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Victory and Defeat in the Induction of a Therapeutic Response through Vaccine Therapy for Human and Canine Brain Tumors: A Review of the State of the Art

Michael R. Olin^{1,*}, G. Elizabeth Pluhar², Brian M. Andersen¹, Rob Shaver¹, Nate N. Waldron¹, and Christopher L. Moertel¹

¹Department of Pediatrics. University of Minnesota, Minneapolis, MN 55445

²Department of Veterinary Medicine, College of Veterinary Medicine. University of Minnesota, St. Paul, MN 55108

Abstract

Anti-tumor immunotherapy using tumor lysate-based vaccines has made great advances over recent decades. Cancer vaccines aim to elicit adaptive immune responses through various pathways by providing tumor and tumor-associated antigens with an immune stimulant or adjuvant. These anti-tumor vaccines are therefore developed as personalized treatments. Utilizing tumors as a source of vaccine antigens in immunotherapy has demonstrated promising results with minimal toxicity. However, to date, researchers have failed to overcome the overpowering immune suppressive effects within the tumor microenvironment. Immune suppression occurs naturally via multiple mechanisms. These mechanisms serve an important homeostatic role restoring a normal tissue microenvironment following an inflammatory response. Due to these suppressive mechanisms and the inherent heterogeneity of tumors, it is imperative to then elicit and maintain a specific tumoricidal response if vaccine therapy or some other combination of reagents is chosen. In this review, we focus on the historical use of tumors as a source of antigens to elicit a tumoricidal response and the limitations encountered that prevent greater success in immunotherapy. We describe the advantages and disadvantages of various vaccines and their ineffectiveness due to tumor-induced immune suppression.

Keywords

Tumor lysate; immunosuppression; canine glioma model; CTLA-4

I. INTRODUCTION

Discovery of cancer immunotherapy dates back over 100 years. In the 1890s, William Coley, a surgeon in New York, noted in records that dated back to the 1700s that cancer patients with post-operative streptococcal infections had spontaneous regression of their tumors.¹ To test his observations, Coley injected *Streptococcus* and *Serratia* bacteria into his

*Address all corresponding to: Michael R Olin; University of Minnesota, 3-136 Cancer and Cardiovascular Building, 2231 6th St SE, Minneapolis MN 55455; Tel.: 612-626-3123; Fax: 612-624-3514; Olin0012@umn.edu.

patients' tumors, inducing spontaneous regression in greater than 10% of the cases.¹ More than 120 years later, many new cancer immunotherapy approaches have been created by applying our new knowledge of cancer, immunology, and vaccinology.

Understanding the relationship between the immune system and cancer formally began in the late 19th century, when the effect of inflammation against pathogens and against tumors was established.² Decades of studies using animal models led to the immunosurveillance theory, which postulates that tumor cells can be recognized and destroyed by the immune system.³ We now know that tumors express self- and neo-antigens from their aberrant genetic programs, making them immunologically distinct from normal tissue.⁴

Current treatments such as chemotherapy and radiotherapy have shown beneficial effects in some cancers, particularly those of hematopoietic origin,^{5,6} but these benefits have been more limited in solid tumors. Because tumor recurrence is a common event in patients treated with surgery alone, it is imperative that we generate more effective adjuvant therapeutics that are less invasive and produce fewer adverse effects. Thus, cancer immunotherapy is an important and exciting field that is currently producing signs of efficacy in hundreds of clinical trials,^{7,8} although response rates remain low.

Immunotherapeutic approaches are extremely important. To achieve a proper tumoricidal response, immunotherapy must provide the correct mechanism(s) of treating cancer by harnessing the immune system. These mechanisms will allow for the elimination of cancer cells that are unable to be completely removed via resection or radiotherapy due to their anatomic relationships or location.^{9,10} This is especially true in the deadliest of primary brain tumors, malignant glioma.¹¹ We have yet to see any significant progress with non-immunotherapeutic strategies against glioma.⁹⁻¹¹ Here, we review the various types of immunotherapy and how they specifically relate to central nervous system tumors. In addition, we focus on why we are failing to achieve greater response rates with this therapeutic approach.

II. DEVELOPMENT OF TUMOR VACCINES

A. Tumors as a Source of Antigens

Studies designed to discover the immunological roles that various immune cells play in cancer have led to the development and exploration of anticancer vaccines.¹² Cancer vaccines aim to elicit adaptive immune responses by providing tumor-associated antigens (TAA) in conjunction with an immune stimulus or adjuvant. Tumor cells are frequently used as a source of personalized immunotherapy by specifically targeting multiple, patient-specific tumor antigens.¹³ Vaccines utilizing tumor cells as the sources of antigen include tumor lysate-pulsed dendritic cells, dendritic cell-tumor cell fusions, tumor-derived heat-shock proteins, cytokine-secreting tumor cells, and direct injection of tumor cell lysate. Unfortunately, tumor cell extracts are typically poorly immunogenic as evidenced by the suppression of dendritic cell maturation, which in turn, inhibits the priming of T cells.^{14,15} Although various strategies have attempted to increase tumor cell immunogenicity (e.g., heat shock, irradiation, genetic engineering),¹⁶ the limited efficacy of these cancer vaccines in randomized clinical trials demonstrates the need for novel approaches.

B. Complications in the Development of Tumor Vaccines

The development of anti-tumor vaccines is often complicated. It remains unclear how tissue culture might affect antitumor immune responses evoked by tumor cell vaccines. It has been reported that primary human glioma cells cultured in serum-containing media are genetically and phenotypically different from the primary tumor; whereas culture of the same cells under serum-free conditions produces cells that more closely reflect the native tumor and appear to enrich for a tumor stem cell phenotype.¹⁷ In a murine model, glioma cell lysates generated in serum-free conditions were shown to be more effective than those derived from serum-containing media when employed in tumor lysate-pulsed dendritic cell (DC) vaccines.¹⁸ In addition to tissue culture medium components, studies have demonstrated that culture conditions, such as a low oxygen environment, can significantly enhance immunogenicity.^{19,20} Our laboratory characterized the differential immune response to tumor lysate vaccines using a murine glioma model. We identified oxygen concentration as an “immunologic switch” that affects both cell-mediated and humoral immune responses elicited by tumor cell lysates.¹⁹ We compared tumor cells generated from primary tumor cells snap frozen in the operating room to cells that were cultured at 5% and 20% (atmospheric) O₂. We observed a significant difference in the expression of more than 3,000 genes between the 20% O₂ cultured cells and the *in situ* tumor cells.²⁰ Of these, 77 genes were differentially expressed between the 5% and 20% O₂ cultured cells, trending towards the mRNA expression levels observed in the 5% O₂ cultures reflecting the *in situ* tumor. Tumor cells grown at physiologic oxygen tension are inherently more capable of priming CD8 T cells by a mechanism that appears to be independent of increased major histocompatibility complex (MHC) I/II, CD80/83/86, IL-6, IL-8, IL-10, or IL12p70 expression in DCs.²⁰ These alterations in the culturing of tumor cells have a profound impact on tumor antigen expression. The source of tumor antigens is important for the development of a tumoricidal response and can dramatically vary the initiation of an immune response. Cell-based approaches likely provide a diverse pool of antigens triggering concurrent CD4 and CD8 T-cell responses along with tumor-reactive antibodies proving effective in multiple cancer types across several species.²¹

1. Tumor cell lysates—Tumor-derived cell vaccines can be (1) autologous tumor cell lysates of tumor cells from the patient receiving the vaccine or (2) allogeneic lysates, which are produced from one or more tumor cell lines that are similar enough to the patient’s tumor to induce an immune response when given to the patient. We will address more differences between autologous and allogeneic sources of tumor antigens later (Table 1). An advantage of whole tumor cell lysates is the ability to utilize multiple tumor associated antigens (TAA) such as GnT-V,²² survivin,²³ IL13Rα2,²⁴ tyrosinase-related protein 1 (Trp-1) and Trp-2,^{25,26} Gage,²⁷ human melanoma-associated antigens p97/GP100,¹⁰ EphA2/Eck,²⁸ Aim-2,²⁹ Sart-1,³⁰ Her2/nu³¹ and Mage-1³¹ to elicit an immune response.³²

Although vaccines consisting of whole cell tumor lysates have shown some signs of successful therapy, they failed to receive FDA approval in the United States. An example of this is Melacine®, a melanoma allogeneic vaccine consisting of lysates from two melanoma cell lines combined with the adjuvant DETOX®. When given to 198 patients in phase II/III trials, Melacine® resulted in a 6% overall response rate (five complete and seven partial

responses).³³ The four patients with complete responses were alive and disease-free 7–10 years post-treatment; implying long-term disease control through memory responses. Interestingly, a retrospective analysis showed that the response rate improved to 38% in patients expressing HLA-A2 and HLA-C3.³³ Due to a better safety profile and comparable complete response rates as chemotherapy or high-dose IL-2, Melacine® has been approved in Canada for treatment of metastatic melanoma.

Nephrectomy was performed in patients with organ-confined disease and lysate vaccines were produced from autologous tumor cells stimulated with interferon gamma (IFN γ). Use of Reniale® led to an increase progression-free survival compared to nephrectomy with no adjuvant therapy (Hazard Ratio=1.58, 95% C.I. = 1.05 to 2.37 at 5 years).³⁴ Subsequently, a review with 10-year follow-up showed that 5-year overall survival of patients with UICC stage 3 disease was 71.9% in patients receiving autologous vaccine versus 60.3% for those who had surgery alone ($p=0.008$).³⁵

2. Apoptotic bodies—The study of the contents of tumor cells and the cellular processes and life cycle of these cells have shown the existence of apoptotic bodies.³⁶ Apoptotic bodies are enclosed vesicles that are formed by cells undergoing the process of apoptosis or programmed cell death. Apoptotic bodies have been used as a source of TAA in the treatment of B-cell leukemia,³⁷ melanomas,³⁸ and brain tumors.³⁹ Apoptotic bodies may be relevant in the treatment of primary brain cancers like astrocytoma, anaplastic astrocytoma, and glioblastoma multiforme (GBM) because they may envelop potential toxins or cellular contents that are immunogenic.⁴⁰ Apoptotic bodies that have undergone endocytosis by autologous dendritic cells can elicit anti-tumor T-cell responses²¹ and therefore can be considered as viable candidates for experimentation *in vivo* for cancers across multiple species.^{41,21}

C. Peptide Vaccines

A meta-analysis accounting for 3444 patients in 173 clinical trials indicated that whole tumor cell-based vaccines had higher response rates (~8%) than vaccines consisting of synthesized antigens (~4%).⁴² Despite these data, following thousands of clinical trials using peptide vaccines Provenge® became the first FDA-approved cancer vaccine. Provenge® was designed to treat men with metastatic castration-resistant prostate cancer utilizing a fusion protein of prostatic acid phosphatase and granulocyte-macrophage colony-stimulating factor (GM-CSF).⁴³ A phase III trial that included more than 500 patients extended median survival by 4.1 months, 25.8 months versus 21.7 months in the placebo group.⁴⁴ Another example of a peptide vaccine is that derived from the melanoma-associated antigen, MAGE-3. MAGE-3 vaccination generated the first clinically validated response to peptide-based immunotherapy.⁴⁵ Phase I and II trials testing MAGE-3-derived peptides in melanoma and non-small cell lung carcinoma (NSCLC) patients have been completed, and a phase III study in individuals affected by NSCLC is currently underway.^{46,47}

Combinatorial approaches have been employed to increase the efficacy of peptide-based therapies. Schwartzentruber *et al.* working with stage IV metastatic melanoma patients, administered gp100-derived peptides mixed with incomplete Freund's adjuvant in

combination with high-dose IL-2.⁴⁸ The results obtained from this randomized phase III clinical trial were encouraging and showed that the progression-free survival time of advanced melanoma patients receiving the peptide vaccine combined with IL-2 is longer than that of patients treated with IL-2 alone.⁴⁸ The impetus to develop peptide vaccines has increased with the identification of numerous other tumor-associated antigens.

