Immune-based tumor regression induced by Fc-OX40L in a murine model of glioma

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Dedication

This dissertation is dedicated to my family and friends who have seen me through these years in graduate school. I am eternally thankful for the love and support you have given me.

Abstract

Glioblastoma multiforme (GBM), the most aggressive form of glioma, is a lethal brain tumor; despite current standard of care interventions the median survival remains a dismal 15-19 months. Immunotherapy has been shown to be a promising adjuvant therapy for malignant brain tumors although there are significant hurdles that need to be overcome in order to mount an effective immune response capable of tumor elimination. These challenges include lack of adequate endogenous antigen presentation and the immune suppressive nature of the tumor microenvironment. Vaccination with tumor-derived antigen has sought to overcome the issue of inadequate antigen presentation. Despite these attempts tumor reactive immune cells have been inefficient at complete tumor elimination. A possible explanation for these disappointing results is the effect of anergy, which results in functionally inactive immune cells and is often induced when antigen is recognized by immune cells without the proper level of costimulatory signals. The focus of this research has been on creating a more effective treatment and elucidating the role of the immune cells involved utilizing mouse tumor models. To test if additional costimulation was necessary to achieve a functional immune response a panel of costimulatory molecules was screened in combination with tumor vaccine (tumor lysate and CpG adjuvant). The costimulatory molecules screened included 41BBL, CD80, GITRL and OX40L, which were fused to the Fc portion of human immunoglobulin. Fc-OX40L in combination with a lysate-based vaccine yielded the most potent

response resulting in complete tumor regression in the majority of animals. Upon tumor rechallenge these animals were capable of rapidly clearing the tumor, suggesting treatment generated immunological memory. Additionally, this therapy has been tested in combination with the standard of care chemotherapy resulting in regression of 100% of glioma tumors, however 80% of these animals developed fatal secondary lymphoid malignancy. These data demonstrate Fc-OX40L has unique and incredibly potent activity against experimental gliomas relative to the other molecules tested. In addition it reveals a potential hazard in combining mutagenic chemotherapeutics with immunotherapy.

Further analysis has aimed to understand the mechanisms that contribute to tumor clearance in this treatment model. Lymphocytes isolated from the draining lymph nodes of vaccine/Fc-OX40L treated animals had enhanced cytolytic activity compared to controls. In addition, T cells infiltrating the brain of vaccine/Fc-OX40L treated animals showed an increased proliferative capacity, indicated by Ki67 staining. Additional analysis has demonstrated a CD4 T cell, Natural Killer, and B cell dependent, and CD8 T cell independent mechanism of action. The presence of tumor reactive antibody in the serum of treated mice and deposition of antibody at the tumor site generated interest in the role that antibody mediated mechanisms play in tumor clearance, which was enhanced by the observation that loss of the Fc receptor negatively affected the ability of animals to control tumor growth. Tumor infiltration of perforin expressing NK, NK

T cells, and neutrophil-like cells in vaccine/Fc-OX40L treated animals may provide a possible mechanism of tumor killing.

These data aim to elucidate the immune mediated mechanisms of tumor clearance. The results indicate a mechanism independent of CD8 killer T cells, which is contrary to conventional theories. Further understanding the necessary effector cells will aid in the design of future immunotherapy approaches.

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List of Abbreviations

WHO	World Health Organization
GBM	Glioblastoma multiforme
EGFR	Epidermal growth factor receptor
PDGFR	Platelet derived growth factor receptor
Rb	Retinoblastoma
BBB	Blood brain barrier
TMZ	Temozolomide
VEGF	Vascular endothelial growth factor
FDA	Food and Drug Administration
TKR	Tyrosine kinase receptor
APC	Antigen presenting cell
DC	Dendritic cell
MHC	Major histocompatibility complex
TCR	T cell receptor
PD	Programmed death
Th	T helper
IFN	Interferon
IL	Interleukin
BCR	B cell receptor
ADCC	Antibody-dependent cell-mediated cytotoxicity

FcR	Fc receptor
CTL	Cytotoxic T lymphocyte
Treg	T regulatory cell
TGF	Transforming growth factor
IDO	Indoleamine 2,3-dioxygenase
MDSC	Myeloid derived suppressor cell
iNOS	Inducible nitric oxide synthase
NO	Nitric oxide
ROS	Reactive oxygen species
Her2	Human epidermal growth factor 2
ACT	Adoptive cell therapy
PAMP	Pathogen associated molecular patterns
TLR	Toll-like receptor
CNS	Central nervous system
Da	Dalton
CSF	Cerebral spinal fluid
GM-CSF	Granulocyte macrophage colony stimulating factor
CLTA-4	Cytotoxic-lymphocyte antigen 4
TNF	Tumor necrosis factor
TRAF	TNF receptor-associated factors
NK	Natural Killer
Tem	Effector memory T cell

Tcm	Central memory T cell
Ig	Immunoglobulin
IACUC	Institutional Animal Care and Use Committee
HBSS	Hanks Balanced Salt Solution
IP	Intraperitoneal
PBS	Phosphate buffer saline
ODN	Oligodeoxynucleotide
i.d.	Intradermal
BIL	Brain infiltrating lymphocytes
IHC	Immunohistochemistry
BLI	Bioluminescence imaging
DLN	Draining lymph node
TNT	Tumor necrosis treatment
LEC	Liver expressing chemokine
NFDM	Non-fat dry milk
TTBS	Tris-Tween Buffered Saline

Chapter 1

Introduction

Background on Brain Tumors

There are approximately 22,000 newly diagnosed cases of malignant primary brain tumors every year in the United States (1). Malignant gliomas account for the majority of these tumors and arise from the glial cells that compose the "gluey" or supportive structure of the brain (1). Depending on the subtype of glial cell they are derived from gliomas can be astrocytomas, ependymomas, oligodendrogliomas, or mixed gliomas. Astrocytomas are the most common malignant glioma and classified by World Health Organization (WHO) standards into four categories; pilocytic (grade I), diffuse (grade II), anaplastic (grade III), and glioblastoma multiforme (grade IV) (2). Grade III and IV are malignant tumors characterized by high cellularity, vascular proliferation, the presence of mitotic bodies, and necrosis (2). Median survival for glioblastoma multiforme (GBM) is 15-19 months and anaplastic astrocytomas 2-5 years (1).

Brain tumors that present at first diagnosis as GBMs are referred to as *de novo* GBMs and occur most commonly in adults. Typically, pediatric brain tumor patients present with low-grade gliomas that can develop into high-grade gliomas, referred to as secondary GBMs. Secondary and *de novo* GBMs are genetically distinct; *de novo* GBMs are most often associated with mutations and amplifications of epidermal growth factor receptor (EGFR), loss of heterozygosity of chromosome 10q, and deletions of PTEN and p16; while secondary GBMs are generally associated with mutations in p53, overexpression of the platelet derived

growth factor (PDGF), aberrations in the retinoblastoma (Rb) and p16 pathway and loss of heterozygosity of chromosome 10q. Despite these differences the morphology of these tumors are comparable and respond similarly to current standard of care therapies (1, 3).

Currently standard of care therapies include surgical resection (when possible), radiotherapy, and chemotherapy. Options have been limited due to the unique nature of the brain. The blood brain barrier (BBB, discussed later) can exclude certain drugs, making some of the pharmaceutics developed for other tumors useless. In addition surgical resection of the entire tumor has proven to be impossible. Even when the bulk tumor is surgically accessible tumor cells are able to infiltrate into normal brain tissues (4). Even in extreme cases where full hemispherectomies were performed the tumor ultimately recurred in the contralateral hemisphere (5). Temozolomide (TMZ) is the chemotherapeutic of choice for brain tumors due to its ability to penetrate the BBB (6). TMZ works as an alkylating agent, causing DNA damage resulting in mutations, leading to double strand breaks and apoptosis of affected cells (6). A recent study has shown that TMZ in combination with radiotherapy was more effective at extending survival than radiotherapy alone. The combination therapy resulted in an increase in progression free survival of 11% at two years compared to 1.8% for radiotherapy alone. However, 5-year progression free survival for the combination therapy is still a dismal 4.1% (7). Even when therapy is successful

at extending survival the side effects can be devastating. All the current methods utilized can result in damage to the surrounding normal tissues and cause long-term neurological problems, especially in pediatric patients whose developing nervous system is particularly susceptible to this bystander effect (8, 9).

The poor prognosis of these tumors and lack of progress made toward improving the outcome for these patients has led many researchers to pursue options outside of traditional cancer treatment protocols. GBMs are highly vascularized tumors, which overexpress vascular endothelial growth factor (VEGF), an angiogenesis mediator, leading some investigators to pursue angiogenesis as a drug target. Bevacizumab (Avastin) is a humanized monoclonal antibody against VEGF that has been used in a variety of cancers including, colorectal, breast, and lung. This drug has recently gained approval from the Food and Drug Administration (FDA) for use in recurrent GBM patients based on several studies that have shown modest increases in progression free survival in patients that received Bevacizumab as a salvage therapy, after conventional therapies failed (10). Other therapies have focused on targeting the pathways that are commonly mutated.

Some of the common mutations described previously involve activation of an autocrine loop in growth factor signaling. Mutations that cause the overexpression of growth factor ligands, such as PDGF, EGF, or VEGF, or

mutations that cause the receptors to be constitutively active are responsible for this phenomenon. PDGFR and EGFR are tyrosine kinase receptors (TKR) that activate the PI3K, RAS, and SRC pathways. PI3K activates mTOR through AKT, which is inhibited by PTEN. Targets for TKRs and the many players in the signaling cascade have been developed and tested in clinical trials for a variety of cancers, including gliomas. Clinical trials with targets for EGFR (gefitinib, erlotinib) or PDGF (imatinib) for high-grade gliomas have failed to induce survival beyond that observed with TMZ (11).

Despite these pursuits, there have been no true advances in patient outcome. Another field of study for glioma treatment is immunotherapy. This therapy has the potential to selectively eliminate tumor cells with limited toxicity and control recurrence through the generation of immunological memory. Many investigators are pursuing this field in a variety of divergent approaches. Although currently there are no FDA approved methods for brain tumors, pre-clinical and early clinical trials show promise that demand closer investigation into the complexity of tumor immunology.

Tumor Immunology

The immune system has a critical role in the development of cancer. It has been recognized since very early on that an intact immune system was necessary for the control of cancer. In 1909 Paul Ehrlich suggested that without the immune

system cancer would be much more common in long-lived organisms (12). Since then many additional studies have supported this observation with numerous mouse models demonstrating that without an intact immune system animals were more susceptible to tumor development and thus the cancer immunosurveillance hypothesis was adopted (12). Schreiber and colleagues have more recently revised this theory and developed the cancer immunoediting hypothesis that proposed that the immune system not only controls tumor quantity, but also quality (12). This process is broken down into three phases; elimination, equilibrium, and escape. The elimination phase involves the innate and adoptive immune system recognizing danger signals or tumor antigens and eliminating the tumor, in many cases before the tumors would be detectable by standard diagnosis (12). In the equilibrium phase the adaptive immune system is responsible for preventing tumor outgrowth. This has been noted in several cases of cancer being transferred between patients through organ donation. In these cases an immunosuppressed patients that received organ donations from immune-competent patient in cancer remission succumbed to malignant disease, suggesting that an intact immune system is capable of controlling tumor development even when it is not completely eliminated (12, 13). Immune escape is necessary for the development of clinical disease and can occur by decreased expression of immune reactive antigens, downregulation of MHC complexes that are necessary to present antigens, and immunosuppressive microenvironment (12). The current understanding that immune evasion is one of the hallmarks of

cancer makes the manipulation of the immune system a prime target for cancer therapeutics.

