVATE/ACT II),[30] median progression-free survival (mPFS) and mOS for patients receiving Rindopepimut were 14.2 and 24.6 months, respectively. Among recurrent GBM (rGBM) patients, 82% no longer exhibited detectable EGFRvIII expression. This smaller phase 2 trial was followed up with a much larger multi-institutional, single-arm phase 2 trial (ACT III) with 65 nGBM patients.[29] mPFS and mOS from diagnosis were 12.3 and 24.6 months, respectively, and EGFRvIII was not detectable in 4/6 (67%) of tumor samples obtained after >3 months of treatment.

Based on these results, a randomized blinded phase 3 trial (the ACT IV study) was carried out in nearly 200 locations worldwide, with more than 700 patients.[31] The trial compared survival in EGFRvIII-positive nGBM patients who were treated with standard of care (SOC) radiation/temozolomide (TMZ) and then received either the EGFRvIII vaccine (Rindopepimut) plus GM-CSF or placebo. There was no significant survival benefit for Rindopepimut over the control group, with the control group showing an OS of 20.1 months, and the treatment group, 20.0 months. Explanations for this trial failure are included in Table 2. This relative increased survival of 20.1 months in the control group (that historically had been reported to be 14 months) points to the issue that eligibility criteria selecting for subjects with more favorable demographic, imaging, and performance characteristics will also lead to mOS longer than what is expected from historical data. A somewhat unexpected finding was that loss of EGFRvIII expression by reverse transcriptase-polymerase chain reaction (RT-PCR) was observed in both the Rindopepimut and control group. Although only a fraction of patients were available to study this, one implication may be that EGFRvIII could have been lost during the precedent SOC before randomization to trial, perhaps underscoring the temporal plasticity of GBM as a function of treatment.[31] The results of the trial also suggest that immunotherapies targeting single antigens are unlikely to observe survival improvements due to the inherent heterogeneity[32] as well as immune escape related to loss of expression or downregulation of target antigen.

Toca511: Replicating Retroviral Gene Therapy

The Toca511 trial utilized a replicating retroviral vector that carried the gene for yeast cytosine deaminase (yCD), endowing chemosensitivity to the prodrug 5-fluorocytosine (5-FC; Table 1).[33] Preclinical studies showed that the cytotoxic cell death that ensued with rejection of tumors was due to a cytotoxic CD8+T-cell antitumor response.[34] In addition, 5-fluorouracil (5-FU) derived from the conversion of 5-FC was also shown to be directly cytotoxic to myeloid-derived suppressor cells (MDSCs) in tumors.[35] The initially published multi-institutional phase 1 trial enrolled 43 subjects that underwent a craniotomy to

Table 1. Recent Randomized Phase 3 Clinical Trials of Immunotherapy in GBM

Trial/Treatment Name, Agent, Year of Publication	Targeted Tumor	Trial Type	Design	Efficacy	Serious Adverse Events	Additional Results
ACT IV, Rindopepimut, EGFRvIII peptide vaccine linked to KLH (2017)	Newly diagnosed EGFRvIII+ GBM	RCT, double-blind, multicenter	Maximal surgical resection and standard chemoradiation without progression. Then randomized 1:1 Rindopepimut +TMZ (n = 371) vs. KLH placebo + TMZ (n = 374), administered intradermally.	Study terminated for futility after preplanned interim analysis due to no significant difference in mOS	Seizures, brain edema, pulmonary embolus	- Robust humoral responses in the Rindopepimut group. - EGFRvIII expression after treatment undetectable in 57% of Rindopepimut and 59% of control patients
Toca511/FC, replicating retrovirus that delivers yCD to sensitize tumors to 5-FC (2020)	First or second recurrence of GBM or AA.	RCT, open-label, multicenter	Randomized at time of resection 1:1 to Toca511/FC (Toca511 administered in tumor cavity followed by intravenous 5-FC, n = 201) or SOC (investigator choice of lomustine, TMZ, or Bev, n = 202)	No significant difference in mOS in the ITT population.	Neurologic deficits	- Patients at second recurrence or with AA or with IDHmut with possible improved mOS - IDHmut tumors with higher baseline levels of immune cells and less immunosuppressive cells. - Median cycles of 5-FC posttreatment were only two. due to tumor progression.
Checkmate 143, nivolumab, (2020)	First recurrence of GBM after standard chemoradiation	RCT, open-label, multicenter	Randomized after chemoradiation to nivolumab (n = 184) or bevacizumab (n = 185)	No significant difference in mOS	No significant safety events attributable to nivolumab alone	- Hazard ratio for patients with no baseline steroid use was lower for the nivolumab group. - mOS was increased in patients with no baseline steroids and methylated MGMT promoter
Checkmate 498, nivolumab (2022)	nGBM with unmethylated MGMT promoter	RCT, open-label, multicenter	Randomized to nivolumab + RT (n =280) or TMZ + RT (n = 280)	Statistically significant survival benefit of SOC TMZ + RT over nivolumab + RT	Cerebral edema, suddent death, respiratory failure	- PD-L1 expression did not predict responses.
Checkmate 548, nivolumab (2022)	nGBM with methylated MGMT promoter	RCT, open-label, multicenter	Randomized to nivolumab + RT +TMZ (n = 355) or Placebo + TMZ + RT (n = 354)	No significant difference in mOS or mPFS	Respiratory failure/distress, pancytopenia, pneumonia	- PD-L1 expression did not predict responses
DCVax-L, Autologous DCs loaded with tumor lysates (2022)	N and rGBMs	Prospective, externally controlled nonrandomized multicenter	DCVax-L + SOC (n = 232) vs. contemporaneous matched external controls treated with SOC (n = 99)[A]	Increase in mOS for treatment compared to control[A]	Cerebral edema	- tail of long-term responders

[A]Refer to main text regarding limitations of design and data analyses/interpretation.

resect recurrent HGG and at the same time the peritumoral cavity was free-hand injected with escalating doses of the agent.[36] After 6 weeks elapsed to allow for sufficient tumor transduction, subjects started the prodrug 5-FC. In the efficacy evaluable population, OS for recurrent HGG subjects was 13.6 months. In this phase 1 trial, the number of CD4+ T cells increased in treated subjects. In addition, genomic analyses of tumors showed that subjects who survived the longest also expressed transcripts associated with neuronal function.