Peptide-based vaccines have several advantages over whole-cell vaccine strategies (Table 1). In particular, synthetic peptides (1) can be easily and inexpensively produced in clinical grade, (2) can be easily administered to patients, (3) are relatively non-toxic, and (4) aid in the monitoring of antigen-specific antitumor immune responses (reviewed in Hayes *et al.*⁴⁹). A major disadvantage of this approach is that peptides are restricted to specific HLA alleles. Ideal candidates for peptide vaccines would therefore be HLA-compatible peptides that are derived from TAA expressed exclusively on the tumor cells and can induce a cytotoxic T-cell response upon immunization. Only a few TAA are expressed on the surface of tumor cells (e.g., HER2, MUC1)^{49,50} and represent valid therapeutic targets. Another major limitation for this therapeutic approach stems from the concept of “tumor escape.” Tumor cells can undergo antigenic variation or lose the expression of immunogenic antigens and/or HLA molecules, thereby avoiding the recognition by the immune system, also known as cancer immunoediting. In this setting, antigen-negative tumor variants will be positively selected under the pressure of T cells targeting their antigen positive counterparts.

III. THE ROLE OF IMMUNE CELLS IN IMMUNOTHERAPY

While many cells of the innate and adaptive immune system help eliminate tumors, the adaptive arm, comprised of B and T lymphocytes, is considered the most capable of tumor elimination and long-term survival.⁵¹ Therefore, it is important to use tumor antigens that induce an adaptive tumoricidal response. Researchers have focused on the initiation of an anti-CD8 T-cell response because T-cell responses are able to eliminate tumor cells independent of their proliferative state and because of their demonstrated resistance to chemotherapy.

A. Natural Killer Cells

Natural killer (NK) cells are effector cells that play a major role in the immune response to viral infections and different types of cancers.⁵² The mechanism by which NK cells eliminate virally infected and cancerous cells is not exactly defined, but it is likely independent of the presentation of target cell antigens. Instead, it is thought that signals derived from interactions between the NK cell and the target cell generates both stimulatory and inhibitory signals leading to NK cell activation.⁵³ Through advances in research studying the mechanisms by which NK cells identify and kill select tumor and virally infected cells, NK cells are now known to be a powerful component of innate anti-tumor immunity. It has been hypothesized that after resection of the bulk of tumor, the remaining tumor microenvironment represents a therapeutic challenge for the delivery of targeted small molecules and other chemotherapeutic drugs, as well as for NK cells to locate and identify residual tumor cells.⁵⁴ However, the tumor microenvironment may influence NK-mediated defenses by a number of immunosuppressive strategies. Tumors can suppress NK functional activity similar to T cells due to the down-modulation of activating NK receptors.⁵⁵

B. Activation of Tumor-Reactive B Cells and Antibodies

B cells are lymphocytes each of which express a somatically mutated, unique, membrane-anchored antibody molecule, known as the B-cell receptor (BCR).⁵⁶ B cells develop in the bone marrow, but they reside in the follicles of lymph nodes in their naïve state awaiting direct contact with antigens from the lymphatic drainage.⁵⁶ Unlike CD8 T cells, B cells do not require antigen-presenting cells to classically engage antigen with their BCR. The size of epitopes that can stimulate B cells is thought to be variable, although likely still a small fraction of the originating molecule. Like CD8 T cells, B cells require costimulatory and cytokine signals for full activation. Antigen binding to the BCR triggers internalization and subsequent digestion of antigen into peptide fragments. Some fragments bind MHC class II (MHC II) receptors and are presented in the peptide-MHC II complex on the surface of the B cell. Simultaneous with this antigen processing, the B cell begins to migrate to the periphery of the follicle bordering the T-cell zone.⁵⁷ Costimulation arises when a CD4 T helper cell recognizes its cognate peptide-MHC II complex on the surface of the B cell,⁵⁸ after which T-cell CD40L-CD40 interactions are required for full activation of the antibody response.^{59,60} CD4 TCR engagement of peptide-MHC II triggers cytokine (IL-2, IL-4, IFN γ , IL-5) production by the helper cell that then act locally on the B cell.⁶¹ Upon receiving these three signals (antigen binding, CD40 stimulation, and cytokine binding), B cells undergo immunoglobulin class switch DNA recombination to produce a specific isotype of membrane-bound antibody/BCR (IgM to IgG, IgA, or IgE). B cells then differentiate and undergo one of three fates: (1) short-lived plasma cells that secrete antibodies,⁶² (2) germinal center B cells that mutate their BCRs to evolve into higher affinity receptors for eventual differentiation into memory B cells or long-lived plasma cells,⁶¹ or (3) long-lived memory B cells.⁶³ Following tumor cell vaccination, antibodies produced by activated B cells circulate through the blood and bind to tumor antigens present within the blood or the tumor site. Antibody binding to intracellular antigens released by dead tumor cells may opsonize the antigens and facilitate their uptake by antigen presenting cells. Antibody binding to surface antigens on living tumor cells can trigger a number of antibody-mediated effector activities that result in tumor cell death. Effector functions triggered by antibody binding to its cognate epitope include: (1) neutralization of the target protein's function,⁶⁴ (2) phagocytosis leading to clearance and/or adaptive immunity,⁶⁵ (3) complement dependent cytotoxicity,^{66,67} (4) chemoattraction of leukocytes or (5) antibody-dependent cell-mediated cytotoxicity.⁶⁷

C. Role of Antigen-Presenting Cells as a Means of Immune Induction

The dendritic cell was identified by Ralph Steinman and Zanvil Cohn in 1973.⁶⁸ Ten years after their discovery, DC was confirmed as the most potent of cross priming cells, as DC depletion results in the drastic reduction of killing in a mixed leukocyte reaction.⁶⁹ Antigen presentation to CD8 T cells is imperative to stimulate a tumoricidal response⁷⁰ and DCs present antigens more efficiently than any other cell type.⁶⁹ Upon injection of tumor cells or cell components, DCs take up these materials and process them as they patrol the skin and secondary lymphoid organs. For optimal priming of CD8 T cells, DCs must provide three signals: (1) antigen cross-presentation in the form of a peptide-MHC I complex, (2) co-stimulatory molecules on their surface, and (3) secreted cytokines.⁷¹ Additional complexities, such as the DC subset of interest,⁷² DC trafficking,⁷³ tissue of antigen

challenge,⁷⁴ and optimal CD4 T helper cell differentiation,⁷⁵ profoundly influence CD8 T-cell activation. It is now clear that once optimally activated, CD8 T cells can specifically target brain tumors in human patients.⁷⁶

Dendritic cells are naturally able to stimulate an antitumor immune response and generate immunological memory in the course of this response.⁷⁷ Clinical trials demonstrate the possibility of using DCs loaded with antigenic peptides to induce peptide-specific responses in patients with lymphoma, malignant melanoma, prostate cancer, and glioblastoma multiforme.^{39,78–80} Alternatively, DCs pulsed or loaded with autologous tumor lysates have been used to induce stronger and longer immune responses against tumors.^{81–83} This strategy has the advantage of providing tumor antigens capable of being presented in association with MHC class I and class II molecules by DCs, thus reducing the potential for tumor escape.

D. Cross Priming in Active Immunotherapy: Activating Tumoricidal CD8 T cells

The “altered self” hypothesis states that CD8 T cells distinguish self from non-self (such as tumor neoantigens) by recognition of the complex of peptide antigen and MHC I.^{84,85} Each CD8 T cell undergoes somatic mutation to develop a unique T-cell receptor specificity for a given peptide sequence and MHC I.⁸⁶ At first, immunologists debated how and where peptide-MHC I complex formation occurs. Does the CD8 T cell recognize the MHC I-peptide complex on the surface of the injected tumor cells or cell fragments or do the antigens transfer to another antigen presenting cell? While both scenarios are possible, Michael Bevan demonstrated the transfer of antigen to a host immune cell.⁸⁷ He coined the term “cross priming” to describe the process by which exogenous antigens are taken up and loaded on MHC I receptors rather than expressed directly.⁸⁷

E. Cross Presentation

Identifying and understanding the function of the cell type responsible for cross priming is crucial to understanding how to best elicit a CD8 T-cell response. Cross presentation of tumor antigens is necessary for CD8 T-cell-mediated tumor rejection; it also requires additional DC-CD8 T-cell signals. Early studies that sought to reconstitute T-cell priming were hampered by the unresponsiveness of T cells after stimulation of the MHC I receptor with peptide alone. Jenkins and Schwartz demonstrated that antigen-loaded antigen presenting cells that were chemically fixed presented peptide to CD4 T cells, but these T cells could not proliferate without a co-stimulatory short-range signal.^{88,89} Complementary to these findings was the discovery that at steady state, DCs induce CD8 T-cell antigen tolerance.⁹⁰ The missing signals in the case of CD4 and CD8 T cell activation were CD80 and CD86; DC-expressed ligand for the CD28 co-stimulatory receptor on T cells. CD28 signaling results in the amplification of the TCR signal, thereby inducing a proliferative response and IL-2 synthesis by the activated T cell.⁵¹

Attempts to recapitulate cross priming *in vitro* by incubating naïve CD8 T cells with microbeads containing MHC I-peptide complexes and CD80/86 were partially successful.⁹¹ These stimuli triggered several rounds of T-cell division and temporary T-cell effector function, but the T cells were unable to reach full effector function, survive, and develop

memory.⁹¹ When interleukin 12 (IL-12) or interferon alpha/beta (IFN α/β) were added to these cultures, full CD8 activation was achieved.⁹¹ Further studies of adoptive transfer of IL-12R^{+/+} CD8 T cells into IL-12R^{-/-} recipients demonstrated that the IL-12R on CD8 T cells was sufficient for this signal to occur.⁹² Tumor cell vaccines should therefore stimulate the production of IL-12 or IFN α/β to provide adequate signal 3 (cytokine secretion) for optimal cross priming. IL-12 secretion *in vivo* can be stimulated when the CD40L on CD4 T cells binds to CD40 expressed by DCs, a key trigger for IL-12 release.⁹³

A few methods for inducing signal 3 have shown promise, including providing exogenous IL-12 via genetic engineering (e.g., IL-12 producing tumor cells)⁹⁴ or co-injection of recombinant IL-12 cytokine.⁹⁵ The use of type I interferon-promoting adjuvants as inducers of signal 3 also show promise for tumor cell vaccine therapy, as these adjuvants augment signals 1 and 2 by enhancing DC maturation.