An effective anti-tumor response, endogenous or induced by immunotherapy, requires a fully developed immune response against the tumor (14). This necessitates that all the components of the immune system are functional; any disruption in this process, from priming to effector function can abrogate the effect of the immune system and lead to tumor outgrowth. Antigen presenting cells (APCs) are the first line of defense in immune surveillance and responsible for recognizing antigen that is either located in peripheral tissues or lymph nodes. Dendritic cells (DCs) are the most potent APC capable of activating the cellular immune response. Naïve DCs are able to engulf and process antigen; these cells become activated when a danger signal, generally associated with viral or bacterial infections or apoptotic cells, are sensed resulting in maturation, causing upregulation of costimulatory molecules, such as CD40, CD80, and CD86. Mature DCs can migrate to lymphoid tissue where they can present processed antigen through the major histocompatibility complex (MHC) to cognate T cells (15). Classically, endogenous proteins are processes and presented via MHC I molecules, whereas MHC II molecules mainly present exogenous antigen. However, pathways exist that allow for crosstalk between these pathways and thus exogenous antigen can be presented on MHC I, and endogenous antigen on MHC II (16). CD8 T cells recognize antigen presented via MHC I and CD4 T

cells by MHC II, through T cell receptors (TCR). When a T cell recognizes its cognate antigen through the MHC-TCR complex it can undergo clonal expansion and adopt an effector function or become tolerant and/or die (17). This outcome is generally determined by the level of costimulation present. The costimulatory ligands CD80/86 are expressed on activated, mature DCs and bind to the receptor CD28 on naïve T cells. This initial round of costimulation results in the upregulation of additional costimulatory receptors on T cells (GITR, OX40, 41BB) and ligands on the surface of DCs (GITRL, OX40L, and 41BBL). The additional coactivation results in pro-survival signals, proliferation and effector function adaptation. If the proper level of costimulation is not achieved these cells do not become effector cells and either become anergic (non-functional) or die.

CD8 T cells are cytotoxic “killer” T cells that can directly induce cell death in target cells. CD8 T cells activated in secondary lymphoid organs require reactivation at the site of effector function by interacting with the antigen/MHC I complex on the target cell. CD8 T cells can induce death through multiple mechanisms, including the production of perforin proteins that create membrane channels in the target cell resulting in leakage of cytoplasmic contents from the cell and allow granzyme proteases to enter into the target cell and induce apoptosis (18). Additionally, ligands such as FasL and programmed death ligand-1 (PD-1L) can induce apoptosis upon ligation to the corresponding receptor on the target cell (reviewed in (19)). CD8 T cells have been the focus

on the vast majority of tumor immunotherapy due to the fact that every nucleated cells in the body expresses MHC I and continually processes and presents antigen through this complex for the purpose of immune surveillance (20). This does indicate that CD8 T cells would be the prime target for an anti-tumor response, however there are many other components of the immune system that have recently shown to be important for a competent anti-tumor immune response.

CD4 helper T cells (Th) can develop into multiple subsets, including Th1 or Th2 cells, upon activation. The Th1 cells are responsible for providing helper function to CD8 cytotoxic cells, which are responsible for direct killing of target cells. Th2 cells on the other hand lead to the activation of the humoral immune response, by activating B cells and inducing antibody production. The helper functions are largely conducted through secretion of cytokines such as interferon (IFN)-γ (Th1) or interleukin (IL) -4 (Th2) (21).

B cells can bind antigen through the B cell receptor (BCR). For full activation the BCR-antigen complex is internalized, antigen is processed, and presented on MHC II to CD4 Th cells (22). The costimulatory molecule CD40L on Th cells is recognized by the corresponding receptor on the B cells and provides the proper level of costimulation to induce activation of the B cell to undergo somatic hypermutation and class-switching (22). Activated B cells have the potential to

become memory B cells or terminally differentiate into antibody-secreting plasma cells. The effector phase of the humoral response requires additional help from the innate immune system to carry out its function. Antibody class-switching is promoted by the cytokine milieu present during activation and can greatly influence the effector function of the antibody response. Antibody coating of target cells can increase phagocytosis, induce complement-mediated cytotoxicity, or antibody-dependent cell-mediated cytotoxicity (ADCC). Certain antibody isotypes are complement fixing, meaning they bind to complement proteins and trigger a cascade of proteolytic cleavage the results in the generation of a membrane attack complex capable of generating holes in the membrane of the target cells resulting in apoptosis (23). Additional classes of antibody have the capacity to bind to Fc receptors (FcR) on the surface of innate immune cells, such as Natural Killer (NK) cells, monocytes, macrophages, and neutrophils, through the Fc portion of the immunoglobulin and induce ADCC (22). NK cells are the main effector cell responsible for ADCC and induce cell death through secretion of perforins and granzymes as described for cytotoxic T lymphocyte (CTL) activity above (22).

Suppressive Function of the Immune System

In addition to all the ways the immune system as evolved to be activated and attack perceived threats, multiple mechanisms of immune suppression are in place and also an important aspect of the immune response. A fine tuned

immune response requires a delicate balance of activation and suppression; too weak of a response results in tumor outgrowth and ultimately death, whereas too aggressive of a response results in autoimmunity and destruction of healthy tissue.

The effector immune cells described previously have adapted ways to control their function. IL-10 is an inhibitory cytokine that is secreted by many immune cells, including CD4 and CD8 T cells and acts to inhibit both the innate and adaptive immune responses (24, 25).

In addition to autoregulatory cytokines, a subset of immune cells exists that have regulatory function as their principle purpose. Regulatory T cells, Tregs, are a subset of CD4 T cells that have the capacity to negatively regulate the immune response. These cells are vital for maintaining homeostasis and preventing uncontrolled immune responses and autoimmunity. However, they also play a role in the immune suppressive tumor microenvironment and therefore it is important to understand how these cells function. Two populations of Tregs exist, naturally occurring Tregs (nTregs) that develop as Tregs in the thymus and inducible Tregs (iTregs) that develop as Th cells and are induced to develop regulatory function in the periphery.

Tregs can exert their suppressive function by secreting inhibitory cytokines (IL-10, transforming growth factor (TGF) -β). Cell-cell contact between Tregs and effector T cells can induce cytolysis through perforins and granzymes. Tregs are also able to modulate the metabolic activity of the effector T cells. Expression of CD25 (IL-2R) is a hallmark of Tregs and it is believed Tregs are able to deplete the IL-2 in the environment through expression of this receptor and that this effectively deprives effector T cells of IL-2, which is necessary for survival (26). In addition, Tregs are believed to act on DCs as well, presumably by inhibiting their maturation, decreasing the expression of costimulatory molecules, and inducing a tolerogenic phenotype by stimulating the production of indoleamine 2,3-dioxygenase (IDO) in targeted DCs (26).

Myeloid derived suppressor cells (MDSCs) are another regulatory subset of immune cells that have a variety of mechanisms for suppressing the immune response. These cells express high levels of the enzymes arginase and inducible nitric oxide synthase (iNOS) both of which use L-arginine as a substrate (27). Reduction of L-arginine availability in the microenvironment by arginase breakdown can inhibit T cell proliferation through decreased expression of the CD3ζ chain and cell regulatory cyclins (27). In addition, iNOS metabolism of L-arginine produces nitric oxide (NO), which can interfere with the Jak/Stat pathway and induce apoptosis in T cells. Reactive oxygen species (ROS) can also be produced by MDSCs and can be induced by factors commonly produced

by tumors, such as TGF-β, PDGF, and IL-10 (27). MDSCs can also induce the nitration of TCRs by peroxynitrate rendering affected T cells unresponsive to their cognate antigen (27).

Ways that Tumors Escape Immune Surveillance

Despite the multitude of ways the immune system has adapted to eradicate tumors, which is effective in the majority of individuals, some tumor cells are capable of evading the immune system. Immune evasion requires a multifactorial approach on the part of the tumor cells. Many tumor cells secrete suppressive factors, such as IL-10, TGF-β, and Prostaglandin $E_{2,}$ that can act to inhibit infiltrating immune cells, rendering them inactive, even if proper priming and trafficking occurs (19, 28). Secretion of IDO by glioma cells degrades tryptophan leading to cell cycle arrest in T cells (19). VEGF is an important growth factor for tumor development known predominately for its role in angiogenesis. However, VEGF is also able suppress the immune response by restricting the maturation of DCs and thus reducing antigen presentation and T cell activation (29). Many GBMs secrete the chemokines CCL-2 and CCL-22, which are responsible for recruitment of Tregs to the tumor site (30). There is evidence that tumor endothelial cells express the FasL, which upon binding to the receptor, Fas, expressed on T cells can induce death. This finding suggests that tumor vasculature may prevent T cell infiltration and action by inducing apoptosis in responding cells (31). Similarly, PD-1L (B7-H1) has shown to be

upregulated on glioma cells and can exert immune regulatory function upon binding the receptor PD-1 on T cells (19, 32).

Additionally, many tumors, including both human and mouse models of glioma, downregulate the expression of MHC (33). As described previously, activated CD8 T cells require engagement of the TCR with antigen/MHC I complex on the target cell in order to initiate killing. The absence of MHC on the tumor surface will render most infiltrating CTLs ineffective.

Immunotherapy

In the 1800's it was observed that cancer patients that developed post-operative infections had better outcomes than those that did not. Based on this observation in 1891 William Coley began injecting bacteria intratumorally in cancer patients to recapitulate these results (34, 35). Although this type of therapy has not become mainstream it opened the door to exploring the immunological response to tumors. Immunotherapy aims to harness the power of the patient's immune system to recognize and selectively eliminate tumors. This therapy is attractive for several reasons; it in theory is capable of being very specific, eliminating the need for destructive radiation and chemotherapy. Additionally, the immune system retains memory that should be capable of responding to and quickly eliminating any recurrence. A variety of approaches to

stimulating the tumor reactive immune response have been examined, including cytokine therapy, passive immunotherapy and active immunotherapy.

Cytokines have the potential to modulate the immune response and have been utilized in tumor patients to activate the immune response. Some examples include IL-2, IL-4, IL-12, IL-23, IFN-α, and IFN-γ. Although pre-clinical animals models demonstrated efficacy, there has been limited success in clinical trials. Delivery of these molecules has been one hurdle. Intratumoral injection of recombinant cytokines resulted in toxicities with no increase in survival compared to controls (36). Currently, viral vectors, plasmid delivery through liposomes or polymer-based delivery systems, and genetically modified cells producing cytokines have been studied for local delivery (36). Cytokines therapy is non-specific, however delivery of these molecules to the tumor site may be able to overcome the immune suppress microenvironment and tip the balance in favor of the anti-tumor immune response.

Passive immunotherapy bypasses the patient's own immune system through the delivery of antibodies against a specific antigen or adoptive transfer of antigen specific cells. Serotherapy, the administration of antigen specific antibodies, has been utilized to target known tumor associated antigens. Human epidermal growth factor 2 (Her2) is overexpressed in approximately 30% of breast cancers (37). Administration of the Her2 antibody (Herceptin) has shown efficacy in Her2

positive breast cancer patients (37). A limitation to this method is the lack of immunological memory formation, which is critical to guard against tumor recurrence. Adoptive cell therapy (ACT) involving transfer of tumor reactive T cells isolated from the patients tumor, stimulated and expanded *ex vivo*, and readministered to the patient has had success in the field of melanoma immunotherapy (38). The limitation to passive immunotherapy is the lack of breadth of coverage. Antibodies or adoptively transferred cells that are specific to one or a few tumor-associated antigens may not be enough for complete tumor eradication. Tumors are often heterogeneous and targeting one subset is generally not enough to eliminate all tumor cells. Additionally, tumors acquire and lose mutations rapidly and this evolution can contribute to immune evasion (39).

Active immunotherapy involves activating the patient's anti-tumor immune response *in vivo*. This process may involve vaccination of the host with tumor cells or lysate. Due to poor antigen presentation, many researchers have turned to loading dendritic cells *ex vivo* with tumor antigen (whole tumor cells, tumor lysate, or peptides) and then administering those activated DCs to the host (36). This approach seeks to allow appropriate antigen presentation to tumor reactive T cells, a key step in activating the anti-tumor immune response. Many vaccines are administered with adjuvants, which aim to enhance the activation and antigen presentation of dendritic cells. As mentioned previously, DCs recognize danger

signals as part of the activation pathway. Many of these signals are pathogen associated molecular patterns (PAMPs) that bind toll-like receptors (TLRs) expressed by DCs. Adjuvants used in the clinical setting attempt to model PAMPs that would be recognized during infection, such as bacterial unmethylated DNA (CpG) or viral dsRNA (PolyI:C), and bind to TLR 9 and 3 respectively (40).

Immunology of the CNS

Immunotherapy for the treatment of brain tumor has been viewed as especially challenging due to the specialized relationship between the central nervous system (CNS) and the immune system. Studies from the 1920's gave the first insights into the different immunological environment of the brain, noting that rodent tumors that were readily rejected in the peripheral tissues were protected from immunological rejection in the brain (reviewed in (41)) . From these early studies the concept of the "immune privileged" brain emerged. The observation that the brain lacks lymphatic vessels and the presence of the blood brain barrier provided an explanation to these early observations. However, work that has been done since then has generated a much more complex image of the tightly regulated workings of the immune system in the CNS and the concept of "immunologically specialized" sites has been adopted.

