THE ROLE OF OXYGEN IN THE IMMUNOGENICITY OF BRAIN TUMOR CELL VACCINES

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Dedication

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Abstract

Tumor cell vaccination is a promising strategy for patients with primary brain tumors. Cell-derived vaccines have been developed and tested in patients with many cancers, resulting in objective responses in a minority of cases. Tumor cells are often harvested from tissue culture, where phenotype can shift in response to many factors. Oxygen affects expression of thousands of genes, yet its impact on immunogenicity in culture was unknown until recently. Compared to atmospheric oxygen levels, physiologic (5%) oxygen increases the adjuvant properties and efficacy of glioma vaccines. These studies determined the impact of oxygen on cell-mediated and humoral immunity with regard to its potential for improving efficacy in brain tumor patients. Immunogenicity and oxygen were first investigated over a broader oxygen range, with 5% oxygen still yielding the greatest extension of survival. With the effort to maximize immunogenicity, primary autologous meningioma cells from pet dogs were cultured in 5% oxygen for vaccine production. Dogs vaccinated with lysate/adjuvant vaccines based on these cells induced robust antibody responses and survived a median of three-fold longer than historic controls. Further studies in mice led to the discovery that glioma cells cultured in 5% oxygen upregulated a "danger" signal, annexin II. Monomeric annexin II enhanced dendritic cell cross presentation, CD8 T cell priming, and extended survival of murine breast carcinoma and plasmacytoma. Enriching for endogenous danger signals by decreasing tissue culture oxygen is a simple means to enhance the immunogenicity of brain tumor cultures.

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CHAPTER ONE

INTRODUCTION[1]

Brain Tumors: Epidemiology and Types

It is estimated that there are 688,000 people living with a primary brain or central nervous system tumor, of which 124,000 are malignant (1). Primary brain tumors can be divided into six histological categories, in order of decreasing incidence: (1) tumors of the meninges, the membranes surrounding the brain and spinal cord (35%); (2) neuroepithelial tumors, including glial, choroid plexus, and primitive neuronal tumors (34.5%); (3) tumors of the sellar region such as pituitary adenomas and craniopharyngiomas (13.5%); (4) tumors of the cranial and spinal nerves (8.7%); (5) unclassified tumors such as hemangioma (5.5%); and (6) primary CNS lymphomas (2.5%) (2). Due to the high prevalence of clinically aggressive glioma and meningioma, alternate approaches for these tumors have potential to improve survival and quality of life in a large number of patients.

Meningiomas

Meningiomas are derived from meningothelial arachnoid cap cells, which reside in locations where the cerebrospinal fluid is resorbed into the systemic circulation (3). Meningiomas impair neurologic function by compressing the brain, leading to seizures as

[1] Reprinted in part with permission from Andersen BM and JR Ohlfest. "Increasing the efficacy of tumor cell vaccines by enhancing cross priming." **© Elsevier Inc. Originally published in *Cancer Letters*. 2012.** Dec 28;325(2):155-64. PMID: 22809568

the presenting symptom in most cases (4). Initial workup involves imaging pre- and – post-contrast MRI, which typically reveals meningiomas as homogenously contrast-enhancing since they are usually outside the blood-brain barrier (5). A connection to the meninges is often seen, which is known as a "dural tail."

Meningiomas are classified by their typical histologic appearance of fusiform cells that are positive for epithelial membrane antigen (EMA) and negative for glial markers such as glial fibrillary acidic protein (GFAP) (6). The World Health Organization assigns a grade of I-III to meningiomas to predict outcomes. Grading is based on the number of mitotic figures, invasion into brain, histologic subtype, and the presence of necrosis (7, 8). While 90% of meningiomas are benign tumors that are cured by surgical resection (9), recurrent, atypical (WHO II), and malignant (WHO III) meningiomas affect over 6,000 patients a year (2, 9).

Several molecular aberrations have been associated with meningiomas. Loss of heterozygosity of chromosome 22q occurs in 60% of meningiomas, and early steps in tumorigenesis are thought to be due to loss of the *NF2* gene (3). The precise mechanism of tumor formation is not known. *NF2* encodes the protein Merlin, which is involved in Hippo signaling and contact inhibition (3). Recent genomic analysis has divided meningiomas into *NF2* and non-NF2 tumors, with NF2 loss-of-function tumors more likely to be aggressive. Other mutations identified were in the TRAF7, AKT, and SMO genes, with SMO mutations in non-NF2 meningiomas arising from the skull base (10). Numerous other gene mutations have been identified including those in angiogenic pathways, MAP kinase signaling, and many growth factors and cytokines (3).

The standard-of-care treatment for all meningiomas is maximal surgical resection, which is followed by radiation in the case of WHO II and III tumors. Brain-invasive meningiomas become "a disease of the entire brain," since tumor cells intermix with parenchymal architecture, bearing a worse prognosis, (7, 11). No treatments have been established in randomized trials for salvage therapy for meningioma, although radiation, stereotactic radiosurgery, and hydroxyurea are commonly used (4, 9, 12, 13, 14).

Gliomas

Gliomas are neoplasms thought to arise from glia and are malignant in 67% of patients (2, 11). While lower grade gliomas often present with seizures, high-grade gliomas present more often with focal neurologic dysfunctions such as hemiparesis, visual deficits, hemi-sensory loss, or aphasia (15). The highest grade (WHO IV) and deadliest glioma is glioblastoma multiforme. Median age of newly diagnosed glioblastoma patients is 65, whereas those of WHO grade III astrocytomas and oligodendrogliomas are 41 and 48, respectively (16, 17).

Appearance of gliomas on MRI is variable, with the most classic glioblastomas showing a "ring" enhancement pattern following contrast administration. The "ring" is due to disruption of the blood-brain barrier by the tumor, with a central necrotic region (18). WHO grade III astrocytomas and oligodendrogliomas have variable appearance on MRI, with irregular, heterogeneous contrast enhancement (19). As many as 30% of cases of these two grade III tumors, however, are non-contrast-enhancing (20). Lower grade astrocytomas and oligodendrogliomas are usually non-contrast-enhancing, as the blood-brain barrier is still intact, with the tumor lying behind this barrier (15).

Glioblastomas can arise from previously lower-grade gliomas (secondary) or arise *de novo* (primary). Virtually all low grade tumors will progress to grade III or IV (malignant) status over time (15). Malignant gliomas (WHO grade III and IV) can be further broken down into histologic subtypes after the normal cells they are thought to resemble, the most common being astrocytoma and oligodendroglioma. Grade III astrocytomas can be identified by expression of GFAP, increased cellularity, nuclear atypia, increased mitotic activity, and micro-vascular proliferation (6). Glioblastomas (WHO grade IV astrocytomas) also bear these characteristics except that they have a less differentiated, pleomorphic morphology, in addition to "pseudopallisading necrosis" (6, 15). Grade III oligodendroglioma histologically appear to have a "fried egg" appearance, basophilic cells that are consistent in appearance, with a cleared cytoplasms (21). Oligodendrogliomas contain calcifications more often than astrocytomas, and often with finely branching capillaries known as "chicken wire vasculature (6)."

Considerable progress has been made with regard to understanding of genetic aberrations of gliomas. The Cancer Genome Atlas reported gain of function in the Ras/receptor tyrosine kinase pathway in 88%, loss of function of P53/MDM2 signaling in 87%, and loss of Retinoblastoma signaling in 73% of glioblastomas (22). Molecular subtyping of high-grade gliomas revealed considerable molecular heterogeneity and dramatically different prognoses, despite similar histologic appearance (23). The proneural gene expression profile bears a positive prognosis, with median survival more than double that of the mesenchymal and proliferative subtypes (23). Considerable progress has been made in understanding and targeting the initiating events of proneural

glioblastoma, which is most often in younger patients with secondary glioblastoma (24-27). In this subtype, an oncometabolite, 2-hydroxyglutarate, is produced by mutant isocitrate dehydrogenase 1 ($IDH1^{R132H}$) (24-28). As this mutation is found in many grade II and III astrocytomas and oligodendrogliomas, decreasing production of this oncometabolite could prevent transformation to a higher grade (24-27). Moreover, oligodendrogliomas with deletion of chromosomes 1p and 19q are known to have better response to chemoradiotherapy and better prognoses (29).

High-grade gliomas are treated by surgical resection followed by neoadjuvant chemoradiation therapy, consisting of temozolomide alkylating chemotherapy and focal radiation (30). Median survival for WHO grade IV glioma is approximately 14.6 months, with only a few patients living longer than five years (31). Persons whose high-grade gliomas recur receive treatment with the monoclonal antibody targeting VEGF, bevacizumab (32) or salvage chemotherapy such as the mustard agent lomustine (33). Patients survive a median of 8 months following these treatments (34). Therefore, glioma as well as meningioma patients with aggressive disease need more effective treatments to extend survival and improve or maintain their quality of life.

Cancer Vaccines: Historical Perspective and Successes

The less effective aseptic surgical techniques of 18th century medicine led to post-operative tumor resection infections, ultimately revealing that the immune system is capable of eliminating tumors. In the 1890's, William Coley, a surgeon in New York, noted, in records dating back to the 1700s, that cancer patients with post-operative streptococcal infections sometimes had spontaneous regression of their tumors. He

therefore injected *Streptococcus* and *Serratia* bacteria into his patients' tumors, inducing regression in more than 10% of cases (35, 36).

120 years later, basic knowledge of cancer, immunology, and vaccinology has brought about many cancer immunotherapy approaches. Tumors express self- and neo-antigens from their aberrant genetic programs, distinguishing themselves immunologically from normal tissue. As tumors enlarge, they evade and suppress the immune system, often overcoming spontaneous immune responses (37). Vaccines aim to activate adaptive immune responses by providing antigens in conjunction with an immune stimulus or adjuvant. Thousands of cancer patients have been treated with tumor cell-based vaccines, with evidence of efficacy. Provenge® is the first United States FDA-approved cancer vaccine, intended for men with metastatic castration-resistant prostate cancer. It consists of autologous antigen presenting cells incubated with a protein fusion of prostatic acid phosphatase and granulocyte-macrophage colony-stimulating factor (GM-CSF; (38). A recent phase III trial that included over 400 patients extended median survival by 4.1 months (25.8 versus 21.7 months in placebo; p=0.02; ref. (39). Although subunit and peptide vaccines have several advantages, a recent meta-analysis accounting for 3,444 patients in 173 clinical trials indicated that tumor cell-based vaccines had higher response rates (~8%) than vaccines consisting of synthesized antigens (~4%) (40). Cell-based approaches likely provide a diverse pool of antigens that can trigger concurrent CD4 and CD8 T cell responses with tumor-reactive antibodies, and thus broader antigenic coverage.

Vaccines consisting of materials from cultured tumor cells show some success but have yet to receive approval in the US. Melacine® is a melanoma vaccine consisting of lysates from two allogeneic melanoma lines combined with the adjuvant DETOX®. When given to 198 patients in phase II/III trials, it induced five complete responses and seven partial responses (overall response rate 6.0%; ref. (41). While the overall response rate was similar to (if not lower than) that of Coley, four patients with complete responses were still alive and disease free at 7-10 years post-treatment, implying long-term disease control through memory responses (41). Interestingly, retrospective analysis showed that the response rate was 38% in patients expressing HLA-A2 and HLA-C3 (41). With a better safety profile and comparable complete response rate to chemotherapy or high-dose IL-2, Melacine® was approved for treatment of metastatic melanoma in Canada. A tumor cell lysate vaccination known as Reniale® improved renal cell carcinoma outcomes in a recent phase III study (42). Nephrectomy was performed in patients with organ-confined disease, and lysate vaccines were produced from autologous tumor cells stimulated with IFNγ. Reniale® led to increased time-to-progression and survival compared to nephrectomy monotherapy (Hazard Ratio=1.58, 95% C.I. = 1.05 to 2.37 at 5 years; (42). Recently, 10-year follow-up survival of patients with aggressive stage III disease was 58.9% in patients given Reniale® versus 36.2% in surgery monotherapy (43).

Brain Tumor Cell Vaccines

Despite the improvement of survival in brain tumor patients over the last decade, these tumors often continue on a devastating course. Therefore, great hope for brain

tumor care has sprung from the early proof-of principle evidence in clinical trials testing brain tumor cell-based vaccines. Yamanaka and colleagues reported efficacy from treatment of 24 recurrent glioma patients with intratumoral and/or intradermal injection of autologous glioma lysate-pulsed dendritic cells. One patient had a partial response (>50% volume reduction), three had "minor" shrinkage of tumor (25-50%), and ten others exhibited stable disease lasting at least four months (44). Moreover, seven of these fourteen patients who benefitted from the vaccine showed increases in tumor-reactive T cells by ELISpot (44). Another more-recent phase II study of autologous tumor-pulsed dendritic cell vaccination achieved an impressive 54% three-year survival. Responding patients showed increased CD8 T cells into tumors following vaccination (45). While these results have drawn enthusiasm and hope, overall response rates must be improved to benefit larger patient populations. And to increase overall response rates, we must scrutinize the mechanisms by which brain tumor cell vaccinations act.

Mechanisms of Anti-Brain Tumor Immunity

There are many key effector cells and molecules involved in anti-brain tumor responses. While many cells of the innate and adaptive immune system help eliminate tumors, the adaptive arm, comprised of the B and T lymphocytes, is considered the most capable of tumor elimination and long-term tumor control (46). The advantages of polyclonal antigen-specificity and memory make them more precise, adaptable to resistance mechanisms, and longer-lasting than conventional chemotherapies (47). Understanding the mechanisms of B and CD8 T cell activation following vaccination will help to identify the key hurdles to successful brain tumor immunotherapy.

Dendritic Cells: Immune Sentinels and Presenters of Tumor Vaccine Antigens to CD8 T Cells

Antigen presentation to CD8 T cells is imperative for igniting a tumoricidal response (48), and dendritic cells (DCs) present these antigens more efficiently than any other cell type (49). 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: (i) antigen cross-presented in the form of a peptide—MHC I complex; (ii) co-stimulatory molecules on their surface; and (iii) secreted cytokines (50). Additional complexities, such as the DC subset of interest (51), DC trafficking (52), tissue of antigen challenge (53), and optimal CD4 T helper cell differentiation (54) also 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 (55).

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 neo-antigen) by recognition of the complex of peptide antigen and the major histocompatibility complex (MHC) I (56, 57). Each CD8 T cell undergoes somatic mutation to develop a unique T cell receptor specificity for a given peptide sequence and MHC I (58). 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 cell or cell fragment, or do antigens transfer to another cell? While both scenarios are possible, Michael Bevan demonstrated that transfer of antigen

to a host immune cell occurred (59). He coined the term "cross priming" to describe the process by which exogenous antigens are uptaken and loaded on MHC I rather than expressed directly (59).

Identifying and understanding the function of the cell type doing the cross priming is crucial to understanding how to best elicit a CD8 T cell response. The DC was identified by Ralph Steinman and Zanvil Cohn in 1973 (60). It took ten years from their discovery to confirm the DC as the most potently cross priming cell, as DC depletion resulted in the drastic reduction of killing in the mixed leukocyte reaction (49).

What features of DCs make them adept at cross priming? The most crucial is known as signal one, or cross presentation. Antigens are recognized and taken up by DCs through endocytosis, macropinocytosis, or phagocytosis, degraded into peptide fragments 8-10 amino acids in length that are loaded onto MHC I and shuttled to the cell surface. The mechanisms of cross presentation are partially understood, but recent studies indicate that immune stimuli such as those discussed later induce changes in the routing of antigen.

While 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 unresponsiveness of T cells after mono-stimulation with MHC and peptide. 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 (61, 62). Complementary to these findings was the discovery that at steady state,

DCs induce tolerance to CD8 T cell antigens (63). The missing signals in the case of CD4 and CD8 T cell activation were CD80 and CD86, DC-expressed ligands for the CD28 co-stimulatory receptor on T cells. CD28 signaling results in the amplification of the TCR signal, inducing a proliferative response and IL-2 synthesis within the activated T cell. Following CD28 signaling, additional co-stimulatory receptor-ligand interactions are important in driving optimal T cell activation including, ICOSL-ICOS, OX40-OX40L and CD137-CD137L among others (46).

Attempts at recapitulating cross priming *in vitro* by incubating naïve CD8 T cells with microbeads containing MHC I-peptide complexes and CD80/86 were partially successful (64). These stimuli triggered several rounds of cell division and temporary effector function, but cells were unable to reach full effector function, survive, and develop memory (64). When interleukin 12 (IL-12) or interferon alpha/beta (IFNα/β) were added to these cultures, however, full CD8 activation was successful (64). 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 (65).

Tumor cell vaccines should therefore stimulate the production of IL-12 or IFNα/β to provide adequate signal 3 for optimal cross priming. IL-12 secretion *in vivo* can be stimulated when CD4 T cells present CD40L to CD40 expressed by DCs, a key trigger for IL-12 release (66). Multiple methods of inducing signal three have shown promise, including providing the exogenous cytokine via genetic engineering (e.g., IL-12 producing tumor cells) (67, 68) or co-injection of recombinant cytokine (69). The use of type I interferon-promoting adjuvants as inducers of signal 3 show promise for tumor cell

vaccine therapy, as they augment signals 1 and 2 by enhancing DC maturation.

The Activation of the Tumor-Reactive B Cells and Antibodies

B cells are lymphocytes that each express a somatically mutated, unique membrane-anchored antibody, known as the B cell receptor (BCR) . Each B cell clone is therefore responsive to a specific cognate antigen and capable of initiating antibody responses. B cells develop in the bone marrow, but they reside in the follicles of lymph nodes in their naïve state, awaiting direct contact with tumor cell antigens from the lymphatic drainage (70).

The molecular specificity of B cells arises from the specific antigen to which their membrane-bound antibodies, are tuned. Antigen-BCR binding is the first of two required signals for full activation that leads to antibody formation and development of a humoral memory response. Unlike CD8 T cells, B cells require no antigen presenting cells to classically engage antigen with their BCR. The size of B cell epitopes is thought to be more variable, although still a small fraction of the originating molecule. Epitopes for BCRs include proteins, carbohydrates, lipids, and nucleic acids.

Like CD8 T cells, B cells require costimulatory and cytokine signals for full activation. Antigen binding to BCRs triggers internalization and subsequent digestion of antigen into peptide fragments. Some fragments bind MHC class II and are presented in the peptide—MHC II complex on the surface of the B cell. Simultaneous to this antigen processing, the B cell begins to migrate to the edge of the follicle bordering the T cell zone (71). Costimulation arises when a CD4 T helper cell recognizes its cognate peptide—MHC II complex on the surface of the B cell (72), after which (T cell) CD40L--

CD40 interaction are required for activation of the antibody response (73-75). As well, CD4 TCR engagement triggers local cytokine (IL-2, IL-4, IFNγ and IL-5) production by the helper cell to act locally on the B cell (76). Upon receiving these three signals, B cells undergo class switch recombination to produce a specific isotype of membrane-bound antibody/BCR (IgM to IgG, IgA, or IgE). B cells then differentiate into one of three fates: (1) short-lived plasma cells, that secrete antibodies (77); (2) germinal center B cells, which mutate their BCRs to evolve higher affinity receptors for eventual differentiation into memory B cells or long-lived plasma cells (76); or (3) long-lived memory B cells (78).

After B cell activation following tumor cell vaccination, antibodies then circulate through the blood and bind to tumor antigens within the blood or at the tumor. Antibody binding to intracellular antigens from dead cells may assist in antigen uptake by APCs, and surface binding to living tumor cells can trigger a number of effector activities that result in tumor cell death. Effector functions triggered by antibody binding to cognate epitope include: (a) neutralization of the target protein's function (79), (b) phagocytosis leading to clearance and/or adaptive immunity (80, 81), (c) complement dependent cytotoxicity (82, 83) (d) chemo-attraction of leukocytes, or (e) antibody-dependent cell-mediated cytotoxicity (83). The isotype of a given antibody determines the structure of Fc region and thus the recognition of the Fc region by specific Fc receptors. Class switching of antibodies by B cells (for example, from IgM to IgG) alters the antibody Fc regions and thus the effector functions that can impact the cell bearing the epitope.

Molecular Pattern Detectors: Keys to Dendritic and B Cell Activation

Under steady-state conditions DCs are immature. If they encounter antigen in isolation, tolerance ensues due to weak signal 1 and the lack of signals 2 and 3 to fully activate T cells (63). To mature DCs in culture and break tolerance, early DC immunologists added medium from gamma globulin-treated monocytes, which operated indirectly to trigger cytokine release (84, 85). *In vivo*, however, DCs sense the local cytokine milieu in combination with molecular patterns of pathogens and tissue damage.

The discovery of *Toll*, a gene in *Drosophila* associated with immunity from fungal infection (86), marked the explosion of the molecular pattern field. Toll-like receptors (TLRs) were found to be sensors of molecules conserved among many species of pathogens. At the same time, DC pioneers discovered maturation could occur through addition of lipopolysaccharide, a cell wall component of gram-negative bacteria (87). TLRs thus link the innate and adaptive immune systems through the maturation program. Upon ligation of their leucine rich repeat-containing extracellular domains with pathogen-associated molecular patterns (PAMPs), TLR cytosolic domains cluster together (sometimes with a different TLR) which allows adaptor proteins to bind and signal through NFκB and AP-1 (88). Within minutes of recognition of a microbial pattern, multiple signal transduction arms (transcriptional and post-translational) enact secretion of pro-inflammatory cytokines and increased surface expression of pre-made co-stimulatory and MHC molecules (89). Importantly, co-administration of TLR ligands with antigens increases cross presentation (signal 1) by altering the intracellular processing route of antigen (90).

TLR ligands, in particular the TLR9 agonist hypomethylated cytosine-phosphodiester-guanine (CpG) oligonucleotides from bacteria, also induce the activation of B cells when co-delivered with antigen (91). The potency of CpG stimulation is often strong enough to induce plasma cell differentiation in the absence of CD4 helper cells (92), but also increase B cell proliferation (91), class-switching (93), upregulation of costimulatory molecules (94), and cytokine release (91).

TLRs are joined by many receptors that sense PAMPs and other patterns, and all these receptors have been appropriately named pattern recognition receptors (PRRs). Diverse molecular structures are therefore capable of working alone or in combination to activate dendritic and B cell activation enhance the adaptive immune response against brain tumors.

Death and Danger Sensors in Dendritic Cells and B cells

All cells undergo stress responses in situations of environmental hostility, including mechanical trauma, nutrient starvation, temperature extremes, DNA damage, infection and hypoxia. These insults result in the release of pre-made molecules or the production of stress-related molecules that are subsequently released. Active or passive death can lead to the release of "danger" molecules, and growth conditions before death directly impact the expression of molecules to be released by these processes. Numerous studies document the production and biologic function of stress-associated molecules in malignant and non-malignant cells. Such studies have laid the foundation for identifying the internal arsenal of molecules within tumor cells that may enhance the anti-tumor adaptive immune response under the proper conditions.

Shortly after the discovery of TLRs, Matzinger and Fuchs proposed that the concept of the immune system's discrimination between self and non-self was inaccurate (95, 96). After all, this dichotomy failed to explain autoimmunity (95, 96). They argued, rather, that the immune system's "switch" was sensitive to danger signals, be they from pathogens or from pathologically dying self-cells (95, 96). Endogenous danger signals from stressed, dying, or dead cells were termed the damage-associated molecular patterns (DAMPs), and these molecules are largely detected by the same pattern recognition receptors as PAMPs (97). Since their inception, their identification has been provocative, arduous and controversial.

In 2000, Rock and colleagues reported the intrinsic adjuvant activity of cytosolic proteins within multiple syngeneic cell lysates. The lysates enhanced CD8 T cell killing following co-injection with model antigens such as chicken ovalbumin (98). These findings reveal many key properties of DAMPs:

1. Cells passively release pre-made DAMPs upon instantaneous death (necrosis) or apoptosis;
2. UV irradiation, chemotherapy, or heat shock can boost adjuvant activity; and
3. Malignant and non-malignant cells can release DAMPs.

Shortly thereafter, B cell activation was observed by synergy between BCR and self-DNA-mediated TLR9 activation in a murine model of rheumatoid arthritis (99). These studies were corroborated by similar findings in human B cells (100).

In addition, these studies set the stage for the molecular identification of DAMPs themselves. We now know that DAMPs constitute a diverse group of proteins

and non-proteins that activate TLRs, nod-like receptors, purinergic receptors, and scavenger receptors (101, 102). Excellent reviews on the many effects on sites of inflammation abound (101, 102). Relevant to the use of tumor cell vaccines are the effects of these molecules on the adaptive immune response, and how tissue culture can enrich for these molecules.

The currently characterized DAMPs make up diverse groups of molecules from all cellular compartments. Most important to cross priming are their target receptors, known for their pattern recognition and connections with signals 1,2, and 3. Less-well studied DAMP sensors are the C-type lectin receptors (CLRs), RIG-I-like receptors (RLRs) and nod-like receptors (NLRs). They synergize with TLRs, and their effects on cross priming are less well understood. These receptors could exert powerful biologic effects and demand attention for their potential as tumor vaccine targets.