After this trial, a randomized phase 3 trial was completed.[37] Patients with recurrent HGG were randomized 1:1

Table 2. Reasons for Failures and Lessons Learned for the 6 Phase 3 Immunotherapy Clinical Trials for GBM

Rindopepimut (EGFRvIII peptide vaccine)
1—**Reason for failure:** The control group mOS fared better than what had been shown in the historical control group used in the earlier phase trials. **Lesson learned:** Use prospective and concomitantly accruing control groups, perhaps with propensity score matching, in early trials to maximize the validity of OS.
2—**Reason for failure:** The single target was effectively eliminated but this was not sufficient to inhibit tumors from growing via other pathways. **Lesson learned:** GBM's high degree of intratumor heterogeneity makes it very unlikely that single-target therapy can be successful, even if the target seems to be a potent driver of tumor growth.
3—**Reason for failure:** The target (EGFRvIII) was lost in both control and treatment groups. **Lesson learned:** GBM's evolution and adaptation even to standard therapies may lead to loss of the targeted antigen.
Toca511 (immunogenic replicating retrovirus)
1—**Reason for failure:** The trial encompasses all high-grade glioma populations. **Lesson learned:** Post hoc analyses of earlier phases of the modality and of the phase 3 trial itself revealed that benefit was more likely for slower growing gliomas, such as IDHmut ones, and not for GBM, perhaps because time was needed for vector integration into tumors and generation of effective adaptive immune responses.
2—**Reason for failure:** Trial design used injection of the vector into the cavity of resected tumors. **Lesson learned:** Since retroviral vector integration and delivery of immunogenic transgene requires cell division, the number of residual tumor cells in the resected peritumoral cavity may have been too low for efficient gene delivery.
The 3 Checkmate Trials (monoclonal antibodies against PD-1)
1—**Reason for failure:** Little preexisting biomarker data to study trial failure. **Lessons learned:** Encompass biomarker data during the trial itself or in early-phase trials via "window of opportunity" or (even better) longitudinal sampling trials. Therefore, the 6 lessons below are relatively speculative: **a**—The immunosuppressive GBM TME does not allow penetration and/or persistence of activated antitumor lymphocytes. **b**—There are multiple immune checkpoint and immune-evasive signals employed by GBM to evade immunotherapy. Targeting only one (PD-1) axis is not sufficient. **c**—The timing of anti-PD-1 administration with surgical resection or other therapies is important due to the complex temporal kinetics that the PD-1 signaling axis has on T cells' life cycle. **d**—PD-1 and PD-L1 may co-exist on same cells or not in different tumors and therapy outcomes may depend on this interplay. **e**—The balance between PD-1 blockade activating CD4+ regulatory T vs. CD8+ cytotoxic T cells dictates effectiveness of the therapy for different tumor types.
DCVax-L (dendritic cell vaccine pulsed with autologous GBM lysates)
1—**Reason for failure:** The clinical trial primary endpoint was PFS based on MRI imaging. **Lesson learned:** With immunotherapies, tumor inflammation can make MRIs difficult to interpret in terms of progression.
2—**Reason for failure:** The clinical trial allowed for subjects in placebo group to "cross over" to treatment group upon suspected progression. **Lesson Learned:** With a high degree of "crossover," the statistical power to evaluate OS in the placebo group was lost. Current attempts to include external control groups may be limited in being accepted by medical and regulatory community.
3—**Reason for failure:** Absence of biomarker data inhibits our understanding of the reasons behind the "tail" of long-term survivors in the trial. **Lesson learned:** Need to include biomarker data via longitudinal sampling during the trial.

to receive Toca511/FC (n = 201) or SOC control (n = 202). For the SOC control group, patients received investigators' choice of single therapy: lomustine, TMZ, or bevacizumab. There was no difference in outcomes with a mOS of 11.10 months for the Toca511/FC group and 12.22 months for the control group. Post hoc analyses of small subsets of patients showed that patients enrolled at second recurrence appeared to have an increase in mOS compared to the control group. Further, subjects with IDHmut tumor or grade 3 anaplastic astrocytoma (AA) did better with Toca511 (n = 8 for IDHmut and n = 7 for AA) than control (n = 11 for IDHmut and n = 6 for AA). Genomic analyses showed that the IDHmut tumors appeared to have less of an immunosuppressive TME which could have explained a preferential benefit. It is not clear from the trial whether the AAs had genetic mutations characteristic of GBM (and thus expected to behave like GBM) or not. It thus seems that there was perhaps an effect of Toca511 for multiply recurrent IDHmut or possibly slower growing AAs, but the trial was not powered or designed to test this. A possible explanation for this is that a slower rate of growth may have helped not only the extent and distribution of gene transduction but also provided more time for administration of multiple cycles of 5-FC. Although these analyses were carried out post hoc, they could have informed subsequent prospective trial eligibility to IDHmut gliomas, before proceeding with a large RCT targeting all HGG, including GBM.

The Checkmate Trials: Immune Checkpoint Monoclonal Antibodies Against PD-1

The Checkmate Trials were a series of clinical trials centered around the usage of nivolumab, a human IgG-4 PD-1 immune checkpoint inhibitor, which reached phase III targeting GBM patients in the recurrent setting (Checkmate 143),[38] or in nGBM patients with unmethylated MGMT promoter (Checkmate 498)[39] or in nGBM patients with a methylated MGMT promoter (Checkmate 548; Table 1).[40] The

rationale for two different trials targeting the unmethylated vs. methylated MGMT promoter nGBMs was that in the former case TMZ, which can cause lymphopenia and immunosuppression, was not administered as per the Stupp regimen. Overall, results were largely disappointing, with OSs not different from that measured in the placebo arm of each of the 3 trials. The impact of the trials was also reduced by limited biomarker analyses both pre- and posttreatment.