Ehrlich demonstrated in 1885 that intravital dyes injected into an animal dispersed throughout the various organs but left the brain unstained (reviewed in (42)). This observation was one of the earliest in detailing the existence and function of the blood brain barrier. It is now known that the previous observations were caused by the unique physiology of the capillaries in the brain. Endothelial cells that form the brain capillaries are distinct morphologically from those in the periphery; the lack of fenestrations and the presence of tight junctions between endothelial cells contribute to the physical barrier, as do the astrocyte foot processes and pericytes that envelop the endothelial cells of the capillaries (43). Capillary endothelial cells of the brain show reduced pinocytosis compared to peripheral capillaries and the presence of numerous efflux transporters (43). The existence of these barriers restricts passive diffusion to molecules less than 400 Daltons (Da) (43). Misconceptions surrounding the BBB and the exclusionary properties, following Ehrlich's early work, led many to believe that the BBB effectively eliminated any immune surveillance by blocking both immune cells from reaching the brain (efferent arm) or antigen draining to peripheral lymph nodes, stimulating an immune response (afferent arm). However, subsequent work demonstrated that in fact the efferent and afferent arms of the immune system were intact for the CNS (44, 45). More recent studies have described in great detail the route of efflux (of proteins and potential antigens) out of the CNS (reviewed in (41)). These studies have determined that fluid leaving the brain can enter the blood or drain to the ipsilateral cervical lymph nodes, and that the

concentration of CNS derived proteins is much greater in the cervical lymph nodes than the blood (reviewed in (41). Due to the preferential drainage to the cervical lymph nodes many studies involving immunotherapy for CNS tumors have focused on vaccinations near to this site. Priming at the cervical lymph nodes in theory would boost natural antigen presentation that is occurring as well as take advantage of the pre-existing proximity and bias to the brain. The cervical lymph nodes and the response to CNS antigen have been studied in great detail. Interestingly, cervical lymph nodes have a clear bias in the type of immune response they induce, which has been largely ignored by tumor immunologist in the field of brain tumors. Cervical lymph nodes preferentially induce a Th2 type immune response, which is responsible for the activation of the humoral immune response, but largely ignores the CD8 cytotoxic response (41). It has also been observed that although CD8 T cells in the cervical lymph nodes do respond to antigen by rapidly proliferating, direct CTL activity was not observed, suggesting that the polarized Th2 response prevents development of these cells into fully active killer cells. This is due to the production of TGF–β, a cytokine that suppresses the CTL response, in the CNS (41). Cerebral spinal fluid (CSF) that drains to the cervical lymph nodes contains this cytokine and may be partly responsible for the polarized response. Additionally, the CNS production of TGF-β can also suppress CTLs within the brain rendering this response ineffective (41). This biased response, that appears to be CNS specific, is likely to have evolved due to the physical nature of the brain. The

skull, although protective, does not allow for much room for swelling and thus inflammation in this organ can be catastrophic. Therefore, it is reasonable to believe that the tight regulation on the inflammatory immune response is an adaptation meant to limit damage to this very important site.

Immunotherapy in Brain Tumors

Despite the complex nature of CNS immunology, immunotherapy for the treatment of brain tumors is still an active area of research. Multiple approaches have been taken to the clinic and entered Phase I-II studies. Adoptive transfer of autologous lymphocytes, isolated from peripheral blood and tumor infiltrating lymphocytes, activated *ex vivo*, and reinfused to the patient has been attempted in several clinical trials (46). Active immunotherapy trials have included injection of irradiated, autologous tumor cells. A large number of active immunotherapy studies have focused on DC vaccines. DCs isolated from the patient have been pulsed with tumor lysate, tumor peptides, tumor mRNA, or whole tumor cells, and administered to patients (46). These clinical trials have been well tolerated by the patients and shown promising signs of immune response correlating with radiographic response and modest improvements in overall survival. However these studies have failed to meet the burden of proof of efficacy necessary to achieve phase III trials (47). A series of clinical trials attempted to utilize a common mutation, EGFRvIII, associated with glioma and leads to the constitutive, ligand independent activation of EGFR. The in-frame deletion of

exons 2-7 results in a novel peptide sequence that serves as a neoantigen and is present in approximately 30% of GBM patients. Studies evaluated the efficacy of vaccinations of a peptide-based vaccine and peptide pulsed DC vaccines again showed promising signs of efficacy, however upon recurrence 82% of patients had lost EGFRvIII expression (48). This suggests that immunoediting is at work in response to these therapies. It also indicates a need to target multiple antigens in vaccines in order to avoid immune evasion.

Immunotherapy in Other Tumors

Immunotherapy has been utilized successfully and given FDA approval for prostate cancer and melanoma. A prostate cancer immunotherapy known as Provenge, involves activating autologous APCs with a recombinant fusion protein (PA2024) consisting of the prostate antigen, prostatic acid phosphatase, and granulocyte macrophage colony-stimulating factor (GM-CSF), an immune adjuvant. This therapy showed success and received FDA approval (49). Ipilimumab (Yervoy) is an immune modulatory reagent that recently received FDA approval for the treatment of metastatic melanoma. Yervoy is a humanized monoclonal antibody against cytotoxic-lymphocyte antigen 4 (CTLA-4), a regulatory receptor on the surface of activated T cells. CTLA-4 is upregulated on T cells following T cell activation and competes with the CD28 receptor (also on the T cell) for the ligands CD80/86 which are presented on APCs, in fact CLTA-4 has a higher affinity than CD28 for the corresponding ligands (50). CTLA-4

inhibits the T cell response induced by TCR and CD28 signaling pathways, by blocking IL-2 production and preventing proliferation. By blocking the CLTA-4 receptor with Yervoy, the intent was to allow signaling through CD28 to occur without the inhibition induced by CTLA-4 and eliminate the tolerogenic effects on tumor reactive T cells. In practical clinical trials this end was achieved, many patients saw increase in progression free survival and rarely complete response, however, the majority of patients also development severe autoimmune complications (51). These unwanted side effects warrant further examination before implementing these immune modulatory drugs into general use for cancer treatment.

Costimulatory Molecules in Immunotherapy

The FDA approval of ipilimumab for the treatment of melanoma has opened a new and exciting door in the field of immunotherapy. The use of immune modulatory agents for cancer treatment has been rapidly incorporated into pre-clinical immunotherapy models. Blocking CLTA-4, as ipilimumab does, effectively removes the brakes from the immune response and does so in a non-specific manner. The subsequent licensing of all targeted T cells is likely the cause of the adverse immune related toxicities observed. However, there are many more immune modulators that are promising targets. As previously mentioned, following TCR-MHC engagement and CD28 ligation by CD80/86 several additional costimulatory molecules are upregulated on the surface of the

APC and T cell. Targeting these secondary costimulatory signals is appealing for many reasons, including the fact that these cells have already recognized cognate antigen and received permissive signals through CD28 ligation. This in theory would target only activated T cells and potentially reduce the negative effects seen with non-specific licensing by ipilimumab.

These additional costimulatory molecules include members of the tumor necrosis factor receptor (TNFR) family, such as 41BB, OX40, and glucocorticoid-induced TNFR (GITR). The engagement of these receptors on the T cell by the corresponding ligands (41BBL, OX40L, and GITRL) on APCs results in recruitment of adaptor proteins TNFR-associated factors (TRAFS) which signal through JNK and NF-κB and results in modulating the expression of factors regulating proliferation, cytokine production, and survival signals (52). Signaling through these costimulatory molecules leads to T cell expansion, upregulation of effector cytokine production, and can break tolerance. Despite these similarities there are difference in kinetics of expression and cell subset preference. 41BB (CD137) is expressed on activated T cells, NK cells, NKT cells, DCs and nTregs peaking 48 hours after TCR engagement. Expression occurs more rapidly on CD8 T cells compared to CD4 T cells, however there doesn't appear to be a difference in the proliferative response between the two subsets (53). GITR expression peaks at 24 hours post TCR engagement and is primarily expressed

on nTreges, B cells, macrophages, DCs, natural killer (NK) cells, and activated T cells (53).

OX40 is another costimulatory receptor that is the main focus of this thesis work. Therefore, more attention will be given to the known roles that OX40 plays in immune response. OX40 expression is restricted to activated T cells (preferentially CD4 T cells) and Tregs (53). Some data suggest that CD4 T cells are biased to Th2 response, although more recent data suggests that OX40 signaling does not bias the polarity of CD4 T cells (54). OX40 expression peaks 24-48 hours following engagement and returns to baseline by 120 hours post engagement (53, 54).

When OX40L-OX40 engagement occurs the cell receives several signals necessary for survival. OX40 stimulation results in increased proliferation, cell survival, and effector function presumably through the modulation of several gene expression profiles. Notably, decreased expression of Fas, CTLA-4, and MAD4 followed OX40 ligation (55). Fas ligation leads to cell death and this observation is important not only for the initial rounds of activation but also in the effector phase of these cells. It has been observed that T cell leukemia cells that have been activated through OX40 ligand are resistant to Fas induced cell death (56). This may become important for T cell response in the highly immunosuppressive environment of tumors. Additionally, MAD4 inhibits c-myc,

which is necessary for cell cycle progression and therefore proliferation (55). The downregulation of CTLA-4 following OX40 engagement may be important in the ability of OX40 to break tolerance, by effectively removing inhibitory signals. In addition to signaling through the receptor the OX40L presenting cell also receives a signal resulting in the production of IL-12 by DCs and enhanced B cell differentiation (55).

OX40 has proven to be valuable in the expansion and survival of T cells during primary encounter with antigen; moreover, there is evidence to suggest that OX40 plays a critical role in the generation of memory as well as the recall response of these memory cells. Distinct subsets of memory CD4 T cells exist and can modulate the response generated to re-encounter with antigen. Effector memory (Tem) and central memory (Tcm) T cells differ in location and recall responsiveness. Tcm cells are located in the secondary lymphoid tissues and provide long-term protection to antigen. However, the recall response rate is slower than Tem cells which reside in extra-lymphoid tissues (57). In the absence of OX40 CD4 T cells fail to generate Tem cells as efficiently, while Tcm generation is unaffected (58). This may suggest a bias in the production of Tem CD4 T cells upon OX40 stimulation. The accumulation of Tem cells could potentially result in a more rapid recall response upon recurrent antigen stimulation. Accumulating evidence also exists for OX40 involvement in the

recall response as blocking OX40 signaling hinders the induction of proliferation of memory CD4T cell upon restimulation (reviewed in (57)).

OX40 is also expressed on Tregs. However, in this context OX40 ligation plays a suppressive role on the target cell. In this case, OX40 stimulation results in reduced regulatory functions of the Treg, which in the context of an immune response would allow for the survival and function of effector T cells (57). Additionally, T cells can be induced to become Tregs in the presence of certain cytokines, however, OX40 signaling reduces the conversion of T cells to Tregs, which is also beneficial to a productive immune response (57).

Researchers have sought to manipulate these costimulatory molecules in animal models of various diseases. Control of autoimmune/inflammatory diseases may be achieved by inhibiting costimulatory signaling, while enhancing signaling is the goal in cancer models (59). To achieve activation of these signaling pathways multiple approaches have been taken. Researches have utilized both agonizing antibodies that bind their respective receptors inducing signaling and ligand fusion proteins, where the corresponding ligands are fused to immunoglobulin (Ig) proteins for systemic deliver *in vivo* (53).

In reality, to combat the multifactorial approach tumors have utilized to evade the immune system (lack of antigen presentation and costimulation, immune

suppressive cytokines and recruitment of Tregs) immunotherapy should offer an equally broad response. To date the majority of pre-clinical and clinical approaches have been aimed at studying individual approaches. One benefit of using costimulatory molecules in tumor models is the inherent dual role they play in immune modulation. In addition to being potent stimulators of effector T cell function, the same costimulatory signal on a Treg has inhibitory properties (60-62). Potentially these molecules could play dual roles in reducing the immune suppressive tumor environment and providing the necessary pro-survival signals for a vigorous effector T cell function.

Thesis Statement

The goal of this work is to make improvements to the already established vaccine based immunotherapy approach. In addition to creating a more effective treatment I have sought to elucidate the role of the immune cells involved. Below I will describe how I successfully identified a costimulatory molecule that has proved efficacious for treatment of a murine model of glioma and the insights gained into the effector functions at play.