CLRs are scavenger receptors that serve as a recognition and entry point for specific glycosylated antigens (103). Closely linked to entry is the endocytic routing of an antigen taken up after binding CLRs; therefore, recognition by CLR significantly influences in the intracellular route of the antigen within the cell. As tumor antigens are frequently alternatively glycosylated (104), the role of CLRs in tumor antigen processing is a potential mechanism for selective priming against tumor antigens. For example, some CLRs expressed by DCs such as DC-SIGN recognize malignancy-associated glycosylation patterns selectively (105). As discussed later, CLR binding by itself often leads to tolerance (106), but its potential to contribute to cross priming via antigen uptake and routing in combination with other maturation stimuli is relatively unexplored.

RLRs and NLRs are cytosolic receptors that bind diverse ligands, including single- and double-stranded RNA, double-stranded DNA, and cathepsin enzymes that are released as a result of phagosome rupture. These receptors play an integral part in vaccine components empirically established in the past, ranging from live attenuated viruses to aluminum (107, 108).

Providing ligands for these pattern recognition receptors in purified form and in combination with tumor antigens in various forms has yielded inconsistent results. An alternative to this exogenous source is to make use of tumor cells in culture that produce their own ligands for pattern recognition receptors. A wealth of literature indicates there are many avenues one might use to boost cross priming through induction of these endogenous ligands. Understanding how to optimize the production of the molecule or molecules that best enhances adaptive immunity will not be a trivial task; however, such knowledge is required to move the field forward and enhance the efficacy of tumor cell vaccines.

Rational Design of Tumor Cell Vaccines Aimed at Inducing Adaptive Immunity

Recent technology has enabled the identification of many tumor-associated antigens and the production of synthetic antigen (e.g., peptide, subunit) vaccines (109, 110). Despite these efforts, to date tumor cell-based vaccines have triggered double the response rate (~8%) relative to vaccines consisting of synthesized antigens (~4%) (40). At least part of this difference in success lies in the challenge of variable antigen expression among cells in a given tumor and between tumors of different individuals. Vaccinating with too few antigens will result in selection of antigen loss variants and

tumor progression. This has been elegantly demonstrated in animal models by Schreiber and colleagues, indicating loss of single immunodominant CD8 T cell epitope can be adequate for immune escape (111). In humans, a notable example includes glioblastoma patients vaccinated with a peptide from epidermal growth factor receptor variant III (EGFRvIII), in which recurrent tumors stopped expressing the protein despite being positive at enrollment (112). To induce a broad response, vaccines must provide adequate quantities of multiple antigens (40).

At the time of writing this thesis, clinicaltrials.gov contained over 1,000 search hits for "tumor cell vaccines," the majority of which place great faith in *ex vivo* tumor cell culture to provide such a diverse pool of antigens. As in the past, the success or failure of these trials will likely depend on all-too-often neglected aspects of tissue culture and vaccine production that affect the three required signals for B and CD8 T cell activation. With new knowledge of endogenous adjuvants, working stepwise is the most rational approach for moving beyond the current modest success.

Tissue culture differs from *in vivo* tissues in many aspects, including nutrient supply, dimensionality of substrate, and oxygen tension. These differences result in many changes, including divergence in antigen expression, decrease in heterogeneity, and loss of stromal cells to which immunization can target. Studies such as that by Lee and colleagues have sparked optimism that tissue culture can be more immunogenic once we identify crucial factors that govern cross priming and B cell activation. They observed dramatic and irreversible gene expression changes in glioma cells grown in serum-containing media (113). Culturing cells in neural stem cell (NSC) media with basal

fibroblast growth factor and epidermal growth factor resulted in a relatively preserved phenotypic and genotypic profile (113). When glioma cells from NSC media were used for vaccine immunotherapy of glioma-bearing mice, they resulted in superior tumoricidal CD8 T cell responses and increased survival compared to vaccines from serum-containing culture (114).

Tissue culture is therefore a double-edged sword in that it risks inducing an artificial phenotype, yet proper manipulation may result in a vaccine superior to the phenotype of the tumor itself. As discussed below, oxygen tension in culture can enrich for antigen and adjuvant expression during tumor culture (115).

Oxygen

Oxygen (O_2) regulation is crucial for survival of metazoan organisms. In the mammalian body, O_2 delivery homeostasis occurs through sensing of the molecule in the blood vessels in the lungs and kidneys, but all cells sense changes in oxygenation (116, 117). O_2 sensors affect gene expression, phenotype, and behavior in response to changes in oxygen availability in a graded and temporally complex manner (118). Upon a decrease in O_2, neurons and glia react through short- and long-term mechanisms to adapt and attempt to restore O_2 supply (119). On the scale of seconds to minutes, decreases in O_2 cause diminished in neuronal and glial excitability through altered membrane channel conductivity such as that of TASK potassium channels (120). As well, short-term suppression of gene transcription and translation occurs to conserve energy (121, 122). Longer-term adaptation to hypoxia results in changes in gene expression through redox-sensitive transcription factor activity, such as that of nuclear factor kappa B (NFkB),

activator protein 1 (AP-1), early growth response protein 1 (EGR-1), and hypoxia-inducible factors (HIFs) (123-125). It is believed that hundreds of genes transcriptionally regulated by O_2, but these changes vary greatly among cell types (126).

The best understood cell-autonomous O_2 sensors are the HIFs, whose alpha subunits are targeted for degradation in normoxia (127). Upon a decrease in O_2 below ~6%, which is within the physiologic range of brain parenchyma (128), degradation ceases, and HIFs become active as transcription factors (129). HIF-responsive genes can be identified by the presence of hypoxia response elements (HREs); however, dozens of O_2-regulated genes lack HREs and are altered by HIF indirectly or by HIF-independent means (130).

Oxygen levels within the healthy brain range from 1-5% (128), with levels in glioma ranging widely, from 0.1-10 % (131). HIFs vary in expression level exponentially between 1 and 6% O_2 (132), but we lack a deep understanding of how variability in this activity modulates gene expression and phenotype. Moreover, tissue culture incubators for glioma culture often regulate CO_2 but not O_2. As a result, atmospheric O_2 levels (~20%) induce degradation of HIFs and shift gene expression away from that of the tumor *in situ* (133).

Conversely, we recently found that transfer of glioma cells from 20% to physiologic (5%) O_2 (for at least 2 weeks) led to a drastic shift in gene expression, bringing gene expression closer to the tumor *in situ* (133). Lowering O_2 induced increases in expression of HER2, SOX2, EphA2 and IL-13Rα2, all glioma-associated antigens with defined CD8 T cell epitopes (134). Lysates from 5% O_2 cultures also

primed CTLs with superior tumoricidal functions against gliomas *in vitro* using primary human samples and *in vivo* using murine models (133).

Upregulation of stress-related molecules during hypoxia is a common response seen in many cell types (118); however, it was unknown what immunologic effect if any would be seen by decreasing O_2 from a supraphysiologic to a physiologic range. Intriguingly, lysates from 5% O_2 possessed unique adjuvant activity (that was absent in 20% O_2 lysates), inducing superior responses to exogenous antigens as measured by CD8 T cell proliferation and cytokine production (133, 135). Weekly vaccination of 5% O_2 lysate-containing vaccines resulted in a 41% increase in survival of glioma-bearing mice compared to vaccination with lysate from the same cells grown at atmospheric levels (135). Five percent O_2 lysate co-delivered to splenic DCs with chicken ovalbumin also increased the number of peptide—MHC I complexes on the cell surface, indicating an enhancement of cross presentation (signal 1) (135). In addition, serum from mice vaccinated with lysates from 5% O_2 reacted with fewer glioma antigens than serum from mice vaccinated with lysates from 20% oxygen (135). Glioma-reactive antibodies were not investigated further.

These data formed the basis for allogeneic glioma cell vaccines cultured in 5% O_2 that were recently tested in a phase I clinical trial for recurrent glioblastoma patients (NCT01171469; Olin *et al.,* in preparation). The mechanisms behind O_2's influence on immunogenicity and its breadth of application, however, remain unknown. This thesis contributes to brain tumor vaccine immunology by investigating these unknowns.

THESIS OBJECTIVES

1. Determine if decreasing oxygen dose below 5% results in further increased adjuvant activity of glioma lysates;

2. Assess whether lysate vaccines from low oxygen are feasible, safe, immunogenic and effective in a preclinical large animal model of aggressive meningioma; and

3. Test whether the increased adjuvant activity of glioma lysates from 5% O_2 is due to upregulation of a damage-associated molecular pattern.

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CHAPTER TWO

IMMUNOGENICITY TRANSITIONS IN GLIOMA CELLS ACROSS VARIOUS OXYGEN TENSIONS

SUMMARY

Immunogenic plasticity is an important feature of cultured tumor cells, as shown by the finding that transfer of glioma cells from atmospheric (~20%) to tissue-physiologic (5%) oxygen (O_2) increases their immunogenicity. While lysate vaccines are more effective when made from cells cultured in 5% O_2, it was unknown whether immunogenicity would increase further as O_2 levels were decreased below 5%. Here, studies investigate how immunogenicity varies across a wide range of O_2 tensions, suggesting that more than one "immunogenicity transition" exists along the O_2 continuum. These studies first examine the originally described transition from 20% to 5% O_2 that results in the shift from immune-suppressive to immune-stimulatory (133, 135). Reversing suppression with desferrioxamine, which increases levels of hypoxia inducible factors (HIF) 1α and 2α, suggests that HIFs are at least partially responsible for changes in immunogenicity. Next, we tested whether additional transitions in immunogenicity existed as O_2 was decreased below 5%. Cross presentation enhancement *in vitro* by glioma lysates from cells cultured in 2% O_2 was increased beyond levels seen from 5% O_2-derived lysate, but lysate from 1% O_2 was less effective. This second immunogenicity transition is likely due to expression changes in many molecules. For

example, we observed an upregulation of heat shock proteins (HSPs) 27 and 70, which have been implicated in immune suppression. Interestingly, preliminary data indicate that lysates from cells cultured in 2% O_2 fail to extend survival of glioma-bearing animals. These studies predict that immunogenicity varies with O_2 tension due to multiple variables that affect processes other than cross presentation, and that O_2 tensions less than 5% may not yield more immunogenic glioma cell vaccines. Further studies therefore focused on lysates from 5% O_2.

INTRODUCTION

It is estimated that 1.5% of the genome is transcriptionally regulated by oxygen (O_2 (126), but how regulation differs in various cell types and finer fluctuations are poorly understood. While many mechanisms underlie O_2-induced gene regulation, the best-studied sensors, the hypoxia-inducible factors (HIFs), exemplify the complexity of expression changes following a shift in O_2 level across a relatively narrow range (127). HIF protein levels vary exponentially between 1 and 6% O_2 (132), the tensions that are present within the healthy brain and within gliomas themselves (128, 136). As HIF levels increase, so does their DNA binding activity, with the half-maximal binding response between 1.5 and 2% O_2 and maximal response at 0.5% O_2 (132). In gliomas, O_2 levels are in constant flux due to variation in vascular diameter, neo-angiogenesis, and HIF-driven motility and invasion of glioma cells (137). We lack a complete understanding of how physiologic variations in O_2 tension (1-5%) modulate the immunogenicity of glioma cells, and this knowledge could lead to more effective glioma cell vaccines through manipulation of tissue culture O_2.

Decreased O_2 is associated with expression changes in many immune modulatory molecules that could shift the overall immunogenicity of glioma lysate vaccines. Immune modulatory proteins upregulated under decreased O_2 include: adenosine, which inhibits differentiation of dendritic cells and diminishes T cell responses through many mechanisms (138, 139); heat-shock proteins, whose immunogenicity is likely context dependent but is currently associated with regulatory T cells and chaperone function (140, 141); and the annexin A2—S100A10 tetramer, which activates macrophages and probably other leukocytes through TLR4 (142, 143). Undoubtedly many other molecules contribute to immunogenicity in a complex manner, and the convolution of the response to change in O_2 requires empiric testing. Head-to-head comparisons of glioma cells in 20% versus various O_2 conditions would increase understanding of low O_2-induced immunogenicity for improved vaccine efficacy.

The following experiments tested the immunogenicity of lysates from glioma cells cultured in a broad range of O_2 tensions. In addition to O_2 tension, pharmacologic hypoxia was induced in GL261 glioma cells from which lysates were made. These lysates were then tested for their abilities to increase cross presentation and cross priming. With the understanding that hundreds-to-thousands of molecules are altered upon changes in O_2, these experiments are a starting point for dissecting dominant immunogenic players that call for additional testing to further delineate the mechanisms of positive and negative immunogenicity in glioma cell cultures. They also demonstrate the magnitude of immunogenic alterations within a relatively narrow O_2 range, and the importance of empiric testing of multiple conditions to optimize immunogenicity.

METHODS

Cell culture

GL261 murine glioma cells were cultured in media containing DMEM/F12 (1:1) with L-glutamine, sodium bicarbonate, penicillin/streptomycin (100 U/mL), B27 and N2 supplements, and 0.1 mg/mL Normocin. GL261 cells were supplemented with rhEGF and rhFGF (20 ng/mL; Peprotech) semiweekly. Unless otherwise stated, cells were cultured in the indicated O_2 tension for at least two weeks before use. For desferrioxamine use, compound was added at 130 μM for 48h as previously described (144).

Splenocyte-mediated cross priming

For measurement of IFNγ, C57BL/6J splenocytes were plated at a concentration of 2×10^5 cells/well in a 96-well plate in complete RPMI 1640 media. Splenocytes were pulsed with 10 μg tumor lysate with or without 10 μg human (h) $gp100_{25\text{-}33}$ peptide (KVPRNQDWL) or ovalbumin protein, and incubated for 24 hours. $CD8^+$ T lymphocytes were purified from Pmel (135) or OT1 mouse splenocytes using negative immunomagnetic selection according to the manufacturer's protocol (Miltenyi Biotec). Purity was greater than 95% by flow cytometry. 2×10^5 purified CD8 T cells from a Pmel or OT1 mouse were co-cultured with pulsed splenocytes. Forty-eight hours after co-culture, IFNγ was quantified in the tissue culture supernatant using a flow cytometric bead array according to the manufacturer's protocol (BD Biosciences).

Splenic dendritic cell cross presentation

For measurement of presented chicken ovalbumin (OVA) peptide, BL/6 mouse splenocytes were plated at a concentration of 4×10^5 cells/well in a 96-well plate in complete RPMI 1640 media. Splenocytes were pulsed with 10 μg indicated tumor lysate with or without 10 μg peptide containing the core OVA-derived SIINFEKL/ H-$2K^b$ epitope (EVSQLEQLESIINFEKLTEEWTSSNVM) and incubated for 24 hours. Cells were harvested, stained for CD11c, MHC II, and SIINFEKL-H-$2K^b$, and analyzed by flow cytometry as described below.

Western immunoblot

GL261 murine glioma cells were washed, pelleted, and lysed in radio-immunoprecipitation assay buffer containing protease and phosphatase inhibitors (Pierce). Protein concentration was determined, and 40 μg were loaded per lane and separated on a 4% to 12% SDS-PAGE gel. Gels were transferred to nitrocellulose, blocked, and incubated in 1:1000 anti-human HSP60 (cross-reacts with mouse, polyclonal, Santa Cruz Biotechnology), 1:1000 anti-human HSP70 (cross-reacts with mouse, clone N27F34, Abcam), , anti-human HSP27 (G3.1, Santa Cruz Biotechnology), and anti-mouse b-actin (Abcam) in blocking buffer for 1 hour. Membranes were then washed six times before incubation in 1:20,000 anti-goat, mouse, or rabbit IgG HRP (Jackson ImmunoResearch) in blocking buffer for 1 hour. Nitrocellulose was incubated in ECL Plus chemiluminescent substrate (GE) for 5 minutes, drained, and exposed to HyBlot CL Autoradiography film (Denville Scientific) for 30 seconds.

Flow Cytometry

Analyses of stained dendritic and CD8 T cell suspensions were performed on a custom Canto II(BD) and analyzed with FlowJo software (Tree Star, Inc.). Fluorochrome-conjugated mAbs used were CD11c—FITC (eBioscience, clone N418), SIINFEKL-H-2K^b—PE (eBioscience, clone 25-D1.16), and I-A/I-E—eFluor450 (MHC II; eBioscience, clone M5/114.15.2). The corresponding isotype controls were also purchased from eBioscience.

Glioma Model

C57B/6J mice were purchased from Jackson Laboratories and maintained in specific pathogen-free facilities at research animal resources at the University of Minnesota. The murine GL261 glioma model was established as described previously (133, 145). Briefly, 15,000 luciferase-expressing GL261 cells in 1μL PBS were stereotactically injected into C57BL/6J female mice (6-8 weeks of age). The site of implantation was the right striatum, 2.5 mm lateral, 0.5 mm anterior to bregma and 3.0 mm deep from the cortical surface of the brain.

Immunization Protocol

GL261 cells were dissociated with non-enzymatic cell dissociation buffer (Sigma), washed three times in PBS, resuspended in 500 μL PBS, and frozen initially by placing in –80°C overnight. Cells were further lysed by five cycles of freezing in liquid nitrogen and thawing in a 56°C water bath. The protein concentration of the supernatant was determined using a Bradford assay. Pellets were resuspended, and lysates were stored at –80°C until use for vaccination or *in vitro* experiments. Each tumor-bearing mouse was vaccinated with 65 μg protein lysate mixed with phosphorothioated type-B CpG ODN

685 (5′-tcgtcgacgtcgttcgttctc-3′; SBI Biotech; Tokyo, Japan) in a final volume of 100 μL injected intradermally. Vaccine was injected (50 μL) in the lower neck and (50 μL) in the left inguinal region.

RESULTS

Lysates from atmospheric oxygen are immune-suppressive

In effort to understand the changes that enable increased efficacy of lysate vaccines from physiologic oxygen, we focused on the outcome of cross priming. *In vitro* experiments of cross priming demonstrate that decreasing O_2 levels by four-fold leads to increased dendritic cell cross presentation and increased IFNγ production by CD8 T cells (135). These medium-throughput assays were used to further investigate properties of lysates from 5% and 20% O_2. First was the finding that lysates from 20% O_2 suppress cross presentation and cross priming compared to treatment with antigen alone, strongly suggestive of the presence of immune suppressive molecules (Fig. 2.1 A&B). However, it was unknown what mechanism(s) of suppression occurred.

Pharmacologic hypoxia reverses immune suppression

Cells possess many mechanisms to adjust to changes in oxygenation in their environments. The most well known mediators of altered gene expression from lowered O_2 are hypoxia-inducible factors (HIFs). GL261 cells, like almost all cells, upregulate HIF 1α and 2α below 6% O_2 (137). We added desferrioxamine (DFX) to GL261 cultures in atmospheric O_2 as a method to mimic low O_2 culture, as it is known to increase the expression of HIF 1α and 2α in many cell types, including gliomas (129). Lysates from

cells treated with DFX in 20% O_2 were better able to induce cross priming compared to untreated cells, implicating HIFs in the immunogenic response (Fig 2.2).

Cross presentation is maximally increased at 2% O_2

Culture of glioma cells in 5% O_2 in previous studies was chosen for practical reasons: due to ease of proliferation and the ability to maintain large incubators at this tension for large-scale culture. Oxygenation levels in gliomas range from 0.1 to 8% O_2 (136), and it is unknown which levels are most immunogenic. GL261 cells were cultured in 20%, 5%, 2%, and 1% O_2, for at least two weeks, harvested for lysate production, and lysates were co-delivered with an ovalbumin peptide fragment to splenic dendritic cells to assess cross presentation. Surface levels of peptide—MHC I complex were greatest in cells pulsed with lysate from 2% O_2, suggesting efficacy of vaccines could be further improved (Fig. 2.3). Moreover, lysates from 1% O_2 were about as effective as those from 5% O_2, suggesting a peak of immunogenicity is present within the physiologic O_2 range (Fig. 2.3).

Decrease to 1% O_2 causes increases in heat shock proteins 27 and 70

Many molecular changes could underlie the differential responses seen in lysates from 20, 5, 2, and 1% O_2. These lysates were probed for several stress-related proteins that have been implicated in immune responses. We observed expression changes through titration of O_2 (Fig. 2.7). While heat shock protein 60 showed appearance of a doublet in 5% and 2% O_2, its levels did not change appreciably across O_2 levels. In contrast, heat shock proteins 27 and 70 dramatically switched on expression following culture in 1% O_2 (Fig. 2.4).

Vaccines from 2% O_2 fail to extend survival in murine glioma

The immunogenicity of lysates from 2% O_2 was tested by vaccination of GL261-bearing mice with these lysates in combination with CpG 685 ODN, the adjuvant used in previous studies (135). Interestingly, these vaccines failed to extend survival; however, efficacy of 5% O_2 lysate vaccines in this study failed to reach statistical significance ($p=0.11$; Fig 2.5).

DISCUSSION

These studies are a starting point for optimizing of glioma cultures for increased cross priming. While lysates from 5% O_2 act in an adjuvant manner to increase cross presentation and cross priming, those from 20% O_2 act suppressively. Many immune-suppressive molecules have been described in glioma cells and could be responsible for suppression in 20% O_2. Several examples are: (1) TGFβ, one isoform of which was originally discovered in gliomas (146). Interestingly, membrane expression of latent associated peptide (LAP) of TGFβ1 was greater in primary human glioma cells cultured in 5% O_2 than those in 20% O_2, decreasing the likelihood of its role here (133); (2) butyrophilin 1A1, which was overexpressed in 20% O_2-cultured primary human gliomas as measured in Illumina microarray (133), and is known to suppress T cell responses (147). Attempts at western blotting BTN1A1 in GL261 were unsuccessful due to lack of antibody reactivity (data not shown); (3) CD200, the expression of which is upregulated in primary human glioma cultures from 20% O_2 (133). Recent studies also demonstrate that antagonizing the CD200 axis in GL261 vaccines improves cross priming and survival (data not shown). Numerous other mechanisms or combinations of mechanisms

could contribute to immune suppression. A logical next experiment for determining the role of each molecule or mechanism would be to test the immunogenicity in GL261 cells with a loss of function in the suspected molecules (in 20% O_2), and/or in dendritic or CD8 T cells with losses of function in the receptors or suppressive pathways involved.

Non-protein molecules cannot be ruled out when considering mechanisms of immune stimulation or suppression. Immune-stimulatory or -suppressive lipids or carbohydrates have variable half-lives, and their production kinetics are less well understood (148). Investigation of these molecules in loss of function experiments is more difficult as genetic ablation is often not able to result in precise loss of one molecule. Neutralizing antibody experiments may prove more fruitful in the future if non-protein candidates are identified in screens.

Evidence for HIF-regulated control of immunogenicity is suggested by the increase in cross priming of glioma cells from atmospheric oxygen, treated with the iron-chelator, desferrioxamine (DFX). DFX binds to Fe(II) within prolyl hydroxylase enzymes, inhibiting their ability to hydroxylate HIF α subunits for degradation (144, 149). Additional studies in with HIF1α or HIF2α loss function in GL261 cells would be necessary to demonstrate the requirement for HIFs in immunogenicity.

By repeating previous cross presentation studies with additional lysates from 2% and 1% O_2 we observed that immunogenicity varies enormously as glioma cultures decrease O_2 by small increments. Importantly, cells were cultured for a two-week period, and all cultures continued to show cell division during this time. While lysates from 2% O_2 doubled the efficiency of cross presentation, those from 1% O_2 were equally effective

at cross presentation as those from 5% O_2. However, lysate vaccines from 2% oxygen failed to extend survival as those from 5% oxygen in one survival study. This survival study must be repeated for definitive conclusions to be made, as the sample size is small and mice treated with lysate from 5% O_2 within the same group did not benefit from vaccination as much as in previous studies. Loss of immunogenicity one gained may be due to HSP27 or 70 upregulation, but many other mechanisms are also possible.

This study describes the highly dynamic immunogenicity of glioma cells across a a broader O_2 range. Analysis of the differential gene expression in lysates from different O_2 tensions may enable identification of important immunogenic players, known or unknown, for improvement of glioma lysate vaccines. We chose to focus on lysates from 5% O_2 due to their consistent efficacy in vaccines (135). Further studies sought to assess mechanisms related to feasibility, safety, and mechanisms of immunogenicity in a large animal model of another brain tumor, meningioma, and to characterize one molecule's contribution to adjuvant immunogenicity in 5% O_2 culture.