More specifically, Checkmate 143 compared nivolumab with bevacizumab in rGBM patients, resulting in comparable mOS and worse mPFS in the experimental group, but potential modest benefit in rGBM, MGMT-methylated patients.[38] Checkmate 498 compared radiotherapy (RT) + nivolumab versus RT + TMZ in MGMT-unmethylated nGBM: improved mOS was reported in the control group compared to the immune checkpoint inhibitor group.[39] Checkmate 548 compared TMZ + RT + nivolumab versus SOC (RT + TMZ + placebo): mOS was not different for placebo compared to nivolumab.[40]

Explanations for the failures of these trials have varied, but in general all have suffered from a relative lack of scientific biomarker data (Table 2):

1 The first explanation is that GBMs are highly lymphocyte-depleted and have been described as immune-deserted for T cells.[21,41] Therefore, systemic activation of T cells by inhibition of PD-1 would not ensure that these T cells could traffic to and engage GBM cells thriving in a milieu of immunosuppressive cells and factors.
2 Another explanation is that the PD-1/PD-L1 pathway may not be the only immune checkpoint pathway involved in maintaining GBM evasion. Surprisingly, an exploratory phase I clinical trial combining nivolumab and ipilimumab,[42] a CTLA-4 inhibitor, showed a lack of benefit over bevacizumab in contrast to preclinical experiments that showed an anti-PD-1, anti-CTLA-4 combination therapy capable of curing 75% of GBM immunocompetent mice,[43] somewhat emphasizing the gap between murine models and clinical translation. In addition, we know that there are multiple other immune checkpoint signals[44–48] and epigenetic signals[49,50] that maintain GBM immunoevasion.
3 A third explanation is that the timing of immune checkpoint inhibitor administration may matter. In fact, a small phase 2 trial comparing adjuvant versus neoadjuvant pembrolizumab in subjects with rGBM showed significantly improved survival in the latter group.[51] However, this study was amended to include an additional 25 patients to the neoadjuvant pembrolizumab arm and no survival benefit was observed.[52] Thus, the question of improved survival associated with neoadjuvant PD-1 blockade remains unclear. This trial was coupled with extensive biomarker correlates showing that the neoadjuvant regimen led to a significant increase in tumor expression of IFNγ-associated transcriptomes with a decrease in tumor cell cycle gene expression signatures. Two other trials also examined tumor signatures of subjects with apparent responses to PD-1 blockade. In one study,[53] genomic and transcriptomic analyses of tumor tissue showed a significant enrichment of PTEN mutations associated with immunosuppressive gene signatures in nonresponders, while there was enrichment of MAPK pathway alterations (PTPN11, BRAF) in responders. Single-cell RNA sequencing of one of the PTEN-mutant, nonresponder tumors showed an immunosuppressive signature primarily from CD44 + tumor cells, a marker of tumor cell invasion into brain. In the second study, Schalper et al.[54] showed the presence of more diversity in T-cell receptor (TCR) sequences (a marker of T cell clonotypes) in subjects treated with nivolumab compared to historical untreated tumors and TCR clonotype diversity was also associated with survival. These small studies suggest the importance of tumor biomarker analyses missing from most patients in the Checkmate Trials which could have led to improved selection of subjects and to possible explanations related to trial failures.
4 Another explanation may also be entertained based on a recent study that found that PD-1 to PD-L1 binding can occur in cis in antigen-presenting cells (APCs). With PD-1 binding to PD-L1 on APCs, more vacant PD-1 molecules on T cells could lead to T-cell activation.[55] This has correlated with better patient prognosis in patient tumor tissue expressing both PD-1 and PD-L1 at higher levels.
5 A perhaps final explanation may be the recent finding that PD-1 blockade can also activate FoxP3+ CD4+ T cells, which have an immunosuppressive regulatory function.[56] Therefore, the response or lack thereof of a tumor to PD-1 blockade results from the balance of immune-activating PD-1 blockade on cytotoxic CD8+ T cells versus immune-suppressing PD-1 blockade on regulatory CD4+ T cells.

DCVax-L: Autologous Dendritic Cells Pulsed With Autologous GBM Lysates

DCVax-L[57] was the first phase 3 clinical trial for GBM with a personalized component (Table 1). In this trial, patients underwent SOC for GBM and then received several doses of autologous dendritic cells processed and activated using patient tumor lysate as loaded vaccines.[58] The injected dendritic cells then theoretically induced immune recognition of tumor antigens and increased intratumoral antitumor immunity, potentiating immune memory.

There have been 2 reports related to the results from the phase 3 trial. The first preliminary report of 331 subjects randomized after surgery and chemoradiation to receive DCVax-L and TMZ ($n = 232$) versus placebo and TMZ ($n = 99$) reported a mOS of 23.1 months for the former.[58] Two hundred and fifty out of 1599 screened patients were excluded per protocol if there was MRI evidence of early progression or pseudoprogression after standard chemoradiation, a trial exclusionary criterion that is fairly routine. This exclusion (both for the placebo and the DCVax-L group) would ensure that aggressive tumors able to evade chemoradiation would not be in the trial and perhaps make OS and PFS times longer than one would expect from a general GBM population. The mOS for the placebo group was not reported because 90% of subjects crossed over from the placebo to the DCVax-L group upon determination of progression (based on MRI) from treatment. In fact,

the trial design initially allowed this crossover because the primary endpoint was PFS and not mOS. The crossover allowed in the trial underscores the need for appropriate prospective statistical designs in RCTs allowing for this occurrence. The absence of reporting of data for the placebo group also is not a common occurrence in RCTs. In this trial approximately 30% of the subjects (n = 100) initially were reported to show extended survival. This was not fully explained by known prognostic factors: only 29% were younger than 50 years of age, 65.9% had methylated MGMT, 71% had a complete resection, and only 8% of these patients had all 3 positive prognostic factors. However, the status of IDH was not reported presumably because the trial was conducted at a time (2007–2015) when IDH status was not yet fully available at all centers, and this would confound the interpretation of this survival data.