Chapter 2

An in vivo immunotherapy screen of costimulatory molecules identifies Fc-OX40L as a potent reagent for the treatment of established murine gliomas

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We tested the combination of a tumor lysate vaccine with a panel of costimulatory molecules to identify an immunotherapeutic approach capable of curing established murine gliomas. Glioma-bearing mice were primed with a tumor lysate vaccine, followed by systemic administration of the following costimulatory ligands: OX40L, CD80, 4-1BBL, and GITRL which were fused to the Fc portion of human immunoglobulin. Lymphocytes and mRNA were purified from the brain tumor site for immune monitoring studies. Numerous variations of the vaccine and Fc-OX40L regimen were tested alone or in combination with temozolomide. Lysate vaccinations combined with Fc-OX40L led to the best overall survival, yielding cure rates of 50-100% depending on the timing, regimen, and combination with temozolomide. Cured mice that were rechallenged with glioma cells rejected the challenge, demonstrating immunological memory. Lymphocytes isolated from the draining lymph nodes of vaccine/Fc-OX40L-treated mice had superior tumoricidal function relative to all other groups. Vaccine/Fc-OX40L-treated mice exhibited a significant increase in proliferation of brain infiltrating CD4 and CD8 T cells, as indicated by Ki67 staining. Fc-OX40L had single agent activity in transplanted and spontaneous glioma models and the pattern of inflammatory gene expression in the tumor predicted the degree of therapeutic response. These data demonstrate that Fc-OX40L has unique and potent activity against experimental gliomas and warrants further testing.

Translational Relevance

Protein biologics that activate immunostimulatory pathways or inhibit immunoregulatory pathways are entering the clinic at an unprecedented rate. Comparative studies of this class of agents, given alone or in combination with vaccines and chemotherapy, should inform rational clinical trial design. We performed a screen in glioma-bearing mice to identify a lead candidate for further optimization with the following costimulatory ligands: OX40L, CD80, 4-1BBL, and GITRL. OX40L proved to be most efficacious and synergized with vaccines to cure well-established tumors. The combination with temozolomide, the standard of care chemotherapy for glioma patients, was very efficacious but was associated with secondary malignancies, warranting caution and further study. Inflammatory gene expression signatures in different glioma models provided insight as to how the pre-existing immune response at the time of treatment may predict responders and non-responders. Together, these studies reveal exciting prospects and challenges in applying this class of immune modifiers to clinical trials.

Introduction

Glioblastoma multiforme (GBM) is a lethal brain tumor with a median survival of 15-19 months (63). There is an urgent need for more targeted and effective therapies. Recent studies have shown vaccines to be a promising adjuvant therapy for GBM (reviewed in (47)). Major challenges in the field include the immunologically specialized nature of the brain and the immune suppression documented in GBM patients (2, 64, 65). Vaccines (peptide, dendritic cell (DC), and lysate-based) have been used to overcome inadequate natural antigen presentation by increasing the frequency of tumor-reactive T cells. When tumor antigen is presented to T cells in the absence of proper levels of costimulation, functionally anergic cells can be primed that have poor tumoricidal function. T cell costimulation occurs secondary to T cell receptor (TCR) recognition of antigen by MHC presentation. One of the first costimulatory signals occurs through the CD28 receptor, which is constitutively expressed on naïve T cells, recognizing its ligands, CD80 or CD86. Members of the tumor necrosis factor receptor superfamily are upregulated on T cells following this initial activation. These receptors include glucocorticoid-induced TNFR-related protein (GITR), 4-1BB, and OX40 (53). Engagement of these receptors by their corresponding ligands leads to enhanced T cell survival and differentiation into effector and memory cells.

Prior studies have shown that it is possible to break anergy by delivering agonist antibodies or recombinant ligands that activate costimulatory receptors present on T cells (reviewed in (66)). Blockade of inhibitory checkpoints such as Cytotoxic T-Lymphocyte Antigen 4 (CLTA4) on T cells is another strategy that has gained attention. The recent FDA approval of ipilimumab (a CTLA4 antagonist) for the treatment of metastatic melanoma is a first in class success that has invigorated the field of immunotherapy (67). However, ipilimumab is not specific to tumor-reactive T cells, resulting in severe and long-lasting adverse events in an appreciable fraction of patients (68); this has led some to challenge the rationale for its use (51). Indeed, many questions remain unresolved about the optimal use of these novel immunomodulatory drugs. In the case of drugs that activate T cell costimulatory receptors, what are the relative potencies of agonist antibodies versus recombinant activating ligands? How should such drugs be combined with the standard of care chemotherapy or experimental vaccines? The details of combination therapy, such as dose, timing, and frequency of administration are important considerations that can be preliminarily addressed in preclinical models. Laying this basic foundation of knowledge will enable rational clinical trial design.

We previously characterized immunomodulatory proteins consisting of the C-terminus of the Fc portion of immunoglobulin fused to costimulatory ligands: CD80, GITRL, OX40L, or 4-1BBL (69-72). Comparative studies of an anti-OX40

agonist antibody with Fc-OX40L demonstrated Fc-OX40L was far more potent on a per molecule basis (71), establishing the rationale to develop this class of Fc-ligand fusion proteins. In this prior work, varying the dose and frequency of each drug led to the development of optimized single-agent treatment regimens. These regimens were capable of triggering tumor-reactive T cells and tumor regression in murine models of colon and renal cell carcinoma (69-72). Nonetheless, such single agent approaches rely on endogenous tumor-reactive T cells as targets, which may be too limiting, justifying the investigation of vaccines that increase the frequency of responder T cells. We previously demonstrated the efficacy of a tumor cell lysate /toll-like receptor (TLR) agonist vaccine to treat mice bearing intracranial gliomas. The vaccine consisting of tumor cell lysate and CpG, a TLR 9 agonist, cured 50% of glioma-bearing mice through a mechanism requiring CD4 and CD8 T cells (73). However, when this tumor cell vaccine was prepared in standard tissue culture conditions (e.g., atmospheric 21% oxygen) and modeled at clinically scalable antigen doses, the efficacy of vaccination was abolished despite evidence of immune priming (74). One goal of this study was to determine if additional costimulation was necessary to achieve efficacy following vaccination with lysate/CpG. To address this we screened a panel of Fc-ligand fusion proteins in combination with the tumor lysate/CpG vaccine and identified Fc-OX40L as the most potent, demonstrating both single agent activity and synergy with the CpG/lysate vaccine. The optimal frequency, timing, and combination of Fc-OX40L with vaccination were

established in mice bearing implanted GL261 gliomas. The efficacy of the optimized regimen was generalizable to other vaccine adjuvants (PolyICLC, a TLR3 agonist), other tumor models (breast carcinoma), and effective in combination with the standard of care chemotherapy for GBM, temozolomide. Comparative studies in two glioma models revealed inflammatory gene expression signatures that were associated with response rates to Fc-OX40L as a single agent. Collectively, these studies provide a wealth of information that can be exploited when considering the design of clinical trials.

Materials and Methods

Animal Models and Cell Lines. The culture conditions for GL261-Luc and EMT6 have been described elsewhere (73, 75). All animals were maintained in a specific pathogen free facility in accordance with the University of Minnesota Institutional Animal Care and Use Committee (IACUC) guidelines. For the implanted glioma model, seven-week-old C57BL/6 (B6) mice were purchased from Jackson Laboratory. Gliomas were established by intracranial inoculation of 15,000 GL261-Luc cells in 1 μL of Hank's balanced salt solution (HBSS) (Gibco) to syngeneic B6 mice. Animals were anesthetized with a ketamine/xylazine cocktail (54.7 mg/mL ketamine and 9.26 mg/mL xylazine) prior to surgery. Cells were implanted into the right striatum at coordinates 2.5 mm lateral, 0.5 mm anterior from the bregma, and 3 mm ventral from the surface of the brain (73). Cells were delivered over 5 minutes at a rate of 0.2 μL/min. Tumor implantation

was confirmed by bioluminescence imaging 3 days following inoculation; animals received 100 μL Luciferin (Gold Biotechnology) by intraperitoneal (IP) injection and were imaged with an IVIS50 system (Caliper Life Sciences). The *de novo* glioma model was induced by transposon-mediated gene transfer, as we have previously described (76). The following plasmids were injected into the brains of B6 mice at 1-2 days following birth, pT2/C-Luc//PGK-SB13 (0.07μg), pT/CAGGS-NRASV12 (0.14 μg), and pT2/shP53/GFP4/mPDGF (0.14 μg). Mice that became moribund where humanely euthanized.

The breast carcinoma model was induced in BALB/c mice that were purchased from Taconic. Breast carcinoma was induced by injecting 4 $x10^6$ EMT6 cells in 100 μL of HBSS cells into the upper right mammary fat pad of syngeneic BALB/c mice while under sedation from isoflurane gas (75). Tumor burden was determined with calipers by measuring the greatest longitudinal diameter (length) and greatest transverse diameter (width). When a measurement in either direction reached 20 mm the animal was humanely euthanized.

Quantitative RT-PCR for immune-related gene expression. Total RNA was isolated from tumor-bearing brain or control tissue (normal brain) using Qiagen's RNeasy mini kit (Valencia, CA) and DNase treated using Turbo DNase (Applied Biosystems, Foster City, CA) per manufacturer instructions. For quantitative RT-PCR, 100 ng RNA was amplified with gene specific primers using one-step

Power SYBR green RNA-to-Ct kit (Applied Biosystems) and run in an MX3000P Stratagene thermocycler. Data were acquired and analyzed using MxPro software (Stratagene). Samples were run in triplicate and gene expression was normalized to the housekeeping gene GAPDH. Primer sequences were obtained from the NIH qRT-PCR database [http://primerdepot.nci.nih.gov] and synthesized by the USC Microchemical Core Facility.

Vaccine Production and Delivery. Cells were collected and washed 3 times with phosphate buffered saline (PBS), resuspended in PBS and flash frozen with liquid nitrogen. Cell lysis was induced by 5 cycles of freezing in liquid nitrogen and thawing in a 37°C water bath, vortexing after each round. Cell death was verified by trypan blue dye exclusion. Lysates were stored at -80°C until use. Protein concentration was determined using Pierce BCA Assay kit (Thermo Scientific). Purified CpG 1826, an unmethylated oligodeoxynucleotide (ODN) sequence (5'-tccatgacgttcctgacgtt-3') with a full phosphorothioate backbone was obtained from Integrated DNA Technologies (IDT, Coralville, IA), and verified to be endotoxin free. CpG 1826 was resuspended in 1x TE at a concentration of 10 mg/mL and stored at -80°C until use. PolyICLC was provided by Oncovir Inc. The final vaccine was generated by combining 65 μg of cell lysate with 50 μg CpG 1826 or 10 μg PolyICLC in a 100 μL final volume (adjusted with saline). Animals received 100 μL of vaccine or saline by intradermal (i.d.) injection above the shoulders.

Costimulatory Fusion Proteins Production and Delivery. Costimulatory fusion proteins were genetically engineered, biochemically characterized, and functionally validated as described previously (69-72, 77). Previously published optimal doses for each costimulatory fusion protein were used: Fc-4-1BBL (150 μg/dose), CD80-Fc (40 μg/dose), Fc-GITRL (25 μg/dose), Fc-OX40L (50 μg/dose) and LEC/chTNT3 (20 μg/dose). Each costimulatory fusion protein was brought to a final volume of 100 μL per dose with PBS and delivered by IP injection.

CTL Assay. GL261 cells were labeled with 10 μM/mL CFSE (Invitrogen) for 15 minutes then thoroughly washed with PBS. Cervical lymph nodes were harvested, dissociated by passing through a 70 μm filter, and incubated with CFSE labeled GL261 cells for 6 hrs. Following incubation, cells were stained with 7-AAD (ImmunoChemistry Technologies) and analyzed by flow cytometry. The percentage of CFSE labeled target cells that incorporated 7-AAD was plotted as the percent lysis.

Flow Cytometry. Cervical lymph nodes were passed through a 70μm filter to generate a single cell suspension. To harvest brain-infiltrating lymphocytes (BIL), mice were first perfused with PBS to flush the capillaries. The brains were removed and minced into very fine (< 1mm) pieces with a razor, passed through

a 70 μm filter, and subjected to a two layer Percoll gradient (70% and 30%) and centrifuged at 400xg for 30 minutes. The lymphocyte interface was harvested by aspiration. Cells were counted using trypan blue dye exclusion and a hemocytometer. For these studies antibodies against CD3 (clone 17A2), CD4 (clone GK1.5), CD8 (clone 53-67) (ebioscience), and Ki67 (clone B56) (BD Pharminogen) were used for phenotypic analysis. Intracellular Ki67 staining was achieved by using the ebioscience Foxp3/transcription factor intracellular staining kit according to the manufacturer's instructions. A BD Bioscience FACSCanto was used for analysis of cells and data were analyzed using Flowjo software (Tree Star). The total number of stained cells was determined by multiplying the percentage of stained cells by the total number of viable cells determined previously by trypan blue dye exclusion.