FIGURES

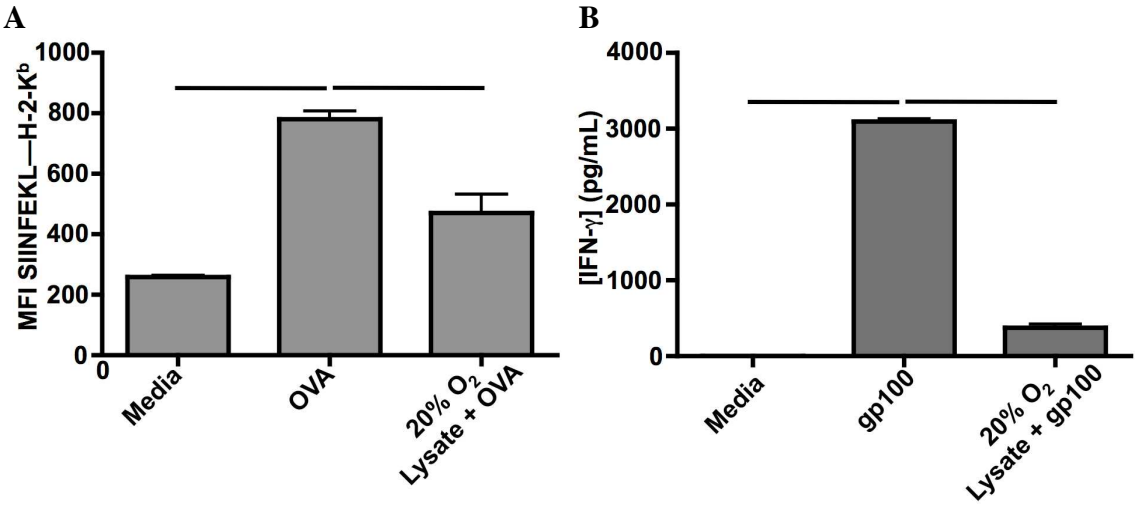

Figure 2.1: Suppression of cross presentation and cross priming by GL261 lysates from atmospheric oxygen. A, splenocytes were pulsed with a peptide fragment of chicken ovalbumin containing SIINFEKL alone or in combination with lysate from 20% O_2. Cross presentation in $CD11c^+$ cells was measured 24 h later with a fluorochrome-conjugate antibody recognizing SIINFEKL—H-$2K^b$. B, Splenocytes were pulsed with a peptide of the Pmel-1 CD8 T cell epitope alone or in combination with lysates from 20% O_2, responder Pmel CD8 T cells were added and IFNγ was measured by bead array 48 h later. Error bars, SEM; Lines, $p<0.05$ by unpaired t-test.

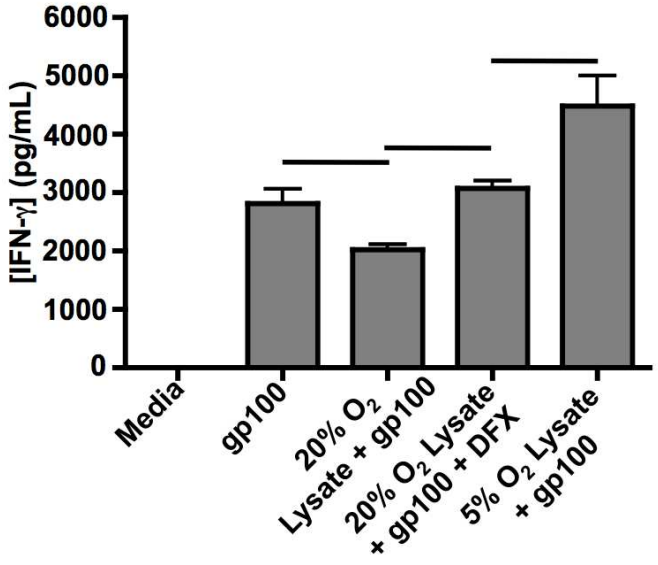

Figure 2.2: Enhancement of cross priming by GL261 lysates from atmospheric oxygen treated with a HIF-stabilizer, desferrioxamine (DFX). Splenocytes were pulsed with a peptide of the Pmel-1 CD8 T cell epitope alone or in combination with lysates from 20% or 5% O_2, responder Pmel CD8 T cells were added and IFNγ was measured by bead array 48 h later. Error bars are SEM; *, $p<0.05$ by unpaired t-test.

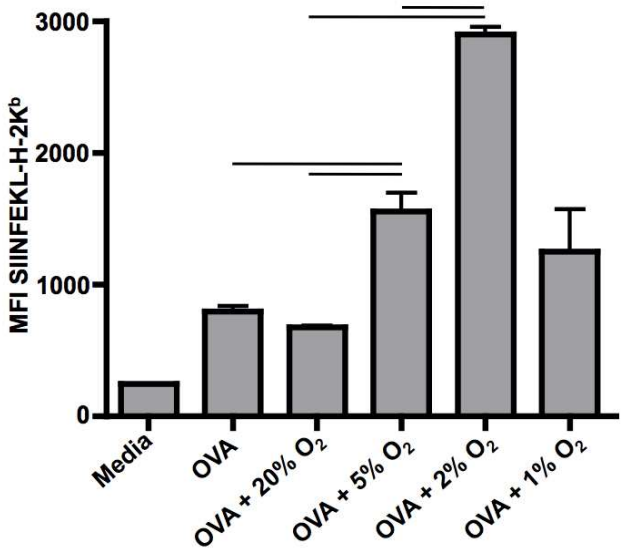

Figure 2.3: Increased numbers of peptide—MHC I complexes on dendritic cells pulsed with lysates from 5%, 2%, and 1% O_2. Splenocytes were pulsed with a peptide fragment of chicken ovalbumin containing SIINFEKL alone or in combination with GL261 lysate from 20%, 5%, 2%, or 1% O_2. Cross presentation was measured 24 h later by labeling with a fluorochrome-conjugated antibody recognizing the SIINFEKL—H-2K^b complex. Cells were gated on the dendritic cell ($CD11c^{hi}MHCII^{hi}$) population. Error bars are SEM, and lines show $p<0.05$.

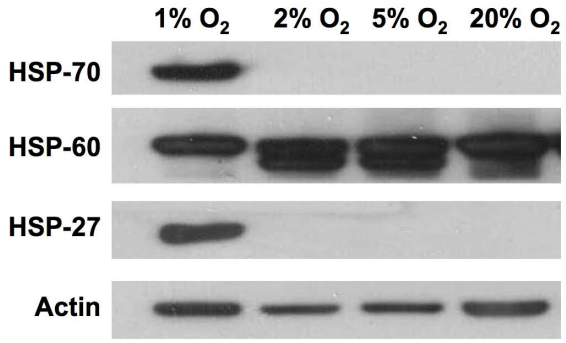

Figure 2.4: Differential expression of stress-related proteins in GL261 cells cultured in four oxygen concentrations. GL261 cells were cultured in the indicated oxygen concentrations for at least two weeks, and aliquots were taken for vaccination and half of each aliquot was flash-frozen and blotted for the indicated proteins following SDS-PAGE.

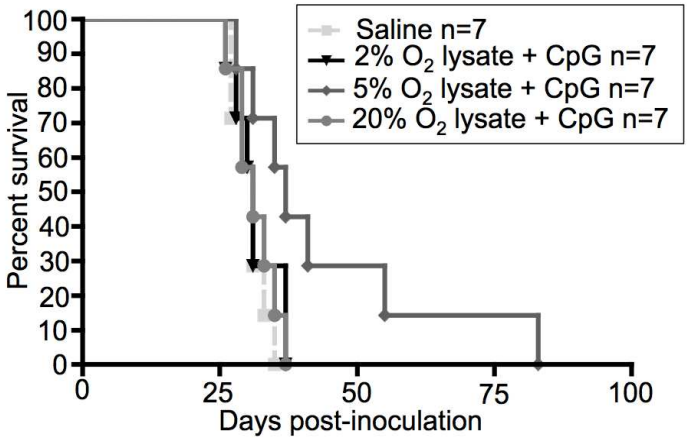

Figure 2.5: Lysate vaccination from cells cultured in 2% O_2 is ineffective. Kaplan-Meier plot of mice vaccinated with saline or GL261 lysate from the indicated O_2 tension and CpG 685 ODN (n=7 per group). p=0.11 by log-rank.

CHAPTER THREE

VACCINATION FOR INVASIVE CANINE MENINGIOMA WITH AUTOLOGOUS LYSATE FROM PHYSIOLOGIC OXYGEN INDUCES *IN SITU* PRODUCTION OF ANTIBODIES CAPABLE OF ANTIBODY-DEPENDENT CELL-MEDIATED CYTOTOXICITY[2]

SUMMARY

Malignant and atypical meningiomas are resistant to standard therapies and associated with poor prognosis. Despite progress in the treatment of other tumors with therapeutic vaccines, this approach has not been tested preclinically or clinically in these tumors. Spontaneous canine meningioma is a clinically meaningful but underutilized model for preclinical testing of novel strategies for aggressive human meningioma. We treated 11 meningioma-bearing dogs with surgery and vaccine immunotherapy consisting

[2] Adapted with permission from Brian M. Andersen, G. Elizabeth Pluhar, Charles E. Seiler, Michelle R. Goulart, Karen S. SantaCruz, Melissa M. Schutten, Joyce P. Meints, M. Gerard O'Sullivan, R. Timothy Bentley, Rebecca A. Packer, Stephanie A. Thomovsky, Annie V. Chen, Dominik Faissler, Wei Chen, Matthew A. Hunt, Michael R. Olin, and John R. Ohlfest. "Vaccination for Invasive Canine Meningioma Induces In Situ Production of Antibodies Capable of Antibody-Dependent Cell-Mediated Cytotoxicity" Reprinted from **© 2013 The American Association for Cancer Research. Originally published in *Cancer Research*. 2013. 73:2987-2997.**

of autologous tumor cell lysate combined with toll-like receptor ligands. We attempted to enhance immunogenicity by culturing autologous tumor cells for vaccination in 5% oxygen, which showed a trend for the best therapeutic benefit in murine glioma in Chapter Two and previous publications. Therapy was well tolerated, and only one dog had tumor growth that required intervention, with a mean follow up of 585 days. Interferon gamma elaborating T cells were detected in the peripheral blood of two cases, but vaccine-induced tumor-reactive antibody responses developed in all dogs. Antibody responses were polyclonal, recognizing both intracellular and cell surface antigens, and heat shock protein 60 was identified as one common antigen. Tumor-reactive antibodies bound allogeneic canine and human meningiomas, demonstrating common antigens across breed and species. Histological analysis revealed robust infiltration of antibody-secreting plasma cells into the brain around the tumor in post-treatment compared to pre-treatment samples. Tumor-reactive antibodies were capable of inducing antibody dependent cell-mediated cytotoxicity to autologous and allogeneic tumor cells. These data demonstrate the feasibility and immunologic efficacy of 5% O_2-derived vaccine immunotherapy for a large animal model of human meningioma and warrant further development towards human trials.

INTRODUCTION

Meningioma is the most common primary brain neoplasm, with over 100,000 patients diagnosed in the United States between 2004 and 2008 (17). Newly diagnosed tumors are managed by surgical resection alone. Roughly 6,000 patients will need additional treatment in the United States every year due to recurrence (9), which often

occurs with invasive or malignant disease (150, 151). Current salvage approaches include re-operation, radiation, radiosurgery and chemotherapy; there is controversy regarding the perceived clinical benefit of these interventions (12, 152-154). The three-year recurrence rate in re-operated World Health Organization (WHO) grade I meningioma is 50% (155), with risk of recurrence even greater in grade II and III tumors (8, 13, 156). Radiation, while modestly effective in benign disease, has been associated with cognitive deficits, secondary malignancies, and the transformation of the tumor to a higher-grade neoplasm (9).

One challenge in treating aggressive meningiomas is the paucity of animal models with which to test combinations of surgery and systemic therapy in a meaningful manner. Spontaneous meningiomas in dogs make up 45% of primary canine brain tumors (157) and are an under-utilized resource for preclinical studies. Canine and human meningiomas share many features, including histological resemblance, overexpression of growth factor receptors, deletion of chromosomal segments, and losses of function in tumor suppressor genes (158-160). Over 40% of canine meningiomas were atypical or malignant in one study (161). Dogs develop many other cancers that are prevalent in humans, and tumors progress 5-7-fold faster in dogs relative to humans (162). We hypothesized that preclinical studies in canine meningioma would enable accurate and rapid testing of immunotherapy for aggressive meningioma.

Cancer vaccines have been tested in multiple malignancies, including gliomas, with evidence of clinical activity (45, 55). A meta analysis covering over 100 clinical trials revealed that response rates to vaccination with peptides containing defined T cell

epitopes were less than half of that achieved with whole cell-based vaccines (40). The higher response rate achieved with whole cell vaccines (as used in this study) could be due to greater antigenic coverage or the potential to induce tumor-binding antibodies. Meningiomas are not subject to the same principles of immune privilege as cells within the brain parenchyma such as gliomas (163). Relative to gliomas, meningiomas may be better candidates for immunotherapy because they: (1) are not insulated by the endothelial tight junctions that limit large molecule (e.g. antibody) diffusion; (2) are in direct contact with cerebrospinal fluid that drains to the venous circulation and cervical lymph nodes for antigen presentation to T and B lymphocytes; (3) lack the T cell trafficking checkpoints present in the Virchow-Robin space (e.g., glia limitans); and (4) are relatively slow growing tumors that may be more susceptible to adaptive immune responses. However, invasive, atypical, and malignant meningiomas can infiltrate the brain parenchyma, requiring penetration of antibodies and/or lymphocytes for control of post-surgical, microscopic disease.

Tumor cell lysates mixed with synthetic toll-like receptor (TLR) ligands function as effective vaccines to induce anti-tumor immune responses in glioma-bearing animals (164, 165). The mechanisms of lysate/TLR ligand vaccines have been thoroughly characterized and tested in many cancer patients; however, the activity of this type of vaccine against meningioma is unknown. TLR activation on antigen presenting cells facilitates the induction of adaptive immune responses by enhancing antigen presentation, expression of co-stimulatory molecules, cytokine production, and homing to secondary lymphoid organs. The TLR 9 ligand CpG olidgodeoxynucleotide induced clinical

responses in select melanoma and renal cell carcinoma patients (166, 167). The FDA-approved TLR 7 ligand imiquimod exhibited adjuvant activity in cancer clinical trials (45, 168), with excellent efficacy as a single topical agent against skin cancers (169).

We conducted a vaccine immunotherapy trial for pet dogs with symptomatic, spontaneous meningiomas. Dogs underwent surgery followed by vaccination with autologous tumor cell lysate from 5% O_2 culture that was combined with imiquimod or CpG. Herein we report safety, robust extension of survival, homology among antibody epitopes between dogs and humans, and vaccine-induced, local antibody production in the brain. Vaccination for canine meningioma reveals promising avenues for further development towards human trials.

METHODS

Surgery, Vaccination Production, and Administration

Dogs were enrolled after obtaining owner consent according to an approved protocol from the University of Minnesota IACUC. A presumptive diagnosis of meningioma was based on the MRI characteristics of a solitary extra-axial mass in the brain, with heterogeneous T1W signal, usually isointense, homogenous T2W signal, sharply defined borders, homogenous contrast enhancement, evidence of a dural tail, that may have associated cysts, peritumoral edema and falx-shift. Surgical resection was performed under general anesthesia using the appropriate approach based on tumor location. Dogs were hospitalized with supportive care for 1-2 days after surgery. Corticosteroids used to minimize peritumoral edema were discontinued by 48 hours

before vaccination. Part of the tumor specimen was used for histological diagnosis, and the remainder of the tumor was cultured for vaccine production.

Cultures were established by mincing specimens with scalpels and digestion at 37 °C for 15 minutes in suspension with TrypLE™ Express (Invitrogen/Life Technologies). Cell suspensions were filtered through a 100 mm filter, washed with PBS, and placed in culture in 10 cm culture plates pre-coated with 1:10 Matrigel™ (BD Biosciences), in serum-free neural stem cell media consisting of DMEM:F12 (1:1), with L-glutamine, sodium bicarbonate, penicillin/streptomycin (100 U/mL), B27 and N2 supplements (Gibco), and 0.1-mg/mL Normocin™ (InVivoGen). Semi-weekly, cells were given 20 ng/mL rhEGF and rhFGF (R&D systems) and were cultured at 5% O_2 and 5% CO_2. Harvest for vaccination involved scraping cells from one 10 cm dish. Cells were washed thrice in PBS, and underwent five freeze-thaw alternations by transfer from warm water bath to liquid N_2 followed by radiation (200 Gy). Protein was measured by Bradford assay with standard Coomassie reagent (Pierce), and lysates were stored at -80 °C. GMP-grade CpG 685 ODN was provided by SBI Biotech Co., Ltd (Tokyo, Japan), and imiquimod cream was acquired through the University of Minnesota Boynton Health Services Pharmacy. In five dogs, CpG (2.0 mg) was mixed with thawed lysates immediately before intradermal injection at two sites in the back of the neck. Six other dogs received imiquimod cream (5%; 0.5 g) at two intradermal injection sites in the back of the neck 15 minutes prior to lysate injection. The maximally achievable lysate protein concentration was given to each dog, which varied by tumor volume and the ability of the tumor to proliferate in culture. Lysate doses ranged from 200 to 1,500 μg protein

(average of 595 μg), and average dose did not vary significantly between CpG and imiquimod-treated dogs.

Tumor Volume Measurements

Twenty consecutive surgical human cases (of MAH) were analyzed for volume by MRI, as calculated by [Length x width x height]/ 2 for tumor and intracranial volume. The same procedure was carried out for the eleven dogs in this study and the results were compared as described in supplementary methods.

Western Immunoblot

Cultured tumor cells or homogenized flash-frozen tumors were used for blotting. Cells were lysed, protease and phosphatase inhibitors (Calbiochem, San Diego, CA) were added, and for SDS-PAGE, lysates were diluted in laemmli reducing sample buffer, heated, and centrifuged. Protein standards (BioRad, Hercules, CA) were loaded next to each 40 μg lysate and resolved on NuPAGE 4–12% Bis/Tris gels (Invitrogen). Proteins were transferred to nitrocellulose (Amersham) at 5 V constant voltage using semi-dry transfer (BioRad). The membranes were blocked in 5% NFDM/TTBS at room temperature for 1 hour and cut appropriately into identical blots, each with a molecular weight standard (Bio-Rad) run adjacent to lysate. Each membrane was incubated at room temperature for 1 hour in normal, pre- or post-vaccination sera diluted 1:1000 in 5% NFDM/TTBS, washed six times for 10 min each in TTBS, followed by room temperature for 1 hour in rabbit anti-canine IgG HRP-conjugated secondary antibody (Jackson ImmunoResearch) at 1:50,000 in 5% NFDM/TTBS. Bands were detected using ECL

Western Blotting Detection System (Amersham) and HyBlot CL autoradiography film (Denville Scientific). Densitometry was performed by the Gel Analysis tool in ImageJ 1.45s software (NIH), and values were normalized by dividing by heavy and light chain densities (areas under the curve) from prevaccination lanes.

Detection of Tumor Cell Surface-Reactive Antibodies

A Becton Dickinson Custom Canto three-laser flow cytometer was used for data acquisition. Tumor cells were removed from Matrigel™ -coated 10 cm culture plates by scraping or BD™ Dispase (BD Biosciences), washed with PBS thrice, and incubated with heat-inactivated normal dog, prevaccination, or 3-month post-vaccination serum at 1:100 dilutions at 4° C for 30 minutes. Cells were then washed thrice in PBS and incubated with 1µg anti-canine IgG (H+L)—FITC (American Qualex) at 4° C for an additional 30 minutes before washing and analyzing.

Immunohistochemistry Staining and Quantification of Lymphocyte Infiltration

For lymphocyte analysis, 5µm tissue sections were cut, prepared with standard procedures, and the following antibodies were used: CD3 (AbD Serotec) at 1:2000, CD20 (Thermo Scientific) at 1:2000, and IgG (H+L; Jackson ImmunoResearch) at 1:2000. Tissue sections were incubated with the primary antibodies for 1 hour, rinsed, and a biotinylated secondary antibody was applied for 30 minutes. CD20 and IgG antibodies were followed with undiluted Rabbit Link (Covance), and a biotin conjugated donkey anti-rat IgG (H+L) (Jackson ImmunoResearch) at 1:500 was used with CD3. Sections were rinsed, incubated in hydrogen peroxidase, and a tertiary streptavidin horseradish

peroxidase link (Covance) for 30 minutes. The immune complex was visualized using 3,3′-diaminobenzidine as the chromogen. Sections were lightly counterstained with hematoxylin, dehydrated, coverslipped, and scanned using the Aperio Scanscope XT.

All surgical resection specimens and all necropsy blocks containing tumor or inflammation as seen by H&E staining were included in counting of $CD3^+$ and $CD20^+$ cells. Samples were blinded, and all 10X fields in slides were captured and saved as image files. Automated cell quantification was conducted with a customized macro and the particle analysis tool in Fiji software (ImageJ 1.46j, NIH). Counts underwent statistical analysis as described in supplemental methods before unblinding.

Antibody-Dependent Cell-Mediated Cytotoxicity

Dog blood from healthy donors was collected in anti-coagulant tubes, lysed osmotically, and peripheral blood leukocytes (PBLs) were washed thrice with PBS. PBLs were resuspended in complete RPMI 1640 (supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 0.1 mg/mL Normocin™ from InVivoGen) in a 96-well plate at 2.5 x 10^6 cells/mL, and stimulated 14 hours with 30 μM of the TLR7/8 ligand resiquimod before being washed and co-cultured with antibody-coated tumor cells. Primary meningioma cultures were coated with antibody as described in the cell surface binding procedure. Two washes were performed before addition to PBLs at an effector:target ratio of 25:1. Tumor cell lysis was determined by measurement of lactate dehydrogenase (LDH) activity as indicated by the manufacturer's protocol after seven hours of co-culture (Roche Applied Science). Percent specific lysis of tumor cells was calculated by: [Sample- (tumor only + PBL only)] / (lysed tumor + PBL only) x 100.

Statistical Analysis

Samples were analyzed by unpaired t-test (tumor/brain volume, flow cytometry, LDH activity). Histological cell counts were analyzed by an unpaired t-test with Welch's correction for unequal variances with 95% confidence intervals. Survival was analyzed using a logrank test with 95% confidence intervals. All statistics were conducted using GraphPad Prism version 4.0c for OS X (GraphPad Software, San Diego CA, www.graphpad.com).

RESULTS

Canine Meningiomas Model Aggressive Human Disease

Meningiomas from dogs treated in our study share histological features with human tumors of the same subtype (Fig. 3.1A), in addition to features that signify poor survival in humans. Brain invasion is an independent negative prognostic factor that prompted the reclassification of otherwise WHO grade I meningiomas as grade II (7). Two canine cases exhibited gross tumor invasion into the brain at resection, and three additional cases showed brain invasion upon postmortem analysis (Fig. 3.1B, left, and Table 3.1). Tumor invasion into the skull was also present in two cases (Fig. 3.1B, right). In addition, canine meningiomas occupied more than twice the volume of the brain relative to human tumors (Fig. 3.1C). All dogs were symptomatic at the time of diagnosis, and in ten dogs, the presenting clinical sign was seizures. The rapid recurrence and progression of this disease in canines is consistent with the common appearance of invasion into brain, which, in humans, predicts poor outcomes.

Extension of Survival Following Vaccine Immunotherapy

Eleven dogs underwent craniotomy for tumor removal after radiographic diagnosis of an intracranial neoplasm consistent with meningioma. Following histological confirmation, dogs were administered vaccinations biweekly with meningioma cell lysate from 5% oxygen culture in combination with CpG or imiquimod (Fig. 2A). Mean follow-up time was 585 days, with 36% (4/11) of dogs alive (Table 3.1; Fig. 3.2 B&C). Relative to historical controls, median survival is extended in the immunotherapy cohort (645 versus 222 days, $p<0.05$), with no censoring of dogs that died from other causes (Fig. 3.2B and Table 3.2). Neither vaccination cohort contained a case of frank tumor progression, although more deaths occurred in the CpG cohort (Fig. 3.2C). One CpG-treated dog developed two meningiomas, of which the second was not identified before vaccination due to small size. The second tumor was subsequently removed after its growth caused breakthrough seizures, and four other vaccines were given using tumor lysate of the second tumor.

Tumor-Reactive Antibodies Bind Cell Surface Antigens and Cross-React with Human Meningiomas

Immune responses before and after vaccination were measured in peripheral blood mononuclear cells (PBMCs) and sera. Interferon gamma elaborating tumor-reactive T cells were significantly increased in post-vaccination PBMCs in two of nine dogs tested, suggesting a low frequency of circulating T cells three months after surgery (Table 3.3). In contrast, polyclonal tumor-reactive antibody responses to whole tumor lysate (Fig. 3.3A) and cell surface antigens (Fig 3.3B&C) were detected in all eleven

dogs (Table 3.3). Probing of allogeneic tumor cells and lysates indicated recognition of common tumor antigens among dogs, some of which were present on the cell surface (Fig. 3.4A-C). Post-vaccination antibodies recognized recombinant human HSP60 and HSP60 from autologous and allogeneic tumor (Fig. 3.4A), demonstrating the common expression and recognition of this antigen. Remarkably, post-vaccination canine serum recognized human meningioma tissue, indicating antigen conservation between species (Fig. 3.4D).

