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Immunomonitoring in glioma immunotherapy: Current status and future perspectives

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Abstract

Given the continued poor clinical outcomes and refractory nature of glioblastoma multiforme to traditional interventions, immunotherapy is gaining traction due to its potential for specific tumor-targeting and long-term antitumor protective surveillance. Currently, development of glioma immunotherapy relies on overall survival as an endpoint in clinical trials. However, the identification of surrogate immunologic biomarkers can accelerate the development of successful immunotherapeutic strategies. Immunomonitoring techniques possess the potential to elucidate immunological mechanisms of antitumor responses, monitor disease progression, evaluate therapeutic effect, identify candidates for immunotherapy, and serve as prognostic markers of clinical outcome. Current immunomonitoring assays assess delayed-type hypersensitivity, T-cell proliferation, cytotoxic T-lymphocyte function, cytokine secretion profiles, antibody titers, and lymphocyte phenotypes. Yet, no single immunomonitoring technique can reliably predict outcomes, relegating immunological markers to exploratory endpoints. In response, the most recent immunomonitoring assays are incorporating emerging technologies and novel analysis techniques to approach the goal of identifying a competent immunological biomarker which predicts therapy responsiveness and clinical outcome. This review addresses the current status of immunomonitoring in glioma vaccine clinical trials with emphasis on correlations with clinical response.

Keywords

glioma; glioblastoma; immunotherapy; immunomonitoring; vaccine

Introduction

Glioblastoma multiforme (GBM) is the most commonly diagnosed malignant brain tumor in adults. Despite advances in microsurgery and adjuvant chemoradiation, the prognosis remains poor with median overall survival of 14.6 months.[1] Immunotherapy is a promising therapeutic approach aimed at stimulating a specific and sustained antitumor response.

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Overall survival (OS) currently serves as the primary endpoint in clinical immunotherapy trials for GBM. However, immunomonitoring assays, aimed at tracking the effects of immunotherapy upon the patient's immune system, can ideally help identify additional clinical biomarkers that efficiently reflect treatment efficacy. Additionally, these assays can accelerate the development of immunotherapeutic agents by providing insight into the complex interactions between the tumor microenvironment, the immune system, and immunologic interventions.

The primary goals of immunomonitoring in glioma immunotherapy trials include (1) verifying intended immunologic effects of therapeutic interventions, (2) characterizing the effects of immunotherapy on immune cell populations known to be involved in effector and/or regulatory antitumor immune responses, (3) determining functional antitumor responses evoked by immunotherapy, and (4) investigating potential biomarkers of clinical benefit due to immunotherapy. Currently, however, no immunomonitoring technique has been shown to reliably predict clinical outcome, relegating immunological markers to exploratory endpoints. This is likely a consequence of limitations in current techniques and knowledge that prevent a comprehensive understanding of the interactions between tumor, tumor microenvironment, and immune system. Despite this complexity, immunomonitoring techniques provides the opportunity to understand the complex effects of immunotherapy on the immune system and moves the field closer to the goal of a prognostic biomarker.

Paralleling the development of immunotherapies for GBM, several generations of assays have successively approached the goal of a surrogate clinical endpoint that allows monitoring of vaccination responses. Initial immunomonitoring techniques focused on *ex vivo* lymphocyte proliferation and *in vitro* function of cytotoxic T-lymphocytes, effectively characterizing bulk immune responses. However, these assays could not distinguish among the specific immune populations involved. Subsequent generations of assays evaluated antigen-specific T-cell frequency and *ex vivo* cytokine production. While this narrowed investigation to the single-cell level, these techniques still failed to link immunological phenotypes with function. The most recent immunomonitoring techniques have coupled characterization of immune cell phenotype with functional properties, increasing understanding of the functional roles of various cellular phenotypes in cancer immunotherapy.[2]

At present, the ability to reliably correlate clinical outcomes with phenotypic and functional shifts at the immune-tumor interface remains elusive. However, future techniques aim to advance single-cell and multiparameter analyses to define patient-specific immune profiles that may better represent the complex and dynamic nature of the immune system. Here we review and evaluate the current status and limitations of immunomonitoring techniques in glioma immunotherapy, including delayed type hypersensitivity, lymphocyte proliferation, functional cytotoxic T lymphocyte assays, cytokine profiling, antibody titer monitoring, and lymphocyte phenotyping. Moreover, we discuss techniques that may be utilized by the field in the near future, alongside novel biomarkers gaining favor in checkpoint inhibition studies and from outside the field of neuro-oncology.

Delayed Type Hypersensitivity (DTH)

The primary goal of vaccination involves induction of adaptive immune responses against tumor-specific antigens. As the sole *in vivo* immunomonitoring assay, DTH has found widespread application as an attempt to measure successful immunological activation[3–19] (Table 1). In response to intradermal challenge of tumor antigen, local antigen-presenting cells (APCs) release chemokines that recruit $CD4^+$ effector memory T-cells which, in turn, activate macrophages and $CD8^+$ cytotoxic T lymphocytes (CTLs). At intradermal challenge sites, positive responses are characterized by induration measured 42–72 hours following injection. In addition to induration, the presence of antigen-specific T-cells in DTH skin biopsies can also be utilized to distinguish responders from non-responders.[4, 20] While the primary advantage of DTH is accessibility, it lacks a standardized challenge dosage and thresholds for response discrimination which contribute to its variability. Glioma vaccination trials employing DTH have reported correlations between DTH responses and OS,[3–6] progression-free survival (PFS),[7, 8] increased number of tumor infiltrating $CD8^+$ T-cells at recurrence,[9] or decreased computed tomography contrast enhanced lesion area.[10] Others, however, have described poor clinical correlations with both PFS and OS.[11, 13–15, 17] These contradictory reports likely stem from divergent immune stimulatory and regulatory signals encountered within tumor microenvironment and peripheral tissue. Ultimately, the low specificity engendered by DTH limits its role to a preliminary screen which may support additional immunological findings. As an assay relying on an immune response by memory T-cells, DTH techniques are most likely to be effective in demonstrating successful vaccination efforts relying on T-cell mediated responses and less effective for therapeutic strategies relying on humoral responses.

T-Cell Proliferation

A successful vaccination response encompasses antigen presentation to naïve T-cells, resulting in the evolution of memory T-cells that possess the potential to undergo rapid clonal expansion following antigen re-exposure. Consequently, early assays focused on *in vitro* bulk T-cell proliferation as an estimate of *in vivo* antigen-specific T-cell induction.[21] Typically, T-cells or peripheral blood mononuclear cells (PBMCs) are exposed to antigen to induce clonal expansion. Methods of determining the resulting proliferation have included [^{3}H] thymidine incorporation[22] and dye dilution proliferation assays (DDPAs)[23] (Table 2).

[^{3}H] thymidine assays rely on incorporation of radiolabeled nucleotides into DNA of dividing cells following *in vitro* stimulation. Subsequently, the increased amounts of [^{3}H] thymidine present in antigen stimulated samples reflect the magnitude of lymphocyte clonal expansion. However, thresholds describing response discrimination remain unstandardized[21] and failed to predict time to progression (TTP), PFS, or OS in a phase I trial of a DC vaccine targeting brain tumor initiating cells.[12] Moreover, the assay is limited by its bulk nature, preventing analysis of single cell participation in the proliferative response.