The second and final report was recently published.[59] In this manuscript, the data were reanalyzed and now the DCVax-L group exhibited a mOS of 19.3 months from randomization and 22.4 months from surgery. To increase statistical power to the small number of control group patients that had not crossed over, the authors supplemented these with an external control population that exhibited a mOS of 16.5 months. There have been several criticisms leveled to the design and analyses of this trial, most notably the fact that the originally stated primary endpoint of PFS was not different between the groups, presumably because the possibility of vaccine-induced inflammation would have obscured the estimate of MRI-based progression.[60,61] In addition, flaws of the external controls including lack of propensity matching may confound the interpretation of the results. Lastly, the relatively prolonged time that occurred between the end of the trial and this final report is also problematic because of changes in classification of disease and patient and provider expectations related to perceived benefits of a therapy without possibility of critical peer-reviewed evaluation of the data.

Like the other phase 3 studies, though, the relative absence of biomarker data from subject tissues during study therapy limits the ability of the scientific and clinical community to know whether a biologic and immunologic effect did occur in some subjects like the ones who survived the longest (11% at 30 months). It should be noted though that the authors did show increased TILs and other biomarkers of immunological response in tumors treated with DCVax-L which correlated with survival in previous small phase 1 trials.[62–65] With this knowledge, it may have been possible to try and design trials based on this biologic and immunologic biomarker data.

What Is on the Horizon? Other Advanced Immunotherapy Trials for GBM

Current Phase 2 and 3 Trials Recruiting or in Process

Supplementary Table 1 lists multiple relatively advanced phase 2/3 trials of immunotherapy in process or recruiting. These include peptide or DC vaccines, adoptive T-cell therapies, new immune checkpoint inhibitors, oncolytic viruses, vaccines against different peptides expressed in GBM (survivin, CMV, etc.) as well as several others. This shows continued interest in trying multiple immunotherapy modalities for this intractable cancer.

Immunotherapies in Early-Phase Clinical Trials

Continuing along the line of personalized immunotherapy, several patient-based vaccines have been developed to diversify targeted tumor characteristics. A recent clinical trial tested a personalized neoantigen vaccine where tumor neoepitopes were determined via whole exome and RNA sequencing, and synthetic peptides and then manufactured as vaccines to prime the immune system against the tumor.[66] The GAPVAC trials also treated patients with synthetic "off-the-shelf" unmutated antigens matching tumor HLAs, followed by synthesizing peptides against newly discovered mutated tumor epitopes, which induced mostly a CD4+ response.[67] Both trials had well-tolerated responses from patients and showed the emergence of vaccine-specific TILs. The GAPVAC trials showed a mOS of 29.0 months with a mPFS of 14.2, and some long-term persistent epitope-specific CD8s. For the personalized neoantigen vaccine, epitope-specific TILs demonstrated markers of exhaustion and there was evidence that dexamethasone use limited the therapeutic effect.

The IDH mutation also provides a tumor-specific epitope. IDH1-vac, a IDH1-specific peptide vaccine, was shown to be well-tolerated by patients. It led to a 3-year progression-free rate of 63% and 3-year death-free rate of 84% in grade 3 and 4 IDH1mut astrocytoma. Though specific immune data are limited, increased vaccine efficacy was shown in patients with vaccine-specific immune responses.[68] Targeting cytomegalovirus proteins expressed in GBM has also been proposed, where an autologous dendritic cell, pp65-antigen targeting vaccine with DI (dose-intensified)-TMZ, showed a significant difference in survival of 41.1 months compared to 19.2 months in the control group.[69] Although increases in peptide specific IFNγ were noted to correlate with survival, concurrent usage of TMZ may have led to significant Treg expansion and its prolonged use may have limited the vaccine's effects at later timepoints, with the study suggesting that dosing optimization may lead to even better results. New research, however, has suggested Treg plasticity as a phenomenon, with Tregs demonstrating a T_H1 phenotype under IFNγ presence, which may explain the surprising efficacy of the vaccine.[70] Overall, these data suggest that patients may have to be studied more in depth with regards to biologic and immunologic variables that could stratify those with an improved immune response to the applied immunotherapy.

Other novel approaches include the use of gene therapy—one trial injected a vector to induce tumor-specific IL-12 transcription only under the presence of the activator ligand Veledimex to trigger an intratumoral immune response.[71] The activator ligand was shown to cross the BBB through oral administration, resulting in dose-dependent inflammatory response by IFNγ level increase, as well as persistent intratumoral CD8 T cells. These immune responses suggest the development of an inflamed

TME. Due to results also demonstrating increased PD-1 and PD-L1 expression by T cells posttreatment, a combinatory study of the IL12 gene therapy with a PD-1 inhibitor was initiated.[72] Surprisingly his combination did not lead to improved survival effects: possible explanations are several and could range from timing of immune checkpoint blockade administration to the need to block other immune-evasive pathways in GBM. Another series of gene therapy trials utilized an adenoviral vector, aglatimagene besadenovec (AdV-tk, CAN-2409), containing the herpes simplex virus (HSV) thymidine kinase gene. The expression of this gene endows chemosensitivity to nucleoside analogs, such as valacyclovir, ganciclovir, and acyclovir, that lead to DNA damage and cytotoxic immunogenic cell death. This also synergizes with the DNA damage from radiation.[73] CAN-2409 was injected peritumorally at the time of craniotomy in nGBM patients and this was followed by the nucleoside analog with concomitant chemoradiation.[74] A multi-institutional phase 2 clinical trial with a concurrent prospectively matched external database cohort showed that mOS was improved in the treatment group, but what was more interesting was that the significant improvement in mOS (increased by 8.1 months!) and mPFS occurred in subjects who had undergone a gross total and not subtotal resection, consistent with the rationale that immunotherapies may work best with minimal residual disease.[5]