Vaccination Induces T, B and Plasma Cell Infiltration into Peritumoral Brain

We conducted analyses of B and T cell infiltration in surgical and necropsy tissue in the four cases in which it was available. Three CpG-treated dogs died within three weeks of the final vaccination and exhibited robust, focal increases in B (with relatively less T) cell infiltration into peritumoral brain (Figs. 3.5A&B; Cases 1, 3, and 5). One imiquimod-treated dog developed acute lymphoblastic lymphoma and was euthanized 25 weeks after the final vaccination. This dog showed mild B cell infiltration (and no T cell infiltration) into the brain (Fig. 3.5B; case 8). As mentioned above, case 3 presented with a second meningioma in the contralateral hemisphere between the fifth and sixth vaccinations, which was subsequently resected. Western analysis revealed a profound increase in IgG penetration into the second tumor relative to the first, pre-vaccination tumor (Fig. 3.5C&D). Moreover, staining for canine IgG exposed plasma cells in the brains of three CpG-treated dogs (Fig. 3.5E). While previous studies have reported plasma cell entry into the brain in antibody-mediated autoimmune (170) and anti-pathogen (171) responses, this is the first account of induced plasma cell homing into

brain tumors. *In situ* antibody production was further suggested by focal areas of extracellular IgG staining seen in plasma cell-containing areas of brain tissue but not others (data not shown).

Recognition of Non-Neoplastic Brain and Meningeal Antigens by Post-vaccination Sera

Two dogs (cases 3 and 5) were euthanized seven and twenty days after the most recent vaccination. Both animals presented with uncontrollable seizures and tumor recurrence was assumed. At necropsy, case 3 had a microscopic focus of residual tumor and case 5 had no evidence of tumor. To evaluate vaccine-induced autoantibody production, sera from these and three other dogs was probed against normal dog brain, arachnoid/pia mater, and dura mater. Secondary antibody revealed heavy and light chain IgG and IgM deposited in meninges, but not brain parenchyma (Fig. 3.6A, left). These results are consistent with the physiologic permeability of immunoglobulin into these tissues. Serum from cases 3 and 5 reacted to arachnoid/pia and brain parenchyma, respectively. Consistent with the autoreactivity of case 5 sera, analysis of necropsy brain tissue from this dog revealed IgG accumulation on or in neurons distal to the site of resection (Fig. 3.6B). The binding of the sera to normal brain antigens in these dogs sets them apart from two CpG treated dogs and one imiquimod treated dog that remained healthy and had non-reactive sera (Fig. 3.6A).

Postvaccination Sera are Capable of Antibody Dependent Cell-Mediated Cytotoxicity

Anti-tumor effector activities of antibodies encompass multiple mechanisms, including antibody-dependent cell-mediated cytotoxicity (ADCC). Since antibodies reacted with cell surface antigens (Fig. 3.3B&C; Fig. 3.4 B&C; Fig 3.7 A&B), and antibody production *in situ* could enable opsonization of invasive meningioma cells behind an intact blood brain barrier, we tested whether tumor-reactive antibodies could trigger ADCC. Peripheral blood leukocytes killed few tumor cells when co-cultured with meningioma cells, or when tumor cells were pre-incubated with pre-vaccination serum; however, ADCC occurred when tumor cells were incubated with post-vaccination serum (Fig. 3.7C). Post-vaccination serum also triggered ADCC against allogeneic meningioma cells (Fig. 3.7D), demonstrating that allogeneic vaccination may be an effective strategy in canines with meningioma.

DISCUSSION

As many as 57,000 dogs a year develop meningiomas in the US (157, 172), and these dogs are an under-utilized resource for preclinical study. Because the prognosis for canine meningioma is dismal (173), both dogs and humans could benefit from these studies. Our data show the canine model resembles many histological subtypes observed in humans and has features associated with poor prognosis in humans. The large size of the canine brain allows for surgery as a component of therapy, enabling a more realistic clinical interpretation of systemic interventions.

The survival outcomes of this study call for further investigation into the predictive potential of the canine model, especially with regard to tumor and immune biology in the two species. Different responses to vaccination may occur due to

differential antigen expression, TLR expression in leukocyte cell types, tumor growth kinetics, the relationship between histologic subtype, biological behavior and clinical outcome.

Addition of lysate/adjuvant vaccination to surgery resulted in favorable survival compared to historical controls, but a prospectively designed, randomized study is required to make firm conclusions on therapeutic efficacy. Nonetheless, only case 3 experienced tumor growth despite treatment. The survival data may underestimate the benefit of vaccination because censoring was not used (Fig. 3.2B&C). Two dogs included in the analysis died of other malignancies, and two dogs lacked postmortem analysis but were assumed to die of age-related causes (Table 3.1). The three other deaths were due to euthanasia after dogs presented with uncontrollable seizures assumed to be caused by tumor recurrence. Postmortem analysis in these CpG-treated dogs found minimal or no tumor burden, but immunoreactivity to normal brain structures may have been treatment related and contributed to the acute onset of neurologic symptoms (Fig. 3.6A&B). Similar seizure activity in glioma-bearing dogs in clinical trials at our institution has been controlled using additional anti-convulsants, corticosteroids or induction of general anesthesia (data not shown). It is therefore possible that the seizures in meningioma-bearing dogs could have been controlled. Whether CpG or imiquimod is more efficacious is still unresolved from the current study due to the small number of dogs and many non-tumor-related deaths. Given the high level of tumor control and the superior safety profile of imiquimod, however, a prospective randomized trial to compare

surgery alone and in combination with vaccines of tumor lysate and a novel TLR 7/8 ligand was initiated.

Our study is a starting point for investigation of immunotherapy for meningioma. The data indicate that B cell activation and antibody production is the predominant mechanism of immunity induced by vaccination. Tumor-reactive antibodies were detected in the serum of all dogs regardless of lysate dose, demonstrating feasibility of autologous lysate/TLR vaccine production. Antibodies exhibited considerable inter-case and –species cross reactivity (Fig. 3.4 A&D; Table 3.3). B cell infiltration outnumbered T cell infiltration in postmortem brain tissue adjacent to the resection cavity (Fig. 3.5B). In contrast, only two dogs had increased tumor-reactive T cell responses as measured by interferon-gamma ELISpot (Table 3.3). However, it is likely that reactive CD4 T cells were primed following vaccination because antibody responses often require CD4 T cell help. Tumor-reactive T cells in the blood may not represent what occurs in the tumor draining lymph nodes or tumor site. A limitation of our study was that ELISpot was carried out only in pre-vaccination and 3-month post-vaccine blood samples. Nevertheless, infiltrating $CD3^+$ cells were observed in dogs that died within two weeks of the final vaccination (Fig. 3.5 A&B), suggesting some T cell activation. Future studies will examine T cell responses in greater detail.

Tumor antigens in lysate vaccines could include over-expressed normal antigens, mutated (neo-) antigens, oncofetal antigens (expressed during development), or tumor-specific carbohydrates, glycoproteins, or lipoproteins. That two dogs with severe post-vaccine seizure activity had auto-reactive sera to brain and meninges suggests that severe

autoreactivity is possible but infrequent (Fig. 3.6 A&B). Lysates may be depleted of these autoantigens to yield greater tumor specificity and less risk. Since most dogs had no autoreactivity or refractory seizures, risk of autoimmunity must be weighed against the threat to life posed by aggressive meningiomas.

Identification of meningioma antigens recognized by post-vaccination sera will enable the discovery of crucial epitopes for fully synthetic vaccine strategies in more widespread application. We identified HSP60 as one antigen recognized by sera following vaccination. HSP60 can translocate to the cell surface upon stress or malignant transformation (174), but it was not expressed on the tumor cell surface of case 3 (data not shown), arguing against cell surface protein as the functional target of this antibody. Sera that recognized HSP60 also reacted with a human meningioma, and reactivity was observed at 60 kDa; however, more study is needed to determine if HSP60 is a relevant target in human meningioma.

Antibody-mediated autoimmunity is well characterized, with autoimmune conditions in the CNS such as multiple sclerosis being re-evaluated in light of clinical benefit seen from B cell depleting therapies (175). Brain tumor immunotherapy, once focused on T cell-dependent mechanisms, is similarly expanding its breadth of effector mechanisms. Orthotopic mouse models of glioma indicate that survival benefit from immunotherapy is absent in B cell deficient, μMT tumor-bearing mice (176) and unpublished results). The importance of B cells and antibodies in brain tumor immunity are relatively unstudied. Our study documents for the first time the induction of plasma cells homing to brain tumors as a consequence of therapeutic intervention. B cell and

plasma cell infiltration into the brain also indicates promise for immunotherapy to act against invasive cells that exist behind an intact blood brain barrier. ADCC due to *in situ* antibody production in brain is a novel immune effector mechanism relevant to any brain tumor. Our data suggest that vaccination can induce a "Trojan horse"-like infiltration of plasma cells that can in turn trigger ADCC, and thus create excitement for translation of this approach to human meningioma and other CNS cancers.

FIGURES

Case Number	Age/Sex	Breed	Histological Subtype at Biopsy	Brain Invasion*	Vaccine Adjuvant	MRI Response	AB Response	Cause of Death
1	7 /M.I.	Boxer	Papillary	No	CpG	Stable	1,4	Abdominal Tumor
2	10 /F.S.	Laborador Mix	Papillary	n/a	CpG	Stable	1,3	Unknown (Age-Related)
3	11 /F.S.	Laborador Mix	Meningothelial	Necropsy	CpG	Second Tumor, Reoperation Stable	1,2,3,4	Euthanasia (seizure activity)†
4	9 /F.S.	Doberman Mix	Psammomatous	Necropsy	CpG	Stable	4	Euthanasia (paresis)†
5	7 /M.N.	Golden Retriever	Transitional	No	CpG	Stable	1	Euthanasia (seizure activity)†
6	6 /F.S.	Afghan Hound	Transitional	n/a	Imiquimod	Stable	1,3,4	Alive
7	12 /F.S.	Weimaraner	Meningothelial	Necropsy	Imiquimod	Stable	1,3	Lymphoma
8	13 /M.N.	Poodle	Meningothelial	Surgery	Imiquimod	Stable	1,3,4	Alive
9	7 /M.N.	Great Dane	Psammomatous	n/a	Imiquimod	Stable	1,3,4	Alive
10	11 /F.S.	Schnauzer Mix	Transitional	n/a	Imiquimod	Stable	1,4	Alive
11	8 /M.N.	Beagle	Psammomatous	Surgery	Imiquimod	Stable	1,4	Alive

S: Spayed; N: Neutered; I: Intact
*Surgery: brain invasion seen in surgical pathology; Necropsy: brain invasion seen in necropsy
Antibody reactivity: 1 to autologous tumor by western blot; 2. to allogeneic tumor by western blot; 3. to autologous surface antigens by flow cytometry; and 4. to allogeneic surface antigens by flow cytometry.
† Neurologic symptoms were thought to be due to recurrence; euthanasia was chosen, and at postmortem analysis, minimal or no tumor was present.

Table 3.1: Information on dogs receiving autologous lysate/adjuvant vaccination for meningioma.

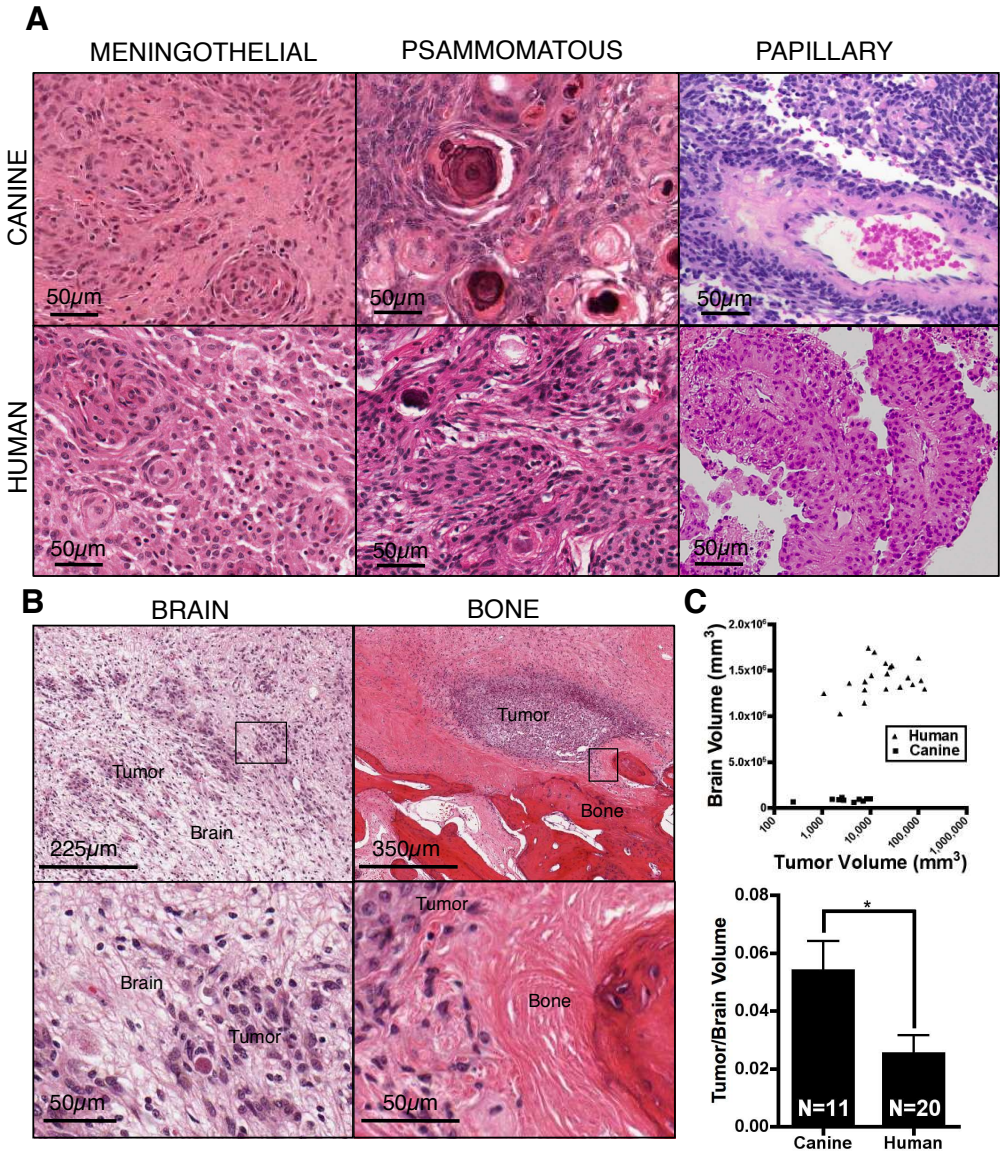

Figure 3.1: Canine meningiomas model aggressive human disease. A, meningioma surgery specimens stained with H&E, left to right, from cases 3, 4, and 1. B, necropsy specimens from 2 dogs with tumors that, in humans, are categorized as WHO grade II on biopsy. C, tumor volume and brain volume of the 11 dogs treated with surgery plus immunotherapy and 20 consecutive intrainstitutional patients with human meningioma. 0, $P < 0.05$.

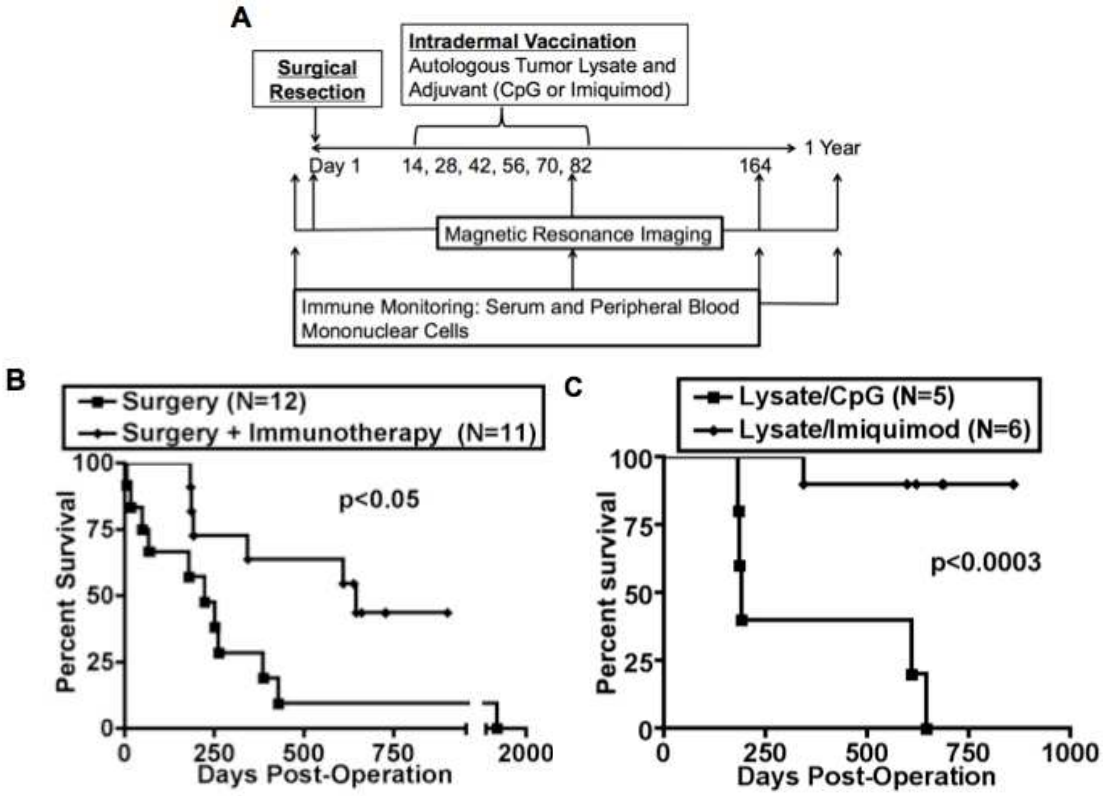

Figure 3.2: Biweekly tumor lysate/adjuvant vaccination schedule results in prolonged survival of spontaneous canine meningioma. A, study design and immune monitoring scheme. B, surgery controls were conducted at 4 institutions, and all cases were confirmed to be meningioma by histopathology. C, comparison of CpG with imiquimod treatment groups.

Case Number	Survival (Days post-operation)	Histological Subtype
12	6	Transitional
13	17	Anaplastic
14	48	Transitional
15	67	Transitional
16	69*	Fibrous
17	178	Meningothelial
18	222	Epitheliomatous
19	252	Meningothelial
20	262	Meningothelial
21	386	Unspecified
22	428	Transitional
23	1855	Unspecified

Cases were seen at the University of Minnesota, Tufts University, Purdue University, and Washington State University. *Denotes dog that is still alive.

Table 3.2: Survival of canines with meningioma following surgery monotherapy.

Dog Number	Fold IFN-g ELISpot Increase	Number Reactive Bands by Western Blot	Antibodies Recognizing Cell Surface Antigens (Fold Increase)
1	0	Polyclonal	Allo (26.9)
2	0	Polyclonal	Auto (20.5), Allo (11.37)
3	0	Polyclonal	Auto (68), Allo (9.5)
4	0	n/t	Allo (16.5), Auto n/t
5	4	Polyclonal	Allo (14.4), Auto n/t
6	Inconclusive	Polyclonal	Auto (34), Allo (27.3)
7	0	Polyclonal	Auto-No Increase, Allo n/t
8	0	Polyclonal	Auto (11.7), Allo (9.7)
9	0	Polyclonal	Auto (2.3), Allo (3.7)
10	n/t	Polyclonal	Auto (2.14), Allo (13.65)
11	20	Polyclonal	Allo (4.1), Auto n/t

Auto: autologous tumor cells recognized; Allo: allogeneic tumor cells recognized; and n/t not tested.

Table 3.3: Immune monitoring summary for vaccinated dogs.

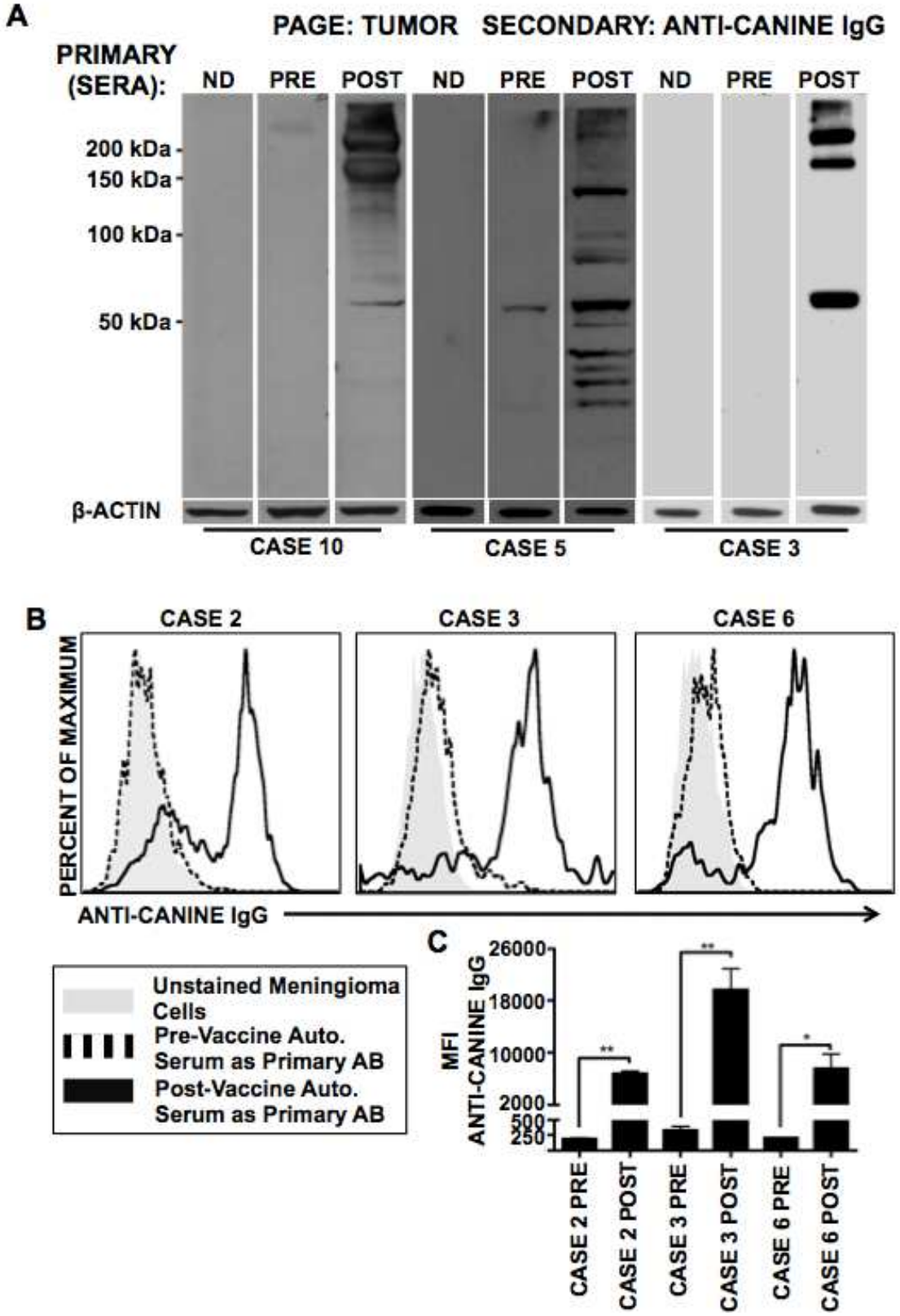
A
PAGE: TUMOR SECONDARY: ANTI-CANINE IgG
PRIMARY (SERA):
ND
PRE
POST
ND
PRE
POST
ND
PRE
POST
200 kDa
150 kDa
100 kDa
50 kDa
β-ACTIN
CASE 10
CASE 5
CASE 3
B
CASE 2
CASE 3
CASE 6
PERCENT OF MAXIMUM
ANTI-CANINE IgG
Unstained Meningioma Cells
Pre-Vaccine Auto. Serum as Primary AB
Post-Vaccine Auto. Serum as Primary AB
C
MFI ANTI-CANINE IgG
26000
18000
10000
2000
500
250
**
**
*
CASE 2 PRE
CASE 2 POST
CASE 3 PRE
CASE 3 POST
CASE 6 PRE
CASE 6 POST

Figure 3.3: Vaccination induces antibody responses to meningioma surface antigens. A, Western immunoblot analysis of autologous tumor cells from culture (cases 10 and 3) or snap-frozen tumor specimen (case 5). Normal dog serum (ND) was used as a control. B, live autologous tumor cells were stained with serum from prevaccination or postvaccination (at 82 days). C, aggregate data of 3 dogs from B. 0, $P < 0.05$; 00, $P < 0.005$.