DDPAs address limitations of [^{3}H] thymidine incorporation as the technique is non-radioactive and facilitates single cell phenotyping. In the assay, carboxyfluorescein diacetate succinimidyl ester (CFSE) acts as a fluorophore that binds to cytoplasmic proteins. Through

flow cytometry analysis, serial halving of cytoplasmic proteins during lymphoproliferation can be monitored as a serial halving of fluorescent intensity. Results from a phase I/II vaccination trial employing autologous tumor derived peptide bound to 96 kD chaperone protein indicate that DDPA monitoring could discriminate between immune responders and the sole non-responder in the cohort, displaying correlation with OS.[24] Contrastingly, in a post-radiation and temozolomide therapy autologous tumor lysate-loaded DC vaccination trial, pre- and post-vaccination comparison of the percentage of proliferating $CD4^+$ and $CD8^+$ cells assayed via DDPA failed to act as single predictors of PFS or OS. However, as components of hierarchical clustering with other immune parameters (CD4+/CD8+ cellular frequency, IFN-γ production, etc.), the proliferative immune response noted in the DDPA contributed to a significant correlation with OS.[15]

For both [^{3}H] thymidine incorporation and DDPA, the major limitation in interpretation across studies involves *in vitro* stimulation and expansion protocols, which remain unstandardized and may not accurately simulate the actual environment in which clonal expansion occurs. Still, T-cell proliferation remains a promising immune marker and techniques such as flow cytometry based Ki67 nuclear antigen detection[25] or telomere shortening analysis through flow cytometry based, fluorescence *in situ* hybridization (FISH)[26] may overcome these obstacles and provide greater relevance to *in vivo* T-cell proliferation by avoiding *in vitro* stimulation protocols. Similar to DTH, T-cell proliferation assays are most viable for therapeutic strategies relying on T-cell mediated responses, as opposed to humoral responses, to demonstrate the induction of a memory immune cell population with the capability for clonal expansion.

Cytotoxic T-Lymphocytes

As a reflection of *in vivo* vaccine antitumor efficacy, cytotoxicity assays determine *in vitro* $CD8^+$ CTL-mediated lysis of target cells or the generation of cytotoxic mediators (Table 3). Cell-mediated cytotoxicity relies on activation of $CD8^+$ CTLs following MHC class I antigen presentation, leading to expression and release of perforin and granzyme proteins for pore formation or caspase cascade induced apoptosis, respectively. The traditional cytotoxicity measures have been the chromium (^{51}Cr) release assay[27] and just another method (JAM) release assays.[28]

In the (^{51}Cr) and JAM release assays, target cells are either loaded with ^{51}Cr or labeled with [^{3}H] thymidine, respectively. Release of these intracellular molecules upon lysis of target cells by CTLs is measured to estimate lytic activity. Clinically, the chromium (^{51}Cr) release assay demonstrated only weak associations with radiographic partial responses in a study of dendritic and glioma cell fusion vaccines[29] and did not correlate with survival in a phase I personalized peptide vaccination trial.[16] In phase I trials of peptide-pulsed[30] and tumor lysate-pulsed DC vaccination,[31] JAM assays demonstrated induction of a lasting antigen-specific lytic response compared to pre-vaccination controls but failed to correlate with OS. Limitations of these assays include requirements of *in vitro* stimulation, radioactive reagents, and non-quantitative, bulk results.

More recent cytotoxicity measures include alamar blue CTL[32] and flow cytometry based assays of apoptotic events.[33–36] Alamar blue serves as fluorescent marker that is an indicator of viable cells. Thus, through measurements of fluorescence, it is possible to determine the fraction of target cells that have been lysed following exposure to CTLs.[32] In a tumor peptide DC vaccination trial, post-vaccination alamar blue CTL assays did not predict objective clinical response or OS, despite negative correlation with disease burden.[37] An additional flow cytometry technique involves assay of CD107a/b CTL cell surface mobilization upon CTL-target cell interaction as a result of cytolysis-associated degranulation.[36] CD107a staining correlated with intracellular lymphocyte IFN-γ responses, predicting a non-significant trend towards increased PFS and OS in a phase I multi-epitope-pulsed DC vaccination trial.[38] Overall, however, there still remain significant questions regarding the mechanistic relevance of *in vitro*, systemic CTL functional activity compared to *in vivo*, CTL responses in the tumor microenvironment. When used, these assays are most amenable for monitoring therapeutic interventions relying on CTL-mediated antitumor activity.

Cytokine Profiles and Antibody Titers

The glioma microenvironment establishes an immunosuppressive niche which promotes Th2, Treg, and M2 anti-inflammatory cellular phenotypes via direct cell-cell interaction and cytokine secretion. Vaccination goals therefore include a therapy-driven shift towards a proinflammatory response conducive of Th1 and M1 polarization. Evaluation of characteristic Th1 and Th2 cytokine release profiles is afforded by methods including enzyme–linked immunosorbent assay (ELISA),[39] enzyme linked immunospot (ELISPOT),[40] intracellular cytokine staining (ICS),[41] and quantitative polymerase chain reaction (qPCR) assays[42] (Table 4).

In vitro ELISAs can quantify bulk immune cell cytokine production in response to antigen challenge. The process involves incubating PBMCs or isolated immune cells with a hypothesized antigen. After incubation, the supernatant is collected and cytokines of interest can be quantified via detection antibodies. Similar techniques can be utilized on peripheral blood serum samples to estimate *in vivo* cytokine production. Employed to monitor pre- and post-vaccination serum proteins in an autologous tumor DC trial, ELISA elucidated a significant inverse correlation between levels of both TGF-β2 and VEGF with PFS and OS.[43] Moreover, quantification of elevated GM-CSF and TNF-α production in a DC vaccine study demonstrated the ability to distinguish stable from non-stable disease.[44] Used in several phase I clinical trials involving personalized peptide,[16] dendritic-glioma fusion cell,[45] and autologous tumor DC vaccines,[46] ELISA monitored a combination of IFN-γ, IL-2, and IL-12 production as a correlate of Th1 induction that demonstrated dose-dependent escalation. Yet, none of the studies demonstrated predictive capability regarding clinical outcomes.

ELISPOT assays possess the ability to determine both the frequency of individual antigen-specific T-cells and their cytokine production. While similar to the ELISA, the ELISPOT instead utilizes a precipitating fluorophore that results in spots representing single cells secreting the cytokine of interest. Spot size and intensity can be quantified and correlated

with cytokine production. Several glioma immunotherapy studies have utilized IFN-γ ELISPOT assays as markers of antitumor immune response.[47, 48] Employed in this manner, the ELISPOT has displayed ability to track long-term vaccine-induced antigen-specific IFN-γ production up to 3.5 years following final vaccination.[9, 13] Positive IFN-γ ELISPOT results have been correlated with minor clinical responses and stable MRI results in a phase I/II trial of tumor lysate DC vaccination.[10] Similarly, during a phase I α-type-1 polarized DC inoculation trial, increased frequency of IFN-γ producing cells correlated with clinically stable disease.[19] In addition, several DC vaccination trials have demonstrated positive IFN-γ ELISPOT as a prognostic marker of increased survival either as a single assay[3] or as a constituent of hierarchical clustering analysis.[15] However, the ELISPOT remains controversial as several studies describe significant clinical responses in patients with negative IFN-γ ELISPOT results.[13]

In contrast to ELISPOT and ELISA, ICS and multiparameter flow cytometry techniques support assessment of multiple cytokines concurrently with traditional cell surface markers for phenotypic analysis of cytokine-secreting cells. Thus, these techniques allow for the detection of cytokines at the single cell level. Using ICS to determine antigen-specific IFN-γ and TNF-α secretion in CTLs as markers of a Th1 response and therapy-induced cytotoxicity, a phase I trial of a multi-epitope DC vaccine demonstrated a non-significant trend towards increased PFS and OS in patients with increased ICS.[38] This correlation was further elucidated in a phase I trial of autologous tumor derived peptide vaccine where IFN-γ production distinguished immune responders with increased OS from non-responders.[24]

An additional technique to assess cytokine production involves quantification of lymphocyte gene expression through qPCR. IFN-γ qPCR was utilized as a preliminary screen for vaccine response in a phase I/II trial of autologous tumor peptides bound to 96kD chaperone protein and correlated with ICS and DDPA.[24] Utilized in distinguishing vaccine responders from non-responders in a phase II tumor lysate-pulsed DC trial, IFN-γ qPCR served as a significant prognostic marker of increased PFS and OS. Impressively, IFN-γ qPCR demonstrated a logarithmic correlation with both PFS and OS across periods of concurrent vaccine and chemotherapy administration.[18] However, its main limitations involve a lack of phenotyping capabilities and the fact that mRNA expression is not necessarily equivalent to functional protein.