Temozolomide Administration. Temozolomide (TMZ) was purchased from Toronto Research Chemicals as powder and resuspended in PBS at a final concentration of 10 mg/mL. A dose of 50 mg/kg was administered by oral gavage for 5 days starting on day 7 following inoculation.

Histopathology and Immunohistochemistry. Tissues were fixed in 10% neutral buffered formalin, routinely processed into paraffin using standard histology techniques, sectioned at a thickness of 4 μm, stained with hematoxylin and eosin, and evaluated by light microscopy.

For immunohistochemistry (IHC) preparations, 4 μm formalin-fixed, paraffin-embedded sections of tissue were deparaffinized and rehydrated, followed by antigen retrieval (using 10mM Citrate buffer pH 6.0 or Tris EDTA pH 9.0) in a steamer prior to IHC procedures performed on a Dako Autostainer. IHC for CD3 was performed using a rat anti-human CD3 monoclonal antibody (Clone CD3-12, Serotech) as primary antibody. IHC for B220 was performed using a rat anti-mouse CD45R/B220 monoclonal antibody (Clone RA3-6B2, BD Pharmingen) as primary antibody. IHC for Pax5 was performed using a goat anti-human Pax5 polyclonal antibody (Santa Cruz Biotechnology, catalog number SC-1974) as primary antibody. Detection of bound primary antibody was achieved using the appropriate Rat-HRP or Goat-HRP Polymer Detection systems (Biocare Medical), with DAB as the chromogen.

Statistical Analyses. Statistical comparisons for lymphocyte counts were made by a Mann-Whitney (non-parametric) test. Expression of immune-related genes was compared using a student's t test. Differences in animal survival were evaluated by log-rank test. All tests were performed with Prism 4 software (Graph Pad Software, Inc). P values <0.05 were considered statistically significant.

Results

Comparative Efficacy of Combination Vaccine with Costimulatory Ligands

The efficacy of vaccination with lysate/CpG was tested alone and in combination with Fc-ligand fusion proteins. Mice bearing GL261 gliomas were primed intradermally by three vaccinations (days 3, 7, and 10), followed by daily IP injection of Fc-ligand on days 17-21 following tumor challenge. The rationale for this timing was to first prime tumor-reactive T cells, then provide a sustained (five consecutive days) co-stimulatory signal. Vaccine alone or the combination of vaccine with CD80-Fc or Fc-4-1BBL did not enhance survival compared to saline controls (**Fig. 2-1A-C**). The vaccine/Fc-GITRL combination resulted in a statistically significant, albeit incremental, increase in survival without long-term survivors (**Fig. 2-1D**). The vaccine/Fc-OX40L combination resulted in a significant increase in survival and 50% long-term survivors (**Fig. 2-1E)**. In long-term survivors, the kinetics of complete tumor regression varied from 24-45 days after inoculation as determined by periodic bioluminescence imaging (BLI) (**Fig. 2-2A**). In order to assay for immunological memory, long-term survivors and naïve controls were intracerebrally challenged with GL261 in the contralateral hemisphere relative to initial challenge. No additional treatment was administered. All cured mice rejected rechallenge within 8 days (**Fig 2-2B**) and survived long-term, whereas all naïve animals succumbed to tumor burden (**Fig. 2-1F**).

This initial screen was carried out using previously determined optimal doses of each Fc-ligand fusion protein (range 25-150 μg/dose). For a more direct comparison, the identical screen was repeated by administering the Fc-fusion proteins or an agonist OX40 antibody (OX86) at the same dose of 50 μg. Using this more equivalent dosing scheme, only mice treated with vaccine/Fc-OX40L survived long-term (**Fig. 2-3**), confirming our initial experiments and the superior potency of Fc-OX40L to OX86 that was reported previously in other tumor models (71). The unique ability of the vaccine/Fc-OX40L combination therapy to cause tumor regression and establish immunological memory warranted further mechanistic studies.

T cell response correlates with survival outcome

In order to understand how the various Fc-ligands altered immune responses, animals were treated as described in **Figure 2-1** and euthanized on day 25. This time point was four days following the final Fc-ligand injection and was chosen because it was when initial tumor regression was noted by BLI (**Fig. 2-2**). Lymphocytes from the brain and brain draining lymph node (DLN) were quantified and assayed for tumoricidal function. There was a two-to-three fold increase in the total number of cells in the DLN of vaccine/Fc-OX40L-treated animals compared to all other groups except Fc-GIRTL/vaccine (**Fig. 2-4A**). The difference between the Fc-GIRTL/vaccine and vaccine/Fc-OX40L groups was not statistically significant ($p = 0.15$). However, only cells isolated from the

DLN of vaccine/Fc-OX40L-treated animals had appreciable tumoricidal function in *ex vivo* CTL assays (**Fig. 2-4B)**. These experiments revealed quantitative and functional differences in lymphocytes in the DLN that correlated with outcome; Fc-GIRTL/vaccine followed by vaccine/Fc-OX40L had increasingly superior survival as shown in **Fig. 2-1,** whereas tumoricidal function was unique to vaccine/Fc-OX40L.

Flow cytometry analyses revealed animals in the vaccine/Fc-OX40L treatment group had an increase in the percentage of CD4 and CD8 T cells at the tumor site (**Fig. 2-5A**). However, when absolute numbers were analyzed only 3/5 mice treated with vaccine/Fc-OX40L had increased numbers of CD4 and CD8 T cells at the tumor site (**Fig. 2-5A**). Due to the high animal-to-animal variability, this difference was statistically insignificant, with the exception of brain infiltrating CD4 T cells in two cases. Notably, the percentage of vaccine/Fc-OX40L-treated mice with increased tumor infiltrating T cells (~60%) correlated with the long-term term survival rate of 50%. A more robust assay was Ki67 staining, a marker of proliferation. There was a significant increase in the frequency of CD4 and CD8 brain infiltrating T cells that were mitotically active in vaccine/Fc-OX40L-treated mice (**Fig. 2-5B**). The significance of this finding is unclear, but it may represent an intrinsic difference in the proliferative competence of T cells, consistent with previous studies that demonstrated OX40 agonists could break T cell anergy in tumor models (78). All together, these experiments established a link between

complete response rate, priming in the DLN, tumoricidal function, and tumor infiltration of T cells that was unique to the vaccine/Fc-OX40L combination therapy. Further studies were then undertaken to systematically dissect the contribution of Fc-OX40L and the vaccine and optimize combination therapies.

Determination of the optimal treatment strategy

The potency of Fc-OX40L as a single agent was examined in glioma-bearing animals treated with Fc-OX40L. We treated well-established tumors, beginning treatment on day 17. Impressively, Fc-OX40L was capable of extending survival of tumor-bearing animals and curing 20% (1/5) (**Fig. 2-6A,** Regimen 1). In a further attempt to optimize complete response rates, vaccination was given concurrently with Fc-OX40L on days 7, 10, and 13 followed by 5 daily doses of Fc-OX40L alone (days 15-19). This was reproducibly the most potent treatment, with 70% cure rates (5/7) (**Fig. 2-6B,** Regimen 2). Variations of this treatment were tested to determine the contribution of vaccine and Fc-OX40L. Fc-OX40L alone given early (days 7,10, and 13) (Regimen 3) had a weak therapeutic effect unless given in combination with vaccine (Regimen 4) (**Fig. 2-6A, B).** The efficacy of Fc-OX40L given late (days 15-19; Regimen 5) was improved if vaccination was administered on days 7,10, and 13 (Regimen 6). We focused on the timing and frequency used in regimen 2 in subsequent studies due to the greatest therapeutic index of this regimen.

Contribution of vaccine components (antigen vs. adjuvant) with Fc-OX40L

The components of the vaccine/Fc-OX40L treatment were broken down to determine which is necessary for maximum effect. Lysate, CpG, or the combination "vaccine" had no effect on survival (**Fig. 2-7A**). Both lysate/Fc-OX40L and lysate/CpG/OX40L were able to significantly extend survival with 50-60% cure rates (**Fig. 2-7B**). Therefore, a TLR agonist is dispensable to achieve complete responses. However, the addition of a TLR agonist (PolyICLC or CpG) to the regimen was associated with a delay in the time of initial death of the first 20-40% of animals compared to lysate/Fc-OX40L (**Fig. 2-7B-C**). In clinical oncology, the closest analogy would be to describe the contribution of the TLR agonist as increasing progression free survival. Additionally, a single animal in the lysate/Fc-OX40L treatment group that was determined to be a complete responder through BLI, subsequently succumbed to tumor recurrence at day 158, a phenomenon not observed in the lysate/CpG/Fc-OX40L treatment group (**Fig. 2-7B**). Increased survival caused by inclusion of the TLR agonist (to lysate/Fc-OX40L) never reached statistical significance compared to lysate/Fc-OX40L alone, but was reproducible and generalizable to both TLR3 (PoyICLC) and TLR9 (CpG). Unexpectedly, CpG or PolyICLC given without lysate antigen but with Fc-OX40L had no effect on survival (**Fig. 2-7B-C**). Thus, apparently TLR agonists administered i.d. near the tumor DLN diminished the efficacy of Fc-OX40L administered IP as a single agent. This finding reveals the importance of co-delivering a source of antigen with TLR ligand adjuvant in settings where Fc-

OX40L is administered systemically. Although the mechanisms of how these combinations are promoting or antagonizing therapeutic efficacy are unclear, the combination of vaccine (TLR agonist plus lysate) with Fc-OX40L consistently yielded highest survival rates. This was a robust finding in the GL261 glioma model and extended to other models; notably the vaccine/Fc-OX40L regimen was curative in 5/5 mice bearing established EMT6 breast carcinoma (**Fig. 2-8).**

Complete responses using temozolomide combination therapy associated with secondary malignancies

TMZ is a DNA alkylating chemotherapy that is part of the current standard of care for GBM (79). Any investigational treatment will likely be delivered in combination with TMZ in the clinic. For that reason we studied the compatibility of the two treatment strategies. We attempted to model a likely scenario in newly diagnosed GBM patients, which historically have received TMZ prior to vaccine immunotherapy (79-81), by administering vaccine or Fc-OX40L alone and in combination with TMZ. TMZ was administered by oral gavage for five consecutive days beginning on day 7 post GL261 inoculation. Vaccination was performed on day 12 and 19, followed by 5 daily doses of Fc-OX40L beginning on day 19. When TMZ was given alone or in combination with vaccine, survival was significantly enhanced, although there were no long-term survivors (**Fig. 2-9A**). The survival of glioma bearing mice was not significantly different between the TMZ/Fc-OX40L and vaccine/Fc-OX40L groups; yielding 20-40% long-term

survivor rates (**Fig. 2-9B**). Strikingly, the addition of TMZ to vaccine/Fc-OX40L resulted in 100% complete tumor regression by day 50 after GL261 challenge (**Fig. 2-9B**). However, 80% of the mice cured by the TMZ/vaccine/Fc-OX40L combination developed lymphoid neoplasia involving multiple organs, killing 80% (4/5) with a latency of 82- 378 days (depicted in **Fig. 2-9B**).

Immunohistochemistry revealed CD3 positive neoplastic cells consistent with a T cell lineage in 2 animals, whereas it appeared that there was neoplasia involving both T and B lineages (CD3, B220 and Pax5 positive, respectively) in the other 2 animals (**Fig. 2-10**). Glioma progression was the cause of death in all other animals in this experiment with the exception of a single mouse in the TMZ/Fc-OX40L group that died with evidence of microscopic disease on day 170. In all other experiments glioma progression was also the cause of death, as documented by neurologic deterioration and histological analysis of treated brains, except for two mice in the lysate/Fc-OX40L group (**Fig. 2-7**) that died of unknown causes (data not shown).

Analysis of single agent Fc-OX40L in transplanted and spontaneous glioma models

It remained unclear how generalizable these data were to autochthonous glioma models. We therefore investigated the efficacy of Fc-OX40L in a *de novo* glioma model that is induced by oncogene transfer into neonatal mice as previously

described (76). Plasmids encoding three oncogenes and a *firefly luciferase* reporter were delivered intracerebrally to transform endogenous brain cells within 1-2 days of birth. Beginning at the time of weaning, animals were imaged to identify when tumor formation occurred. For lack of an adequate cell line to use for lysate generation (equivalent to autologous as in the GL261 system), Fc-mOX40L was administered as a single agent. Treatment began when tumor was first detected by BLI as depicted in **Figure 2-11A.** Fc-OX40L monotherapy caused a statistically significant increase in survival, although this difference was incremental and there were no long-term survivors (**Fig. 2-11B**). This finding was in contrast to Fc-OX40L monotherapy in the GL261 model, which consistently cured 20-25% of mice (even those bearing 17-day established tumors), prompting us to further investigate the microenvironment of both tumor systems.