Oncolytic viruses are also being investigated in the context of GBM. DNX-2401 is an adenovirus with a 24-base pair deletion in the E1A gene restricting viral replication to cells with defective retinoblastoma signaling.[75] The treatment demonstrated a mOS of 12.5 months in a rGBM cohort when combined with pembrolizumab (PD-1 inhibitor),[76] as well as extended survival in a diffuse intrinsic pontine glioma cohort to 17.8 months,[77] a disease where the median survival after diagnosis is conventionally less than 1 year.[78] Patients with a moderate inflammatory phenotype and PD-1 expression at presentation derived the most benefit from the adenovirus–pembrolizumab combination, as increased T-cell exhaustion was found through markers such as TIGIT and LAG3, while insufficient PD-1 expression weakened a possible benefit from pembrolizumab. This reiterates the need for better understanding of the immune landscape before applying immune checkpoint inhibitors as adjunct therapy.[76] Another oncolytic virus is G47Δ, which is a HSV type 1 (HSV-1) with deletions in the viral genes for α4, γ34.5, inactivation of the viral ICP6 gene, and US11 promoter overlap: these were engineered to minimize replication in non-cancer cells which could lead to neurotoxicity. The mOS was reported to be 20.2 months for a cohort of rGBM patients and this has led to approval by the Japanese equivalent of the FDA.[79] In this trial, the authors injected G47Δ using a longitudinal injection design with concomitant biopsies spaced over several months. This allowed them to study the histologic evolution of the tumor as a function of treatment.[80] Single-arm approval of an agent administered to a small cohort of patients is unusual. Most experts agree that use of historical control data to evaluate the benefit of a therapy is also fraught with bias. Another oncolytic HSV also showed immunotherapy effects with reported encouraging survival in a pediatric high-grade glioma patient population[81] and is currently in evaluation in a phase 2 trial.[82] A possible advantage of this agent has been its ability to target the myeloid population[83] which is a major contributor to immunosuppression in the GBM TME. The oncolytic virus discipline has attempted to understand in early phase 1 clinical trials what are the immunologic determinants of possible patient responses. Several immunologic and biologic signatures have been reported to correlate with subject responses in small phase 1 trials of a measles virus,[84] reovirus,[85,86] adenovirus,[87] retrovirus,[88] and poliovirus[89]: the immunologic and biologic signatures of responses could point towards selecting more appropriate patients for more advanced trials of each of these modalities.

Another approach has been the use of stem cells, such as neural[90] or mesenchymal,[91,92] to deliver oncolytic viruses in subjects with GBM. In a first in human clinical trial, neural stem cells were utilized to deliver an oncolytic adenovirus in GBM patients by direct intratumoral injection showing the safety of the approach.[93] These modalities may possibly resolve issues related to delivery of biologic and immunologic agents into GBMs.

Excitement for GBM immunotherapy is also being generated by using CAR T cells.[24,94] CAR T-cell therapy has also been attempted for glioma patients, specifically for H3K27M-mutated diffuse midline gliomas.[95] Though increased intracranial pressure required careful monitoring due to the treatment's potential inflammatory effects, administration through an Ommaya reservoir was reported to show benefit for patients in a very small clinical trial.[95] The challenge of GBM heterogeneity is being addressed in a multifaceted fashion, including the increase in epitopes targeted in vaccination options, such as personalization through matching tumor profiles to an overexpressed or tumor-specific "database" of HLAs,[96] and through peptide vaccines specifically targeting identified neoantigens within resected GBM tumor. Preclinical developments have been made in the realm of CAR T-cell therapy, where new receptor designs have been generated to potentially increase immunological targets. SynNotch CAR T cells[97,98] and LINK CAR T cells[99] represent the next generation of CAR T systems with the potential of targeting multiple antigens through "prime-and-kill" or "logic-gated" CAR T systems that attempt to avoid immune escape. Despite both showing some success in murine models, much evidence is still needed in ascertaining their clinical efficacy.

A Way Forward: Longitudinal Sampling of GBMs During Clinical Trials

The Time Has Come to Longitudinally Sample GBMs and Fluids From Patients on Trial

Although there has been considerable value with "window of opportunity" trials where tissue is collected at one timepoint relatively early after treatment initiation,[100–102] this approach is limited by a single timepoint collection of tumor tissue and by the short interval of collection after the therapeutic perturbation. Window of opportunity trials may be appropriate to understand a relatively simple mechanistic question, such as target engagement by a new drug, or to ascertain drug delivery into enhancing

and nonenhancing tumor components. Nonetheless, complex mechanisms that evolve over time, such as long-term changes in TME induced by immunotherapy, underscore the need for more sophisticated trial designs that could be used to maximize scientific information (Figure 1). The study from Todo et al.[79] has shown that multiple longitudinal biopsies from GBM subjects on trial are possible and relatively well-tolerated. Although these biopsies are composed of small number of cells, technologic advances allow these small samples to be analyzed successfully for sophisticated genomic, immunologic, and biologic assays, such as single-cell RNA sequencing, spatial transcriptomics, and immunopeptidomics as discussed below. We argue that the next phase of clinical trial design should use longitudinal biopsies of GBMs and associated fluids (cerebrospinal fluid and peripheral blood) to study the effect of the immunotherapy and/or other therapies more comprehensively. In this context, via a multi-institutional consortium (Breakthrough Cancer), we have initiated an early-phase trial of an immunotherapy where subjects undergo several longitudinal biopsies of their GBM and associated fluids over several months in the trial (NCT03152318). Understanding how the immunotherapy under study changes GBMs will be critical to decide how to best proceed for the next phase of clinical trials.