Similar to quantifying cytokines via ELISA, it is also possible to measure antibody titers against specific targets. This technique has shown positive results in a recently concluded phase II clinical trial studying a vaccine (rindopepimut) which primes the immune system against EGFRvIII-mutated GBM. After vaccine administration, anti-EGFRvIII antibody titers increased ≥4 fold in the majority of patients. Anti-EGFRvIII titers also increased along with the duration of treatment.[49] However, such assays are only beneficial in strategies in which a significant humoral antitumor immune response is expected, whereas cytokine profiling can be utilized in variety of settings depending on the cytokines selected for analysis.

Lymphocyte Phenotyping

Determination of frequency and function of vaccine-induced, antigen-specific T-cells at the single-cell level may reflect efficacy of the antitumor response (Table 5). The simplest evaluation of lymphocyte phenotypes is afforded by complete differential blood counts. Incorporated into a phase II heat-shock protein peptide complex-96 vaccination trial, pre-vaccination absolute lymphocyte count (ALC) served as a significant predictor of OS, suggesting lymphopenia as a possible negative prognostic marker in selection of immunotherapy candidates.[50] Phenotypic analysis of lymphocyte subsets, in addition to antigen-specific T-cells, elaborates on the competing immune suppressive and stimulatory factors involved in the antitumor response. PBMC flow cytometry allows for evaluation of these various lymphocyte subsets through cell-surface staining of characteristic clusters of differentiation (CD). Clinical trials tracking pre- and post-vaccination shifts in $CD3^+$ T-cell, $CD3^+CD4^+$ helper T-cell, $CD4^+CD8^+$ double positive T-cell, and $CD56^+$ NK cell populations either did not attempt clinical correlation[10, 29, 45, 46] or demonstrated no relationship with PFS or OS.[51] However, a phase I/II autologous DC trial used the technique to demonstrate induction of a desired antitumor memory $CD8^+$ T-cell population.[13]

Of particular relevance to monitoring immune suppression are Tregs, myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs). Correlations with survival have been demonstrated with pre- and post-vaccination alterations in the systemic Treg population. Assessment of a phase I/II tumor peptide-96kD complex inoculation cohort across lymphocyte phenotypes distinguished between vaccine responders and non-responders that correlated with OS. Whereas responders exhibited increased $CD3^+$ T-cells, $CD3^+CD8^+$ double positive T-cells, NK cells, and decreased Tregs correlated with increased OS, non-responders had significantly elevated Tregs related to decreased survival.[24] Several phase I DC-based immunotherapy trials have supported these findings through report of a hazard ratio for death of 3.62 – 7.19 for each unit increase in the ratio of post- to pre-vaccination Treg percentage.[52, 53] However, other studies have failed to demonstrate correlations with Treg populations and clinical outcome.[54] In addition to Tregs, a phase I DC vaccination study also demonstrated a significant relationship between decreased OS and increased CTLA4 expression on helper T-cells and CTLs post-vaccination. In contrast, increased proportions of activated $CD8^+$ T-cells served as a positive prognostic marker for increased survival that approached significance.[53] Moreover, expanded post-vaccination NK cell frequency demonstrated correlations with prolonged PFS and OS in a study of an autologous DC vaccine,[43] demonstrating the potential prognostic capabilities of several lymphocytes subsets in glioma vaccination.

PBMC flow cytometry has the potential to elucidate immune response patterns both during immune induction and longitudinally across therapeutic administration. Moreover, the technique affords not only phenotypic analysis of PBMCs, but also functional determination depending on the combination of ICS and flow cytometry protocols applied. Yet, the assay remains technically limited in the number of accessible parameters that may slow future studies that aim to assess increasingly complicated biomarker combinations.

Emerging Technology

Given limited prognostic power of current immunomonitoring assays, emerging technologies such as phosphoflow[55] and cytometry by time of flight (CyTOF)[56] have been investigated. Unlike traditional flow cytometry, phosphoflow also assesses phospho-signatures characterized by activation and phosphorylation of intracellular signaling pathways such as STAT and MAPK involved in T-cell receptor antigen recognition, cytokine activation, and expression of costimulatory or inhibitory molecules. In a phase I DC vaccination trial, the technique has distinguished patients with increased survival based on increased pSTAT5 and decreased pSTAT1 ratios in $CD3^+CD8^+$ T-cells after an *ex vivo* stimulation protocol following vaccination.[57] In contrast to flow cytometry, emerging CyTOF techniques theoretically possesses the capability to assess 70–100 parameters per cell, expanding the number of biomarkers that can be assessed for a single cell.[56]

Due to increasing number of parameters assayed per cell by these emerging technologies, immunomonitoring is progressing towards immune profiling and phenotyping,[58] which has demonstrated a strong correlation with OS in GBM.[59] Serial monitoring of immune profiles from pre-vaccination baseline allows for evaluation of immunotherapeutic effects on the immune system at large. With increasingly high-dimensional data, unsupervised hierarchical clustering techniques such as spanning tree progression analysis of density normalized events (SPADE)[56, 60] and visualized stochastic neighbor embedding (viSNE)[61] will likely be necessary to avoid bias in manual interpretation and detection of rare cell populations.

Immunomonitoring in Checkpoint Inhibition and Non-Glioma Immunotherapy

Due to the relative infancy of immunotherapy for glioblastoma, the majority of trials utilizing immunomonitoring techniques have been early phase trials that cannot provide conclusive evidence regarding clinical outcomes. In comparison, the field of melanoma immunotherapy is relatively advanced and has successfully investigated several immune biomarkers. One form of immune-based therapy which has demonstrated success in malignancies such as melanoma is that of immune checkpoint inhibition. This therapy consists of antibodies that do not target the tumor proper, but instead bind immunosuppressive proteins, preventing and reversing immune cell exhaustion and anergy. While the clinical data is not yet available for this type of intervention within the field of neuro-oncology, there are ongoing planned clinical trials which will aim to fill this void (NCT02311920 and NCT02017717).

Biomarkers that have been investigated in CTLA-4 checkpoint inhibition (ipilimumab) trials include patient ALC measurements with stratification based on a numerical cutoff and rate of rise.[62] The underlying hypothesis postulates that a higher ALC portends an improved prognosis and is a sign of an activated immune system. Overall, the data remains somewhat inconclusive with some reports noting survival benefit, while others note lack of association between ALC and survival.[63–66] Another potential biomarker for monitoring the immune system involves measuring the population of lymphocytes expressing ICOS (*I*nducible T-cell *COS*timulator). ICOS is a protein expressed on the surface of activated T-cells which plays a

role in proliferation and survival of activated T-cells. When expressivity of ICOS positive T-cells was assessed in melanoma patients receiving ipilimumab, patients with a higher population of $CD4^{+}ICOS^{hi}$ cells demonstrated an increased OS.[67] For PD-1/PD-L1 trials, the degree of expressivity of PD-L1 within the tumor has also been hypothesized to predict response to the therapy. This is highly applicable to the realm of neuro-oncology since GBM is known to express PD-L1 (which binds and signals via the immune checkpoint protein PD-1) and the degree of expressivity has been correlated with worse outcomes.[68] While a study which investigated PD-1 inhibition (nivolumab) in melanoma demonstrated an improved OS regardless of a tumor's PD-L1 status,[69] the correlate of this finding in GBM remains unknown.