Gene expression profiling has proven useful in identifying differences between tumors and has lead to the further classification of gliomas beyond the histological standards to a molecular subtype (82). In an attempt to understand the molecular differences between the two glioma models, we conducted a quantitative RT-PCR analysis of 30 immune-related genes in brains from both glioma models and normal B6 mice. Relative to normal brain, both tumor models exhibited log-fold increases in *CD11c* expression, a DC marker, and *arginase-1* (**Fig. 2-11C**). The arginase enzyme is released by myeloid derived suppressor

cells, locally depletes L-arginine, and consequently impairs T cell proliferation and survival (83). Response to costimulation may be dependent on which costimulatory molecules are absent endogenously. Interestingly, OX40L was the only co-stimulatory ligand of those tested in our models that had lower expression in the tumor relative to the normal brain in either model. The GL261 and spontaneous models differed in several important ways. GL261 tumors exhibited a 15-fold increase in *IFN-γ* expression compared to the spontaneous model, and consistent with this expressed more *PD1-L*, which is induced by IFNγ (84). In addition, *CD3* (a T cell marker) was more highly expressed in the GL261 model, whereas the NK cell marker *CD49b* was more highly expressed in the spontaneous model (**Fig. 2-11C**); although these were not statistically different they were trending toward significance (CD49b p=0.071, CD3 p=0.088).

Discussion

Recent clinical trials employing peptide vaccines or lysate-pulsed DC vaccines have shown promising results in GBM patients (81, 85, 86). These studies have documented immunologic and radiographic responses in select patients, and favorable survival rates that await validation in randomized clinical trials. However, the majority of patients treated by vaccination ultimately experienced tumor recurrence and succumbed to disease, highlighting the need for more potent immunotherapy and the induction of memory cells. Recombinant costimulatory ligand-Fc fusion proteins offer an attractive adjuvant to vaccine

immunotherapy. To this end, the purpose of this study was to screen a panel of Fc-ligands using doses previously optimized in other tumor models (69-72). Fc-OX40L was clearly the most efficacious in the GL261 vaccination model. We are cautious to avoid over-interpreting these data; this does not mean that one Fc-ligand is universally better than the others, considering that the dose, timing in relation to vaccination, and biologic activity of each agent was not systematically optimized prior to the screen that identified Fc-OX40L as our lead candidate. It is likely that the efficacy of each drug could be significantly changed by the temporal relationship to TCR signaling following vaccination. Nonetheless, this study is the first head-to-head screen using Fc-ligand fusion proteins, and the first to clearly demonstrate the therapeutic potency of the combination of vaccine/Fc-OX40L in a brain tumor model.

The reproducibility of our data and that of others using OX40 agonists strongly suggests that OX40 agonists warrant clinical testing in a variety of cancers. The vaccine/Fc-OX40L combination was generalizable to various TLR agonists (TLR9 or TLR3) and other tumor models (GL261 glioma or EMT6 breast carcinoma), yielding cure rates of 50-100% depending on the model and adjuvant used. To our knowledge, no immunotherapy has improved survival in an implanted brain tumor model beyond day 10 (87), whereas Fc-OX40L improved survival of mice treated starting 17 days after tumor challenge (GL261), and in a spontaneous model as a single agent. We noted a curious finding

following Fc-OX40L monotherapy: that treating earlier relative to tumor implantation progressively decreased the median survival (compare regimen 1,3, and 5 in **Fig. 2-6A**). This is the opposite of conventional wisdom, which would argue immunotherapy works better on smaller tumors. We speculate that this is due to a delay in natural antigen presentation caused by tumor cell death, leading to more responder T cells in animals with advanced tumors. Indeed, the appeal of cancer vaccines is to increase the frequency of tumor-reactive T cells. Although vaccination did not alter T cell priming measured in the DLN on day 25 (15 days after last vaccine; **Fig. 2-4A**), vaccination primed equivalent expansion of CD4 and CD8 T cells in the DLN at day 15 relative to the vaccine/Fc-OX40L combination (**Fig. 2-12**). Consistent with a model of vaccination increasing tumor-reactive T cell frequency, vaccination combined with Fc-OX40L synergistically increased survival rates in the GL261 model relative to Fc-OX40L monotherapy. We speculate that an important effect of Fc-OX40L is rescuing T cell anergy, as shown by enhancing tumoricidal function (**Fig. 2-4B**). This hypothesis is consistent with other reports that have studied the OX40/anergy axis in greater detail (78, 88). In addition, Fc-OX40L has been shown to activate tumor vasculature and tumor-associated DCs, revealing the complexity of response to this drug (89). A significant percentage of infiltrating T cells expressed the proliferation marker Ki67 with the vaccine/Fc-OX40L treatment, supporting the notion that this treatment yields a qualitative difference in T cell response (**Fig. 2-5**). Restimulation by antigen presenting cells (APCs) can

induce proliferation in responding T cells, which has been documented to occur at the tumor site, in addition to the lymph nodes (90). It has also been documented that Ki67 has a half life of 60-90 minutes and therefore it is also possible that these cells have been stimulated in the lymph nodes and recently migrated to the brain (91). In either situation, it appears that the infiltrating T cells in the vaccine/Fc-OX40L group have recently experienced a productive encounter with an APC and is a likely explanation for the corresponding increase in tumoricidal function.

The naturally occurring immune response to cancer warrants detailed consideration because there is evidence that it may predict therapeutic response. Untreated GL261 tumors had an inflammatory phenotype, dominated by T cells and strong *IFNγ* expression, whereas the spontaneous glioma model had higher *IL-6* expression and was NK cell dominated (**Fig. 2-11**). This is intriguing because Fc-OX40L monotherapy consistently cured 20-25% of mice in the GL261 model, but only incrementally increased survival in the spontaneous model. These results are reminiscent of those reported by Prins et al. in GBM patients treated by lysate-pulsed DCs and TLR7 or TLR3 agonists (86). There are at least three molecular subclasses of GBM based on microarray signature: mesenchymal, proneural, and proliferative, where mesenchymal is associated with inflammation and has the worst outcome (82). The study by Prins et al. preliminarily demonstrated an increase in survival of vaccinated patients of the

mesenchymal compared to the proneural subclass. Although this study was not powered to detect clinical efficacy, it offers a provocative hypothesis consistent with our data: that the pre-existing immune response and inflammatory milieu might be used to predict responders to specific immunotherapy protocols. This paradigm is quintessentially personalized medicine and warrants testing in prospectively designed, randomized clinical trials. The 30-gene immune phenotyping panel used in this study is a simplified assay relative to microarray that could be useful in such efforts. We are currently evaluating these ideas by conducting a randomized study of vaccine/Fc-OX40L immunotherapy in pet dogs with naturally occurring glioma.

The correlation between reduced efficacy of treatment, as seen for the spontaneous model and a lack of T cell infiltration would suggest that patients with tumors that have a less inflammatory immune signature would not benefit from this type of treatment. Previous work has sought to enhance the tumor infiltration of lymphocytes by conjugating leukocyte recruiting chemokines to antibodies specific to necrotic tissue, which is present in most high-grade tumors. These tumor necrosis treatment (TNT) antibodies conjugated to the liver expression chemokine (LEC) have been shown to promote immune cell infiltration in animal tumor models (77, 92). When tested in the implanted glioma model a modest increase in survival was observed with no long-term survivors (**Fig. 2-13**). It remains to be seen if combining such a treatment with Fc-OX40L

treatment would be effective in enhancing survival in the implanted glioma model and more interestingly overcome the lack of lymphocyte infiltration seen in the spontaneous model and allow OX40 activated cells to carry out an anti-tumor response resulting in increased survival. An important note would be that in previous testing with the LEC/TNT3 treatment, the molecule was delivered systemically; given the large molecular weight of antibodies and the size restrictive nature of the blood brain barrier (BBB) it may be more effective to deliver this treatment through intratumoral injection, thereby bypassing the need for passive diffusion through the BBB. Translationally, use of this molecule would not be ideal for most patients with resected tumor, as surgical debulking eliminates the tumor core and thus necrotic tissues. However, this may prove very useful in cases in which surgical resection is not an option or for recurrent patients who are less likely to undergo additional surgeries.

The high incidence of secondary malignancy in TMZ-treated animals warrants attention. It is possible that TMZ-exposed T cells are generally more susceptible to transformation in B6 mice, rather than requiring a specific Fc-OX40L/TMZ combination. Further investigation into the effect of TMZ on secondary malignancies in the context of immunotherapy is beyond the scope of this report, but demands caution. We are currently investigating delaying TMZ until after vaccine/Fc-OX40L is completed in dogs with naturally occurring glioma in an attempt to address the theoretical risk of accelerated secondary malignancy by

priming TMZ-exposed T cells. The vaccine trials conducted in GBM patients to date have not reported an elevated risk of secondary malignancy, although the caveat is that most treated patients died before meaningful long-term follow up was obtained. As novel immunotherapies are approved that significantly increase patient survival, the topic of secondary malignancy in heavily pre-treated patients may become increasingly important.

In summary, these preclinical studies have laid the foundation for the clinical development of Fc-OX40L for the treatment of GBM and other cancers. The combination of Fc-ligand fusion proteins with vaccines is an appealing strategy worth pursuing. Such combination therapies may represent an important leap forward in the field of cancer immunotherapy.

Acknowledgements. This work was supported in part by NIH grants R01 CA154345, R01 CA160782, the American Cancer Society grant RSG-09-189-01-LIB, and generous support from the Minnesota Medical Foundation, the Hedberg Family Foundation, and the Children's Cancer Research Fund to JRO. We acknowledge the University of Minnesota Masonic Cancer Center Comparative Pathology Shared Resource for histology and immunohistochemistry preparations, and thank Josh Parker for preparation of Figure 2-10. We thank Nicole Feng for assistance with qRT-PCR.

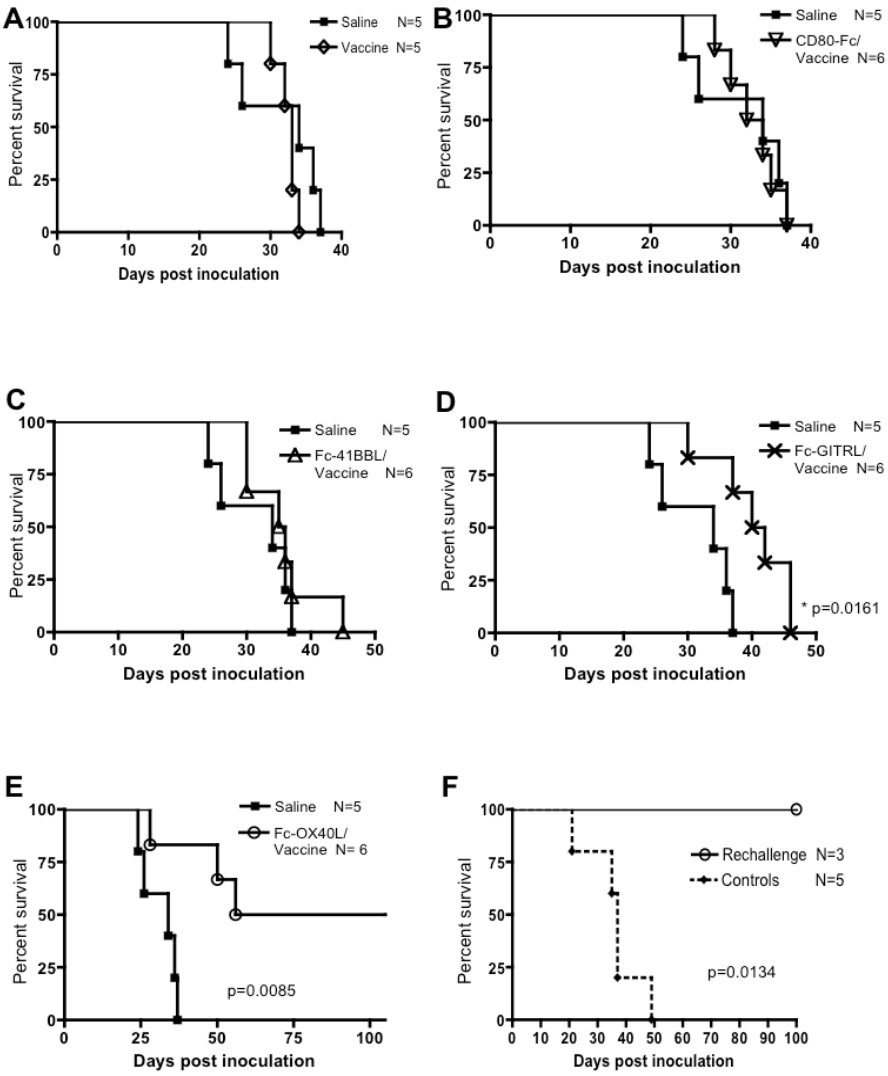


Figure 2-1. Comparative efficacy of combination vaccine with costimulatory ligand fusion proteins. Animals were inoculated with 15,000 GL261-Fluc cells on day 0. Vaccine, lysate/CpG, was administered i.d. on days 3, 7,and 10. On day 17 animals received one of four costimulatory molecules (CD80, 4-1BBL, GITRL, or OX40L) as Fc fusion proteins or PBS control for five consecutive days. Overall survival compared to saline controls was documented for vaccine alone (A), CD80-Fc/vaccine (B), Fc-4-1BBL/vaccine (C), Fc-GITRL/vaccine (D), and Fc-OX40L/vaccine (E). (F) 100 days post inoculation the vaccine/Fc-OX40L-treated survivors were rechallenged with GL261cells in the contralateral hemisphere. In addition, naïve control animals were also inoculated in the same manner.