Several challenges exist in the implementation of a longitudinal sampling trial for GBM. Table 3 attempts to list these as well as possible solutions with the caveat that only experimental implementation will determine if these challenges and solutions are valid and/or if other challenges and solutions exist. While "multi-omic" analyses of serially sampled tissues hold incredible promise for understanding the biological impact of clinical therapies, great care must be taken in the experimental design and analysis of such studies. Different -omic platforms vary in features such as sensitivity, coverage, and reproducibility—requiring robust statistical approaches when combining data from different platforms for use in unified analyses. A number of tools are available for performing such data integration tasks for different types of multi-omic analyses (ie, disease subtyping, biomarker prediction, etc.) using both Bayesian and non-Bayesian approaches.[103]

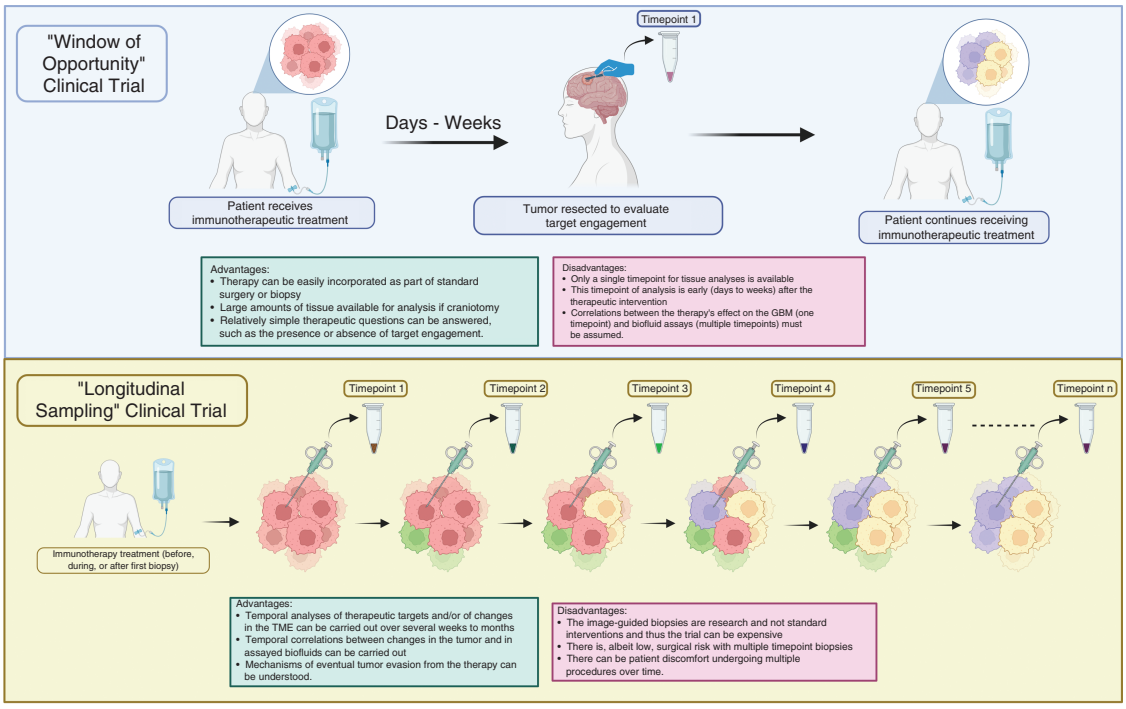

Figure 1. "Window of opportunity" (top row) versus "longitudinal sampling" clinical trial. In the window of opportunity trial (top row), a therapeutic intervention is performed before tumor resection or biopsy. The advantages of this approach are: 1—the therapy can be easily incorporated as part of standard surgery or biopsy; 2—if the surgery is a craniotomy, large amounts of tissue is available for analysis; and 3—relatively simple therapeutic questions can be answered, such as the presence or absence of target engagement. The disadvantages are: 1—only a single timepoint for tissue analyses is available; 2—this timepoint of analysis is early (days to weeks) after the therapeutic intervention; 3—correlations between the therapy's effect on the GBM (1 timepoint) and biofluid assays (multiple timepoints) must be assumed. In the longitudinal sampling trial, multiple timepoint image-guided biopsies are performed before, during, and/or after the therapeutic intervention. The advantages of this approach are: 1—temporal analyses of therapeutic targets and/or of changes in the TME can be carried out over several weeks to months; 2—temporal correlations between changes in the tumor and in assayed biofluids can be carried out; 3—mechanisms of eventual tumor evasion from the therapy can be understood. The disadvantages of this approach are: 1—the image-guided biopsies are research and not standard interventions and thus the trial can be too expensive for the routine research-based funding mechanisms; 2—there is, albeit low, surgical risk with multiple timepoint biopsies; 3—there can be patient discomfort undergoing multiple procedures over time.

Table 3. Challenges and Possible Solutions for Implementation of Longitudinal Sampling Clinical Trial

Challenge	Possible Solution(s)
Ethics of multiple longitudinal surgical biopsies (outside of SOC) for tissue analyses	Perform a few initial "proof-of principle" and "proof-of-safety" trials with intratumoral therapy at same time as biopsy, before trials assessing systemic therapies.
Expense related to multiple longitudinal stereotactic biopsies not covered by third-party or pharmaceutical sponsors	- Attract philanthropic or governmental support to show value of longitudinal biopsies in new therapy evaluation - Implement processes and workfows that make each stereotactic surgical biopsy a less expensive outpatient procedure
Surgical morbidity (infections, hemorrhage)	- Minimize foreign material (hemostatic agents, multilayered closure) in wound. - Careful preoperative planning for needle trajectories targeting tumor regions for biopsy
Sampling bias due to tumor heterogeneity	- Target different areas of tumor (Enhancing and FLAIR) with 6–8 core biopsies using routine neurosurgical techniques
Determination of number of biopsies required for each timepoint to determine responses to the investigational agent	- Perform pilot experiments with 1–2 biopsies during the first few timepoints to determine if selected "-omic" assays are providing useful data.
Quality control and adequacy of biopsied tissue for "multi-omic" assays	- Same as above
Causal determination of changes due to the therapeutic agent	- Compare analyzed changes to those known to occur with tumor evolution and determine if changes are consistent with purported mechanism of action of the investigational agent[A]
Temporal spacing and number of longitudinal biopsies	Determination would depend on the mechanism of action of the investigation agent[B]
Analyses of complex longitudinal "multi-omic" data	Refer to several recent reviews of complex biostatistics and bioinformatic platforms for analyses of "multi-omics" data.