Interestingly, checkpoint inhibition studies in melanoma have also demonstrated a potential role for immunogenetics in predicting patients likely to benefit from checkpoint inhibition.[70] Utilizing whole-exome sequencing of tumor DNA, a unique collection of mutation-derived neopepitopes that could drive T-cell anti-tumor activity were discovered to occur overwhelmingly in patients benefitting from anti-CTLA4 checkpoint inhibition. Moreover, in studies of anti-PD-1 checkpoint inhibition including a phase II trial in mismatch-repair deficient colorectal cancer[71] and an investigation of non-small cell lung cancer,[72] increased tumor mutational burden was correlated with clinical benefit. With increasing mutational burden, greater antitumor neoantigens are produced which stimulate an endogeneous immune response, shackled only by a subsequent increase in immunologic checkpoints. These findings have resulted in the hypothesis that checkpoint inhibition is most effective in the setting of an immunogenic tumor and suggest the possibility of screening endogenous patient tumor immunogenicity as a predictor of response to checkpoint inhibition therapy. Within the realm of glioma immunotherapy, basic immunogenetic monitoring has already been employed in a phase I dendritic cell vaccine trial demonstrating a potential increased benefit of vaccination in the mesenchymal subtype of GBM compared to other subtypes.[54] Within tumors of this subtype, greater expression of pro-inflammatory genes and increased $CD3^{+}CD8^{+}$ lymphocytic infiltration are observed, along with increased survival following vaccination compared to historical controls. Together, these studies indicate the potential importance of tumor immunogenetics in the selection of patients likely to benefit from immunotherapy and support increased investigations into this form of immunomonitoring.

Of note, immunomonitoring in vaccination efforts and checkpoint inhibition may differ in some regards and overlap in others. Whereas checkpoint inhibition may perform optimally in the setting of an endogenously immunoreactive tumor, vaccination efforts may show the most promise in the setting of a relatively less immunogenic tumor, whose immunogenicity must be enhanced. In these situations, immunomonitoring efforts may assist in the selection of appropriate therapeutic options for patients. As the field of immunomonitoring evolves along with immunotherapy, the similarities and differences between immunomonitoring in vaccination and checkpoint inhibition therapies will require increased investigation.

Immunomonitoring Limitations

The interpretation of immunomonitoring results currently faces several limitations including (1) our current incomplete understanding of the complex interactions between the tumor

microenvironment and the immune system, (2) biomarkers that may simply be surrogate measures of overall patient condition, and (3) lack of assay standardization and consistency across immunotherapy trials. Based on evolving research, our interpretation of immunological findings may continue to shift and explain why some of the past and present immunomonitoring assays have failed to correlate with overall survival. For example, *in vitro* assays of immune cell function have largely failed to take into account the variety of forms of immunosuppression encountered in GBM. Although isolated immune cell subsets may demonstrate effective antitumor activity *in vitro*, these functions are likely to be inhibited *in vivo* by factors including immunological checkpoints and immunosuppressive lymphoid populations. In this regard, the inability to correlate an immune response with survival may not be the failure of an assay, but a result of our incomplete understanding of cancer immunology. Moreover, studies reporting increased ALC, among other biomarkers, as a beneficial prognostic marker may simply be identifying patients who are capable of mounting effective antitumor responses and surviving regardless of the therapy administered. Patients failing to respond with an increase in ALC may simply be nutritionally compromised or in a poorer overall condition, confounding interpretations of treatment effect. Together, these limitations may help explain some of the results observed in the literature and provide implications for the development of future immunomonitoring efforts.

Increasing the reliability of immunomonitoring assays also remains a goal of all future trials incorporating such techniques. Harmonization and standardization efforts are currently underway to address the issue of interpretation of results across studies. Whereas harmonization strives to determine crucial variables affecting assay performance while permitting laboratories freedom over their protocols, standardization is stricter and prescribes central facilities or specific protocols and reagents in order to ensure precision. As some of the most commonly employed immunomonitoring assays, ELISPOT and ICS techniques each possess standardization and harmonization guidelines,[73] along with recommendations for minimal reporting metrics.[74] Since patients inherently demonstrate large variation in their immunological status, such measures to reduce assay variability are vital to appropriate analysis and interpretation of data.

Conclusion

Immunomonitoring assays possess the potential to elucidate immunological mechanisms of antitumor vaccination, monitor disease progression, and assess potential candidates for immunotherapy. While some techniques have demonstrated promising correlations with clinical outcome, the majority of immunomonitoring assays to date have failed to reliably serve as prognostic markers in glioma immunotherapy. Moving forward, encouraging immunomonitoring approaches are attempting to elucidate patient-specific immune profiles encompassing multiple phenotypic and functional facets of the immune system that can better correlate with clinical responses to immunotherapy. Yet, differences in systemic and tumor microenvironments remain a significant obstacle in the interpretation of immunomonitoring results. Continued improvement of immunomonitoring remains essential to increased understanding and design of immunotherapeutic interventions. As each current immunomonitoring technique possesses inherent limitations, the selection of an assay for

future investigations must rely on the specific goals of the investigators. Depending on the mechanism of action of the immunotherapeutic intervention, techniques monitoring a humoral response (antibody titers) may be desired over techniques that assess antigen-specific T-cell induction and function (T-cell proliferation, CTL assays, ELISPOT). On the other hand, lymphocyte phenotyping serves as a highly flexible technique with the ability to assess both effector and regulator immune cell populations that affect a patient's response to immunotherapy and is highly amenable to a wide variety of interventions. Given the rapidly emerging role of immunogenetics in studies outside of neuro-oncology, it is likely that these techniques will soon be used in GBM to investigate which patients are most likely to benefit from specific immunotherapeutic interventions. However, as the field currently stands, the most holistic approach to monitoring a patient's response to immunotherapy likely involves the combination and analysis of several independent biomarkers concurrently, which together, may depict an immune phenotype that is greater than the sum of its individual parts. While the role of prognostic immune biomarkers in future clinical endeavors for glioma immunotherapy remains to be seen, they have proven to be important avenues in need of additional attention, research, and development to investigate the mechanisms of immunotherapy, responses to therapeutic interventions, and prediction of patients most likely to benefit from therapy.

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References

1. Stupp R, Mason WP, Van Den Bent MJ, et al. Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. New England Journal of Medicine. 2005; 352:987–996. [PubMed: 15758009]
2. Keilholz U, Martus P, Scheibenbogen C. Immune monitoring of T-cell responses in cancer vaccine development. Clinical Cancer Research. 2006; 12:2346s–2352s. [PubMed: 16609057]
3. Yamanaka R, Homma J, Yajima N, et al. Clinical evaluation of dendritic cell vaccination for patients with recurrent glioma: results of a clinical phase I/II trial. Clinical Cancer Research. 2005; 11:4160–4167. [PubMed: 15930352]
4. Clavreul A, Piard N, Tanguy J-Y, et al. Autologous tumor cell vaccination plus infusion of GM-CSF by a programmable pump in the treatment of recurrent malignant gliomas. Journal of Clinical Neuroscience. 2010; 17:842–848. [PubMed: 20466548]
5. Sampson JH, Heimberger AB, Archer GE, et al. Immunologic escape after prolonged progression-free survival with epidermal growth factor receptor variant III peptide vaccination in patients with newly diagnosed glioblastoma. Journal of Clinical Oncology. 2010; 28:4722–4729. [PubMed: 20921459]
6. Sloan AE, Dansey R, Zamorano L, et al. Adoptive immunotherapy in patients with recurrent malignant glioma: preliminary results of using autologous whole-tumor vaccine plus granulocyte-macrophage colony-stimulating factor and adoptive transfer of anti-CD3-activated lymphocytes. Neurosurgical Focus. 2000; 9:1–8.
7. Muragaki Y, Maruyama T, Iseki H, et al. Phase I/IIa trial of autologous formalin-fixed tumor vaccine concomitant with fractionated radiotherapy for newly diagnosed glioblastoma: clinical article. Journal of neurosurgery. 2011; 115:248–255. [PubMed: 21568657]