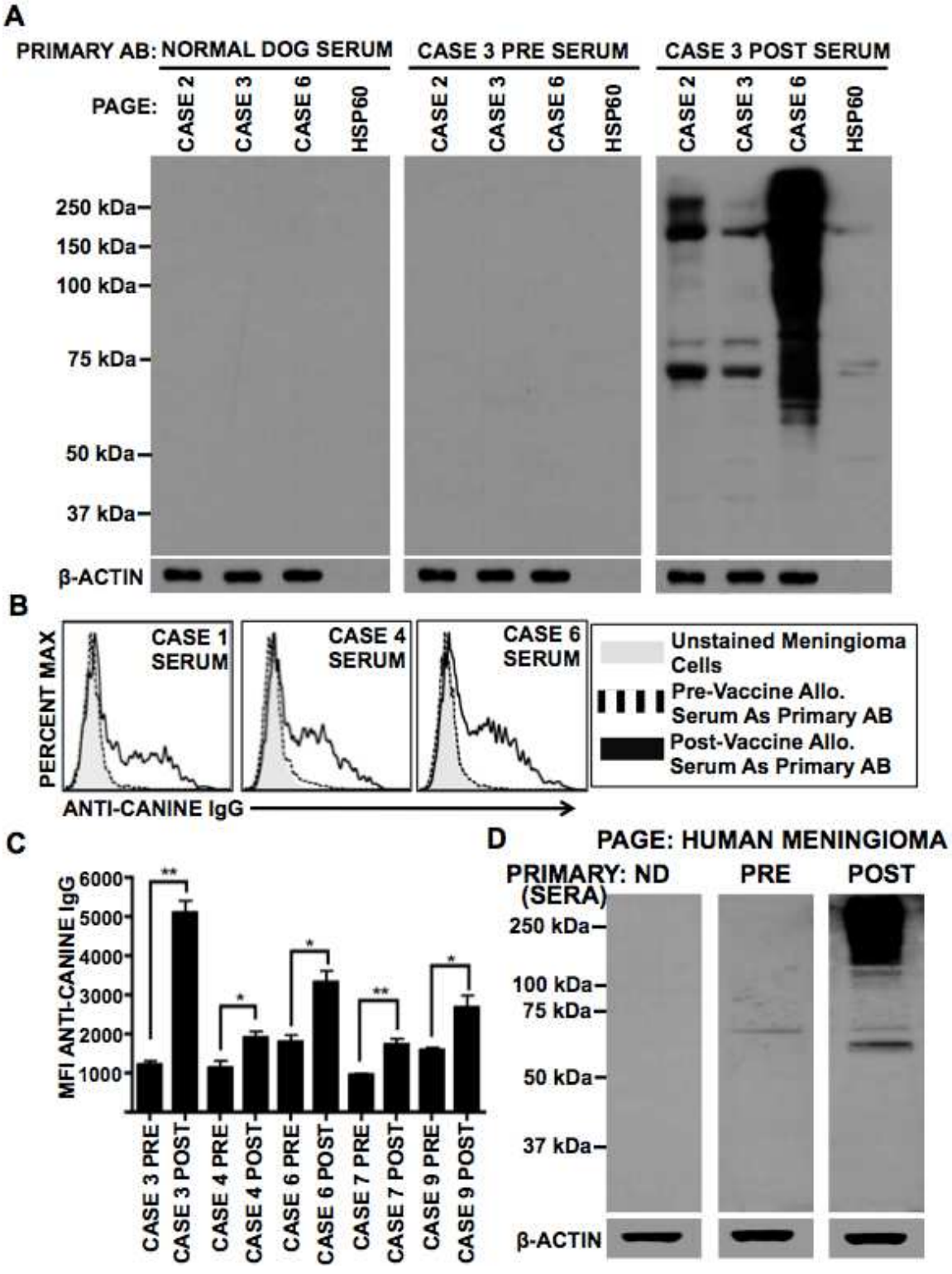
A
PRIMARY AB: NORMAL DOG SERUM
CASE 3 PRE SERUM
CASE 3 POST SERUM
PAGE:
CASE 2
CASE 3
CASE 6
HSP60
250 kDa
150 kDa
100 kDa
75 kDa
50 kDa
37 kDa
β-ACTIN
B
PERCENT MAX
CASE 1 SERUM
CASE 4 SERUM
CASE 6 SERUM
ANTI-CANINE IgG
Unstained Meningioma Cells
Pre-Vaccine Allo. Serum As Primary AB
Post-Vaccine Allo. Serum As Primary AB
C
MFI ANTI-CANINE IgG
6000
5000
4000
3000
2000
1000
CASE 3 PRE
CASE 3 POST
CASE 4 PRE
CASE 4 POST
CASE 6 PRE
CASE 6 POST
CASE 7 PRE
CASE 7 POST
CASE 9 PRE
CASE 9 POST
D
PAGE: HUMAN MENINGIOMA
PRIMARY: ND (SERA)
PRE
POST
250 kDa
100 kDa
75 kDa
50 kDa
37 kDa
β-ACTIN

Figure 3.4: Antibodies recognize allogeneic dog and human meningiomas. A, Western immunoblot analysis of tumor cells from 3 cases and recombinant human HSP60. B, representative histograms of cell surface binding of primary tumor cells from case 7. C, quantification of serum antibody surface binding to an allogeneic papillary (nonstudy dog— recognized by case 3) and a meningothelial meningioma (case 7— recognized by cases 4, 6, 7, and 9). D, Western immunoblot analysis of a human meningioma probed with postvaccination serum from case 3. 0, $P < 0.05$; 00, $P < 0.005$.

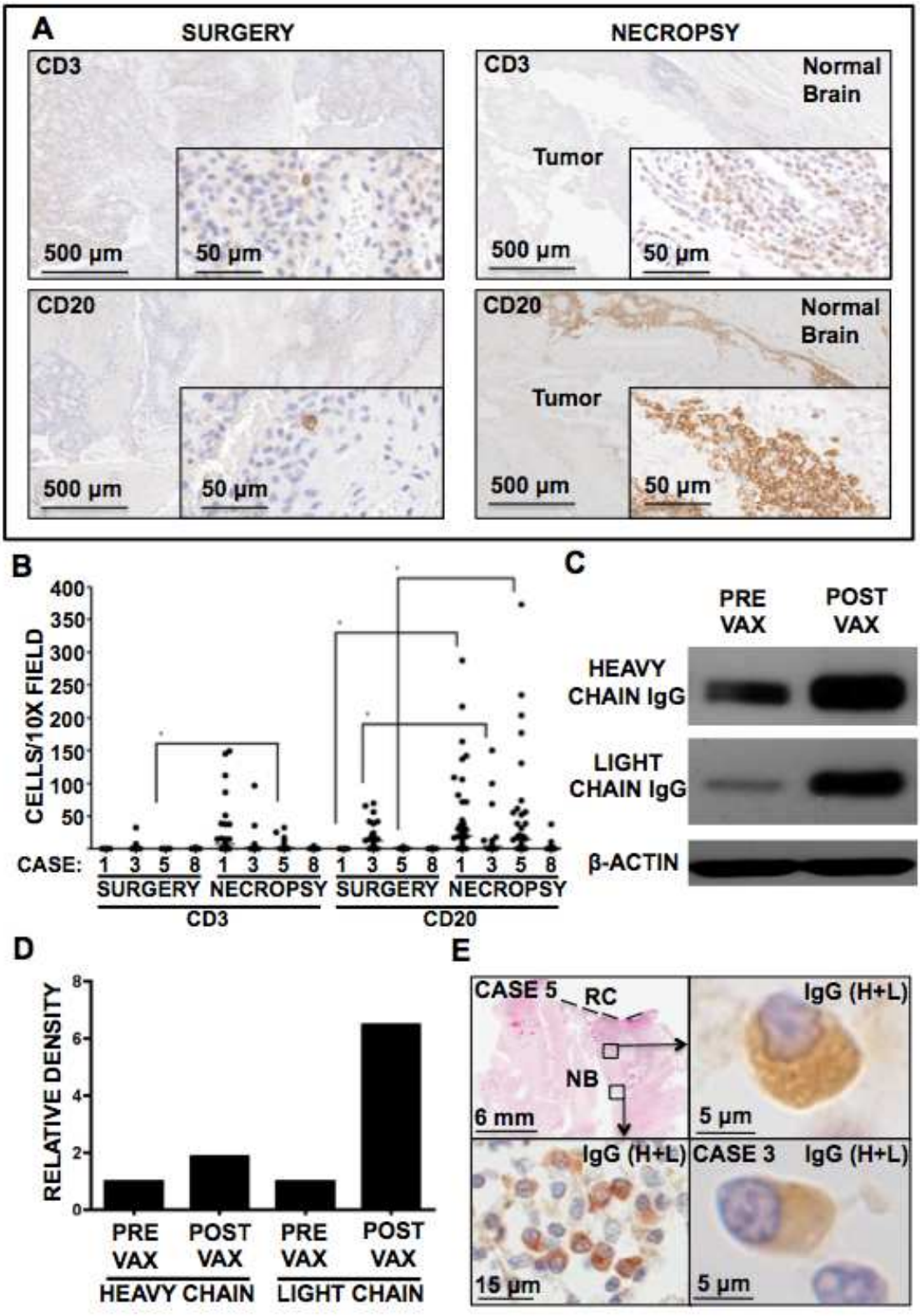
A
SURGERY
NECROPSY
CD3
Normal Brain
Tumor
500 µm
50 µm
CD20
B
CELLS/10X FIELD
CASE: 1 3 5 8 1 3 5 8 1 3 5 8 1 3 5 8
SURGERY NECROPSY SURGERY NECROPSY
CD3
CD20
C
PRE VAX
POST VAX
HEAVY CHAIN IgG
LIGHT CHAIN IgG
β-ACTIN
D
RELATIVE DENSITY
PRE VAX
POST VAX
HEAVY CHAIN
LIGHT CHAIN
E
CASE 5
RC
NB
6 mm
IgG (H+L)
5 µm
15 µm
CASE 3

Figure 3.5: Vaccination induces B- and plasma cell infiltrates in peritumoral brain. A, representative images from CD3 and CD20 immunohistochemistry of biopsies and necropsies from case 1. B, quantification of CD3 and CD20 stains from cases 1, 3, 5, and 8. C, IgG blotted on case 3 primary tumor (prevaccination) and contralateral tumor (postvaccination). D, densitometry of heavy and light chain bands from C. E, immunohistochemical stain of canine IgG (H+L), indicating plasma cell infiltrates in normal brain areas of cases 3 and 5. *, $P<0.005$.

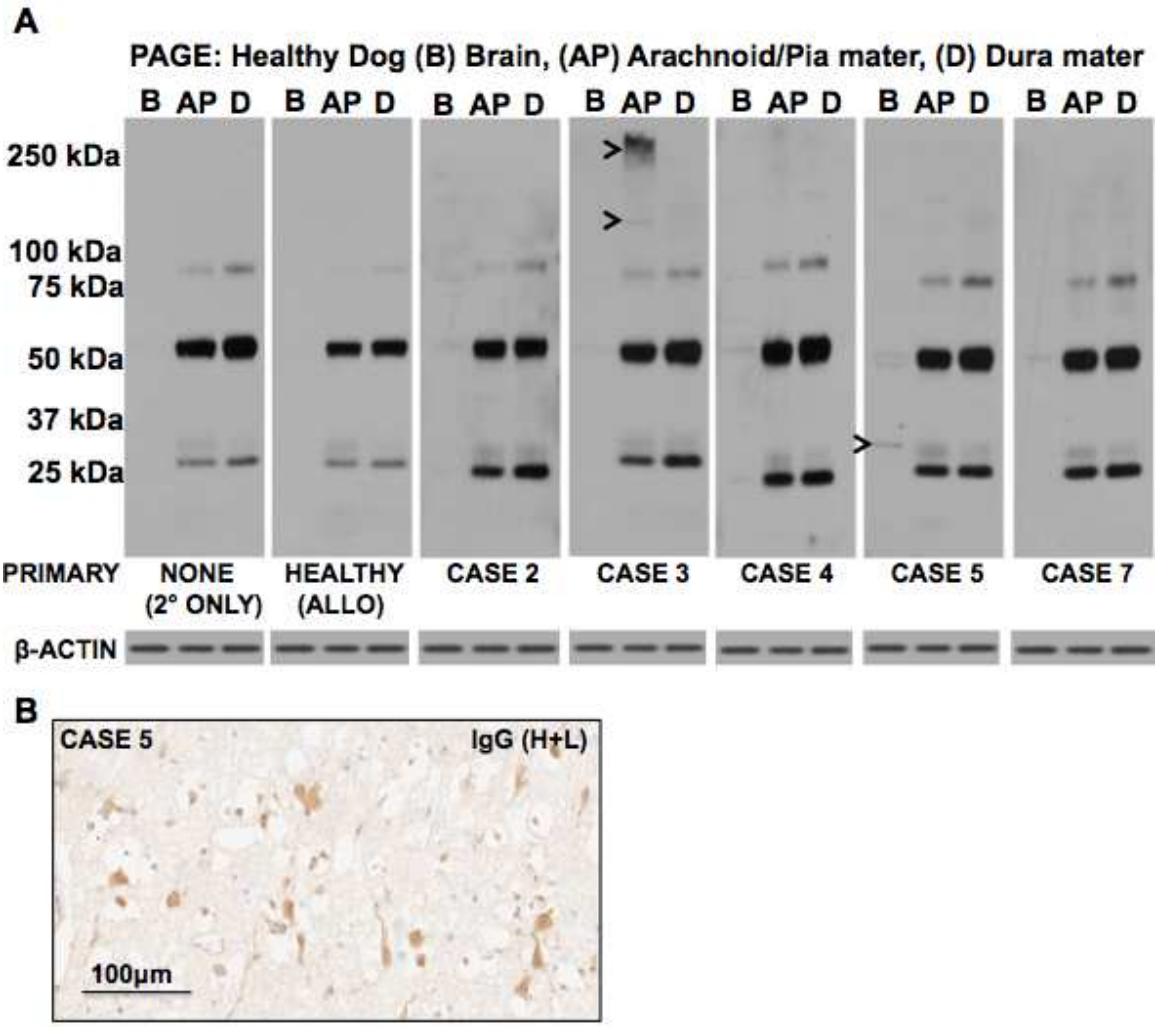

Figure 3.6: Reactivity with normal brain correlates with neurologic symptoms in CpG-treated dogs. A, brain, arachnoid/pia mater, and dura mater were probed with secondary anti-canine IgG (H+L) alone, with serum from an allogeneic healthy dog, or postvaccination sera from 5 cases. Arrows indicate reactivity in dogs with neurologic symptoms following vaccination. B, necropsy specimen from case 5 stained for canine IgG (H+L).

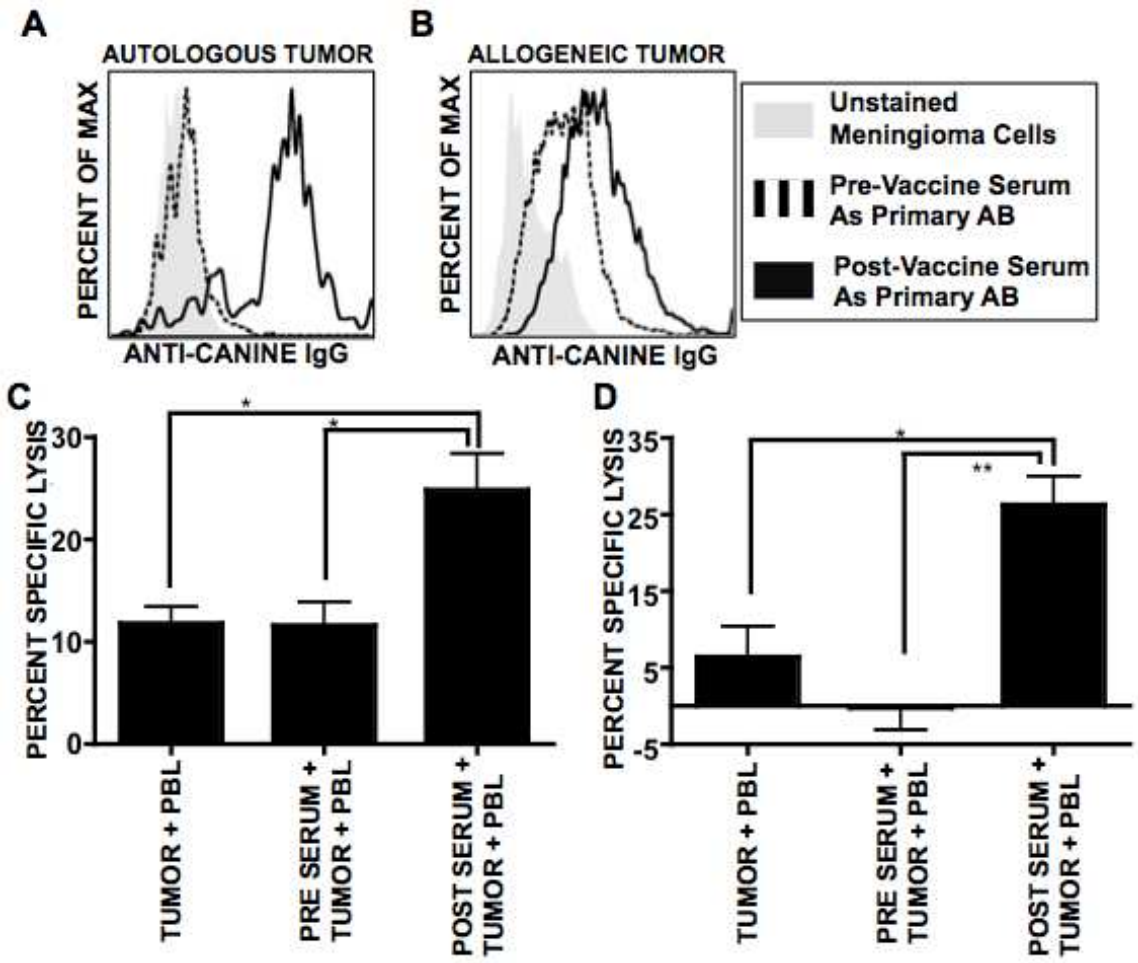

Figure 3.7: Postvaccination serum enables ADCC. A and B, case 3 postvaccination serum antibodies bound autologous tumor (A) and allogeneic tumor (B) of papillary histology from a nonstudy dog. Postvaccination serum also enabled killing of autologous (C) and allogeneic (D) tumors when combined with allogeneic PBL. *, $P < 0.05$; **, $P < 0.005$.

CHAPTER FOUR

ANNEXIN II IS AN OXYGEN-REGULATED DAMAGE-ASSOCIATED MOLECULAR PATTERN

Brian M. Andersen, Charles E. Seiler, Xia Junzhe, Peisheng Hu, Alan L. Epstein, Wei Chen, Bruce R. Blazar, Christopher A. Pennell, John R. Ohlfest, and Michael R. Olin.

SUMMARY

Previously, we reported that culturing glioma cells at physiologic (5%) oxygen increases their immunogenicity when used as therapeutic vaccines. However, the underlying mechanism(s) behind the enhanced immunogenicity were unknown. Here we demonstrate that annexin II (ANXA2) is upregulated in glioma cells cultured at 5% oxygen. Knocking down ANXA2 completely eliminated the therapeutic activity of vaccines from 5% oxygen cultured cells, while adding monomeric ANXA2 (ANXA2m) enhanced the efficacy of vaccines derived from glioma cells cultured in atmospheric (20%) oxygen. This activity is Toll-like receptor 2-mediated. Moreover, a fragment of the N-terminus of ANXA2 is sufficient for TLR2 activity and can be fused to antigens in single-agent vaccines. These data indicate that ANXA2m is a vaccine adjuvant and a potential danger signal within the body.

INTRODUCTION

Dendritic cells (DCs) are the leukocytes most adept at cross priming CD8 T cells by presenting exogenous antigens (49). In a process called cross presentation, DCs internalize exogenous proteins and degrade them into peptides that are routed to various compartments, where they may bind to nascent MHC class I molecules. Stable peptide/MHC I complexes are then delivered to the cell surface for presentation to CD8+ T cells. From this basic understanding, many approaches have been devised to direct tumor cell components to DCs to stimulate tumoricidal CD8 T cells in cancer patients (177).

Whether DCs are targeted directly within patients or are matured ex vivo (85, 178), three signals are required for maximal cross priming: i) sustained cross presentation, ii) upregulation of co-stimulatory molecules on the DC membrane, and iii) cytokine release (179, 180). Signaling initiated by ligation of pattern recognition receptors (PRRs), which detect conserved molecules from damaged cells or pathogens, initiates DC maturation and the enhancement of these three cross priming signals (86, 181). Pathogen-associated molecular patterns (PAMPs) such as nucleic acids (91), cell wall components (86, 182), and bacterial motility proteins (183) are PRR ligands that have been widely tested as tumor cell vaccine adjuvants (184).

Other adjuvant molecules that induce DC maturation through PRRs are the damage-associated molecular patterns (DAMPs), released from injured self-cells (185, 186). Understanding the cellular mechanisms of injury and the specific DAMPs released by injured tumor cells in tissue culture has led to several methods to increase the

immunogenicity of tumor cell vaccines. DAMPs from tumor cells are released or exposed by irradiation (187), anthracyclines (188), cardiac glycosides (189), platinum agents (187), or the combination of proteasome and lysosome inhibitors (190). DAMPs that improve anti-tumor CD8 T cell responses include calreticulin (191), high mobility group box protein 1 (187), adenosine triphosphate (192), and filamentous actin (193, 194). Maximizing the release of DAMPs and preventing their degradation during cell death are popular strategies of enhance the adjuvant properties of tumor cell vaccines. Armed with increased quantities of DAMPs, in addition to a broad array of antigens, new incarnations of DC based tumor cell vaccines bring hope for increasing vaccine efficacy in the clinic.

We have shown that decreasing tissue culture oxygen to physiologic levels (5%) leads to enhanced adjuvant activity of glioma cells in therapeutic vaccines (135). Here we demonstrate that DAMP upregulation is the predominant mechanism of increased immunogenicity from tumor cells cultured in physiologic O_2. Host expression of Toll-like Receptor 2 (TLR2), a PRR that recognizes several DAMPs, was required for the efficacy of vaccines made from 5% O_2 glioma lysates. A subtractive proteomic screen of TLR2-binding partners in glioma cell lysates identified annexin II (ANXA2), a 36 kD calcium- and phospholipid-binding protein expressed in most cell types (145). We characterize for the first time the role of monomeric ANXA2 as a DAMP capable of extending survival of tumor-bearing animals. Intriguingly, the TLR2-active domain of ANXA2 is just fifteen amino acids in length, enabling synthesis of an antigen fusion peptide for use as a single-agent vaccine.

METHODS

Animal models

BALB/cJ, C57B/6J (BL/6) and Tlr2-B6.129-Tlr2tm1Kir/J (*Tlr2*$^{-/-}$) mice were purchased from Jackson Laboratories and maintained in specific pathogen-free facilities at the research animal resources at the University of Minnesota. The murine GL261 glioma model was established as described previously (135). Briefly, 15,000 luciferase-expressing GL261 cells in 1 μL PBS were stereotactically injected into C57BL/6J female mice (6-8 weeks of age). The site of implantation was the right striatum, 2.5 mm lateral, 0.5 mm anterior to bregma and 3.0 mm deep from the cortical surface of the brain. The murine EMT6 mammary carcinoma was established by injection of 10^6 cells in 50 μL PBS into the rostral-most left mammary fat pad of 6-8 week-old BALB/cJ mice (135). The NS0 murine plasmacytoma model was established by injection of 10^6 "empty vector" cells (described below) in 100 μL PBS, intraperitoneally into 6-8 week-old female BALB/cJ mice (195).

Cell culture

GL261 cells for vaccination were cultured in media containing DMEM/F12 (1:1) with L-glutamine, sodium bicarbonate, penicillin/streptomycin (100 U/mL), B27 and N2 supplements, and 0.1 mg/mL Normocin (InvivoGen). GL261 cells were supplemented with rhEGF and rhFGF (20 ng/mL; Peprotech) semiweekly. EMT6 cells, splenocytes, and T cells were cultured in RPMI 1640. GL261 cells for inoculation were cultured in DMEM; both DMEM and RPMI media contained 10% fetal bovine serum, penicillin/streptomycin (100 U/mL), and 0.1 mg/mL Normocin. Two NS0 transfectoma

cell lines were established by transfection with a vector containing glutamine synthetase with or without a sequence of N-terminal His-tagged human ANXA2 ("ANXA2-transfected" and "empty vector"). NS0 cells were cultured in hybridoma medium without glutamine, with 10% dialyzed fetal bovine serum, glutamine synthetase supplement (Millipore), penicillin/streptomycin (100 U/mL), and 0.1 mg/mL Normocin.