F. Brain Tumor Cell Vaccines

Targeted immunotherapy for human brain tumors has taken on many forms and directions since its inception. The recent history of brain tumor vaccine development has focused chiefly on the greatest nemesis, glioblastoma multiforme. Vaccines have been developed using unique tumor-associated proteins as targets via peptide vaccine strategies as a fine scalpel approach and whole tumor lysates as more of a club. The array of weapons ranges from single peptide vaccines and autologous tumor lysate vaccines to multi-peptide vaccines or allogeneic cell lines chosen for their antigen expression and immunogenicity. Immunotherapy adjuvants have varied as well, incorporating polyinosinic-polycytidylic acid (poly(I:C)) stabilized by lysine and carboxymethylcellulose (poly-ICLC),⁷⁶ imiquimod,³⁹ and CpG oligodeoxynucleotides.⁹⁶ Most of the published clinical trials to date allow for no or little concurrent glucocorticoid therapy, while at least one group has allowed up to 4 mg daily of dexamethasone.⁹⁷ Even something as seemingly inconsequential as where to give the vaccine is really not trivial; specified sites are the suprascapular zone,³⁹ the thigh,⁹⁸ the upper arm,⁹⁹ or even directly into lymph-node groups. Because the dendritic cell is logically central to immune processing of tumor antigens and immune signaling, many vaccine manufacturing processes actually include harvesting of dendritic cell precursors, *ex vivo* maturation and processing of the DC-antigen interaction.

Despite early excitement in the medical and business communities, brain tumor immunotherapy has not been as successful as anticipated earlier in this millennium. As we recognize the phenomena of myeloid derived suppressor cells (MDSC) and newly elucidated inhibitory proteins, we hope that resolving these issues will reveal the keys to success and a rediscovery of the “Lost Horizon”.¹⁰⁰ We have selected to present the work of six pioneering groups active in the evolution of brain tumor therapy. This selection is meant to be representative of all research and not minimize the work of many outstanding contributors in this field or over-glorify the work that these groups have done to date. Brain tumors in general, and GBM in particular, are humbling diseases to treat.

IV. AUTOLOGOUS TUMOR VACCINE STRATEGIES

Three groups have been at the vanguard of autologous brain tumor immunotherapy in terms of patient experience and publication:

1. Stefan van Gool leads the group at Catholic University in Lueven, Belgium. Their work culminated in the addition of immunotherapy to the current standard of care as defined by Stupp *et al.* A pilot trial of surgery, chemoradiotherapy, and vaccine with maintenance chemotherapy¹⁰¹ was followed by a phase I/II study of the same design.⁹⁹ Van Gool used whole-tumor lysates manufactured from resected tumors that were then homogenized, filtered, and irradiated (60 Gy). Each patient underwent leukapheresis to obtain autologous immature dendritic cells after surgery and prior to the start of chemoradiotherapy. Vaccine was prepared by loading immature DCs with approximately 200 mg of “tumor proteins.” The vaccine (median 4.1×10^6 DCs) was delivered intradermally in each lymph node region of the upper third of the arms 1, 2, 3, and 4 weeks post-chemoradiotherapy. Subsequently, whole-tumor lysate (400 mL) was given as a boost with the 1st, 2nd, 3rd and 6th temozolomide maintenance cycles. No chemical adjuvant was employed. Patients were not allowed glucocorticoid therapy during the vaccine phase. This strategy was well tolerated in both the pilot and phase I/II trials. Outcomes compared favorably to other contemporary approaches. Not surprisingly, it was clear that patient outcome correlated with the recursive partition analysis (RPA) classification, extent of resection, and O6-methylguanine-DNA-methyltransferase (MGMT) status.¹⁰²
2. John Yu leads the brain tumor immunotherapy group at Cedars Sinai in Los Angeles. Their early work also focused on autologous tumor lysate-pulsed dendritic cells. The lysate was made by exposing resected tumor to three washes, mincing with scissors, and then passing the specimen through metal and nylon meshes. The tumor cells were lysed through four freeze/thaw cycles; larger particles were removed by centrifugation; and the supernatant was passed through a 0.2-mm filter. DCs were obtained by apheresis of mononuclear cells and adherence in tissue culture flasks 1 week before vaccination was initiated. DCs were generated by culturing mononuclear cells in rGM-CSF and rIL4 for 7 days. DC cultures were pulsed with lysate by washing 10^7 – 10^8 DCs with RPMI 1640 and autologous patient serum and then adding 50 mg/ml tumor lysate. The DCs were incubated overnight. 10^7 to 10^8 DCs were given in 0.5 ml of PBS in the deltoid region. Each patient received three vaccines given at 2-week intervals. The vaccine was well tolerated, and the resulting immune response was documented by showing PBMC expression of interferon- γ after vaccine and tetramer staining revealing CD8⁺ T-cell clones against one or more tumor-specific antigen in four of nine patients. Even more dramatic was the median survival time of 133 weeks in the GBM cohort (n=8) compared to 30 weeks for the control patients (n= 26). Interestingly, a relative increase in tumor infiltration was detected by immunohistochemistry in three of six patients who underwent reoperation for recurrence; specifically, CD45RO⁺ memory T cells and CD8⁺ cells were noted.¹⁰¹

This trial was followed by a phase II trial with similar promising results. One unique and cautionary experience emerged when one patient surviving 784 days developed cutaneous glioblastoma with single lymph node involvement at the site of irradiated tumor cell inoculation for DTH testing. This observation serves to remind us that some tumors are radio-resistant, especially at recurrence and that these tumor cells can survive freeze-thaw cycles.²⁷

The results of this phase II trial were reported as vaccine “responders” (n=17) and “nonresponders” (n=15). The responders were defined as those who exhibited interferon- γ levels at least two standard deviations above the pre-vaccine mean after the third vaccination. A survival advantage was reported for those patients who were responders. In the responder group, 8 of 17 were newly diagnosed (47%) versus 3 of 15 (20%) in the nonresponder group. Prior chemotherapy had been given to 47% of responders and 67% of non-responders. Post-vaccine chemotherapy was given to 71% of responders and 60% of nonresponders.²⁸

3. Linda Liao's group at UCLA also used an autologous approach, but they made an effort to further purify their autologous antigen source by culturing the patients' tumor cells, exposing the cells to IFN- γ and IFN- α , and then isolating MHC class I-enriched peptides by acid elution. Primary tumor cell culture took 2 to 14 weeks (median = 5 weeks). The amount of peptide used to pulse autologous dendritic cells was always 100 mg, regardless of the DC number. Fifteen patients were in the “intend to treat” cohort, but cell culture was only successful for 12 of these patients. Tumors were more successfully cultured in newly diagnosed patients. DCs were collected and cultured in GM-CSF and IL-4, then cryopreserved until the day of administration, at which time DCs were pulsed with the tumor cell peptide for 30–60 minutes and, ultimately, given in a relatively large 1 ml intradermal injection below the axilla. The 12 patients in this phase I trial had either newly diagnosed (7) or recurrent (5) tumors. The newly diagnosed patients were treated with surgery and standard course external beam radiation to a maximum dose of 6000 cGy. All patients were off steroids for at least 14 days prior and remained steroid-free throughout the course of vaccine therapy. Three DC dose levels were given: 1×10^6 , 5×10^6 and 1×10^7 cells/ml. The “three vaccine” goal was achieved in four of the six patients due to insufficient numbers of DCs that met phenotype criteria in two of the patients. Clinical manifestations of an induced immune response, local injection site reaction, and regional lymph-node palpable swelling were noted in three patients. In addition, six patients developed new tumor-specific CTL activity after vaccination. All of these patients had minimal and non-progressive disease at the time of vaccination. One newly diagnosed patient with minimal disease and a documented immune response exhibited regression of residual tumor and was a long-term (>5 year) survivor. The authors also postulated that TGF- β 2 expression in the tumor microenvironment was immunosuppressive. Patients with lower TGF- β 2 expression levels had a significantly longer survival time than those with higher levels.¹⁰³

A. Allogeneic Tumor Vaccine Strategies

Our group departed from the autologous tumor cell approach by selecting a cell line (GBM6-AD) grown under hypoxic (5% O₂) conditions that expressed specific antigens favoring a “stem cell-like” or “brain tumor initiating cell” phenotype expressing SOX2, Nestin, CD133, EPHA2, IL13Rα2, and HER2-NEU.³⁹ It has also been shown that the cells grown under hypoxic conditions exhibited increased immunogenicity.²⁰ This cell line could be used as an “off the shelf” antigen source, thereby avoiding the long culture times associated with autologous tumor cells. We incorporated this cell line into the DC vaccine regimen in a pilot trial using autologous DCs that were incubated with GBM6-AD cells that were “heat shocked,” thereby generating so-called apoptotic bodies. The apoptotic bodies were irradiated and incubated with autologous DCs obtained by leukapheresis of mononuclear cells and maturation similar to the processes described above. Vaccines were given subcutaneously at alternating suprascapular sites at three dose levels (5×10^6 , 10×10^6 , and 15×10^6 loaded DCs); all of which were well tolerated. Each vaccine was given with topical imiquimod (a TLR7 agonist) applied at the site prior to vaccination and re-applied 24 hours later. Twelve patients with recurrent CNS tumors entered the trial, but four were unable to receive vaccine due to revision of diagnosis or rapid tumor progression. One patient received one vaccine, but withdrew from the trial to deal with a surgical complication. Six patients were diagnosed with GBM, one with multiple recurrence of medulloblastoma, and one 3-year-old patient with ependymoma. All patients tolerated the vaccine well. One patient had a grade 3 local skin reaction at the vaccine site; otherwise no significant side effects were seen. One patient (a 17-year-old with recurrent GBM) had a complete response (CR) by radiographic criteria. A second patient (a 63-year-old with recurrent GBM) continued on vaccine therapy until evidence of progression. The latter patient had a progression free survival of 39.7 weeks and survived for more than 100 weeks after study enrollment. By post-hoc analysis, we divided our patients into those with “stable” (no progression at week 8 of vaccine therapy) and “non-stable” (progression by week 8) disease. Those with stable disease had a statistically significant decrease in lineage^{neg} (CD3⁻CD14⁻CD16⁻CD19⁻CD56⁻) HLA-DR⁻CD33⁺ MDSCs and monocytic MDSCs (CD14⁺HLA-DR^{lo}). We also observed a statistically significant increase in the percentage of granulocytic MDSCs (CD15⁺CD14⁻) in patients with non-stable vs. stable disease. A trend toward increased IL-17a production was noted in responding patients. Based on this pilot data, we believe that prospective evaluation of MDSC status will aid in detecting those patients most likely to benefit from a vaccine approach.³⁹