8. Ishikawa E, Muragaki Y, Yamamoto T, et al. Phase I/IIa trial of fractionated radiotherapy, temozolomide, and autologous formalin-fixed tumor vaccine for newly diagnosed glioblastoma: Clinical article. Journal of neurosurgery. 2014; 121:543–553. [PubMed: 24995786]
9. Steiner HH, Bonsanto MM, Beckhove P, et al. Antitumor vaccination of patients with glioblastoma multiforme: a pilot study to assess feasibility, safety, and clinical benefit. Journal of Clinical Oncology. 2004; 22:4272–4281. [PubMed: 15452186]
10. Yamanaka R, Abe T, Yajima N, et al. Vaccination of recurrent glioma patients with tumour lysate-pulsed dendritic cells elicits immune responses: results of a clinical phase I/II trial. British journal of cancer. 2003; 89:1172–1179. [PubMed: 14520441]
11. Rutkowski S, De Vleeschouwer S, Kaempgen E, et al. Surgery and adjuvant dendritic cell-based tumour vaccination for patients with relapsed malignant glioma, a feasibility study. British journal of cancer. 2004; 91:1656–1662. [PubMed: 15477864]
12. Vik-Mo EO, Nyakas M, Mikkelsen BV, et al. Therapeutic vaccination against autologous cancer stem cells with mRNA-transfected dendritic cells in patients with glioblastoma. Cancer Immunology, Immunotherapy. 2013; 62:1499–1509. [PubMed: 23817721]
13. Ardon H, Van Gool S, Lopes IS, et al. Integration of autologous dendritic cell-based immunotherapy in the primary treatment for patients with newly diagnosed glioblastoma multiforme: a pilot study. Journal of neuro-oncology. 2010; 99:261–272. [PubMed: 20146084]
14. Ishikawa E, Tsuboi K, Yamamoto T, et al. Clinical trial of autologous formalin-fixed tumor vaccine for glioblastoma multiforme patients. Cancer science. 2007; 98:1226–1233. [PubMed: 17517052]
15. Fadul CE, Fisher JL, Hampton TH, et al. Immune response in patients with newly diagnosed glioblastoma multiforme treated with intranodal autologous tumor lysate-dendritic cell vaccination after radiation chemotherapy. Journal of immunotherapy (Hagerstown, Md: 1997). 2011; 34:382.
16. Yajima N, Yamanaka R, Mine T, et al. Immunologic evaluation of personalized peptide vaccination for patients with advanced malignant glioma. Clinical Cancer Research. 2005; 11:5900–5911. [PubMed: 16115932]
17. De Vleeschouwer S, Fieuws S, Rutkowski S, et al. Postoperative adjuvant dendritic cell–based immunotherapy in patients with relapsed glioblastoma multiforme. Clinical Cancer Research. 2008; 14:3098–3104. [PubMed: 18483377]
18. Wheeler CJ, Black KL, Liu G, et al. Vaccination elicits correlated immune and clinical responses in glioblastoma multiforme patients. Cancer research. 2008; 68:5955–5964. [PubMed: 18632651]
19. Akiyama Y, Oshita C, Kume A, et al. α-type-1 polarized dendritic cell-based vaccination in recurrent high-grade glioma: a phase I clinical trial. BMC cancer. 2012; 12:623. [PubMed: 23270484]
20. de Vries IJM, Bernsen MR, Lesterhuis WJ, et al. Immunomonitoring tumor-specific T cells in delayed-type hypersensitivity skin biopsies after dendritic cell vaccination correlates with clinical outcome. Journal of Clinical Oncology. 2005; 23:5779–5787. [PubMed: 16110035]
21. Clay TM, Hobeika AC, Mosca PJ, Lyerly HK, Morse MA. Assays for monitoring cellular immune responses to active immunotherapy of cancer. Clinical Cancer Research. 2001; 7:1127–1135. [PubMed: 11350875]
22. Piguet PF, Vassalli P. Study of the thymic-derived or-independent nature of mouse spleen cells induced to proliferate in culture by various mitogens and antigens. European journal of immunology. 1973; 3:477–483. [PubMed: 4585311]
23. Lyons AB, Parish CR. Determination of lymphocyte division by flow cytometry. Journal of immunological methods. 1994; 171:131–137. [PubMed: 8176234]
24. Crane CA, Han SJ, Ahn B, et al. Individual patient-specific immunity against high-grade glioma after vaccination with autologous tumor derived peptides bound to the 96 KD chaperone protein. Clinical Cancer Research. 2013; 19:205–214. [PubMed: 22872572]
25. Soares A, Govender L, Hughes J, et al. Novel application of Ki67 to quantify antigen-specific in vitro lymphoproliferation. Journal of immunological methods. 2010; 362:43–50. [PubMed: 20800066]
26. Rufer N, Dragowska W, Thornbury G, Roosnek E, Lansdorp PM. Telomere length dynamics in human lymphocyte subpopulations measured by flow cytometry. Nature biotechnology. 1998; 16:743–747.

27. Brunner K, Mauel J, Cerottini J-C, Chapuis B. Quantitative assay of the lytic action of immune lymphoid cells of 51Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. Immunology. 1968; 14:181. [PubMed: 4966657]
28. Matzinger P. The JAM test A simple assay for DNA fragmentation and cell death. Journal of immunological methods. 1991; 145:185–192. [PubMed: 1765650]
29. Kikuchi T, Akasaki Y, Abe T, et al. Vaccination of glioma patients with fusions of dendritic and glioma cells and recombinant human interleukin 12. Journal of Immunotherapy. 2004; 27:452–459. [PubMed: 15534489]
30. Yu JS, Wheeler CJ, Zeltzer PM, et al. Vaccination of malignant glioma patients with peptide-pulsed dendritic cells elicits systemic cytotoxicity and intracranial T-cell infiltration. Cancer research. 2001; 61:842–847. [PubMed: 11221866]
31. Yu JS, Liu G, Ying H, Yong WH, Black KL, Wheeler CJ. Vaccination with tumor lysate-pulsed dendritic cells elicits antigen-specific, cytotoxic T-cells in patients with malignant glioma. Cancer research. 2004; 64:4973–4979. [PubMed: 15256471]
32. Nociari MM, Shalev A, Benias P, Russo C. A novel one-step, highly sensitive fluorometric assay to evaluate cell-mediated cytotoxicity. Journal of immunological methods. 1998; 213:157–167. [PubMed: 9692848]
33. Liu L, Chahroudi A, Silvestri G, et al. Visualization and quantification of T cell–mediated cytotoxicity using cell-permeable fluorogenic caspase substrates. Nature medicine. 2002; 8:185–189.
34. Vermes I, Haanen C, Steffens-Nakken H, Reutellingsperger C. A novel assay for apoptosis flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled annexin V. Journal of immunological methods. 1995; 184:39–51. [PubMed: 7622868]
35. Schmid I, Krall WJ, Uittenbogaart CH, Braun J, Giorgi JV. Dead cell discrimination with 7-amino-actinomcin D in combination with dual color immunofluorescence in single laser flow cytometry. Cytometry. 1992; 13:204–208. [PubMed: 1547670]
36. Betts MR, Brenchley JM, Price DA, et al. Sensitive and viable identification of antigen-specific CD8+ T cells by a flow cytometric assay for degranulation. Journal of immunological methods. 2003; 281:65–78. [PubMed: 14580882]
37. Liau LM, Prins RM, Kiertscher SM, et al. Dendritic cell vaccination in glioblastoma patients induces systemic and intracranial T-cell responses modulated by the local central nervous system tumor microenvironment. Clinical Cancer Research. 2005; 11:5515–5525. [PubMed: 16061868]
38. Phuphanich S, Wheeler CJ, Rudnick JD, et al. Phase I trial of a multi-epitope-pulsed dendritic cell vaccine for patients with newly diagnosed glioblastoma. Cancer Immunology, Immunotherapy. 2013; 62:125–135. [PubMed: 22847020]
39. Engvall E, Perlmann P. Enzyme-linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. Immunochemistry. 1971; 8:871–874. [PubMed: 5135623]
40. Czerkinsky CC, Nilsson L-Å, Nygren H, Ouchterlony Ö , Tarkowski A. A solid-phase enzyme-linked immunospot (ELISPOT) assay for enumeration of specific antibody-secreting cells. Journal of immunological methods. 1983; 65:109–121. [PubMed: 6361139]
41. Jung T, Schauer U, Heusser C, Neumann C, Rieger C. Detection of intracellular cytokines by flow cytometry. Journal of immunological methods. 1993; 159:197–207. [PubMed: 8445253]
42. Heid CA, Stevens J, Livak KJ, Williams PM. Real time quantitative PCR. Genome research. 1996; 6:986–994. [PubMed: 8908518]
43. Pellegatta S, Eoli M, Frigerio S, et al. The natural killer cell response and tumor debulking are associated with prolonged survival in recurrent glioblastoma patients receiving dendritic cells loaded with autologous tumor lysates. Oncoimmunology. 2013; 2
44. Olin MR, Low W, McKenna DH, et al. Vaccination with dendritic cells loaded with allogeneic brain tumor cells for recurrent malignant brain tumors induces a CD4+ IL17+ response. Journal for immunotherapy of cancer. 2014; 2:1–11. [PubMed: 24829758]
45. Kikuchi T, Akasaki Y, Irie M, Homma S, Abe T, Ohno T. Results of a phase I clinical trial of vaccination of glioma patients with fusions of dendritic and glioma cells. Cancer Immunology, Immunotherapy. 2001; 50:337–344. [PubMed: 11676393]