[A]As examples, for a kinase inhibitor, serial proteomic and phosphoproteomic changes would be analyzed in 2–3 different regions of tumor to determine if there are longitudinal changes in signaling pathways affected by the kinase under question and to determine the magnitude and degree of inhibition of the kinase pathway both spatially and temporally. For a CAR T-cell therapy different assays may be more relevant such as changes in immune cell profiles and multiplex immunofluorescence technologies.
[B]As examples, for an immunotherapy investigational agent where adaptive responses require time, serial biopsies spaced over weeks to months may be required. For a drug with rapid engagement of a tumor target early and more frequent biopsies may be more reasonable.

When studying serially sampled tissue, it is also important to differentiate between observed effects that are caused by technical factors—such as variance from sampling different tumor locations at different timepoints, disease progression between timepoints, and different sampling approaches used at different timepoints—from effects that are caused by treatment. How best to integrate such factors into power calculations for multi-omic studies is still an area of active development.[104] As more interventional studies conduct serial spatial and temporal sampling for multi-omic analyses, the data they generate will provide important insight into the extent to which technical variance can be expected across different omic assays during serial sampling—enabling the development of more robust statistical approaches to deal with these challenges. In the meantime, technical variance can be limited as much as possible by taking such steps as ensuring that timepoints, sampling methods, and tissue processing are consistent across patients, and by assaying tissue from multiple locations within each tumor at each timepoint to control for spatial variability.

As further discussed below, the question arises whether the small amounts of tissues acquired during a biopsy sequentially over time could be sufficient to study effects of therapy.

New Technologies Can Interrogate Even Low Numbers of Cells From a Biopsied Tumor

New technologies that have arisen over the last decade are revolutionizing the capacity to dissect GBMs and its microenvironment, utilizing even small amounts of human tissue.[105] In fact, more and more of the science related to GBM is being conducted in the realm of patient tissues rather than exclusively relying on mouse models. Comprehensive reviews of these technologies have been reported and the integration across several layers of what is now being called "omics" data requires not just sophisticated mathematical and bioinformatic algorithms but also machine-learning and artificial intelligence technologies.[106–108] For the purposes of this review, we briefly describe technologies that already are and could become clinically useful to understand the science of GBMs in the context of a therapeutic clinical trial (Figure 2).

At the DNA level, selected cancer gene exome sequencing (and sometimes whole exome sequencing) is now routinely performed both commercially and at major medical and cancer centers to decipher the most common mutations in oncogenes and tumor suppressor genes. Coupled with analyses of chromosomal allelic loss, rearrangements, copy number variations, DNA methylation,