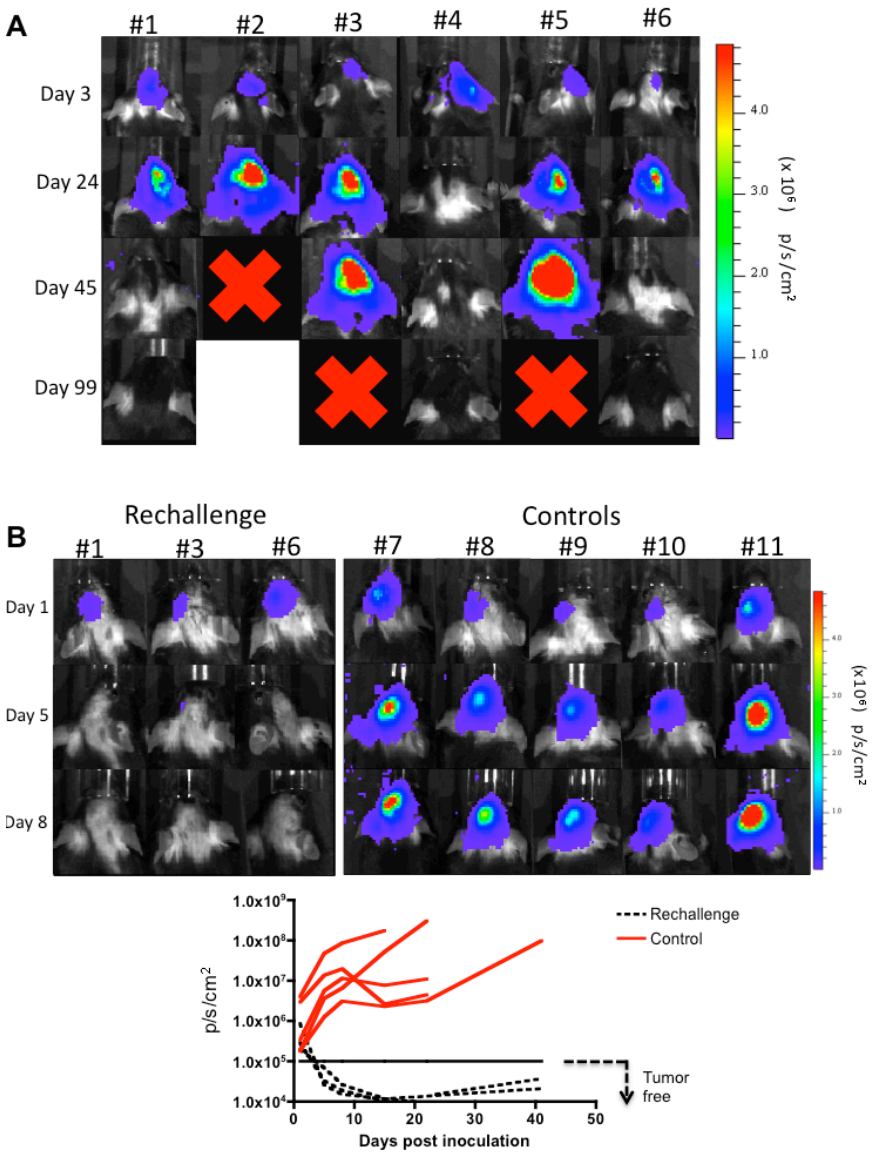


Figure 2-2. BLI reveals kinetics of tumor regression. Bioluminescence imaging was utilized to track tumor progression over time. (A) Glioma-bearing animals were treated with vaccine (days 3, 7,and 10) and Fc-OX40L (days 17-21). Tumor burden was measured by bioluminescence beginning on day 3. (B) Animals that had complete tumor regression as determined in (A) were rechallenged with the same cell line in the contralateral hemisphere; additionally naïve control animals were inoculated likewise and tumor progression was monitored over time. Tumor burden was measured as a unit of photons/second/cm^2 (p/s/cm^2) and plotted over time. A threshold of 1x10^5 was used to distinguish signal from background. Control animals are depicted in solid red lines, rechallenged animals in dashed black, and the threshold as a solid black line.

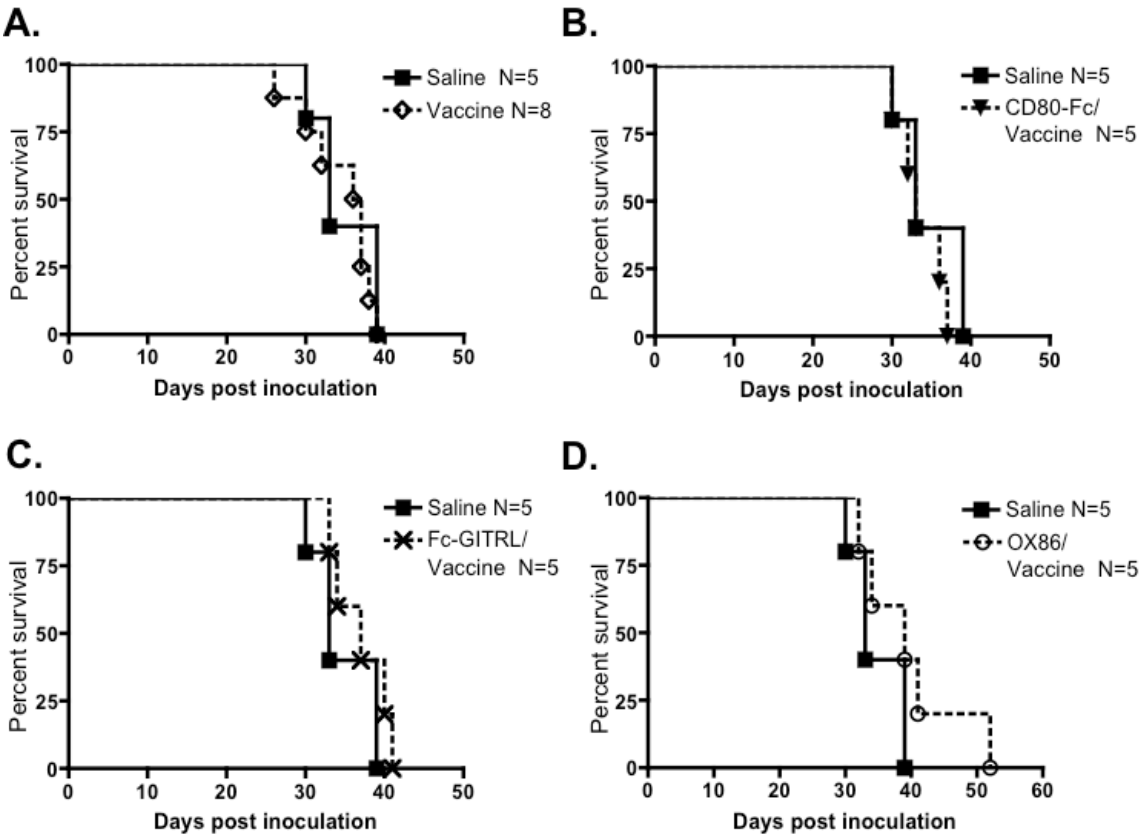


Figure 2-3. Equivalent dosing of costimulatory ligands. To determine if the difference in dosing previously tested played a role in the outcome, the costimulatory molecules were tested at the same concentration per dose as Fc-OX40L (50 μg/dose) in the same manner as described in Figure 2-1. (A) Vaccine alone, (B) CD80-Fc/vaccine, and (C) Fc-GITRL/vaccine survival is shown relative to saline controls. (D) In addition, the agonist antibody for OX40, OX86, was tested at equivalent dosing in order to demonstrate the relative potency.

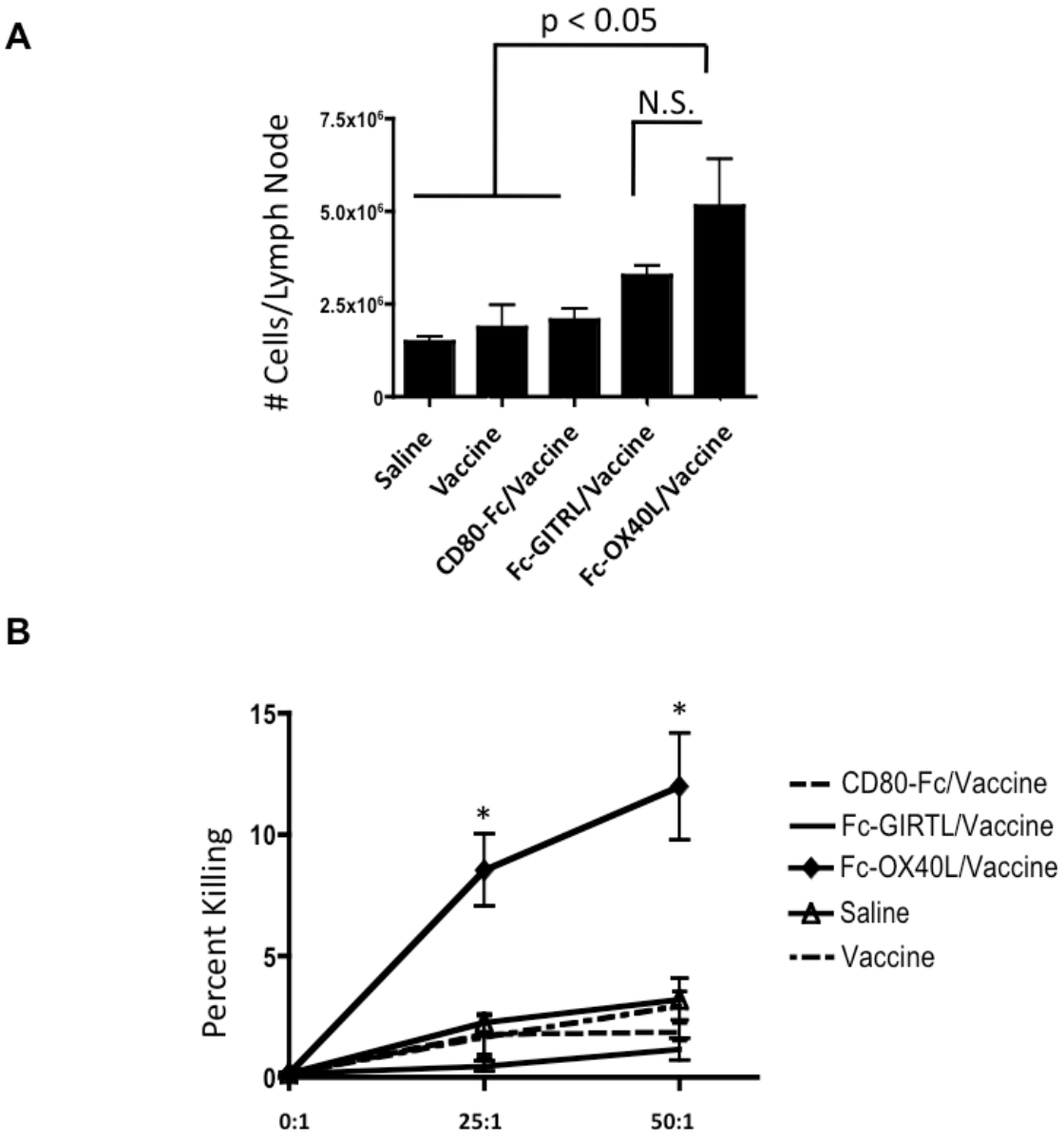


Figure 2-4. Superior priming in the draining lymph nodes of vaccine/Fc-OX40L-treated mice. In order to understand the effect that the costimulatory molecules have on the immune system, animals were treated with the reagents as described in Figure 2-1 and euthanized on day 25. Lymphocytes from the cervical lymph nodes (draining LN) were collected, counted, and assayed for tumoricidal function in CTL assays. (A) Aggregate data showing cell counts in each treatment group. (B) Cell killing plotted as percent lysis. Statistical significance is indicated as $p < 0.05$ (*), or not significant (N.S.). Mean values are shown -/+ SEM; n=4-5/group.