46. Jie X, Hua L, Jiang W, Feng F, Feng G, Hua Z. Clinical application of a dendritic cell vaccine raised against heat-shocked glioblastoma. Cell biochemistry and biophysics. 2012; 62:91–99. [PubMed: 21909820]
47. Okada H, Lieberman FS, Walter KA, et al. Autologous glioma cell vaccine admixed with interleukin-4 gene transfected fibroblasts in the treatment of patients with malignant gliomas. Journal of Translational Medicine. 2007; 5:67. [PubMed: 18093335]
48. Iwami K, Shimato S, Ohno M, et al. Peptide-pulsed dendritic cell vaccination targeting interleukin-13 receptor α2 chain in recurrent malignant glioma patients with HLA-A* 24/A* 02 allele. Cytotherapy. 2012; 14:733–742. [PubMed: 22424217]
49. Schuster J, Lai RK, Recht LD, et al. A phase II, multicenter trial of rindopepimut (CDX-110) in newly diagnosed glioblastoma: the ACT III study. Neuro-oncology. 2015:nou348.
50. Bloch O, Crane CA, Fuks Y, et al. Heat-shock protein peptide complex–96 vaccination for recurrent glioblastoma: a phase II, single-arm trial. Neuro-oncology. 2014; 16:274–279. [PubMed: 24335700]
51. Ardon H, Van Gool SW, Verschuere T, et al. Integration of autologous dendritic cell-based immunotherapy in the standard of care treatment for patients with newly diagnosed glioblastoma: results of the HGG-2006 phase I/II trial. Cancer Immunology, Immunotherapy. 2012; 61:2033–2044. [PubMed: 22527250]
52. Prins RM, Wang X, Soto H, et al. Comparison of glioma-associated antigen peptide-loaded versus autologous tumor lysate-loaded dendritic cell vaccination in malignant glioma patients. Journal of immunotherapy (Hagerstown, Md: 1997). 2013; 36:152.
53. Fong B, Jin R, Wang X, et al. Monitoring of regulatory T cell frequencies and expression of CTLA-4 on T cells, before and after DC vaccination, can predict survival in GBM patients. PloS one. 2012; 7:e32614. [PubMed: 22485134]
54. Prins RM, Soto H, Konkankit V, et al. Gene expression profile correlates with T-cell infiltration and relative survival in glioblastoma patients vaccinated with dendritic cell immunotherapy. Clinical Cancer Research. 2011; 17:1603–1615. [PubMed: 21135147]
55. Perez OD, Nolan GP. Simultaneous measurement of multiple active kinase states using polychromatic flow cytometry. Nature biotechnology. 2002; 20:155–162.
56. Bendall SC, Simonds EF, Qiu P, et al. Single-cell mass cytometry of differential immune and drug responses across a human hematopoietic continuum. Science. 2011; 332:687–696. [PubMed: 21551058]
57. Everson RG, Jin RM, Wang X, et al. Cytokine responsiveness of CD8 (+) T cells is a reproducible biomarker for the clinical efficacy of dendritic cell vaccination in glioblastoma patients. J Immunother Cancer. 2014; 2:10. [PubMed: 24883189]
58. Chang S, Kohrt H, Maecker HT. Monitoring the immune competence of cancer patients to predict outcome. Cancer Immunology, Immunotherapy. 2014; 63:713–719. [PubMed: 24487923]
59. Gustafson MP, Lin Y, LaPlant B, et al. Immune monitoring using the predictive power of immune profiles. J Immuno Ther Cancer. 2013; 1
60. Qiu P, Simonds EF, Bendall SC, et al. Extracting a cellular hierarchy from high-dimensional cytometry data with SPADE. Nature biotechnology. 2011; 29:886–891.
61. Amir, E-aD; Davis, KL.; Tadmor, MD., et al. viSNE enables visualization of high dimensional single-cell data and reveals phenotypic heterogeneity of leukemia. Nature biotechnology. 2013; 31:545–552.
62. Lesokhin AM, Callahan MK, Postow MA, Wolchok JD. On being less tolerant: Enhanced cancer immunosurveillance enabled by targeting checkpoints and agonists of T cell activation. Sci Transl Med. 2015; 7:280sr281.
63. Ku GY, Yuan J, Page DB, et al. Single-institution experience with ipilimumab in advanced melanoma patients in the compassionate use setting: lymphocyte count after 2 doses correlates with survival. Cancer. 2010; 116:1767–1775. [PubMed: 20143434]
64. Berman DM, Wolchok JD, Weber J, Hamid O, O'Day S, Chasalow SD. Association of peripheral blood absolute lymphocyte count (ALC) and clinical activity in patients (pts) with advanced melanoma treated with ipilimumab. J Clin Oncol. 2009; 27:abstract 3020.

65. Simeone E, Gentilcore G, Giannarelli D, et al. Immunological and biological changes during ipilimumab treatment and their potential correlation with clinical response and survival in patients with advanced melanoma. Cancer immunology, immunotherapy : CII. 2014; 63:675–683. [PubMed: 24695951]

66. Postow MA, Chasalow SD, Yuan J, et al. Pharmacodynamic effect of ipilimumab on absolute lymphocyte count (ALC) and association with overall survival in patients with advanced melanoma. J Clin Oncol. 2013; 31:abstract 9052.