B. Tumor Peptide Vaccine Strategies

Heimberger *et al.* discovered the presence of EGFRvIII in 31.5% (54/171) of patients with GBM.¹⁰⁴ Sampson *et al.* noting EGFRvIII tumor specificity, designed a vaccine strategy fusing EGFRvIII (PEPvIII; H-Leu-Glu-Glu-Lys-Lys-Gln-Asn-Tyr-Val-Val-Thr-Asp-His-Cys-OH; Anaspec) with a terminal cysteine that allowed conjugation to keyhole limpet hemocyanin (KLH) (Biosyn; PEPvIII-KLH). A dose escalation phase I trial was carried out in newly diagnosed patients who had completed external beam radiation prior to vaccination. Patients were excluded if they received 2 mg or more of dexamethasone daily. EGFRvIII expression was not an eligibility criterion for the phase I trial. DCs were collected

and matured in culture using a method similar to those described above. At the time of vaccination, DCs were incubated with 500 mg of the peptide PEPvIII-KLH for 2 hours. TNF α , IL-1 β , and IL-6 were then added for another 18 hours. Dose escalation to 1×10^8 antigen-loaded mature DCs was achieved. Each patient received 3 vaccines in equal doses, each 2 weeks apart. The vaccine was given intradermally in the upper thigh 10 cm below the inguinal ligament. Vaccination was received by 12 patients who did not have progressive disease during radiation therapy and was well tolerated by all patients.¹⁰⁵

This group then proceeded to perform a phase II trial that targeted patients with newly diagnosed EGFRvIII-expressing GBM with minimal residual disease. Based on the data of Stupp *et al.*, this trial was modified to include concurrent temozolomide during radiation. Vaccine therapy was initiated 4 weeks after the completion of radiation and followed the administration schedule and site of the phase I trial. Again, no unusual toxicities were noted. Twenty-one patients were initially enrolled and treated, but three patients were later removed from analysis because they did not meet the minimal residual disease criterion (>95% tumor volume resection). The 18 evaluable patients had a median progression-free survival of 14.2 months vs. 6.3 months for the temozolomide treated historical cohort (n=17). This advantage was also seen for median overall survival (OS), which was 26 months for the vaccine cohort and 15 months for the temozolomide cohort. The authors analyzed patient samples for EGFRvIII-specific humoral responses and found that those that developed a humoral response (n=6) had an OS of 47.7 months compared to 22.8 months for those who did not have such a response (n=8). Three patients developed a DTH response, and for those patients, median OS had not been reached by 50 months versus a median OS of 23.1 months for those who did not develop a DTH response (n=14). Finally, immunohistochemical staining for EGFRvIII was performed on 11 samples from patients in the vaccine cohort that had disease recurrence. Nine of 11 (82%) had lost EGFRvIII expression and one had a marked decrease (<1% of cells expressing) compared to baseline.¹⁰⁶

Okada's group at the University of Pittsburgh implemented a phase I/II trial in patients with recurrent high-grade glioma (GBM, anaplastic astrocytoma, anaplastic oligodendroglioma, and anaplastic oligoastrocytoma) to evaluate vaccine safety and induction of anti-glioma-associated antigen (GAA) immunity. The trial employed intranodal injections of α DC1s ($1-3 \times 10^7$) loaded with 4 human GAA peptides (EphA2₈₈₃₋₈₉₁, IL-13R α ₂₃₄₅, YKL-40₂₀₁₋₂₁₀ and GP100₂₀₉₋₂₁₇) in combination with intramuscular injections of poly-ICLC (20 μ g/kg; twice weekly for 8 weeks). Because these epitopes were HLA-A2 restricted, the patient population was limited to those who were HLA-A2 positive. A maximum dexamethasone dose of 4 mg/day was allowed. The dendritic cells were collected by apheresis of PBMC and maturation as described above. The vaccine was prepared by maturation and polarization of DCs with IL-1 β , TNF- α , IFN- α , IFN- γ , and poly-I:C. Two hours before harvest, a pan-DR epitope (PADRE) was added to the cultures. The bilateral intranodal injections were guided by ultrasound into the inguinal and axillary lymph nodes. Vaccine administration was rotated between these lymph node groups. Twenty-two patients were initially enrolled, but only 19 were able to complete the initial schedule of 4 vaccinations, and three were not evaluable due to early progression. The first 10 evaluable patients received 1×10^7 α DC1

per injection, the remainder (n=9) received 3×10^7 α DC1 per injection. Twenty-two patients received at least one vaccination and 19 patients received at least four vaccinations at two α DC1 dose levels at 2-week interval. Patients who demonstrated a positive radiologic response or stable disease without major adverse events were allowed to receive up to 5 booster vaccines at 4-week intervals. Those who continued without major adverse events or tumor progression were vaccinated every 3 months for up to 3 years from the first vaccine in the second booster phase. The first four vaccines induced positive immune responses against at least one of the vaccination-targeted GAAs in PBMCs in 58% of patients. Peripheral blood samples demonstrated significant up-regulation of type 1 cytokines and chemokines, including IFN- α and CXCL10. Nine patients (four GBM, two AA, two AO, and one AOA) achieved progression-free status lasting at least 12 months. One patient with recurrent GBM demonstrated a sustained complete response. IL-12 production levels by α DC1 positively correlated with time to progression. Again, the vaccines were well tolerated. Six of 10 patients at dose level one and five of nine patients at dose level two exhibited immune reactivity to at least one antigen by IFN- γ ELISPOT or tetramer assays. Three patients had an ELISPOT based only on availability of adequate PBMCs for analysis.⁹⁷ These early data for this approach are encouraging, although intranodal injection may not be practical or reproducible at every institution or in all patient groups (e.g., pediatric populations). Likewise, this approach uses HLA-A2-restricted immune epitopes, making this therapy available to ~27% of African Americans and 50% of Caucasian Americans.¹⁰⁷

V. IMMUNOSUPPRESSION

A. Tumor-Induced Suppression in the Draining Lymph Nodes

Immunotherapy for CNS tumors has a variety of obstacles that are currently being addressed. A characteristic problem for patients with GBM is systemic immune suppression.¹⁰⁸ Immunosuppression in cancer patients is a well-known phenomenon. In breast cancer and melanoma patients, T cells isolated from the sentinel lymph nodes (draining lymph nodes (DLN) nearest the tumor) exhibit suppressed responses to various mitogens as compared to T cells isolated from the more distant lymph nodes (Fig. 1).^{109,110} Therefore, vaccinating these patients near the sentinel lymph nodes should induce a less effective tumoricidal response.

As noted above, glioma vaccines have been administered in a variety of sites, including the scapula draining into the axillary lymph nodes,¹¹¹ the anterior upper thigh,¹¹² the upper arm,⁹⁹ and the cervical region^{113,114} for immune activation. Using a murine glioma model, we described the relationship between the proximity of vaccination site(s) to the primary tumor and the strength of the ensuing CD8⁺ T-cell response in the draining lymph nodes.¹¹⁵ In these studies, ovalbumin (OVA)-transfected GL261 glioma-bearing mice were vaccinated with OVA protein plus adjuvant (Poly:ICLC) in the neck, foreleg, or hindleg for drainage into the cervical, axillary, or inguinal lymph nodes, respectively. In all tissues analyzed, there was a stepwise decline in the frequency of OVA-specific CD8⁺ T cells as the vaccination site approached the tumor in the following order: hindleg > foreleg > neck. In addition to a decrease in the number of antigen-primed CD8⁺ T cells, T-cell receptor affinity, effector function, and infiltration into the brain decreased as the vaccination site

approached the tumor. These glioma-induced suppressive effects were not an artifact of the OVA antigen because the same results were observed in mice bearing parental (non-OVA transfected) GL261 tumors.¹¹⁵

B. Mechanisms of Immune Suppression

Multiple mechanisms contribute to immune suppression (Fig. 1). Tumor-elaborated soluble factors, such as TGF β and prostaglandin E2¹¹⁶ can act either within the tumor site or in the DLN to dampen T-cell reactivity. In experimental systems, additional mechanisms of local immune suppression at the DLN include regulatory T-cell-mediated killing of tumor antigen-presenting DCs, T-cell receptor nitration by myeloid derived suppressor cells and tolerogenic tumor-associated dendritic cells (TADCs).¹¹⁷

Tumors induce immune suppression by inducing the development of distinct suppressive immune cell types including MDSCs, type 2 macrophages (M2), TADCs, and T regulatory cells (Treg) and recruiting them to the tumor microenvironment. Tumors recruit these immunosuppressive cells through the expression of chemotactic molecules such as CXCL12. PGE2 is essential in inducing CXCL12 production that results in the recruitment of MDSCs, Tregs, and TADCs into the tumor microenvironment.¹¹⁸ Furthermore, as tumors invade local tissue, PBMCs perceive cancer invasion signals and migrate into the tumor and transform into macrophages.^{119–121} These tumor-associated macrophages (TAMs) are divided into two phenotypes with opposite functions.^{122,123} The classically activated TAMs, the M1 phenotype, can be induced by IFN γ and TNF α and exert a cytotoxic effect on cancer cells.^{122,124} In addition, M1 TAMs release reactive oxygen species, nitrogen intermediates, and inflammatory cytokines (e.g., IL-1 β , IL-6, IL-12, IL-23, and TNF- β) that can facilitate killing of cancer cells. In contrast, the M2 TAMs can be induced by TGF- β , IL-4, and IL-13^{122,123} and secrete factors such as epidermal growth factor, fibroblast growth factor, and vascular endothelial growth factor that promote growth and vascularization of the tumor.^{125–127} In addition, tumors induce MDSCs by producing the cytokines IL-6 and GM-CSF that have been strongly implicated to induce MDSC development via a STAT3 activation pathway.¹²⁸

These immunosuppressive cell types that are induced/recruited to the tumor microenvironment, particularly TADC, can induce tolerance in infiltrating T cells thereby inactivating them. Furthermore, TADC contribute to an immunosuppressed microenvironment by upregulating the immunoinhibitory receptors PD-L1 and CTLA-4 and expressing suppressive cytokines such as TGF- β and IL-10¹²⁹ that further prevent immune activation. In addition to T-cell anergy resulting from negative signaling, T cells may also undergo antigen exhaustion due to prolonged inflammation such as that seen in chronic infections. There is also evidence that T cells undergo senescence after chronic activation and proliferation. Senescent T cells are characterized by decreased cytotoxic activity and can also negatively regulate immune function.¹³⁰