Immunization protocol

Tumor cells were harvested with non-enzymatic cell dissociation buffer (Sigma), washed three times in PBS, resuspended in 500 μL PBS, and frozen initially by placing in −80°C overnight. Cells were further lysed by five cycles of freezing in liquid nitrogen and thawing in a 56°C water bath. The protein concentration of the supernatant was determined by Bradford assay. Pellets were resuspended, and lysates were stored at −80°C until use. Each tumor-bearing mouse was vaccinated with 65 μg protein lysate mixed with or without 50 μg phosphorothioated type-B CpG ODN 685 (5′-tcgtcgacgtcgttcgttctc-3′; SBI Biotech; Tokyo, Japan) or 1.5 μg recombinant human ANXA2 (AbD Serotec) in a final volume of 100 μL injected intradermally. Vaccine was injected (50 μL) in the lower neck and (50 μL) in the left inguinal region for GL261-bearing mice, and (50 μL) in each inguinal region for EMT6-bearing mice. Vaccination for GL261 and EMT6-bearing mice was administered weekly starting three days after tumor implantation, for a total of six doses. NS0-bearing mice received 65 μg lysate in 100 μL PBS, with 50 μL in each inguinal region. The NS0 "priming phase" consisted of vaccination four days in a row, starting three days after tumor implantation. Then, NS0

study mice were given weekly "boost" doses starting ten days after tumor implantation, for a total of six boosts.

Immunohistochemistry

Staining of GL261 tumor specimens from mice was done as previously described. Briefly, Mice were perfused, and standard processing, embedding, and re-hydration methods were employed. Following rehydration, sections are put into ReVeal, 6.0 pH citrate buffer (Biocare Medical) and placed in a steamer followed by a cool-down. Slides were rinsed in tap water and placed into a PBS bath. Sections were blocked and then incubated at 4°C overnight with the primary antibody cleaved caspase 3 (Biocare Medical) at 1:400 dilution. Twenty-four hours later, a biotinylated rabbit link (Covance) was applied for 30 minutes at room temperature followed by a tap water rinse and PBS bath. This was followed with a 4+ streptavidin AP label (Biocare Medical) at room temperature and rinsing with PBS. Vulcan Fast Red (Biocare Medical) was applied at room temperature. The samples were counterstained with Harris hematoxylin, decolorized, rinsed in tap water followed by routine dehydration, and cover slipped using permount. Cleaved caspase 3+ cell number was quantified manually by a blinded review of histologic sections. All tumors were harvested from mice that were sacrificed when moribund (five sections per animal, three animals per treatment group).

Immunoblot

GL261 and human glioma patient cells were washed, pelleted, and lysed in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors (Pierce). Protein concentration was determined, lysates were diluted in reducing sample

buffer and run on 4-12% SDS-PAGE. Gels were then transferred to nitrocellulose, blocked, and incubated in 1:2000 anti-human ANXA2 (BD biosciences) in blocking buffer. Blots were then washed and incubated in 1:20,000 anti-mouse IgG HRP (Jackson ImmunoResearch). After additional washing, nitrocellulose was then incubated in ECL Plus chemiluminescent substrate (GE), drained, and exposed to HyBlot CL Autoradiography film (Denville Scientific).

Immunoprecipitation

Cold GL261 lysate was adjusted to a concentration of 2 mg/mL. 10 µL antibody was added to 500µL lysate followed by overnight incubation at 4°C with rocking. 40 µL Protein A/G Agarose (SantaCruz) was added and incubated at 4°C with rocking. Beads were then washed, putting the final pellet in 80 µL Laemmli reducing sample buffer. Gels were run and blotted using the same antibodies as for precipitation: anti-mouse TLR2 (Clone T2.5, SantaCruz), anti-mouse ANXA2 (Clone 5 BD Biosciences), and anti-mouse IgG HRP (Jackson ImmunoResearch).

Bone marrow-derived dendritic cells and TLR activity

For dendritic cell maturation experiments, bone marrow-derived dendritic cells (BMDCs) were made using a protocol modified from that of Inaba et al (196). Briefly, femurs and tibias were removed and flushed with saline. Erythrocytes were lysed, and remaining leukocytes were washed and placed in 10 cm dishes at 2 x 10^6 cells/10 mL in complete RPMI 1640 and 20 ng/mL GM-CSF (Peprotech). Non-adherent cells were removed and media was replaced every three days. Six days post-bone marrow harvest,

loosely adherent cells were washed and placed at 100,000 cells per well in 200 μL RPMI/GM-CSF, in loosely capped polystyrene FACS tubes.

HEK293 Blue cells stably express an AP-1 and NF-kB inducible secreted alkaline phosphatase gene and hTLR2 or hTLR7 (InvivoGen). HEK293 and BMDC were stimulated with 50 ng/mL Pam_3CSK_4 (Imgenex), 2.5 μg/mL ANXA2 monomer (ANXA2m; AbD Serotec and Aviva Systems Biology), 6.5 μg/mL purified bovine ANXA2 heterotetramer (ANXA2t; Meridian), 5 μg/mL imiquimod (kindly provided by Dr David Ferguson, University of Minnesota), 2.6 μg/mL annexin V (AbD Serotec), or 200 μg/mL ANXA2-OVA peptides, and control peptides were equimolar to ANXA2-OVA. Forty-eight hours following stimulation, BMDCs were stained and analyzed by flow cytometry, and HEK293 Blue supernatant was assayed for secreted alkaline phosphatase activity using QuantiBlue (normalized to media-only treatment; InvivoGen).

Cross priming and cross presentation

C57BL/6J and *Tlr2*$^{-/-}$ splenocytes were plated at a concentration of 2×10^5 cells/well in a 96-well plate in complete RPMI 1640 media. Splenocytes were pulsed with combinations of the following: 10 μg tumor lysate, 2.5 μg/mL ANXA2m (AbD Serotec), or 50 μg/mL human (h) $gp100_{25\text{-}33}$ peptide (KVPRNQDWL) or the core OVA-derived SIINFEKL/ H-2K^b epitope (EVSQLEQLESIINFEKLTEEWTSSNVM). For measurement of presented chicken ovalbumin (OVA) peptide, splenocytes were harvested, stained for CD11c, MHCII, and SIINFEKL-H-2K^b, and analyzed by flow cytometry as described below. For measurement of IFNγ, after 24h, Pmel CD8$^+$ T lymphocytes, that express a Vα1/Vβ13 transgenic T cell receptor specific for a H-2D^b-

restricted epitope corresponding to amino acid positions 25-33 of gp100, were purified from spleen (197) (Miltenyi Biotec), and 2×10^5 per well were co-cultured with original splenocytes. Forty-eight hours after co-culture, IFNγ was quantified in media using a flow cytometric bead array (BD Biosciences).

Cross priming was tested *in vivo* through sequential daily vaccination as described previously (198). Briefly, non-tumor-bearing mice were vaccinated four days in a row with 100 μg chicken ovalbumin protein alone or in combination with the following: (1) 1.5 μg ANXA2m (AbD Serotec), (2) 50 μg CpG 685 ODN (SBI Biotech; Tokyo, Japan), (3) 65 μg GL261 lysate (control or shANXA2(199)); or (4) with 50 μg peptides, with equimolar amounts of control peptides. Mice were boosted three days following the fourth vaccine, and bled the following day for analysis.

Flow cytometry

Analyses of labeled cell suspensions were performed on a custom Canto II (BD) and analyzed with FlowJo software (Tree Star, Inc.). Fluorochrome-conjugated mAbs used were CD11c—FITC (eBioscience, clone N418), CD86—PE (eBioscience, clone GL1), SIINFEKL-H-2K^b—PE (eBioscience, clone 25-D1.16) CD80—APC (eBioscience, clone 16-10A1), CD8a—APC (eBioscience, clone 53-6.7) and I-A/I-E—eFluor450 (MHC II; eBioscience, clone M5/114.15.2). The corresponding isotype controls were also purchased from eBioscience. A PE—dextran-conjugated multimer of SIINFEKL—H-2K^b was purchased from Immudex (Copenhagen, Denmark).

CMV antigen-specific CD8 T cell activation by human monocyte-derived dendritic cells

5 x 10^5 HLA-$A2^+$ immature DCs were pulsed with 2.5 μg/mL ANXANXA2m, with or without addition of 10 μg/mL of $pp65_{495–503}$ (NLVPMVATV) and matured as described previously(133, 196). Following maturation, DCs were washed 3 times, and 5 x10^5 PBMCs from cytomegalovirus (CMV) sera-positive donors were added to DCs. CMV sera-negative PBMCs were used as a control. Cells were incubated for 48 hours, and supernatant was analyzed for IFNγ by cytometric bead array (BD Biosciences).

Peptide synthesis

A 15 N terminal ANXA2 peptide, a 36 amino acid N terminal-ovalbumin peptide fusion ("ANXA2-OVA"; STVHEILCKLSLEGDHSTPPSAYGSVKPYTNFDAE), S100A10-binding domain-truncated form of ANXA2-OVA (ANXA2-OVA-p11; DHSTPPSAYGSVKPYTNFDAEEQLESIINFEKLTEWT), and scrambled ANXA2-OVA (GSCTESIEALHVLELVSPYTKSHNTPDSKGDYPFAEQLESIINFEKLTEWT) were synthesized (Genscript and New England Peptide), and confirmed by sequencing.

Subtractive proteomics

TLR2 binding molecules enriched in 5% O_2 GL261 lysates were identified through precipitation with a in 5% O_2 and 20% O_2 GL261 lysates. Following precipitation, proteins were separated by molecular weight with SDS-PAGE, and protein bands were visualized by silver stain. Immunoprecipitation. GL261 tumor cells cultured in 5% or 20% O_2 were washed, pelleted, and lysed in cold RIPA buffer containing protease and phosphatase inhibitors (Pierce). Protein concentration was determined using BCA colorimetric method (Pierce). Cold GL261 lysate concentration was adjusted to 2mg/ml with 500 μL transferred to microfuge tube. 10 μL TLR2 homodimer—F_c

chimeric protein (R&D Systems) was added to lysate followed by overnight incubation at 4^0C with rocking. 40 µL Protein A/G Agarose (SantaCruz) was added to lysate and incubated 4 hours at 4^0C with rocking. Beads were then washed 8 times in cold TBS putting final pellet in 80ul Laemmli reducing sample buffer. Sample were heated at 95^0C for 5 minutes, centrifuged at 16.1 x g for 1 minute and supernatant was run on 4 – 12% gel. Gel was stained with silver (BioRad) and slices of acrylamide were excised from gel based upon Western and Coomassie staining results. Dry pieces of excised acrylamide treated with Acetonitrile/Water, dried, digested into peptides, extracted and run on LC-MS/MS for protein identification.

Endotoxin testing

Recombinant proteins were assayed for endotoxin using the manufacturer's instructions for two commercially available kits (Genscript and Pierce).

RESULTS

Enhanced immunogenicity in cultures from 5% O_2 is TLR2-mediated.

Increased adjuvant activity in glioma lysates from 5% O_2 cultures led to the hypothesis that glioma cell culture in physiologic oxygen enriches for DAMPs that activate PRRs on DCs (133, 135), and that loss of the cognate PRR should therefore eliminate the benefit of vaccines from 5% O_2 cultured tumor cells. TLR2 is a PRR implicated in inflammation following hypoxia in the brain and kidney (200-202). To determine if TLR2 is required for recognizing DAMPs we measured CD8 T cell responses in the setting of *Tlr2* deficiency. *Tlr2*$^{-/-}$ splenocytes were unable to enhance cross priming *in vitro* after addition of lysate from 5% O_2 (Fig. 4.1A), and vaccination of

glioma-bearing *Tlr2*$^{-/-}$ mice with lysate from 5% O_2 failed to eliminate tumor cells (Fig. 4.1B) or improve survival (Fig. 4.1C). These data strongly suggest that TLR2 recognizes a DAMP that is expressed on glioma cells cultured in 5% O_2.

Monomeric and not tetrameric annexin II exerts adjuvant activity through TLR2

To identify the putative DAMPs responsible for increased vaccine efficacy, we screened glioma lysates for TLR2 binding molecules using immunoprecipitation and mass spectrometry. We found the phospholipid binding protein annexin II (ANXA2) was enriched in the TLR2-binding fraction from 5% O_2 tumor cell cultures (Table 4.1 and Fig. 4.2A). Enrichment of ANXA2 in 5% O_2 (Fig. 4.2A), as well as 2% and 1% O_2 (Fig. 4.2B), was confirmed. Binding of ANXA2 and TLR2 was confirmed by co-immunoprecipitation (Fig. 4.2C). ANXA2 is one of thirteen annexin family proteins that bind negatively charged phospholipids and share 4-6 repeats of "annexin fold" domains (203). ANXA2 exists in cells as a monomer (ANXA2m) or a heterotetramer (ANXA2t) of two monomer molecules bound by two molecules of S100A10 (204). To determine if one or both forms bound TLR2, we pulsed ANXA2m and ANXA2t onto *Tlr*2-transfected reporter cells and measured activation by the secretion of alkaline phosphatase (Fig. 4.3 A). The results demonstrate that TLR2 signaling was induced exclusively by ANXA2m (Fig. 4.3A). Reporter cells transfected with *Tlr7* were not stimulated by ANXA2m or ANXA2t (Fig. 4.3B), demonstrating specificity to TLR2. We next tested whether the absence of TLR2 would affect cross priming. Wildtype and *Tlr2*$^{-/-}$ mice underwent sequential daily vaccination (198) with ovalbumin protein, with or without ANXA2m. Wildtype mice receiving ANXA2m in addition to ovalbumin had a two-fold increase in

the percentage of circulating antigen-specific CD8 T cells compared to vaccination with antigen alone (Fig. 4.3C). However, *Tlr2*$^{-/-}$ mice were unable to expand the population of antigen-specific CD8 T cells above the levels generated by antigen-only vaccination (Fig. 4.3C).

Since TLR2 also binds lipopolysaccharide, we sought to determine whether the responses generated in cell culture were endotoxin-related. We compared levels of TLR2 signaling induced by sampled of ANXA2 and of annexin V (ANXA5) that contained equivalent endotoxin levels (0.65 Endotoxin Units/μg protein; Fig. 4.4A). Equal concentrations of ANXA5 failed to induce TLR2 activity (Fig. 4.3A). TLR2 signaling was also eliminated by exposure of ANXA2m to proteinase or heat (Fig. 4.4B), indicating that immunologic activity originated from a protein and not endotoxin. These findings argue that ANXA2m is a unique DAMP in glioma cultures that increases in physiologic 5% O_2 and boosts cross priming.

Annexin II is required for immunogenicity

To determine if the enhanced cross priming responses are due solely to the upregulation of ANXA2 and not other molecules induced by 5% O_2, we compared responses from wildtype GL261 cells with those from a stable ANXA2 knockdown line (Fig 4.5A) (199). Knocking down ANXA2 significantly decreased the expansion of antigen-specific CD8 T cells in healthy tumor-free mice (Fig. 4.5B) and completely ablated any survival benefit of vaccination in glioma-bearing animals (Fig. 4.5C).

Therefore, we next asked whether the addition of ANXA2m to DC cultures could increase cross priming. To address this question, splenic DCs were pulsed with antigen

and glioma lysate from cells cultured in atmospheric (20%) O_2, with or without ANXA2m. The addition of ANXA2m significantly increased surface levels of peptide—MHC I complexes to levels equivalent to those achieved by lysates from 5% O_2 (Fig. 4.6A). In support of the hypothesis that ANXA2m signals through TLR2, splenic DCs from *Tlr2*$^{-/-}$ mice did not show enhanced levels of OVA antigen-class I complexes following stimulation with lysates from 5% O_2 or ANXA2m (Fig. 4.6B). Moreover, ANXA2m induced robust maturation of bone marrow-derived dendritic cells as evidenced by a significant increase in the surface expression of the co-stimulatory molecules CD80 and CD86 (Fig. 4.7A) and secretion of cytokines IL-12p70 and TNF□ (Fig. 4.7B).

To determine if the addition of ANXA2m could enhance CD8 T cell function, splenocytes were pulsed with antigen and glioma cell lysate from cells cultured in atmospheric O_2, with or without ANXA2m. The addition of ANXA2m boosted IFNγ production by more than eight-fold (Fig. 4.7C), indicating further that ANXA2m has the potential for improving cross priming against tumor antigens. Moreover, the addition of ANXA2m to the vaccines extended survival in the EMT6 breast carcinoma model (Fig. 4.8A).

To assess the therapeutic potential of ANXA2 expressed within mammalian cells, we established a transfectoma cell line from murine NS0 plasmacytoma cells, forcing the expression of high levels of ANXA2. An empty vector transfectoma was used as a control. While 10% of plasmacytoma-bearing animals responded to vaccination with empty vector cell lysate, 80% of animals receiving ANXA2-transfected cell lysate had

long-term tumor control with no evidence of disease (Fig. 4.8B). Surprisingly, however, addition of ANXA2m to GL261 lysate from 20% O_2 cultures failed to increase survival, even in the presence of CpG (Fig. 4.9). Taken together, these data indicate that ANXA2m can be used as an adjuvant in various cancers.

Annexin II purified from mammalian cells fails to mature dendritic cells

Annexin II was then isolated from NS0 cells in an endotoxin-free environment (Fig 4.10A); however immune activity was not observed upon isolation (Fig 4.10 B). Circular dichroism measurement of isolated annexin II in the presence and absence of calcium was classical for alpha-helical structure, suggesting secondary folding was in place (Fig. 4.10 C). Isolation was performed in cells cultured in 5% O_2, in the presence of basal epidermal growth factor and fibroblast growth factor, and in the presence of 3-[(3-Cholamidopropyl) dimethylammonio]-1-□propanesulfonate (CHAPS) detergent. While all isolations were successfully confirmed by Coomassie staining of PAGE products, none successfully activated TLR2, and those tested *in vivo* did not increase cross priming (data not shown).

Annexin II is enriched in human and canine tumor cells

Murine and human annexin II and TLR2 exhibit 98% and 74% amino acid sequence homology, respectively (205). Based on these homologies, we asked if ANXA2m had the same adjuvant activity in a human CD8 T cell cross-priming assay. Given our previous studies demonstrated that human glioma lysates from tumor cells cultured in 5% O_2 enhance antigen-specific CD8 T cell responses (133), we first tested if ANXA2 was upregulated in human glioblastomas cultured in 5% O_2. Expression of

ANXA2 was enchanced in 5% O_2 cultures (Fig. 4.11A). In addition, three canine meningioma cultures from Chapter Three showed enrichment of ANXA2 in 5% O_2 culture (Fig. 4.11B), indicating the upregulation of this DAMP occurs in two additional species and in meningiomas.

Annexin II monomer enhances activation of human CD8 T cells

We then tested whether ANXA2 can increase activation of human antigen-specific CD8 T cells. Therefore, we pulsed monocyte-derived dendritic cells from cytomegalovirus (CMV)-positive and -negative donors with a maturation cocktail and the pp65 CMV antigen in the presence or absence of ANXA2m (133, 206). The addition of patient-matched PBMCs led to a ten-fold increase in IFNγ production following ANXA2m addition, indicating the potential for ANXA2m to augment cross priming in human patients (Fig. 4.11C).

Development of an annexin II—antigen fusion vaccine

These studies provide substantial evidence that ANXA2 is an immunologically significant component of tumor cell vaccines. Aside from cell-based vaccines, many clinical trials have been developed that utilize synthesized tumor peptide antigens (207). In particular, peptide fusion vaccines show the advantage of increasing uptake and cross priming compared to mixtures of unfused molecules (208, 209). The full-length ANXA2 protein is not feasible for these types of preparations, so we sought to determine if a fragment of ANXA2 could be made that would boost immunogenicity in peptide vaccines. It is known that the annexin repeat regions of ANXA2 are shared with other annexin family members such as annexin V (203), which failed to activate TLR2 (Fig.

4.2A) or increase cross priming (data not shown). In contrast, the N terminus of ANXA2 is unique to this protein (203). The 15 N terminal amino acids were capable of enhancing cross priming in a *Tlr2*-dependent manner (Fig. 4.12 A). Further, we synthesized an ANXA2 N terminal-SIINFEKL-based fusion peptide and pulsed this fusion onto *Tlr*2-transfected reporter cells. The ANXA2 N terminal-OVA antigen fusion protein exhibited potent TLR2 activation (Fig. 4.12B). We then vaccinated mice with equimolar amounts this fusion peptide or peptides with equal amounts of antigen, but lacking the 15 N terminal amino acids or scrambled N termini amino acids. These experiments demonstrate that the covalent linkage of an antigen with the N terminal fragment peptide of ANXA2 allowed for the expansion of antigen-specific CD8 T cells (Fig. 4.12C).

DISCUSSION

Unlocking the cross priming potential of the dendritic cell is a cardinal requirement of tumor cell vaccines. Patient-derived immune suppression or poor immunogenicity of cell lysates can limit the impact of co-delivered synthetic adjuvants thereby diminishing dendritic cell activation and cross priming. Our previous studies showed how to overcome poor immunogenicity of glioma cell lysates by maintaining cells for vaccination in 5% oxygen (135). This current study demonstrates that 5% oxygen induced the upregulation of annexin II monomer (ANXA2m), a previously unknown DAMP, in glioma cell cultures. We also found that AXNA2m on glioma cells signals through TLR2 resulting in enhanced cross presentation, costimulation and cytokine release. Knocking down the expression of annexin II from tumor cells cultured

in 5% oxygen led to a loss of adjuvant activity, validating that this DAMP is required for increasing cross priming of glioma antigens.

We were unable to purify immunologically active ANXA2 from mammalian (NS0) cells. While we are unable to explain the reasons behind this lack of activity, we confirmed that the protein was still in solution through circular dichroism. One of many possible post-translational modifications to annexin II may be required for activity that may not have been present in the various isolation conditions attempted. SDS-PAGE strongly suggests that post-translational modifications are not present or required in lysates from 5% O_2, given that there is no band height difference between GL261 lysate from 5 and 20% O_2. However, it is possible that modifications that are sensitive to reducing conditions are required. Moreover, mild reducing conditions result in failure of recombinant annexin II to migrate at all in SDS-PAGE, with congestion in the loading well. Repeating survival experiments or cross priming experiments using NS0 transfectomas that express ANXA2 truncations would further strengthen the case that immunologic activity is ANXA2-derived.

Reducing tissue culture O_2 to 5% enhances the immunogenicity of glioma lysates without compromising the growth or viability of cells (data not shown and refs (133, 135). Regulating the concentration of oxygen in tissue culture is straightforward, compatible with almost all cell vaccine strategies, and has been applied in many studies (133, 135, 210) including two clinical trials (NCT01171469, NCT01400672). Decreasing the concentration of oxygen in glioma cultures could easily precede additional strategies of inducing of DAMP exposure or release through chemical or

radiation-induced injury immediately before harvest. Additional preclinical studies should assess the potential for ANXA2 to synergize with the DAMPs released by these means.

Oxygen sensors and ANXA2 are expressed in essentially all cells (145). Immunogenicity may therefore be improved in other tumor cell cultures through ANXA2 upregulation, either by oxygen regulation or other means. In addition to murine and human gliomas, we observed ANXA2 enrichment in primary canine meningioma (data not shown), an aggressive and uniformly fatal brain tumor in dogs (210). Meningiomas cultured in 5% oxygen for vaccine production resulted in a median survival of 645 days compared to 220 days without vaccination (210) supporting the broad applicability of this model.

This study also provides further support for the development of specific antigen peptide vaccines for cancer immunotherapy. We have identified the TLR2-active domain of ANXA2m and synthesized an ANXA2-antigen fusion peptide. Fusion of antigens with TLR agonists, as opposed to co-delivered but unlinked antigen and adjuvant, generate enhanced immune responses by ensuring that DCs and other leukocytes encounter both structures simultaneously (208, 209). ANXA2-antigen fusion peptides could be developed for patients as "off-the-shelf" agents containing common tumor antigens. Alternatively, they could be "personalized" and synthesized after tumor sequencing to identify immunogenic tumor-specific neo-antigens. As the N terminal 15 amino acids of ANXA2 are required and sufficient to stimulate TLR2 activity, a fusion peptide could be as short as 30 amino acids if one or two CD8 T cell epitopes are fused to

the ANXA2 portion. Future work will address the efficacy of ANXA2 peptide fusions alone and in combination with established TLR agonists to induce synergy in preclinical models of glioma as observed in other vaccines (211).