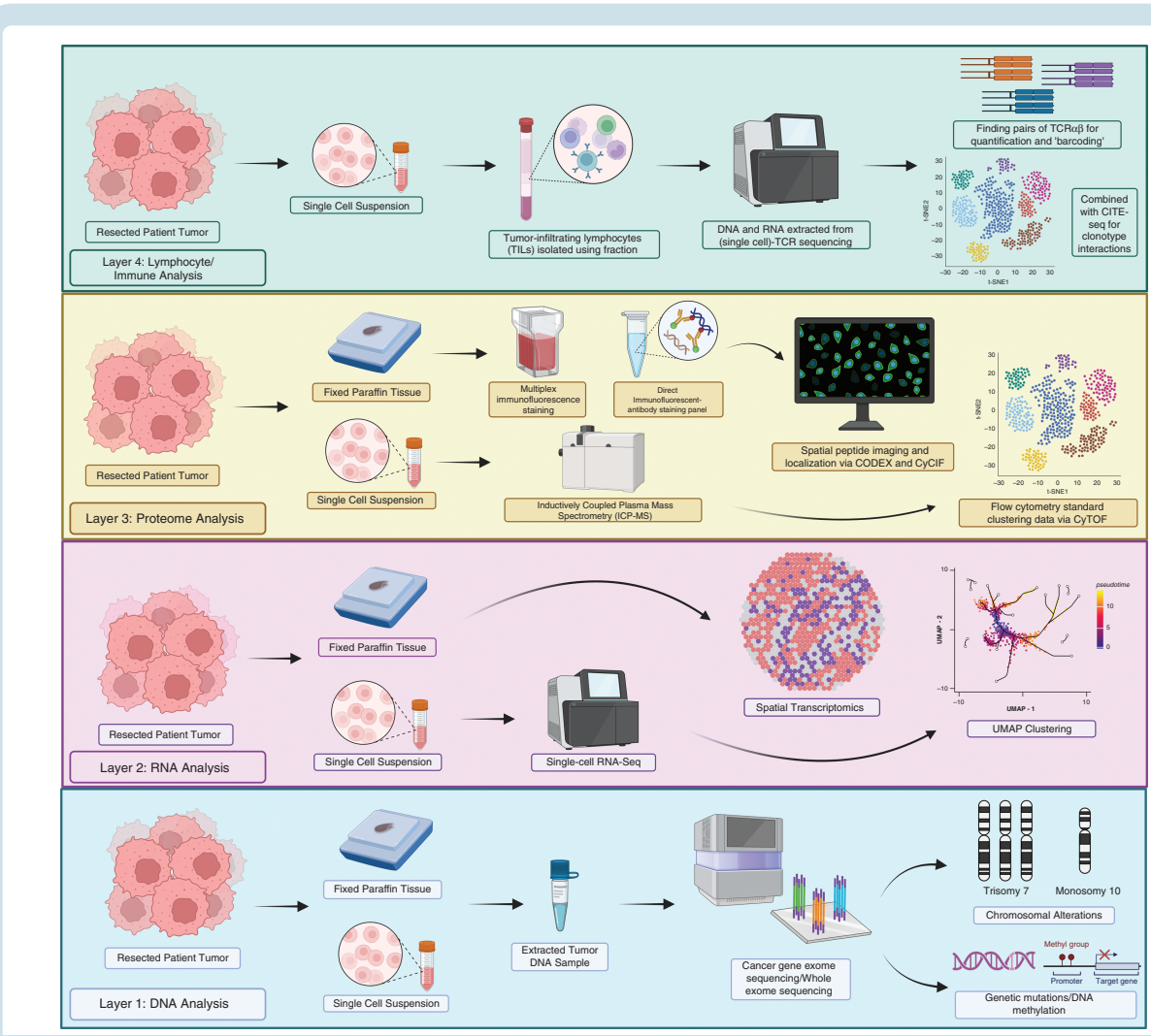

Figure 2. Layers of "omics" analyses of small amounts of tissues available via an image-guided biopsy. A small amount of tumor tissues can now be analyzed via several layers of "omics." At the DNA level, specific or whole exome sequencing, chromosomal alterations, and DNA methylation analyses are routinely performed. At the RNA level, whole and single-cell RNA sequencing and spatial transcriptomics can be utilized. At the protein level, proteome sequencing, phosphoproteomics, immunopeptidomics, and anatomical localization technologies (CODEX, CyCIF, and others) can be used. At the immune cell level, TCR sequencing can be used to understand the evolution of T-cell responses. Coupled with CITE-Seq this can also map the regional changes in T-cell clonotype evolution.

and other analyses,[109,110] these assays are changing the landscape of glioma pathological classification, as discussed,[18] particularly since these can be done with fixed paraffin tissue.

New technologies are also occurring at the RNA/transcription level. Bulk RNA transcriptomics can reveal the wholesale genetic fingerprint of a GBM and when this is performed at the level of single cells (via single-cell RNA sequencing), it provides the scientist/clinician with a deeper understanding of the plasticity and clonal heterogeneity of the tumor during its evolution.[111–115] The spatial analyses on tissue slides of the anatomical location of tumor transcripts (via spatial transcriptomics) provide an understanding of cell-to-cell genotypic relationships, particularly as transcriptome profiles that characterize neuronal, tumor, immune cells and other TME cells are being understood.[116–118] In addition, the epigenome, particularly noncoding RNAs, like microRNAs, is also being sequenced to understand how its expression or lack thereof characterizes cancer evolution,[119,120] although its utility for classification and dissection of therapeutic effects remains in its infancy.

At the protein level, the proteome and the phosphoproteome are being increasingly interrogated with technologies that allow sensitive sequencing even with small amount of material.[121,122] These proteomic technologies can be used to uncover immune-peptidomes,[123,124] opening up the possibility of identifying tumor antigens during immunotherapy. Like spatial transcriptomics, the anatomic localization within tumors of various peptides can now be elucidated using protein barcoding technologies, such as CODEX,[125–127] CyCIF,[128–130] CyTOF,[131–133] or PERTURB-CITE-Seq.[134] These allow the regional visualization of protein–protein interactions within cells of the GBM and can show where these interactions occur as a function

of different areas of the tumor. Combined with imaging, these potent technologies can start to unravel changes in the TME as a function of therapy.

In terms of lymphocytes, the traditional immunologic methods of marking T cells based on their expression of certain markers are now supplemented by TCR "barcoding."[135] Both TCR DNA and RNA sequencing alone or coupled with single-cell TCR sequencing are potent techniques that can barcode TCRs and allow for T-cell clonotype analyses in terms of frequency, diversity and expansion in tumor. Perhaps, with algorithms, AI, and databases of epitopes, the prediction is that these may be sufficient to identify the tumor antigens to which these TCRs react to without having to perform extensive in vitro functional analyses.[136–139] Combined with CITE-Seq technologies,[140,141] visualization of T-cell clonotype interactions with cellular trancriptomic and proteomic programs can add to the richness of understanding the mode of action of the immunotherapy being tested.

Other technologies, such as metabolomics,[142–145] can be used to dissect the downstream effects of therapies on tumor. In addition, GBMs can now be grown as organoids[146–150] and/or patient-derived xenografts[151–158] allowing personalized treatments to be tested and analyzed in vitro.

Conclusion

The discipline of GBM clinical trials, including those that involved immunotherapies, has mostly behaved like a soccer match. In this context, the objective of the game is to get more and more shots on goal to increase the likelihood that a goal will occur and ensure the win. While this approach has been true for most of the history of medicine and for that of oncology, it has not been successful so far for GBM, where multiple phase 3 trials (all those shots on goal) have failed to produce a score. Some of the more recent efforts where early-phase trials have been used to scientifically study specific aspects of patient data to understand the variables of responders versus nonresponders appear promising as a strategy to guide successful drug development forward. In fact, we argue that the discipline may want to consider the Apollo space program that led to the first human walking on the moon in 1969. The space program did not attempt multiple shots on goal, rather gradually and systematically pursued one space mission after another to gradually test each component needed to successfully achieve a lunar landing and a human walk with Apollo 11. In this context, the time may have come to pursue a similar course of action with GBM immunotherapy trials, by pursuing more early-phase clinical trials where longitudinal biopsies of tissues and fluids are embedded in the science of the therapy under study.

Supplementary Material

Supplementary material is available online at *Neuro-Oncology* (https://academic.oup.com/neuro-oncology).

Keywords

image-guided biopsy | multi-omics | phase 0 clinical trial | tumor microenvironment | window of opportunity clinical trial

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Conflict of Interest

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Affiliations

Department of Neurosurgery, Brigham and Women's Hospital, Boston, Massachusetts, USA (E.C., A.L.L., E.A.C.); Center for Neuro-Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA (D.A.R.)

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