C. Immune Suppression in the Central Nervous System

In contrast to the peripheral immune system, the immunologically specialized nature of the brain and its draining cervical lymph nodes must be considered when discussing

immunosuppression and brain tumors. Experiments have demonstrated that vaccinating antigen directly into the brain can trigger higher antibody titers compared with vaccination administered peripherally.¹³¹ In contrast, Th¹-mediated responses are absent or blunted when the antigen is delivered to the brain (reviewed in Harling-Berg *et al.*).¹³² These findings support a model whereby the cervical lymph nodes have an intrinsic Th₂ bias in steady-state conditions. However, it was unclear whether this bias is due to tumor-induced immune suppression, lack of co-stimulation, or an intrinsic bias against CTL development in the cervical lymph nodes. Experiments have shown that CD8 T cells undergo initial expansion following intracerebral tumor cell challenge but fail to differentiate into cytotoxic T lymphocytes (CTLs).¹³³

Immunological synapses between CD8 T cells and glioma cells have been documented in humans.¹³⁴ Despite support for Th₂ immune deviation in the brain DLN, there is evidence that CD8 T-cell responses play a tumoricidal role in human gliomas. Infiltration of CD8 T cells is a positive prognostic factor in glioma patients.¹³⁵ Interestingly, GBM patients receiving autologous tumor lysate-pulsed dendritic cell vaccines had superior survival when their tumor showed the mesenchymal subset of gene expression, which correlated with significantly more infiltrating CD8 T cells at the tumor site as compared to proneural tumors.¹³⁶

Another source of immunosuppression that must be addressed is that in the very vaccines currently used in cancer therapy. Regardless of spontaneous or vaccine-induced T-cell responses, global immune suppression has been widely accepted to occur in glioma patients. Recent data suggest that glucocorticoids and alkylating chemotherapy play a significant role in inducing this global immune suppression. Both drug families are associated with the rapid onset of lymphopenia and an elevation in Treg or MDSC frequency.^{137,138} The severity of lymphopenia following the standard of care (steroids, chemotherapy, and radiation) negatively correlates with overall survival in glioblastoma patients.¹³⁹

D. Overcoming Immune Suppression

1. Checkpoint inhibitors—Clinical trials employing various types of immunotherapy have shown that patients may require a particular immunotherapeutic regime dictated by the immune permissive or immune suppressive tumor microenvironment.¹⁴⁰ While the immune system's main function is to protect the body from harm by pathogens or cancer cells, it sometimes needs a strong trigger to initiate an immune response.⁴ Vaccination using tumor lysates may provide this needed trigger for an innate immune response to develop. On the other extreme, broad, unregulated immune responses can cause significant damage to healthy tissues. Immune checkpoints are inhibitory pathways activated to maintain self-tolerance by modulating the duration of an immune response.¹⁴¹

Due to the myriad of ways tumors can induce immunosuppression in the tumor microenvironment and draining lymph nodes, it is unlikely that any single therapy will be completely effective against CNS tumors. Therapy using a combination of vaccines, adoptive cell therapy, checkpoint inhibitors, and other immune stimulating therapies will likely provide much better results. Accordingly, several immunotherapeutic approaches,

such as those using immune checkpoint inhibitors, have been developed and are under evaluation as potential anti-cancer agents.¹⁴²

T-cell activation is a tightly regulated process that requires two signals,¹⁴² one being antigen presentation. Tumor associated antigens are presented in context with the MHC I or II molecules on specialized antigen-presenting cells (APCs) that then bind to specific T-cell receptors (TCRs). Progression of TCR stimulation into T-cell activation requires a co-stimulatory signal in which B7 molecules on the APC surface bind with CD28 receptors on the T-cell surface. This induces T-cell proliferation, cytokine secretion, and changes in gene expression and cell metabolism.¹⁴³ However, following activation, T cells express CTLA-4, that binds to its ligands CD80 and CD86 with higher affinity than CD28. Several mechanisms of action have been proposed to explain the strong effect of CTLA-4 blockade.^{141,144} CTLA-4 regulates T cells predominantly during their initial activation by dendritic cells and other APCs.¹⁴⁵ Regulatory T-cells and memory CD4 cells also express CTLA-4, and these cells may also be targeted by CTLA-4 blockade strategies.¹⁴⁶

The blockade of CTLA-4 binding or signaling could be an effective means to enhance T-cell responses and develop anti-tumor activity.¹⁴⁷ Ipilimumab is a humanized anti-CTLA-4 antibody that augments T-cell activation and proliferation. In 2011, the FDA approved ipilimumab for all patients with unresectable or metastatic melanoma. Treatment with ipilimumab increased T-cell activation¹⁴⁸ and T-cell proliferation¹⁴⁹ in stage III/IV melanoma patients. Higher levels of T-cell activation were found in 11–13% of patients that correlated with tumor regression or disease stabilization.^{150,151} Ipilimumab was reported to increase overall survival in stage III/IV metastatic melanoma patients.^{150,152} Moreover, approximately 50% of melanoma patients express BRAF-activating mutations,¹⁵³ so the use of BRAF inhibitors, a MEK inhibitor, or a combination of the two also resulted in prolonged progression-free survival.¹⁵⁴

Ipilimumab has been evaluated across a spectrum of patients with locally advanced unresectable or metastatic melanoma.¹⁵⁵ In three phase II studies, ipilimumab monotherapy demonstrated durable responses in approximately 80% of those patients who achieved clinical benefit including complete remission, partial remission, or stable disease.^{156,157} In another phase II trial, the combination of ipilimumab with dacarbazine (DTIC) increased response rates and OS, suggesting that DTIC may provide an additional benefit when used with ipilimumab.¹⁵⁸ Additionally, in two randomized multicenter phase III trials, ipilimumab extended OS in patients with advanced melanoma.¹⁵⁰ In one of the studies, HLA-A* 0201-positive patients with unresectable stage III or IV melanoma were randomized to receive ipilimumab in combination with experimental glycoprotein 100 (gp100) peptide vaccines ($n = 403$), ipilimumab alone ($n = 137$), or gp100 alone ($n = 136$).¹⁵⁰ Compared to gp100 alone, overall survival (OS) was significantly extended with ipilimumab alone (HR for death 0.66; $P = 0.003$) and ipilimumab plus gp100 (hazard ratio (HR) for death 0.68; $P < 0.001$). Median OS in the ipilimumab groups was approximately 10 months, which was notable in this study population with a poor prognosis. In this trial, patients with stable disease for 3 months after week 12 or a confirmed partial or complete response were offered reinduction therapy with their assigned treatment regimen when they had disease progression.^{155,159}

Ipilimumab enhances overall survival in individual melanoma patients. A recent report in 36 heavily pretreated patients with metastatic melanoma found that three of 30 (10%) patients achieving complete remission with ipilimumab had ongoing remission at 34+, 36+, and 41+ months.¹⁶⁰ Moreover, the median duration of response was 16 months in the 11 patients who achieved disease control. In another analysis of 177 patients, all but one of the 15 complete responders had ongoing responses at 54+ to 99+ months.¹⁶¹ Because of the immunologic mechanism of action of ipilimumab, clinicians have observed predictable patterns of response that differ from conventional chemotherapy or radiotherapy.¹⁶² These patterns include shrinkage in baseline lesions without the development of new lesions, durable stable disease (followed by a slow, steady decline in total tumor burden in some patients), response after an increase in total tumor burden, and response in the presence of new lesions that might be perceived mistakenly as disease progression.¹⁶² All response patterns have been associated with favorable survival and indicate that confirmation of true disease progression is essential prior to discontinuation of ipilimumab therapy.¹⁶²

Specific immune-related response criteria have been developed that expand conventional World Health Organization (WHO) and Response Evaluation Criteria In Solid Tumors (RECIST) to account for differences in response kinetics between cytotoxic and immunotherapeutic agents.¹⁶² Long-term follow-up on 177 patients with advanced melanoma treated in three of the earliest ipilimumab trials was conducted at the Surgery Branch of the National Cancer Institute.^{161,163} In these studies, the median duration of response was up to 88 months.¹⁶⁴ On average, complete responses were not achieved until 30 months of treatment; one patient was treated for 6 years until a complete response was achieved. Fourteen of 15 complete responses were ongoing at data cutoff.

In addition to CTLA-4 blockade, monoclonal antibodies targeting Programmed Death-1 (PD-1) and Programmed Death-1 ligand (PD-L1) show similar response rates.¹⁶⁴ PD-1 is a receptor expressed by B and T cells and was initially identified on apoptotic cells.¹⁶⁵ The ligands for PD-1, PD-L1 (B7-H1) and PD-L2, however, have a much broader distribution pattern and are thought to maintain peripheral tolerance by creating a hurdle to immune activation that must be overcome to initiate an effective response.¹⁶⁶ However, when confronted with persistent antigen expression as in chronic viral infections and tumors, the role PD-1 plays in the immune system changes from gatekeeper to veto signal. As briefly described in much greater detail in recent reviews, PD-1 plays a critical role in maintaining tolerance and shutting down ineffective immune responses. On the negative side, PD-1 interferes with vaccination and contributes to T-cell dysfunction in chronic viral infections and cancer. It is unclear exactly how PD-1 mediates these effects. A better understanding of PD-1 mediated signal transduction pathways will broaden the number of therapeutic targets and perhaps reveal novel means of modulating the immune system. However, this has not prevented the effective use of anti-PD-1 antibodies clinically. In a recent phase I trial, nivolumab, an anti-PD-1 antibody, achieved a response rate of 31% in patients with advanced melanoma. Additionally, 18% of patients with NSCLC and 27% with renal cell cancer also responded to this treatment.¹⁶⁷ Furthermore, in another phase Ib study conducted using Merck's IgG4 antibody against PD-1 (MK-3475), 51% of patients with advanced melanoma responded with 9% showing a complete response.¹⁶⁸ And in a smaller study

performed using CT-011, a humanized anti-PD-1 IgG1 antibody, 33% of patients with hematological malignancies responded or sustained stable disease.¹⁶⁸

2. Toxicities Associated with Checkpoint Inhibitors—Clinical testing of the human CTLA-4 and PD-1 antibodies has exposed several toxicities. These are characterized as immunotherapy-related adverse effects (IRAE), and are thought to occur as a result of activation of the immune system. Commonly affected tissues include the skin, liver, bowel, pituitary and other endocrine glands, including adrenal insufficiency (reviewed in Jenkins *et al.*),⁸⁹ with rashes, pruritus, and diarrhea being the most prominent. Rare IRAE included uveitis, conjunctivitis, neuropathy, myopathy, pancreatitis, cytopenia, and nephritis.^{56–58,95,169} Interestingly, these toxicities were less severe in patients receiving PD-1 antibody therapy when compared with those receiving CTLA-4 monoclonal antibodies, ipilimumab, or tremelimumab. This is thought to be due to the alternative-signaling pathway of PD-1 via PD-L2 binding. The majority of these IRAE are readily treatable and respond to temporary interruption or discontinuation of antibody therapy. Not surprisingly, studies performed in animal models of autoimmunity show that treatment with anti-CTLA-4 antibody induces an even more severe disease state.¹⁷⁰ As a result, patients that suffered from any autoimmune disease were excluded from clinical trials testing CTLA-4 blockade treatments.