These studies leave open the question of ANXA2 as a potential DAMP *in vivo*. ANXA2 expression is elevated in many tumors (145), and multiple myelomas expressing high levels of ANXA2 have a worse prognosis (212). These data suggest that immunogenic activity is absent or is overridden by other potentially tumor-promoting functions of ANXA2 (199, 213-215), or by tumor-derived immune suppressive mechanisms such as regulatory T cells or myeloid-derived suppressor cells (54, 216). It is also possible that ANXA2m is not present in great enough quantities, is sequestered, or exists in its tetrameric form that does not bind TLR2. The future may bring novel approaches to increase the immunogenicity of ANXA2 within tumor cells as a strategy to increase anti-tumor immunity *in vivo*.

FIGURES

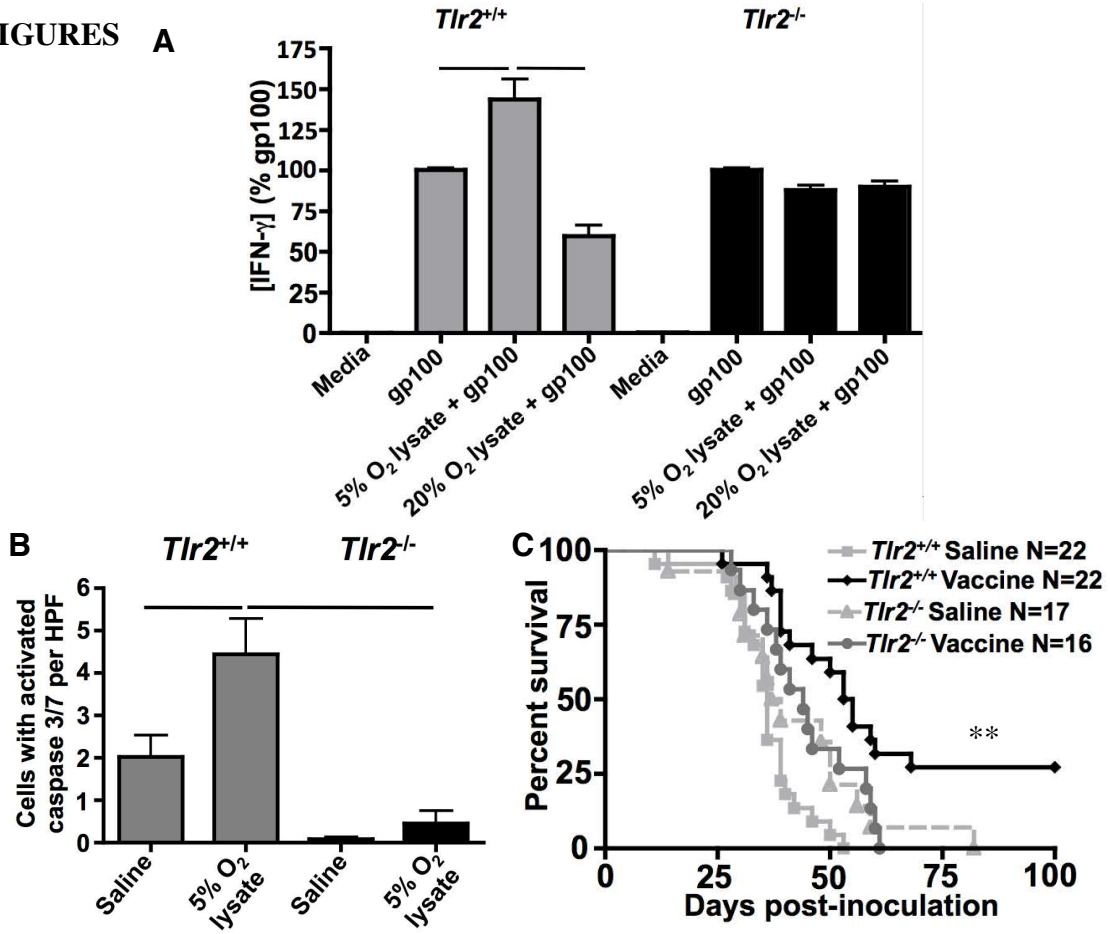

Figure 4.1 Enhanced immunogenicity in cultures from 5% O_2 is TLR2 mediated. A, splenocytes were pulsed with the human $gp100_{25\text{-}33}$ peptide, with or without GL261 lysate from 5% or 20% O_2. Addition of purified pmel CD8 T cells resulted in interferon gamma secretion after 48 h. The experiment was repeated three times with similar results. B, GL261 glioma-bearing mice were given weekly intradermal vaccination of lysate from 5% O_2 culture and CpG ODN, and activated caspase was assessed by immunohistochemistry in tumors at the moribund state. C, survival plot of GL261 glioma-bearing mice given weekly intradermal vaccination of lysate from 5% O_2 culture and CpG ODN. Lines indicate $p<0.05$ by unpaired t-test, and **, $p<0.01$ by log-rank test.

ENRICHED IN 20% O_2	ENRICHED IN 5% O_2
Heat shock protein 9 (73kDa)	Histone cluster 1, H1c (21kDa)
	3-phosphoglycerate dehydrogenase (57 kDa)
	Serum albumin precursor UPS (69 kDa)
	Protein disulfide isomerase associated 3 (57 kDa)
	Ribosomal protein S2 (31kDa)
	Histone cluster 1, H1b (23 kDa)
	Histone cluster 1, H1e (22kDa)
	Chaperonin containing Tcp1, subunit 3 (gamma) (61 kDa)
	Ribosomal protein SA (33 kDa)
	Ubiquitin-activating enzyme E1 (118 kDa)
	Annexin A2 (39 kDa)

Table 4.1: Proteomic hits of TLR2 binding proteins in GL261 lysate. List of proteins identified through LC/MS. Proteins were filtered for those with two or more peptides detected, and identity greater than 90% as determined by Scaffold mass/charge ratio matching.

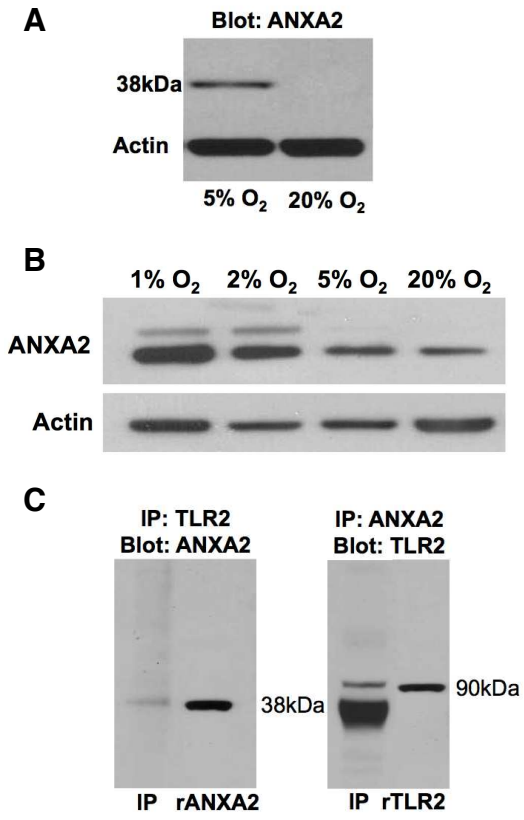

Figure 4.2: Enrichment of annexin II in GL261 cells cultured in four oxygen concentrations. A&B, GL261 cells were cultured in the indicated oxygen concentrations for two weeks, and aliquots were taken for vaccination and half of each aliquot was flash-frozen and blotted for ANXA2 and beta actin as a loading control. C, ANXA2 in GL261 lysates from 5% O_2 culture, co-immunoprecipitated with TLR2 as shown by western immunoblot. Recombinant murine ANXA2 and TLR2 were used as controls.

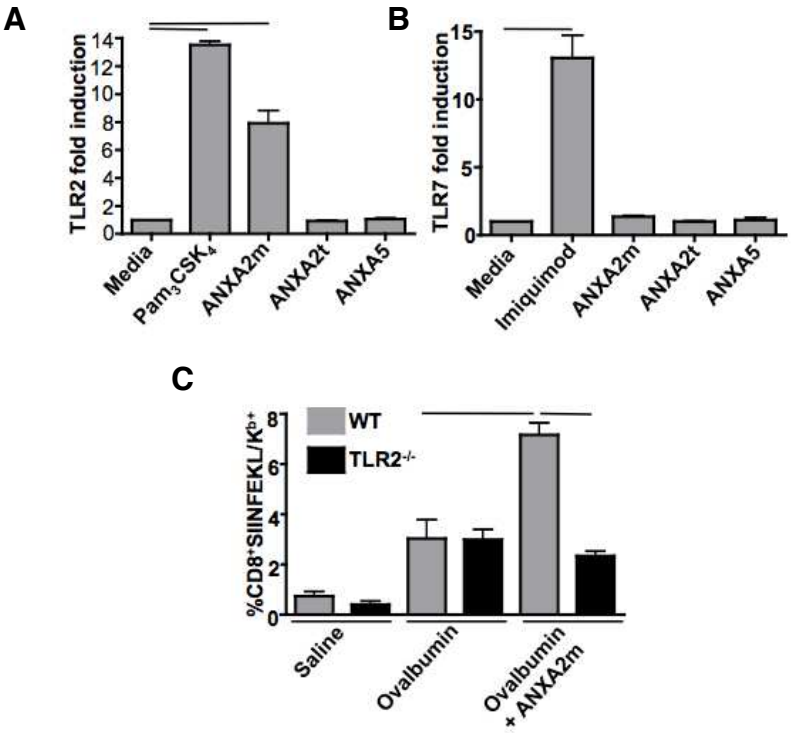

Figure 4.3: ANXA2 monomer increases cross priming through TLR2. Compounds were pulsed onto A, hTLR2- and B, hTLR7-expressing HEK293 Blue® cells, and secreted alkaline phosphatase was measured. C, naïve mice were vaccinated with four sequential daily injections or ovalbumin, with or without recombinant human ANXA2 and boosted once, after three days. Staining of peripheral blood was performed one day after the boost. The experiment was replicated twice with similar results. Bars denote $p<0.005$ by unpaired t-test.

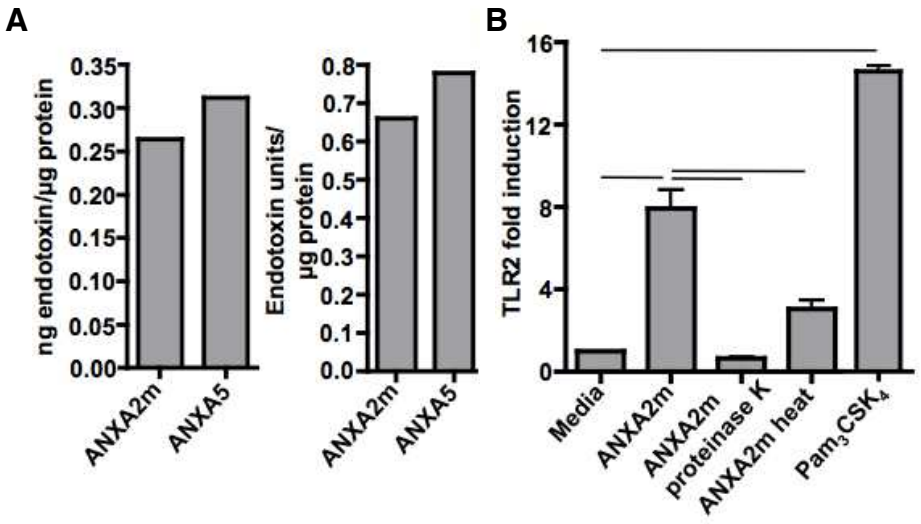

Figure 4.4: ANXA2 monomer activity is protein-derived. ANXA2 monomer activity is protein-derived and induces dendritic cell maturation. A, endotoxin levels were quantified using a Limulus amebocyte lysate kit following the manufacturer's instructions (Genscript). B, ANXA2m and other compounds were exposed to 2 mg/mL proteinase K overnight at 37C or boiling water for 1 hour. Undigested ANXA2m was kept at 37C overnight as a control. Compounds were pulsed onto hTLR2-expressing HEK293 Blue® cells.

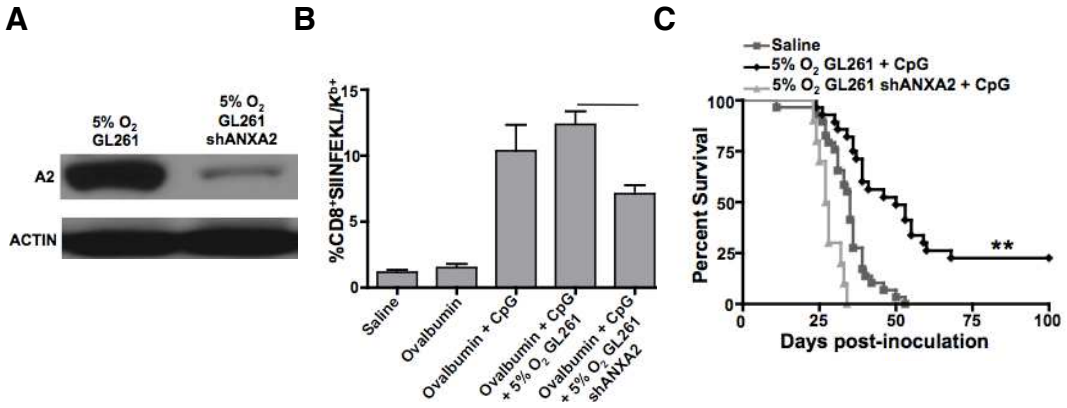

Figure 4.5: ANXA2 is required for adjuvant activity and survival benefit of lysates from physiologic O_2. A, immunoblot confirming knockdown maintenance in 5% O_2. B, naïve mice were vaccinated with four sequential daily injections or ovalbumin, GL261 lysate from two sub-lines, and CpG 685, and boosted once, three days later (n=8, except n=5 for ovalbumin + CpG). Bar shows $p<0.005$ by unpaired t-test. C, Kaplan-Meier plot of weekly vaccination of orthotopic GL261-bearing mice with vaccines from two sublines maintained at physiologic O_2, with CpG 685 ODN (n=30 saline, n=28 5% O_2 + CpG, n=10 5% O_2 shANXA2 + CpG). ** $p<0.0001$ by log-rank test.

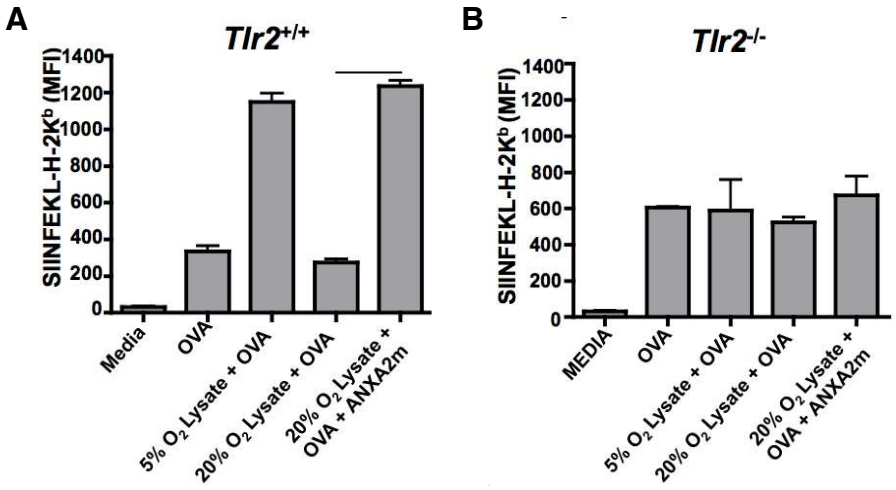

Figure 4.6: ANXA2 is capable of enhancing cross presentation in the presence of poorly immunogenic lysates. A&B, splenocytes were pulsed with the $OVA_{248-274}$ peptide. The SIINFEKL epitope was detected 24 hours later by flow cytometry using an antibody against SIINFEKL/H-$2K^b$. Data represent cells within a $CD11c^+MHCII^+$ gate, and lines represent $p<0.002$.

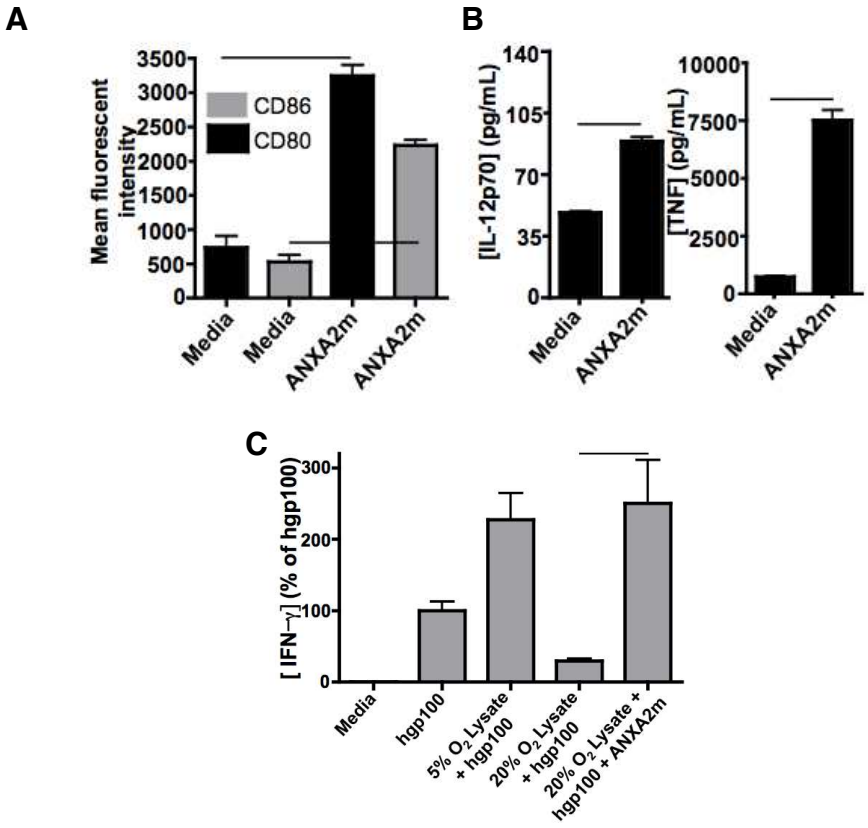

Figure 4.7: ANXA2m induces dendritic cell maturation and increases cross priming. A, bone marrow dendritic cell cultures were pulsed with 2.5 μg/mL commercial recombinant human ANXA2m or media. Cells were harvested and stained after two days. Graphs indicate the $CD11c^{+}MHCII^{hi}$ gated population. B, supernatant from A was collected and assayed using a cytometric bead array. Lines indicate $P<0.01$ by unpaired t-test. C, splenocytes were pulsed with human $gp100_{25\text{-}33}$, with or without GL261 lysate, with or without ANXA2, followed by addition of pmel CD8 T cells. Bars signify $p<0.05$ by unpaired t-test.

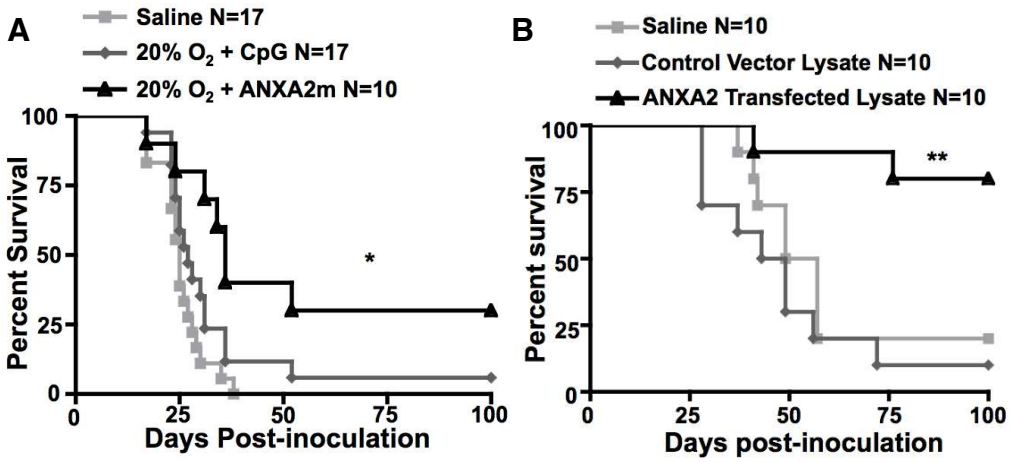

Figure 4.8: ANXA2 is capable of extending survival in the presence of poorly immunogenic lysates. A, Kaplan-Meier survival plot of EMT6 mammary carcinoma-bearing mice vaccinated with the indicated agents (n=18 saline, n=17 20% O_2 lysate + CpG, n=10 20% O_2 lysate + ANXA2m). * $p<0.01$ by log-rank test. B, vaccination of mice bearing NS0 plasmacytomas with lysates from cells containing an empty vector or an ANXA2-expression vector (n=10 per group). ** $p<0.005$ by log-rank.

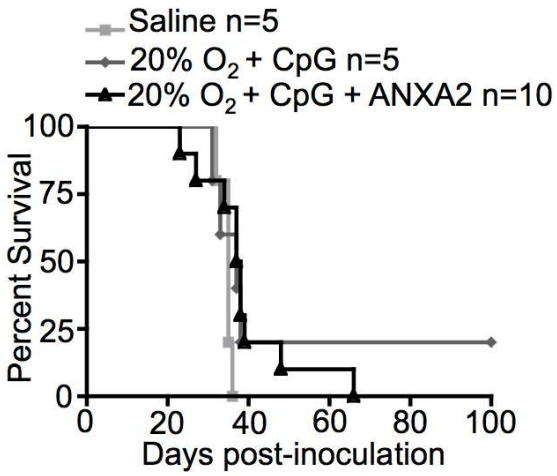

Figure 4.9: ANXA2m fails to extend survival in a murine model of malignant glioma. Orthotopic GL261-bearing mice were vaccinated weekly as in Figures 4.1 and 4.3, but with lysate from atmospheric oxygen, CpG ODN, with or without commercial ANXA2m. Saline was given as a control, and p=0.83 by log-rank analysis.

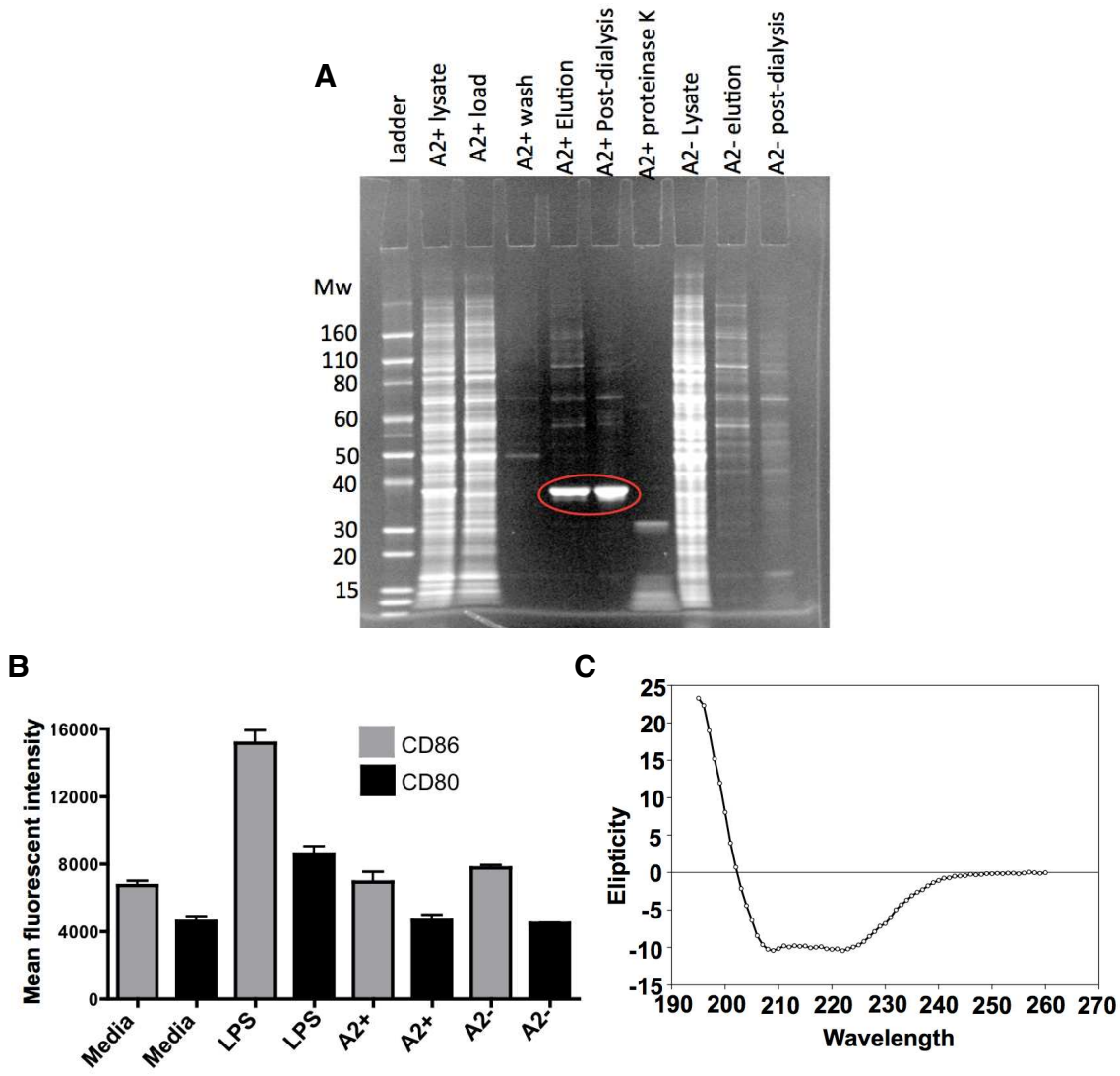

Figure 4.10: Purified ANXA2m from mammalian cells does not induce dendritic cell maturation. A, Coomassie-stained reducing SDS-PAGE of A2 isolation from ANXA2-expressing (A2+) and empty vector-expressing (A2-) NS0 cell lysates from 20% oxygen. B, bone marrow-derived dendritic cell cultures were pulsed with 10 μg/mL isolate from A2+ or A2- cells, LPS, or media. Cells were harvested and stained after two days. Graphs indicate the $CD11c^{+}MHCII^{hi}$ gated population. C, circular dichroism of purified ANXA2 in the presence of calcium, indicating alpha helical structure.