67. Carthon BC, Wolchok JD, Yuan J, et al. Preoperative CTLA-4 blockade: tolerability and immune monitoring in the setting of a presurgical clinical trial. Clinical cancer research : an official journal of the American Association for Cancer Research. 2010; 16:2861–2871. [PubMed: 20460488]

68. Nduom EK, Wei J, Yaghi NK, et al. PD-L1 expression and prognostic impact in glioblastoma. Neuro Oncol. 2015

69. Robert C, Long GV, Brady B, et al. Nivolumab in previously untreated melanoma without BRAF mutation. The New England journal of medicine. 2015; 372:320–330. [PubMed: 25399552]

70. Snyder A, Makarov V, Merghoub T, et al. Genetic basis for clinical response to CTLA-4 blockade in melanoma. The New England journal of medicine. 2014; 371:2189–2199. [PubMed: 25409260]

71. Le DT, Uram JN, Wang H, et al. PD-1 Blockade in Tumors with Mismatch-Repair Deficiency. The New England journal of medicine. 2015; 372:2509–2520. [PubMed: 26028255]

72. Rizvi NA, Hellmann MD, Snyder A, et al. Mutational landscape determines sensitivity to PD-1 blockade in non-small cell lung cancer. Science. 2015; 348:124–128. [PubMed: 25765070]

73. Butterfield LH, Palucka AK, Britten CM, et al. Recommendations from the iSBTc-SITC/FDA/NCI Workshop on Immunotherapy Biomarkers. Clinical cancer research : an official journal of the American Association for Cancer Research. 2011; 17:3064–3076. [PubMed: 21558394]

74. Janetzki S, Britten CM, Kalos M, et al. “MIATA”-minimal information about T cell assays. Immunity. 2009; 31:527–528. [PubMed: 19833080]

Table 1

Delayed-Type Hypersensitivity Assays

Study	Phase	Immunological Outcome	Immunological Correlation with Clinical Outcome
Yamanaka R, et al (2005)	I/II	8/15 patients with positive DTH response	Responders demonstrated increased OS compared to non-responders (p = 0.003); 4/8 DTH responders demonstrated partial-minor responses via MRI
Clavreul A, et al (2010)	I	2/5 patients with positive DTH response	3/5 patients demonstrated prolonged survival; 2/3 patients with prolonged survival were responders
Sampson JH, et al (2010)	II	3/17 patients with positive DTH response	Responders demonstrated increased PFS and OS compared to non-responders (p = 0.03)
Muragaki Y, et al (2011)	I/II	DTH-2 responses varied from 1 to 47 mm	Patients with a DTH-2 response ≥ 12 mm exhibited a trend towards increased OS (p=0.19); Patients with a DTH-2 response ≥ 12 mm had a statistically significant increase in PFS (p < 0.001)
Steiner HH, et al (2004)	Pilot	15/23 patients with positive DTH response	-
Yamanaka R, et al (2003)	I/II	3/6 patients with positive DTH response	DTH responders (2/3) exhibited minor reactions radiographically
Rutkowski S, et al (2004)	Feasibility	6/8 patients with positive DTH response	No correlation between DTH response and survival
Vik-Mo EO, et al (2013)	I/II	1/7 patients with positive DTH response	-
Ardon H, et al (2010)	Pilot	3/7 patients with positive DTH response	No correlation between DTH and clinical response
Ishikawa E, et al (2007)	Pilot	9/12 patients with positive DTH-2 response	Responders exhibited trend towards increased survival (p = 0.12); 5/5 patients with radiologic outcome greater than no change were DTH responders
Fadul CE, et al (2011)	Pilot	0/10 patients with positive DTH response	-
Yajima N, et al (2005)	I	8/11 patients on weekly protocol with positive DTH responses; 3/10 patients on biweekly protocol with positive DTH responses	-
DeVleeschouwer S, et al (2008)	I/II	9/21 patients with positive DTH response	No correlation between positive DTH and survival
Wheeler CJ, et al (2008)	II	0/8 patients with positive DTH responses	-
Akiyama Y, et al (2012)	I	4/9 patients demonstrated positive DTH responses to DC and KLH; 2/4 immune responders demonstrated DTH responses to peptide, DC, and KLH	1/2 patients exhibiting DTH responses to three antigens (peptide, DC, KLH) exhibited clinically stable disease
Sloan AE, et al (2000)	Pilot	17/19 patients developed positive DTH response	Positive correlation between DTH response and survival (p < 0.02)
Ishikawa E, et al (2014)	I/IIa	9/24 patients exhibited DTH ≥ 10 mm	Increased PFS in patients with DTH ≥ 10 mm compared to patients with DTH < 10 mm (p = 0.0071)

Table 2

T-Cell Proliferation Assays

Assay	Study	Phase	Immunological Outcome	Immunological Correlation with Clinical Outcome
[3H] Thymidine Incorporation	Vik-Mo EO, et al (2013)	I/II	7/7 patients with induced T-lymphocyte proliferation	-
	Sampson JH, et al (2009)	I	11/12 patients demonstrated increased proliferation to KLH;10/12 patients demonstrated increased proliferation to PEPvIII	-
DDPA	Fadul CE, et al (2011)	Pilot	Precursor frequency of CD4+ T cells increased post vaccination ($p = 0.004$); CD4+ and CD8+ IFNγ producing cells demonstrated trends towards post-vaccination increase ($p = 0.016$ and $p = 0.105$)	Increased CD4+ and CD8+ precursor frequency along with increased proliferating percentages of CD4+ and CD8+ IFNγ producing cells correlated with a trend towards increased PFS ($p = 0.09$) and significantly increased OS ($p = 0.002$) as elements of hierarchical clustering analysis
	Crane CA, et al (2013)	I	5/6 patients with increased proliferation	Increased proliferation distinguished immune responders with increased OS

Table 3

Cytotoxic T-Lymphocyte Assays

Assay	Study	Phase	Immunological Outcome	Immunological Correlation with Clinical Outcome
Chromium (51Cr) Release	Yajima N, et al (2005)	I	0/16 patients with positive 5 lCr release assay	-
	Kikuchi T, et al (2004)	Pilot	2/8 patients demonstrated increased cytolytic activity following immunization	2/4 patients with radiographic partial responses exhibited increased cytolytic activity following immunization
Just Another Method (JAM)	Yu JS, et al (2001)	I	4/7 patients with positive CTL response	-
	Yu JS, et al (2004)	I	1/1 patients with positive JAM assay	-
Alamar Blue	Liau LM, et al (2005)	I	6/12 patients with positive Alamar Blue CTL response	Responders had stable/minimal residual disease burden (6/6); Responders exhibited increased OS compared to non-responders (p = 0.04)
CD107a+b	Phuphanich S, et al (2013)	I	CD 107a staining correlated significantly with IFNγ responses (r = 0.932, p = 0.001)	Responders (5/15) exhibited a nonsignificant trend towards increased PFS and OS

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Table 4

Cytokine Profiles

Assay	Study	Phase	Immunological Outcome	Immunological Correlation with Clinical Outcome
ELISA	Yajima N, et al (2005)	I	9/11 patients on weekly protocol with increased IFNγ ;5/10 patients on biweekly protocol with increased IFNγ	14/21 patients with increased IFNγ production exhibited trend towards increased survival (p = 0.132)
	Kikuchi T, et al (2001)	I	6/6 of patients demonstrated increased post-vaccination IFNγ	2 clinically minor responses – 2/2 exhibited increased post-vaccination IFNγ
	Jie X, et al (2012)	Prospective case-control	IL-2, IL-12, and IFNγ increased in vaccine vs. control (p < 0.05)	OS increased for vaccine vs. control (p < 0.05); Time to recurrence prolonged for vaccine vs. control (p = 0.04)
	Olin MR, et al (2014)	Pilot	3/9 patients demonstrated significantly increased GM-CSF (p = 0.003) and TNFα (p = 0.04) production; 3/9 patients demonstrated a trend towards increased IL-17a production	Stable disease was associated with higher concentrations of GM-CSF (p=0.003) and TNFα (p=0.04) compared to non-stable disease; No difference in IFNγ production observed between stable and non-stable disease
	Pellegata S, et al (2013)	Pilot	-	Reduced post/pre vaccination ratio of TGFβ2 and VEGF correlated with increased PFS (0.035) and OS (0.02)
ELISPOT	Yamanaka R, et al (2005)	I/II	6/13 patients with positive IFNγ ELISPOT	ELISPOT responders demonstrated increased OS compared to non-responders (p = 0.015); 2/8 ELISPOT responders demonstrated a partial-minor response via MRI
	Steiner HH, et al (2004)	Pilot	3/3 patients demonstrated positive IFNγ ELISPOT	-
	Yamanaka R, et al (2003)	I/II	2/5 patients with positive IFNγ ELISPOT	ELISPOT responders demonstrated minor response or no change via MRI
	Ardon H, et al (2010)	Pilot	5/8 patients with positive IFNγ ELISPOT	No correlation between positive IFNγ ELISPOT and clinical response
	Fadul CE, et al (2011)	Pilot	4/10 patients with positive IFNγ ELISPOT	Increased IFNγ spots on ELISPOT correlated with a trend towards increased PFS (p = 0.09) and significantly increased OS (p = 0.002) as elements of hierarchical clustering analysis
	Okada H, et al (2007)	I	1/7 patients demonstrated positive IFNγ ELISPOT	-
	Iwami K, et al (2012)	I	2/3 patients with positive IFNγ ELISPOT	-
	Akiyama Y, et al (2012)	I	6/8 patients with positive IFNγ ELISPOT	1/6 patients with increased IFNγ production exhibited clinically stable disease
	Okada H, et al (2011)	I/II	11/19 patients with positive IFNγ ELISPOT	-
ICS	Crane CA, et al (2013)	I	7/8 patients with positive IFNγ ICS	Positive IFNγ ICS distinguished responders with increased OS
	Phuphanich S, et al (2013)	I	5/15 patients with positive IFNγ response	Patients with positive IFNγ response (5/15) exhibited non-significant trend towards increased PFS and OS
	Akiyama Y, et al (2012)	I	9/9 patients demonstrated shift towards Th1 response	-