3. Combination Therapy—The clinical experience with CTLA-4 blockade resulted in promising data showing durable responses with PD-1/PD-L1 inhibition suggesting that longterm disease control may be within reach for a subset of cancer patients. The most critical challenge is to substantially increase the proportion of melanoma patients who can enjoy the durable clinical benefits achievable with immune checkpoint blockade therapy. Almost certainly, combinatorial strategies of some sort will be needed to achieve this goal. In BRAF^{V600E} mutant tumors, BRAF/MEK inhibition has revealed promising antitumor activity^{153,154} including in a recent CNS tumor (astrocytoma).¹⁷¹ Importantly, there is emerging evidence for favorable effects of BRAF/MEK pathway inhibition on endogenous anti-tumor immune responses as well as for potential synergies between MAPK-targeted therapy and immunotherapy.^{172,173} Clinical trials combining MAPK pathway inhibition with CTLA-4 and PD-L1 blockade in patients with melanoma are under way.¹⁷⁴

Early clinical data suggest that combined CTLA-4 and VEGF inhibition can also induce durable responses in a substantial proportion of patients with advanced melanoma. Furthermore, a durable complete response rate of 17% was seen with the combination of ipilimumab and high-dose IL-2 in patients with advanced melanoma.¹⁶¹ A notable rate of durable objective response, including four complete responses, was also seen with the combination of tremelimumab and IFN- α in a group of 37 patients with previously treated advanced melanoma.¹⁷⁵ Additional potential complementary immune therapies include adoptive T-cell transfer,¹⁷⁶ vaccines, suppression of Tregs^{177,178} or the blockade of other inhibitory checkpoints such as Tim-3,¹⁷⁹ LAG-3,¹⁸⁰ and B7-H3, among others.

VI. THE CANINE SPONTANEOUS GLIOMA MODEL

Animal models are used extensively to predict response and toxicity of anticancer therapies being developed for human use. Due to the complexity of human tumor environment and host immune interactions, the majority of successful brain cancer therapies administered in mice fail to show the same efficacy in their human counterpart. Furthermore, some drug toxicities observed in humans were never identified in the treated mice. There are several other problems associated with murine models: (1) rodent brains are too small to be appropriate for predicting effects of surgical resection done in patients, (2) induction of tumors using cloned cell lines does not recapitulate the events that lead to glioma formation in humans, (3) the dose of vaccines and chemotherapy given to patients is difficult to accurately model in rodents, and (4) young, immunologically naïve mice implanted with tumor cells may not develop tolerance to tumor antigens seen in humans with spontaneous tumors. Spontaneous tumors in people and dogs evolve over a long period of time, perhaps even years, and maintain continuous interactions with the host. The latter is important for tolerance to tumor antigens and the role that regulatory T cells (Tregs) play in the efficacy of active immunotherapy.

A plausible case can be made that pet dogs with spontaneously occurring tumors recapitulate important features of human disease and would thus provide a superior system for testing novel brain tumor interventions (Table 2). Dogs have a closer evolutionary relationship and share the same environment as humans and have a larger brain size compared to mice.^{181,182} Because treatment regimen has been deemed the “gold standard,” trials of various veterinary treatment in canine cancer are advantageous for early testing of novel therapies.¹⁸³ This idea has recently been made more feasible with completion of the canine genome and development of reagents for immunology and flow cytometry, all recently reviewed.¹⁸⁴ Anti-mouse Fox3P antibody cross reacts with canine Fox3P, and this reagent was recently used to demonstrate that dogs with cancer have nearly double the number of Tregs in peripheral blood compared with normal dogs, similar to humans with cancer.¹⁸⁵ Canine studies can be initiated with IACUC approval and owner consent, and the canine lifespan is approximately 1/10 that of humans, accelerating the pace of translational research. Finally, the expense of canine studies is only a fraction of that of human studies.

Intracranial neoplasia occurs frequently in dogs with a reported prevalence from 0.15 to 4.5% compared with 18.2 cases per 100,000 human.^{186,187} Astrocytoma or glioma account for 20–36% of primary brain tumors in dogs and 25% in humans.¹⁸⁸ Primary canine brain tumors have similar histologic classification as those reported by the World Health Organization for human brain tumors, although the exact incidence of each type and grade is unknown in dogs.¹⁸⁹ Brachycephalic breeds such as Boxers, French and English bulldogs, and Boston terriers have a significantly increased risk of developing glioma.¹⁹⁰ No sex predilection has been reported, and brain tumors are recognized with greater incidence in older dogs and humans.¹⁹¹ Gliomas arise in all areas of the brain but are most common in the telencephalon, especially the frontal lobe and temporal tip in our experience with more than 150 cases. In a series of 987 GBMs from University Hospital Zurich, the most frequently affected sites were the temporal (31%), parietal (24%), frontal (23%), and occipital (16%) lobes, with combined frontotemporal lesions being particularly common.

Clinical signs associated with brain tumors in dogs vary reflecting the location of the tumor. The most common clinical signs associated with intracranial tumors in dogs are similar to those seen in humans and include seizures, mentation changes, vestibular disturbances, hemiparesis, and vision loss. Cerebrospinal fluid findings in animals with astrocytoma are usually nonspecific and include increased protein concentration and mixed-cell pleocytosis that reflect disturbance of the blood-brain barrier.¹⁹¹

There is a high correlation of neuroimaging features with MRI between canine and human glioma. Both human and canine gliomas exhibit iso- or hypointense signal on T1-weighted images with a pronounced mass effect causing a falx shift. T2-weighted images demonstrate a heterogeneous mixture of iso- to hyperintense areas within human and canine glioma. Peritumoral edema is a consistent finding in both human and canine high-grade gliomas.^{192,193} Contrast enhancement is seen in the majority of high-grade gliomas in both species, and ring-enhancement is typical of glioblastoma multiforme (GBM), believed to arise from increased vascular permeability due to tumor angiogenesis and exacerbated by a loss of blood-brain barrier and a central area of necrosis.¹⁹³

The gross and microscopic lesions in canine glioma are consistent with those described in human GBM.^{194–196} Gliomas commonly arise in the subcortical white matter of the cerebral hemispheres. Tumor infiltration often extends into the adjacent cortex or the basal ganglia. When a tumor in the frontal cortex spreads across the corpus callosum into the contralateral hemisphere, it creates the appearance of a bilateral symmetrical lesion, hence the term “butterfly glioma.” Proliferative indices are similar in canine and human GBMs ranging between 5% and 30% using MIB-1 antibody, however it unknown whether this index has reliable prognostic value in either species.^{192,194} Canine and human GBM typically exhibit hypercellularity, nuclear atypia, and pleomorphism, including multinucleated giant cell and cells with aberrant mitotic figures. There are also consistent gross lesions that are focal variegated areas of necrosis and hemorrhage, and cyst formation is common in human and canine GBM. A characteristic feature of human GBM, i.e., presence of multiple areas of necrosis, was found in all canine GBMs. As described in the human disease,^{197–199} canine GBM exhibits prominent serpentine, pseudopalisading necrosis, with thrombosed tumor vessels, extensive hemorrhage, endothelial/microvascular proliferation, and high cellularity.^{192,196} The necrotic foci are typically surrounded by radially orientated, fusiform glioma cells in a pseudopalisading pattern. The presence of microvascular/endothelial proliferation (i.e., multilayered, mitotically active hyperplastic endothelial cells, smooth muscle cells, and pericytes) is a histopathological hallmark of human GBM.^{200,201} Endothelial proliferation, usually close to necrotic areas, with profuse vascularization was observed in dog GBMs.^{192,196}

Tumoral cells infiltrating the normal brain parenchyma are widespread in canine GBM. As in human GBM there is an abundance of cells migrating into the white matter and surrounding neurons and blood vessels.^{192,196} These infiltrates into the normal parenchyma are typically small anaplastic cells; and poorly differentiated cells found in all GBMs express astrocytic intermediate filaments. Although there is no specific marker for GBM cells, many have proteins that are expressed in reactive astrocytes. The expression of GFAP is variable in human and canine GBM and decreases in more anaplastic cells.^{192,196,202}

Finally, macrophage and T-cell infiltration has been reported in human GBMs,²⁰³ and abundant infiltration of these inflammatory or immune cells is also observed in canine GBM.¹⁹⁶

The canine genome exhibits closer evolutionary relationship to the human compared to the mouse counterpart. A recent microarray-based comparative genomic hybridization analysis of 25 canine gliomas showed chromosome copy number aberrations (CNAs) that share evolutionarily conserved synteny with those previously reported in their human counterpart.²⁰⁴ For example, CNAs were found in the canine orthologue to the human 7p chromosome, which contains the EGFR gene; CNAs in this region were present in a large proportion of high-grade canine gliomas, as is the case in people. There were also CNAs in canine chromosomal regions containing the MYC oncogene in 5 of 9 astrocytic tumors. Cytogenetic alterations are more strongly associated with glioma histologic grade than type in both dogs and people.

Epidermal growth factor receptor (EGFR) has received substantial attention because mutations are common in human malignant astrocytomas and seem to be involved in the progression between tumor grades. Activating mutations and gene amplification enhance EGFR signaling in glioblastoma. Increased EGFR activity facilitates the Shc-Grb2-Ras and PI3K pathways, enhancing mitogenesis and angiogenesis while reducing apoptosis.²⁰⁵ Additionally, overexpression of EGFR seems to facilitate glioblastoma leading-edge invasion via upregulation of metalloproteinases and collagens.^{205–208} In a subset of dogs with malignant astrocytomas, EGFR alteration has been associated with tumor invasiveness, but the predictive or prognostic value of EGFR status has not been examined.¹⁸⁸ Increased EGFR expression was recently demonstrated by tissue microarray immunophenotyping in 57% (4 of 7) of GBMs, 40% (2 of 5) of grade III astrocytomas, and 28% (2 of 7) of grade II astrocytomas.²⁰⁹ In people with GBM, the presence of EGFR mutation may be predictive of response to tyrosine kinase blockade, as seen in individuals with other genotypic alterations.^{210–212}

Data from the human literature have demonstrated that vascular endothelial growth factor (VEGF) and its receptor (VEGFR) support tumor neovascularization, vascular permeability, and mitogenesis. These events may enhance peritumoral edema and alter intracranial blood flow. Additionally, the expression of VEGF can be upregulated through a multitude of mechanisms, including EGFR or p53 mutation and activation of the PI3K pathway.^{213,214} VEGF is released by tumor cells and its expression may be facilitated by hypoxia secondary to necrosis.²¹⁵ The effects of VEGF are mediated through VEGFR-1 (also referred to as FLT-1) and VEGFR-2 (also referred to as KDR), which are expressed on blood vessels within astrocytic tumors and at tumor margins.^{216–218} Normal human brain endothelial cells are largely devoid of both VEGFR subtypes.^{216–218} In people, VEGF expression is greatest in high-grade tumors, especially GBM, where immunostaining is intense in glial cells palisading around necrosis.^{219,220} As with humans, the concentration of intratumoral VEGF in dogs with astrocytic neoplasia increases with tumor grade.^{221,222} Three canine VEGF mRNA isoforms (VEGF120, VEGF164, and VEGF188) are recognized in astrocytic tumors, with VEGF164 having the highest relative expression.²²¹ The expression of VEGF mRNA is significantly greater in tumor tissue of dogs with GBM (grade IV) compared with dogs

with grade II astrocytoma.²²¹ High-grade human astrocytic tumors, especially GBM, have greater concentrations of VEGFR-1 and VEGFR-2mRNA, compared with low-grade astrocytomas.²¹⁷ Both VEGFR-1 and VEGFR-2 mRNA have also been identified in astrocytic tumors from dogs, although among 23 kinds of canine astrocytic neoplasia, expression levels did not significantly differ on the basis of tumor grade.²²¹ In people, astrocytic tumor VEGF expression has not been shown to be an independent prognostic biomarker associated with survival.^{213,219,223,224} The value of VEGF and VEGFR as predictive or prognostic biomarkers in dogs with astrocytic neoplasia has not been examined.