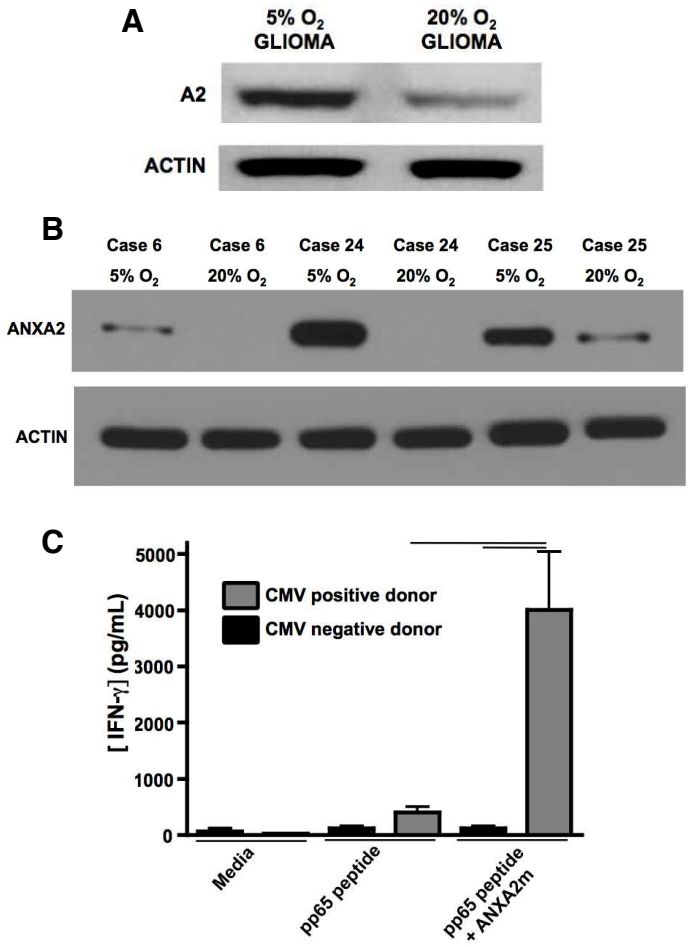

Figure 4.11: ANXA2 enrichment and activity across species. A, western immunoblot of a primary human glioma strain cultured in physiologic 5% or 20% O_2 with anti-human ANXA2. B, primary meningioma cultures from three dogs were kept in 5% or atmospheric (~20%) O_2 for two weeks, cells were harvested, and lysates were separated by SDS-PAGE and probed for ANXA2 and beta actin as a loading control. C, monocyte-derived dendritic cells from CMV sera-positive and –negative HLA-A0201$^+$ donors were pulsed with the $pp65_{495\text{-}503}$ peptide, with or without ANXA2 monomer. After a day cells were washed and autologous PBMCs were added for an additional two days. Interferon gamma was measured by bead array. Bars show $p<0.01$ by unpaired t-test.

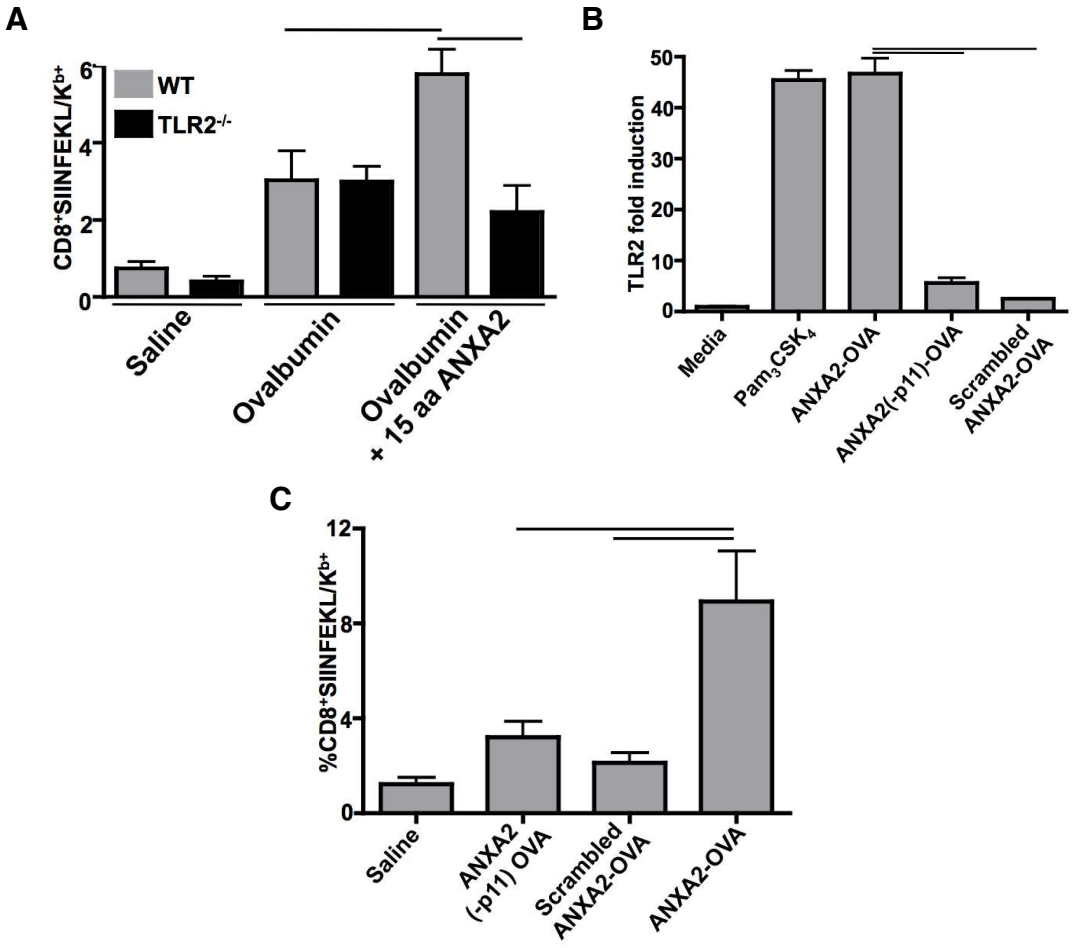

Figure 4.12: N terminal peptides of ANXA2 increase cross priming through TLR2. A and C, naïve mice were vaccinated with the sequential daily injection protocol, with ovalbumin, with or without the ANXA2 15 amino acid peptide or the A2OVA fusion peptide and derivatives. B, peptides of the N-terminus of ANXA2 (ANXA2-OVA fusion, ANXA2-OVA lacking -p11 binding domain, and scrambled ANXA2-OVA fusion) were pulsed onto hTLR2 HEK 293 Blue® cells and secreted alkaline phosphatase was measured. Bars represent an unpaired t-test at $p<0.05$.

CHAPTER FIVE

FINAL DISCUSSION AND FUTURE DIRECTIONS

Contributions and Synthesis of Study Findings

Glioma and recurrent meningioma are two intracranial neoplasms with poor prognoses. Vaccination offers hope to induce long-term control of these tumors with few collateral side effects. While current vaccines show some success in clinical trials, we may improve response rates further if we harness the immunogenic plasticity of cancer cells in tissue culture. Previous to these studies it was known that glioma lysates from 5% O_2 culture increased dendritic cell cross presentation, CD8 T cell priming, and extended survival in tumor-bearing mice. It was believed that certain adjuvant molecules were enriched in 5% O_2 culture, but the molecular mechanisms of O_2 in immunogenicity were unknown. Highlighted herein are important contributions to our knowledge of brain tumor cell vaccines and tissue culture O_2. In addition, connections amongst these chapters have led the formation of testable hypotheses for future work.

Prior to this thesis, the adjuvant effect of glioma cells from 5% O_2 was assumed to be dose-dependent, with the potential to improve efficacy in O_2 tensions below 5% (135). Chapter Two provides a broader perspective by examining four tensions: 20%, 5%, 2%, and 1% O_2. Compared to lysates from 5% O_2, 2% O_2-derived lysates induced greater cross presentation, yet those from 1% O_2 were equally immunogenic to those from 5% O_2. Western immunoblotting revealed that two heat shock proteins, HSPβ-1 (HSP27) and HSP70 increase dramatically between 2% and 1% O_2, which could temper

the adjuvant activity of the lysates. The role of these proteins must also be confirmed by additional studies, but some studies suggest that HSP70 could act as an immune suppressive molecule through regulatory CD4 T cells (139, 217). Annexin II (ANXA2), which binds TLR2 and increases cross presentation and cross priming, was also analyzed. The fact that ANXA2 is further increased from 2% to 1% O_2 appears contradictory to the observed decreased in cross presentation; however, the activity of ANXA2 may be regulated by other mechanisms, for example, through sequestration by a chaperone protein such as HSP70. Lastly in Chapter Two, a glioma survival study demonstrated that lysate from 2% O_2 failed to increase survival, despite higher levels of ANXA2 than those from 5% O_2. These findings suggest further complexity; while ANXA2 can overcome suppression from 20% O_2-derived lysates, it appears that higher levels of ANXA2 in 2% O_2 lysates are inactive or over-powered, either by a modification of ANXA2 itself, or by other molecules induced by 2% O_2. Further investigation into the difference between lysates from 1% or 2% and 5% O_2 may lead to new insights into important structural modifications to ANXA2 or to discovery of novel immune suppressive molecules. In addition, we must also characterize the impact of ANXA2 on the CD4 helper T cell response, the B cell response and other innate anti-tumor responses in the future. The interactions among these responses may shed light on the complex relationship between ANXA2 upregulation below 5% O_2 and inferior vaccine responses.

Findings on the suppressive properties of glioma lysates from atmospheric (20%) O_2 from Chapters Two and Four suggest areas of future investigation. In Chapter Two, lysates from 20% O_2 inhibit cross presentation and cross priming *in vitro*, and

vaccines from 20% O_2 culture are no better than saline (135). Chapter Four shows that the suppressive properties of these lysates depend on TLR2 *in vitro*. Further, mass spectrometry from Chapter Four identified one TLR2-binding protein candidate immune-suppressive molecule—heat shock protein A9 (HSPA9, or mortalin). HSPA9 was enriched with 100% probability in GL261 lysate from 20% O_2 and 0% probability in lysate from 5% O_2. Future studies should test the hypothesis that HSPA9 is an immune-suppressive molecule in GL261 cells from 20% O_2. Lentivirus-mediated HSPA9 knockdown has been achieved in other murine cells (218) and could be attempted in GL261 cells, with the proper lentiviral knockdown controls. This and other loss-of-function approaches should be used to assess the role of HSPA9 in immune suppression. As mentioned previously, CD200 and other putative suppressive molecules must also be investigated. Once we have confirmed the suppressive role of these molecules, future studies could seek to find O_2 tensions or other means to deplete or prevent expression of these molecules in cells harvested for vaccination.

Chapter Two also explored the O_2 sensors within GL261 cells that initiate the shift in immunogenicity. The mechanisms orchestrating increased immunogenicity are mostly unexplored, but with exciting potential for future experiments that could improve glioma cell vaccines. Desferrioxamine (DFX) is a hypoxia-mimetic that chelates iron, inactivates prolyl hydroxylases and stabilizes HIF 1α and 2α. DFX treatment modestly increased immunogenicity of GL261 cells from 20% O_2 within 48 hours. The concentration and duration of DFX used were thought to induce HIF 2α levels comparable to culture in 1% O_2 (129). Given 1% O_2 was less effective at improving

cross presentation, it is possible that further optimization of DFX dose will enable greater immunogenicity. Despite data indicating DFX can rescue GL261 lysates from immune suppression, a clear connection between HIFs and immunogenicity must be established by loss-of-function of HIF 1α or HIF 2α in GL261 cells used for lysate production. If either HIF is required for increased immunogenicity, HIF stabilization may be causing an increase in annexin II (ANXA2). As of yet, however, the direct connection between HIFs and ANXA2 is not established. Binding of HIFs to the ANXA2 promoter and other gene loci was not observed in MCF-7 breast carcinoma cells on which HIF1α and 2α ChIP-seq was performed (219), and no O_2-linked regulators of ANXA2 have been detected by ChIP-seq of several common cell lines analyzed by the ENCODE project (220). Additional evidence, however, supports the possibility that ANXA2 is indirectly regulated by HIFs. Specifically, osteoblasts upregulate ANXA2 in low O_2, and this upregulation can be recapitulated in atmospheric O_2 by exposing cells to VEGF, a known HIF-regulated protein (142). If a clear connection does exist between HIFs and ANXA2 expression, HIF knockdown should also result in decreased expression of ANXA2, and loss of immunogenicity in the absence of HIFs should be rescued by ANXA2 overexpression. These and further experiments may shed new light on the mechanisms of immunogenicity that are switched on within glioma cells cultured in 5% O_2.

The general applicability of these findings to other tumor cell types is warranted from these studies. While preliminary experiments suggest that not all cell types respond to low O_2 culture by upregulating ANXA2 and increasing immunogenicity (data not shown), these data were collected when 5% O_2 incubators were exposed frequently to

atmospheric O_2 by incubator door opening by other scientists. Such interruptions in 5% O_2 culture have been shown by western blot to be correlated with lower ANXA2 expression in GL261 cells. In contrast, when GL261 cells are harvested on Mondays, after a weekend of uninterrupted 5% O_2, they express higher levels of ANXA2 (data not shown). The findings of Chapter Two also present the possibility that various tumor cell cultures, perhaps even gliomas from different patients, could have different immunogenically optimal O_2 tensions. The clear path to establishing whether these findings are more widely applicable is through empiric screening of many tumor cell types and primary cultures from multiple patients.

High-grade meningioma has no standardized salvage treatment after re-operation, nor is there an established meaningful model of high-grade disease. Vaccination is potentially a powerful strategy for salvage therapy, but prior to this thesis, it had not been attempted due to a lack of meaningful model. Chapter Three tests autologous lysate vaccination for spontaneous canine meningioma, a uniformly fatal tumor that is a faithful model of aggressive human meningiomas. Primary meningioma cultures were maintained at 5% O_2, which enriched for ANXA2 compared to cultures from 20% O_2. Vaccination of dogs extended survival more than three-fold compared to historic controls. Based on relative enrichment of ANXA2, we would have expected strong cross priming responses; however, dogs had robust humoral responses against their tumors and rare T cell responses. Antibodies raised from vaccination recognized other meningiomas from other study dogs and one human. These antibody responses were capable of antibody-dependent cell-mediated cytotoxicity. While a direct

comparison with 20% O_2 lysate vaccination is needed to make firm conclusions on O_2's influence on vaccine efficacy, these results indicate that robust induction of antibody responses was induced by vaccines from 5% O_2. In contrast, in murine glioma studies, vaccination with lysate from 5% O_2 augmented CD8 T cell responses and decreased clonality of tumor-reactive antibody responses (135).

Many potential mechanisms could explain the apparent dominance of the B cell and antibody response and minimal T cell response in dogs vaccinated for meningioma, versus mice vaccinated for glioma. First, PBMC samples from dogs were collected two weeks after the most recent vaccination, which is after the contraction of the T cell response in mice and humans (221, 222). In addition it is known that antigen dose can impact the timing and extent of the CD8 T cell response (221). The normalized canine vaccine dose was dramatically lower than that of mice in the O_2 glioma studies (0.02 versus 3.65 mg/kg) (135, 210). Therefore proliferation may have ceased before large numbers of tumor-reactive cells reached a detectible frequency in the blood. It is also possible that meningioma-reactive CD8 T cells were present in other tissues but not the blood. Postmortem samples were analyzed by immunohistochemistry (IHC) following death in glioma-bearing mice and dogs treated for meningioma. Dogs in the meningioma study had an abundance of B and plasma cells, with mild T cell infiltrates, arguing against the theory that the T cells were more abundant in other tissues than the blood. In contrast, in the murine publication that first described the benefit of vaccines from 5% O_2, glioma-bearing mice were euthanized at a moribund state and stained for CD3. These mice were not assayed for B cells; however, mice receiving similar vaccines have shown

B cell and plasma cell infiltration, and a dependence on B cells for vaccination (Murphy *et al*., in preparation). Another potential explanation for the differences in relative T and B cell responses seen by IHC could be that there was essentially no tumor burden in the dogs that were examined, while there was extremely high tumor burden in the mice. Though one would expect that both B and T lymphocytes would increase in the presence of increased antigen, the relative influx of cells may be more similar if antigen loads were more comparable between these two models.

Expression of TLRs likely plays a role in the perceived differential vaccine response between mice and canines to CpG, ANXA2, and imiquimod. Whereas TLR2, the receptor for ANXA2, is expressed broadly in murine cells, including lymphocytes, it is expressed on canine granulocytes, monocytes, and a minor but as-of-yet unidentified sub-population of lymphocytes (223, 224). Murine TLR7 and 9 (which sense imiquimod and CpG respectively) are expressed in myeloid lineage cells (225), including most dendritic cells, canine TLR7 expression is not known, and TLR9 is expressed on canine granulocytes, monocytes, and all lymphocytes (224). Peptide sequence of annexin II, TLR2, TLR7, and TLR9 suggest that the molecular interactions of receptor and ligand are similar. Peptide sequence homology of annexin II is highly similar among human, dog, and mouse (99% and 98% homology to human, respectively) (205). Likewise, dog and mouse TLRs 2,7, and 9 are 67%, 79%, and 75% homologous, respectively (205). While homology comparisons may give a rough picture of the relative similarity is responsiveness to a given ligand, empiric comparisons must be carried out to determine if the quality of the responses truly differ. Further testing of many cytokines and cell

responses under equal experimental conditions would have to be done to draw comparative conclusions.

Despite unclear explanations for the differences between murine glioma and canine meningioma studies, the successful outcomes in this canine study could lead to identification of relevant meningioma antigens and potential damage-associated molecular patterns (DAMPs). One antigen recognized by post-vaccination antibodies in dogs was HSP60. A previous study reported an upregulation of HSP60 upon hypoxia in the brain (226). While this study suggests HSP60 may be enriched in 5% O_2, our blotting of HSP60 in GL261 cells cultured in four O_2 tensions did not show significant O_2 dependence. The significance of HSP60 is unknown, and further loss of function and gain of function cross priming studies need to be carried out in GL261 cells to make definitive conclusions. Moreover, further antigen identification will be investigated from dogs vaccinated for meningioma, using pre- and post-vaccination sera combined with molecular analysis that provides hints as to the more tumor-specific antigens for targeting in the future.

Prior to these thesis studies, the molecules responsible for the enhanced immunogenicity of glioma cells from 5% O_2 were unknown. Chapter Four characterizes the annexin II monomer (ANXA2m) as a key DAMP responsible for increased immunogenicity of lysates from 5% O_2. Proteomic comparisons of whole glioma lysate from 20% and 5% O_2 would generate overwhelmingly large numbers of candidates to test manually. Therefore, a crucial advance came when vaccination was found to be ineffective in hosts null for *Tlr2*. Immunoprecipitation of TLR2-binding molecules in

GL261 lysates therefore enabled distillation to a small list of candidate DAMPs, including ANXA2. Purified recombinant ANXA2m bound and activated TLR2, unlike the ANXA2t, strongly suggesting that ANXA2m operated in lysates from 5% O_2. Knocking down ANXA2 in GL261 cells led to vaccine failure, indicating ANXA2 was required for the efficacy of vaccines from 5% O_2. ANXA2 increased cross presentation, cross priming, and survival of tumor bearing animals when combined with poorly immunogenic lysates from atmospheric O_2, suggesting a dominant effect over putative immune-suppressive molecules. Patient glioma cultures also upregulated ANXA2 in 5% O_2 and showed profound stimulatory activity when ANXA2 was covalently linked to antigen for cross priming.

Following several observations, the immune active domain of ANXA2m was pinpointed to the N terminus through synthesis of N terminal peptide fragments. Binding of TLR2 and enhanced cross priming were accomplished with the 15 N terminal peptides of ANXA2, making feasible synthesized peptide vaccination. Since covalent linkage of adjuvant with antigen has improved immune responses in several systems (208, 209), the N terminus of annexin II was linked to a CD8 T cell epitope of chicken ovalbumin as a proof-of-principle. Following identification of relevant peptide epitopes, off-the-shelf brain tumor vaccines may be synthesized, as peptide fusions with the N terminus of ANXA2.

Additional putative DAMPs were enriched in a proteomic screen of TLR2-binding proteins enriched in 5% O_2. Further studies must be carried out to investigate the roles of these proteins. A logical progression would be similar to that taken during

investigation of ANXA2: confirm enrichment and binding by immunoblot, followed by testing the requirement of such molecules for immunogenicity, then ability to gain immunogenicity upon addition of isolated protein. Given that knockdown of ANXA2 eliminated all survival benefit from vaccination with lysate from 5% O_2, it is unlikely that other proteins will induce a sufficient gain of function upon addition of other putative DAMPs to vaccines with lysate from atmospheric O_2. Such evidence suggests that ANXA2 is dominant within this system.

From a translational perspective, immunoblots of ANXA2 in canine meningiomas highlight inter-tumor heterogeneity among tumors in 5% O_2. ANXA2 levels in three tumors varied greatly, raising the question of whether a standard O_2 tension should be used for culture of cells for vaccination. One alternative is O_2 titration of individual tumor cultures to achieve a high enough level of ANXA2 expression. Given the results of Chapter Two, indicating a "sweet spot" in O_2 level and immunogenicity, empiric murine studies or *in vitro* assays of canine cell lysates must be run to determine which oxygen content yields the most immunogenic lysate among many primary tumors. Immunogenicity should then be traced back to ANXA2 expression and perhaps other candidates that could play a key role as players in vaccine efficacy.

Perspective

Cultured tumor cell components are a source of many unidentified patient-specific antigens and have great potential to prime polyclonal responses. Whole brain tumor cell lysate vaccines can be made immunogenic through various manipulations in tissue culture, thus realizing their potential. These manipulations may enable enrichment

and identification of immunogenic molecules or antigens using new knowledge of the immune system and the immunogenic mediators within tumor cells. The seemingly infinite array of molecules present within tumor cells is, in fact, large; however, it is finite, and better characterization may enable alteration of a small number of relevant immunogenic determinants. We knew previously that decreasing O_2 levels in glioma cell cultures causes enrichment in adjuvant molecules that increase cross priming. This thesis sought to increase understanding of this effect for more rational design of brain tumor cell vaccines and for the potential development of immunogenic structures as purified vaccine components in the future.

For widespread success in immunotherapy, we will need a number of simultaneous approaches. Tumor cell vaccines have shown great promise when combined with immune checkpoint inhibitors to overcome tumor-derived immune suppression. Findings from this thesis and others suggest that we have been introducing suppressive factors within tumor cell vaccines, but removal of these factors or addition of immunogenic factors can turn the tables for increased efficacy. With proper manipulation of O_2 and other tissue culture treatments to release other DAMPs, brain tumor cell vaccines could increase patient survival.

This thesis exemplifies the importance of not simply understanding the tumor to be targeted, or how to best activate the immune system, but how the two entities interact to affect disease outcomes. Increasing knowledge of one's own immune system will reveal new avenues for immune activation and cancer vaccine approaches. As well, increased knowledge of brain tumors and how we can shape their phenotype to our

advantage will enable us to better attack them at their weakest points to induce immunogenic responses in the tumor *in situ*. Knowledge from both sides is indispensible for knowing how to bring vaccination with tumor cells into standard neuro-oncology practice. Through the study of how cultured cells can activate the immune system through culture-induced conditions, this thesis contributes to both aspects at their intersection.

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