Assay	Study	Phase	Immunological Outcome	Immunological Correlation with Clinical Outcome
	Olin MR, et al (2014)	Pilot	3/9 patients exhibited IL-17a production by CD4+ T cells	Stable disease associated with IL-17a production by CD4+ T cells
	Kikuchi T, et al (2004)	Pilot	1/6 patients demonstrated increased IFNγ staining following immunization	1/4 patients with radiographic partial responses exhibited increased IFNγ staining
qPCR	Wheeler CJ, et al (2008)	II	17/34 patients with positive qPCR assay for IFNγ production	Increased IFNγ production correlated with increased TTS (p = 0.041) and TTP (p = 0.015)
	Crane CA, et al (2013)	11/12 patients with positive IFNγ qPCR response	Increased IFNγ qPCR response distinguished responders with increased OS	
	Yu JS, et al (2004)	I	6/10 patients with positive IFNγ qPCR	-
	Pellegata S, et al (2013)	Piolt	In patients with increased NK cell frequency, 3/9 exhibited increased post-vaccination expression of E4BP4 and IFNγ	-
CBA	Prins RM, et al (2011)	I	Increased TNFα and IL-6 detected following vaccination; Th1/Th2 ratios calculated	Heterogeneous and not correlated with clinical outcome; Th1/Th2 ratios not correlated with clinical outcome

Table 5

Lymphocyte Phenotyping.

Assay	Study	Phase	Immunological Outcome	Immunological Correlation with Clinical Outcome
Complete Differential Blood Count	Bloch O, et al (2014)	II	14/41 patients demonstrated absolute lymphocyte count (ALC) above lower limit of normal	ALC ≥ median demonstrated improved survival (p = 0.039); WBC count and absolute monocyte count did not predict survival
	Ishikawa E, et al (2014)	I/IIa	2/23 patients with CTCAE Grade 1 lymphopenia prior to vaccination, 7/23 with Grade 2 lymphopenia, 8/23 with Grade 3 lymphopenia; 6/23 with Grade 4 lymphopenia	Patients with Grade 3 lymphopenia exhibited superior OS compared to patients with Grade 0–2 and Grade 4 lymphopenia (p = 0.024)
PBMC Flow Cytometry	Yamanaka R, et al (2003)	I/II	0/5 patients with changes in CD3 or CD4; 4/5 patients with slight increases in CD8, CD 16, and CD 19; 5/5 patients with changes in CD56 (p < 0.05)	4/5 patients with shift in CD56 had at least a minor response when evaluated by MRI
	Ardon H, et al (2010)	Pilot	6/7 patients demonstrated increased post-vaccination proportion of CD8+CD25+ T cells	No correlation between increased CD8+CD35+ T cells and clinical response
	Crane CA, et al (2013)	I	CD4 and CD8 frequencies stable pre/post vaccination; Expansion of CD3+CD4+CD8+ population in immune responders (11/12); Expansion of CD4+CD25+FoxP3+ Treg population in immune non-responder (1/12)	CD3+CD4+CD8+ population distinguished responders with increased OS; Expansion of CD4+CD25+FoxP3+ Treg cell population distinguished non-responder with decreased OS
	Kikuchi T, et al (2001)	I	No consistent post-vaccination shifts in CD3, CD4, CD8, and CD19; Slightly increased post-vaccination CD 16 and CD56 frequencies in 4/5 pts	2 clinically minor responses observed – 2/2 exhibited increased CD16 and CD56 frequencies
	Jie X, et al (2012)	Prospective case-control	CD3+, CD3+CD4+, CD4+/CD8+, and NK cell populations increased in vaccine vs. control (p < 0.05)	OS increased for vaccine vs. control (p < 0.05); Time to recurrence prolonged for vaccine vs. control (p = 0.04)
	Olin MR, et al (2014)	Pilot	3/9 patients exhibited significant decreases in lineageneg and monocytic MDSCs (p < 0.05); 6/9 patients demonstrated a significant increase in percentage of granulocytic MDSCs	Stable disease associated with decreased lineageneg and monocytic MDSCs vs. non-stable disease associated with increased proportion of granulocytic MDSCs; No difference in Tregs across stable and non-stable disease; Significant increase in CD8+ central memory T cells in stable disease population (p = 0.037)
	Prins RM, et al (2013)	I	Significantly increased NK cell proportion in GAA-DC trial (p < 0.0001)	Decreased Treg ratio (post/pre vaccination) associated with improved survival (p = 0.004); Decreased ratio of activated NK cell (post/pre vaccination) demonstrated a trend towards improved survival (p = 0.081)
	Fong B, et al (2012)	I	No significant pre/post vaccination shifts in CD4, CD8, NK T cells, NK cells, or Treg cell populations; No significant pre/post alteration in PD-1 on any lymphocyte subset	Decreased Treg ratio (post/pre vaccination) associated with improved survival (p=0.0228); Decreased CTLA4 expression by CD3+CD4+ T cells was associated with improved survival (p = 0.0191); Decreased CTLA4 expression by CD3+CD8+ T cells was associated with increased survival (p = 0.0460)
	Prins RM, et al (2011)	I	Vaccinated patients possessed increased CD3+CD4+FoxP3+ and CD3+CD4+CD25+FoxP3+ lymphocytes compared to healthy volunteers	No relevant changes in Treg populations that correlated with clinical outcome

Assay	Study	Phase	Immunological Outcome	Immunological Correlation with Clinical Outcome
	Ardon H, et al (2012)	I/II	Median pre-vaccine/post-vaccine ratios of lymphocyte populations: CD4 =1.01, CD8 = 1.08, NK = 0.89, Treg = 1.28	No significant correlations between NK, Treg, CD4, or CD8 population shifts and PFS or OS; Cluster analysis of Treg and NK cell ratios produced several clusters with non-significant trends towards increased PFS
	Pellegata S, et al (2013)	Pilot	6/14 patients exhibited increased post-vaccination NK cell frequency	Increased post/pre vaccination ratio of NK cells correlated with increased PFS (p = 0.004) and OS (p = 0.02)
	Kikuchi T, et al (2004)	Pilot	7/7 patients demonstrated no significant changes in pre/post vaccination CD3, CD4, CD8, CD16, CD19, and CD56 surface phenotypes	-
Phosphoflow	Everson RG, et al (2014)	I	7/15 patients demonstrated pre/post vaccination pSTAT5 ratio > 1.065 in CTLs	Patients with increased pSTAT5 ratio demonstrated increased OS (p = 0.0015); Patients with survival > 2 years demonstrated increased pSTAT5 ratios (p = 0.015) and lower pSTATl ratios (p = 0.038)