In addition to EGFR, VEGF, and VEGFR, both p53 and platelet-derived growth factor receptor (PDGFR) have been investigated in canine astrocytoma. The p53 tumor suppressor normally functions to halt the cell cycle at G1, induce apoptosis, or arrest proliferation by binding promoter regions of various effector genes.²¹⁸ In people, loss of p53 function appears to be an early event in malignant transformation of astrocytes and is therefore present in low- and high-grade neoplasia.^{218,225} Canine astrocytomas with p53 mutations have been identified, including a tumor-specific somatic mutation (ACTGCT) in codon 253. It is located in a highly conserved region and one of the seven recognized hotspots for mutations of the *p53* gene, although attempts to determine diagnostic, prognostic, or predictive value have not been made.²²⁶ Mutation of p53 or alteration of the p53 pathway in human astrocytoma appears to be of limited diagnostic, prognostic, or predictive value because it is relatively ubiquitous even in diffuse astrocytoma, suggesting an early genetic event in tumor development.^{212,227} PDGFRa is normally expressed on neural stem cells within the subventricular zone and glial precursors; stimulation of these populations with PDGF leads to glioma-like growths in rodents.²²⁸ PDGFRb is found on endothelial cells associated with glioblastoma, and agonists of this receptor may facilitate angiogenesis. Overexpression of PDGFRa in human diffuse and anaplastic astrocytoma has been reported.²²⁹ In contrast, in a population of 11 dogs with varying grades of astrocytoma, minimal increases in PDGFRa mRNA were detected in tumor tissue compared with controls.²²¹ Recently, overexpression of PDGFRa was demonstrated by tissue microarray immunophenotyping in 43% (3 of 7) of GBMs, 20% (1 of 5) of grade III canine astrocytomas, and 14% (1 of 7) of grade II canine astrocytomas.²⁰⁹ In people with astrocytoma, the prognostic and predictive value of PDGFRa expression within tumor tissue is debatable due to inconsistencies in patient populations, analytic methods, and study design. For example, in diffuse astrocytoma, one report showed that high PDGFRa was associated with prolonged survival, whereas another showed that overexpression was correlated with shorter survival.²¹⁴

In addition, overexpression of insulin-like growth factor-binding protein 2 (IGFBP2) was detected in 71% (5 of 7), 60% (3 of 5), and 28% (2 of 7) of GBMs, grade III canine astrocytomas, and grade II canine astrocytomas, respectively.²⁰⁹ The incidence of overexpression of EGFR, PDGFRa, and IGFBP2 in these canine gliomas closely parallels that in human tumors of similar type and grade.²⁰⁹ Overexpression of IGFBP2 in human high-grade astrocytomas is predictive of poorer prognosis. In human GBMs, overexpression

of IGFBP2 and elevated MMP-2 expression are correlated and related to invasiveness and malignancy.

Although canine brain tumors occur fairly frequently, reports of their treatment and outcome are relatively rare. Furthermore, most studies do not separate out the response of specific tumor types, such as GBM. Similar to that in humans, the prognosis for dogs with brain tumors is poor regardless of therapeutic intervention. The median survival time for dogs with astrocytomas that did not receive any type of treatment ranges between 6 and 13 days^{230,231} and between 60 and 80 days in dogs that receive palliative therapy only. Radiation therapy may have increased survival time in one dog with glioma (176 days) compared with corticosteroid therapy in three dogs with glioma (18, 40, and 64 days). Another study examined the effect of surgical resection and lomustine therapy; both dogs diagnosed with GBM died or were euthanized within 2 months of diagnosis.²³² Twenty-nine dogs with intracranial masses, four with glioma, were treated with primary irradiation alone without surgical excision of the mass. The median survival time was 250 days regardless of neuropathologic diagnosis, and most dogs died or were euthanized because of progressive neurological dysfunction.²³³ Another clinical study compared radiation therapy (RT) with and without whole-body hyperthermia in dogs with non-resectable brain tumors that were not biopsied. Adding whole-body hyperthermia to radiation therapy did not improve survival and may have shortened survival due to hyperthermia side effects. Median survival for dogs treated with RT alone was 9.6 months versus 5 months for RT and hyperthermia.²³⁴ There was no specific data for 5 dogs tentatively diagnosed with glioma, but dogs with meningioma had the longest survival times. A retrospective analysis of dogs with brain masses treated with RT did not separate survival data based on tumor type. All intra-axial tumors were presumed to be gliomas, and the median survival time was significantly less in those dogs (40.4 weeks) as compared to those with extra-cranial tumors (49.7 weeks). The median survival for the five dogs that had RT following surgical excision was slightly longer (43.7 weeks) when compared to 29 dogs that had RT only (40.4 weeks).²³⁵ Another retrospective survival analysis of 86 dogs that received a variety of treatments, from nothing to some combination of surgery, radiation, and chemotherapy, found that dogs treated with cobalt-60 radiation regardless of additional therapy lived significantly longer (4.9 months) than dogs that had surgery (0.9 months) or symptomatic treatment (0.2 months).²³⁶

More novel forms of therapy are being tested in dogs. The therapeutic value of brachytherapy was investigated by implanting the brachytherapy applicator, an inflatable balloon catheter, in the resection cavity in dog brain and inflating the balloon catheter with Iotrex containing iodine-125 as a reliable radiation delivery method to target canine brain tumors.²³⁷ Significant beta-galactosidase gene expression by the tumor, but not by surrounding brain tissue, demonstrated successful transduction after a recombinant adenovirus vector bearing the *Escherichia coli* β -galactosidase reporter gene was injected into the blood vessels of a spontaneous olfactory groove meningioma in a dog.²³⁸ Dogs with spontaneous glioma have also been used to study convection-enhanced delivery (CED) that creates a pressure gradient at the tip of the catheter to allow an increase volume of drugs past the blood-brain barrier with minimal toxicity.²³⁹ Stoica *et al.* also identified cancer stem cell (CSC) in dog GBMs and examined the self-renewal ability of these glioblastoma cells with

single colony formation and found both the clone formation rate and the subclone formation rate to be 100%.²⁴⁰ These dog GBM cells expressed CD133, the CSC surface marker reported in human gliomas. To elucidate the ability of CSCs to initiate tumorigenesis upon xenograft transplantation, tumor cells were injected intracranially into nude mice that all subsequently developed tumors characterized with high cellular heterogeneity, neovascularization, and necrosis.

In an effort to validate the use of adenoviral vector (ads) that encodes human soluble fms-like tyrosine kinase 3 ligand (hsFlt3L) to induce conditional cytotoxicity for an immunotherapeutic approach in dogs with spontaneous tumors, canine peripheral blood mononuclear cells were transfected. The dendritic cells generated by this approach had very similar morphology and phenotype to those induced by a cytokine cocktail consisting of IL-4 and GM-CSF.²⁴¹ Furthermore, *in vivo* studies have been conducted to determine the safety and efficacy of adenoviral and nonviral gene transfer into the canine brain.²⁴² These results lead to a clinical trial in pet dogs with spontaneous high-grade glioma to determine the safety and efficacy of adenoviral-mediated TetOn Flt3L and herpes simplex virus type I thymidine kinase (HSV-tk) gene therapy. Fifteen dogs had intraparenchymal adenoviral injections of the combination gene therapy (n=10) or TetOn β -galactosidase gene as a negative control (n=5) around the resection cavity after surgical debulking of high-grade glioma. All dogs were also given valacyclovir, the substrate for HSV-tk; doxycycline to activate expression of the TetOn genes; and temozolomide, initially in a metronomic regimen followed later by five 28-day cycles of high-dose therapy for 5 days. No adverse effects of the intraparenchymal injections or the oral chemotherapy were noted in any dog. All dogs eventually succumbed to recurrent disease, but there were significant increases in both the median progression-free and overall survival times of the dogs given combination gene therapy and temozolomide (195 and 341 days, respectively) compared to temozolomide alone (96 and 117 days, respectively) (unpublished data).

Our group has also published the first documented immune-based treatment of a dog with gemistocytic astrocytoma (GemA) to determine the toxicity and immune responses. Although the incidence and prognosis for canine GemA has not been adequately defined, canine tumors in general progress approximately seven times faster than their human counterparts.¹⁸² The 12-year-old Shepherd cross was diagnosed with a gadolinium-enhancing intra-axial mass in the right frontal lobe by MRI. He was treated using the combination of surgery and immunotherapy. After surgical resection of the tumor, intraparenchymal injections of adenovirus encoding for interferon gamma (IFN- γ) were performed around the resection cavity. A series of five vaccinations of autologous tumor cell lysate mixed with TLR9 ligand, CpG oligodeoxynucleotides (ODN), was given intradermally every 2 weeks starting 2 weeks after surgery. The autologous tumor cells did not proliferate well after culturing for 2 months, so an allogeneic cell line was used for the final vaccines. Interestingly, the dog developed left-sided hemiparesis and blindness in the left eye after these vaccines, and the owner declined the sixth and final vaccine. These neurological symptoms resolved spontaneously within 3 days and correlated with peaks in peripheral levels of tumor-reactive IgG and CD8⁺ T cells. The dog eventually developed severe heart failure and was humanely euthanized 532 days after surgery.²⁴³ No evidence of

tumor recurrence was found in the brain on postmortem examination. The results of the case demonstrate the feasibility of treating dogs with spontaneous glioma using immune-based therapy, and we have since recruited 23 more dogs into a clinical trial using this therapeutic approach, some of which have not completed the 1-year study. In this study, all dogs undergo surgical debulking of the tumor and six vaccinations with autologous tumor lysate vaccine plus CpG ODN, and half of the dogs have been randomly assigned to receive intraparenchymal Ad-IFN- γ gene therapy as well. The tumor cells are cultured at 37°C, 5% CO₂ in a humidified incubator in a specialized neural stem cell medium supplemented with N2, B27, EGF, and β -FGF under low oxygen conditions (5% O₂) that enhance the immunogenicity of glioma cells in therapeutic vaccines.¹⁹ The tumor cells are lysed by multiple freeze-thaw cycles²⁴⁴ and are then irradiated at 200 Gy to ensure there are no live cells in the vaccine lysate that is injected into the dog. Three dogs are still alive in each of the two treatment groups, and there are no significant differences in the overall survival times of the dogs in the two groups (211 versus 212 days with live dogs censored), suggesting no survival benefit of the IFN- γ gene therapy. It is possible that the lack of difference is a type II statistical error due to the low numbers of dogs in the treatment groups. The two long-term survivors, alive 604 and 1308 days since surgery, had low-grade tumors, grade II astrocytoma, and GemA, respectively (unpublished data).

Recruitment is ongoing for another clinical trial in pet dogs with spontaneous high-grade glioma using combinations of immunotherapy and chemotherapy protocols. This trial is structured similarly to the vaccine Ad-IFN- γ study; the tumor is surgically excised and the dogs receive adjuvant therapy of combinations of autologous tumor lysate and CpG ODN vaccines, OX40L, and temozolomide chemotherapy. OX40L, a member of the tumor necrosis factor superfamily, provides a co-stimulatory signal to CD4 and CD8 T cells and inhibits T regulatory cells and thereby has significant anti-tumor activity.²⁴⁵ Thus far, there have been no severe adverse effects of the therapy. No analysis of the survival data has been performed because the dogs are alive. We are still recruiting and blinded to the treatment groups; however the median overall survival time for all dogs treated is 207 days (unpublished data). Administration of the temozolomide between the vaccines may be a potential problem if the chemotherapeutic agent is killing the CD4 and CD8 cells stimulated by the tumor lysate vaccines.

One of the most exciting findings from the canine clinical trials is the efficacy of autologous tumor lysate vaccine immunotherapy against spontaneous meningioma. We believe that these tumors may be even better candidates for immunotherapy than gliomas for the following reasons: (1) meningiomas are not insulated by the blood-brain barrier from antibody penetration and lymphocyte infiltration, (2) meningiomas are typically surrounded by cerebrospinal fluid that ultimately drains to the cervical lymph nodes for more efficient antigen presentation to T and B lymphocytes,²⁴⁶ and (3) meningiomas are relatively slow-growing tumors that may be more susceptible to adaptive immune responses that can take weeks to peak. Although canine meningiomas are very similar to human meningiomas in neuroimaging characteristics, gross and histological appearance, and expression of growth factors and cell surface receptors,^{204,221} the response to surgical excision is not. Local control rates in people with low-grade meningiomas that are completely excised with surgery are 70–80% or better²⁴⁷ and the 5-year recurrence rate is 5% with WHO grade I

meningiomas. The median survival time for dogs treated with surgical excision alone is 4.5 to 7 months.²⁴⁸ Eleven pet dogs with intracranial meningioma were treated by surgical debulking followed by vaccine immunotherapy consisting of a series of six vaccines of autologous tumor cell lysate combined with TLR ligands, CpG ODN in five and imiquimod, a TLR7 ligand, in six. Therapy was well tolerated and there was disease progression or recurrence in any dog. The median overall survival time for all dogs was 690 days. The median overall survival time for dogs that had CpG ODN as an adjuvant was 401 days, whereas those that had imiquimod was 1250 days.²¹ Circulating IFN- γ -elaborating T cells were found in 2 dogs, but vaccine-induced tumor-reactive antibodies developed in all dogs.²¹ Antibody responses were polyclonal, recognizing both intracellular and cell surface antigens, and HSP60 was identified as one common antigen. Tumor-reactive antibodies bound allogeneic canine and human meningiomas, showing common antigens across breed and species. A robust infiltration of antibody-secreting plasma cells was found in the brain parenchyma surrounding the tumor site on post-mortem evaluation that was not evident in the original biopsy samples. Tumor-reactive antibodies capable of inducing antibody-dependent cell-mediated cytotoxicity of autologous and allogeneic tumor cells is a novel immune effector mechanism relevant to any brain tumor.

VII. CONCLUSION

Tumors as a source antigens have been utilized for more than four decades now; however, multiple hurdles remain that we must overcome. With the increasing advances in genetic analysis, researchers are working toward personalized immunotherapies. This will allow us to overcome the major hurdle of tumor heterogeneity. It is now possible to integrate high-throughput genome sequencing to identify immunogenic mutations for the development of personalized therapies.²⁴⁹ However, we still need address to the serious limitation of “antigenic drift” allowing tumors to escape a specialized immune response.²⁵⁰

To develop an effective vaccine, the multitude of complex immune-tumor interactions must be addressed as well. It is not likely that any monotherapy will be effective because of the many mechanisms by which tumors suppress the immune system. Therefore, researchers, including those in our laboratory, are taking a step back and looking at the various mechanisms of immune suppression that tumors utilize to escape immune surveillance. In this article, we reviewed different tumor-derived vaccines and complications associated with the development of tumor lysate vaccines. Utilizing the tumor as the source of antigens has proven to be both beneficial and safe for several tumor types in patients. However, the problems of immune escape and suppression remain major challenges. Therefore, a better understanding of the different tumor-immune interactions in both the tumor microenvironment and the draining lymph nodes are important to gain a better understanding of whole-cell vaccines in the future.

Using better models for brain tumors will enhance research for brain cancer and many other types of tumors as well. The spontaneous canine model we employ provides an excellent opportunity to not only help companion animals but also to test new therapies and treatment regimens. The results that others and we are obtaining with this model are exciting and promising for the future of tumor immunotherapy.

Finally, by combining several immunotherapeutic approaches to create a comprehensive, personalized treatment, we will have much better success treating patients with the aggressive tumor types discussed in this review. Time and time again, promising new immunotherapies provide hope but consistently underperform in clinical trials. These failures allow us to discover new mechanisms that tumors use to avoid immunosurveillance and escape elimination. By merging checkpoint blockade inhibitors, tumor vaccines, and adoptive T-cell therapies we should decrease the tumor's ability to evade elimination and enable us to overcome these deadly cancers.

ABBREVIATIONS

TAA	tumor-associated antigens
DC	dendritic cells
IFNγ	interferon gamma
GBM	glioblastoma multiforme
NK	natural killer cells
BCR	B-cell receptor
MDSC	myeloid-derived suppressor cells
DLN	draining lymph nodes
OVA	ovalbumin
TADCs	tolerogenic tumor-associated dendritic cells
TAMs	tumor-associated macrophages
TCRs	T-cell receptors
APCs	antigen-presenting cells

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FIG. 1.

Immunosuppressive tumor microenvironment. Cancer-associated inflammation up-regulates the production of myeloid cells from hematopoietic progenitors in the bone marrow. Common myeloid progenitor cells give rise to pre-dendritic cells (pre-DCs) and, under the influence of tumor-derived factors (e.g., VEGF, IL-6, and S100A9), myeloid-derived suppressor cells (MDSCs). These myeloid cells migrate into the tumor microenvironment in response to chemokines such as CCL3, CCL12, CXCL12, and β -defensins. VEGF and IL-6 activate STAT3 and C/EBP β signaling in MDSCs, keeping them in an immature state. Tumor cells secrete cytokines/chemokines that recruit suppressive cells such as regulatory T cells (Treg), immature dendritic cells (iDC) MDSCs, and M2 macrophages. M2 macrophages and MDSCs inhibit T-cell responses through nutrient sequestration via arginase-I (Arg-I), reactive oxygen species (ROS) generation and nitric oxide (NO) as well as interference with trafficking into the tumor site. iDC are defective antigen presenters combined with low co-stimulatory molecule expression, they cause T-cell anergy and exhaustion. Tumors drive the formation of regulatory DCs suppressing effector T-cell functions through the secretion of the enzymes l-arginase and indolamine-2,3-dioxygenase (IDO), resulting in (1) the depletion of essential amino acids and the production of the tolerogenic metabolites adenosine and kynurenine; (2) release of immunosuppressive factors such as IL-10 and TGF- β ; and (3) expression of co-stimulatory surface molecules, including PD-L1 and butyrophilins and induce regulatory T cells (Tregs).

TABLE 1

Advantages and disadvantages of synthetic peptide, autologous and allogeneic vaccines.

Peptide Vaccines	<ul style="list-style-type: none"> • Cheaper to make • Able to target specific tumor anti-gens • Vaccine does not require tumor tissue • Decreases risk of autoimmunity due to non-tumor antigens often in autologous vaccines 	<ul style="list-style-type: none"> • Only covers a specific antigen • Greater risk of an immune escape due to clonal expansion of antigen-loss variants • Longevity of MHC-peptide complexes is unknown • Affinity of synthetic peptides for various HLAs varies • MHC-II restricted epitopes for CD4⁺ activation is still scarce
Autologous Vaccines	<ul style="list-style-type: none"> • Provides a panel of personalized class I and class II peptides inducing both CD8 and CD4 T cells • Cell associated antigens result in a more efficient cross presentation compared to soluble antigens • Enhanced cross-priming than peptide antigens • Enhanced DC maturation compared to peptide vaccines 	<ul style="list-style-type: none"> • Takes time to purify and grow tumor cells • Ability to culture enough material for vaccine development in a reproducible and quality-controlled manner is difficult • Risk of losing cells due to contamination • Risk of autoimmune response due to the presence of non-tumor cells • Tumor antigen profile from metastases may be different from primary tumor • Alterations in antigen profile • Addition of suppressive properties to vaccine
Allogeneic Vaccines	<ul style="list-style-type: none"> • Able to derive pure tumor vaccine, reducing the risk of autoimmunity due to non-tumor antigens • Have an off the shelf product • Allogenic HLA molecules induce a potent immunogenic signal, leading to enhanced T-cell response • Capable of developing a cell line with high antigen expression 	<ul style="list-style-type: none"> • Antigen profile may differ from the patients tumor antigens, decreasing an tumoricidal response • Antigen profile may change following extensive passages

TABLE 2

Benefits and difficulties of the use of a canine spontaneous glioma.

<ul style="list-style-type: none"> • Closer genomic evolutionary relation-ship • Spontaneous tumors—more relevant tumor initiation and progression • Infiltrative high-grade tumors • Relatively large size of brain • Relatively high incidence rate • Large numbers of pet dogs • Similar mutations i.e. TP53, EGFR/Chromosome CNAs conserved synteny to human • Relatively easy to surgically debulk tumors • Individual variations/phenotypic diversity similar like humans • Share our environment, exposed to same toxins/mutagens • Recover well from surgery • Similar tumor-induced immunosuppression, i.e., MDSC • Similar anatomic location • Similar histologic subtypes • Similar clinical signs/symptoms • Similar radiologic characteristics • Similar molecular biomarkers/IHC • Clinical trials are less expensive than human trials • Can detect therapy-induced toxicities/adverse effects 	<ul style="list-style-type: none"> • Individual variation leads to higher variance in data • Need consent and compliance of dog owners • No current “gold standard” of care • Few publications about response to treatment • Few veterinary neurosurgeons willing to operate on gliomas • Public resistance to inducing tumors in dogs • Expensive to treat relative to mice • Lower availability compared to mice
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