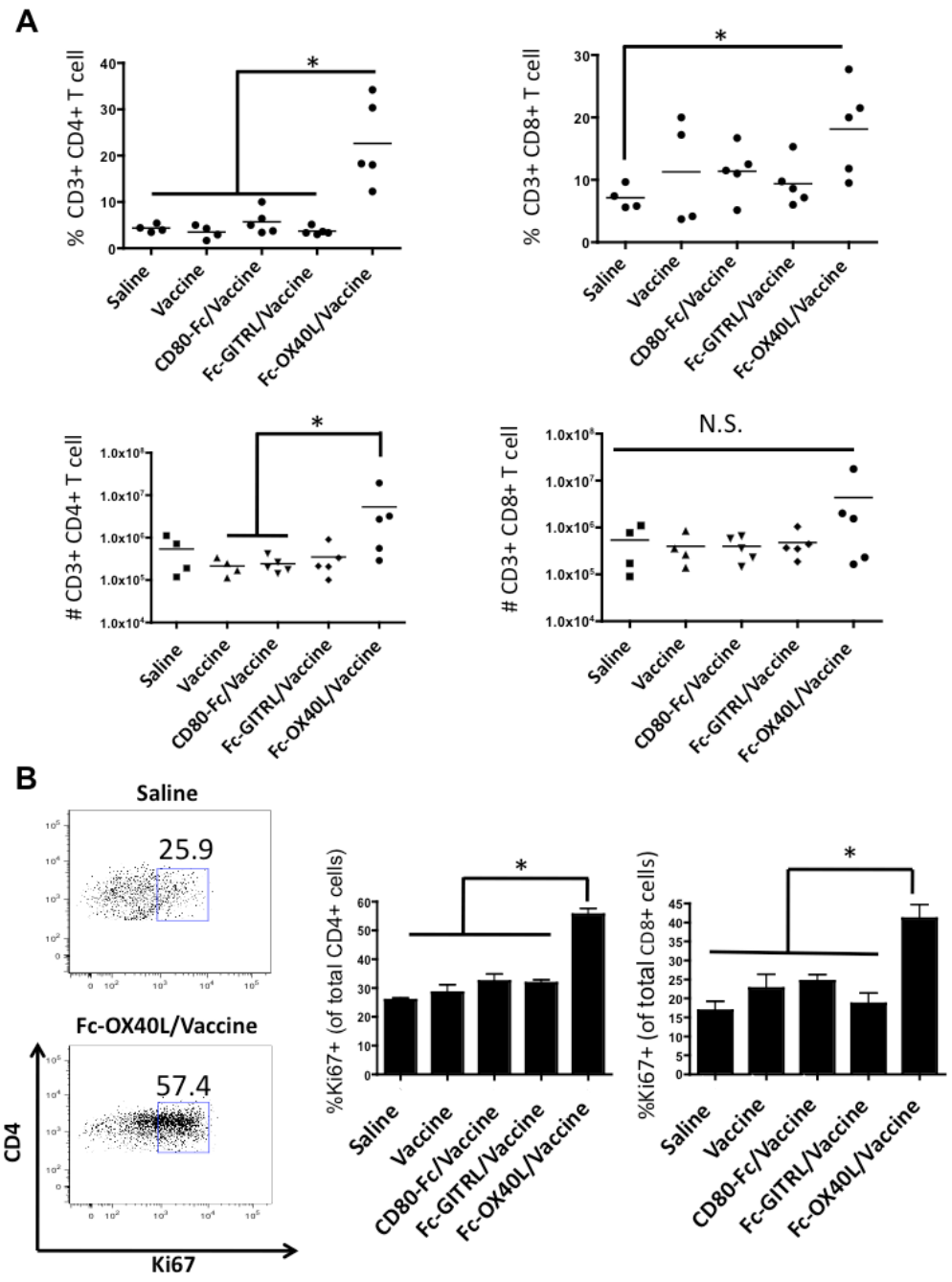


Figure 2-5. Analysis of brain infiltrating lymphocytes. Glioma-bearing animals were treated as described in Figure 2-1 and euthanized on day 25. Lymphocytes were isolated from the brains and analyzed by flow cytometry. (A) The percentage and total numbers of CD4 and CD8 T cells are plotted. (B) The percentage of proliferating CD4 and CD8 T cells was determined by Ki67 staining. Statistical significance is indicated as $p < 0.05$ (*), or not significant (N.S.). Mean values are shown -/+ SEM; n=4-5/group.

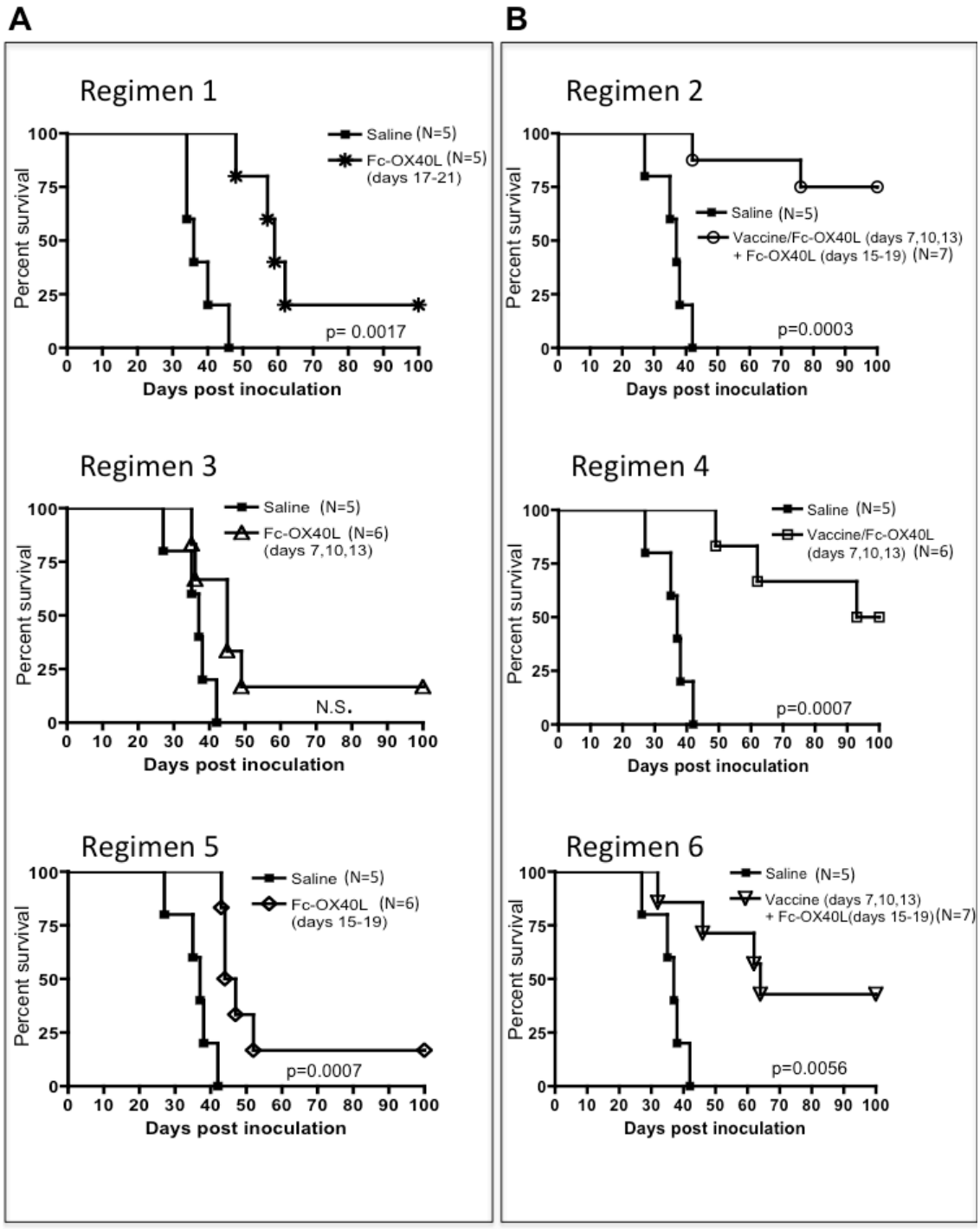
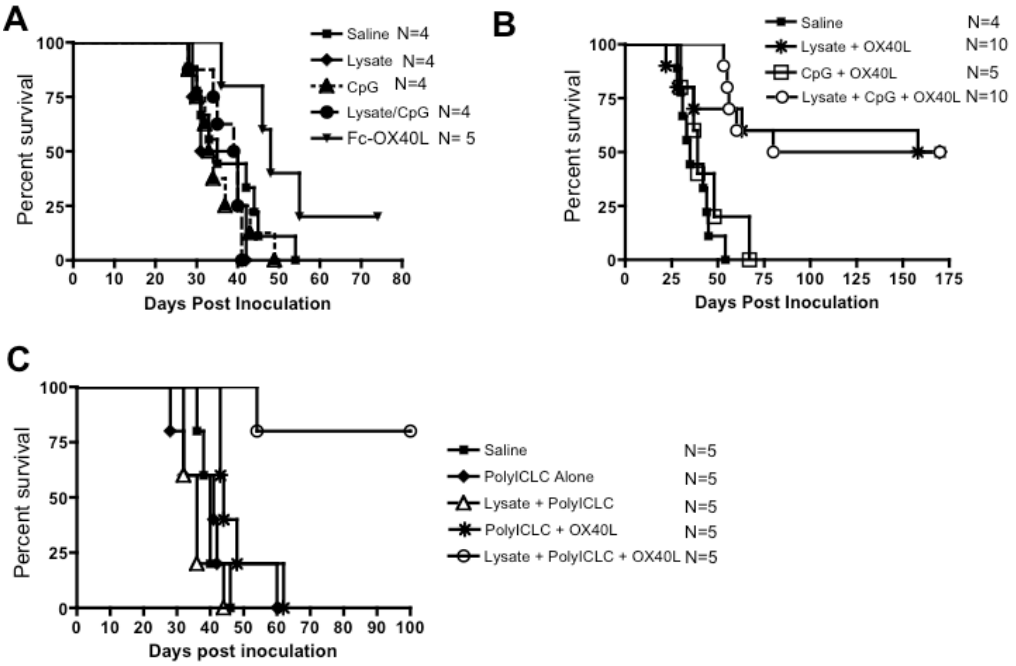


Figure 2-6. Optimization of Fc-OX40L Treatment. (A) The efficacy of Fc-OX40L monotherapy was established at different time points following GL261 inoculation. (B) Treatment with vaccine (lysate plus CpG) and Fc-OX40L was optimized by varying the treatment schedule as indicated.



Group	Median Survival	P value (vs Lysate/CpG/Fc-OX40L)
Saline	35	< 0.0001
Lysate Alone	35.5	< 0.0001
CpG Alone	33.5	< 0.0001
Lysate/CpG	39.5	< 0.0001
CpG + Fc-OX40L	39	0.0022
Fc-OX40L	48	N.S.
Lysate + Fc-OX40L	165	N.S.
PolyICLC Alone	41	0.0017
Lysate /PolyICLC	36	<0.0001
PolyICLC + Fc-OX40L	44	0.0024
Lysate/PolyICLC + Fc-OX40L	undefined	N.S.

Figure 2-7. Antigen vs. Adjuvant. The components of the vaccine/Fc-OX40L treatment were broken down to determine which components were necessary. (A) The components of the vaccine were individually tested. (B) The components of the vaccine were tested in addition to OX40L. (C) In addition, another adjuvant, PolyICLC, was tested for efficacy.

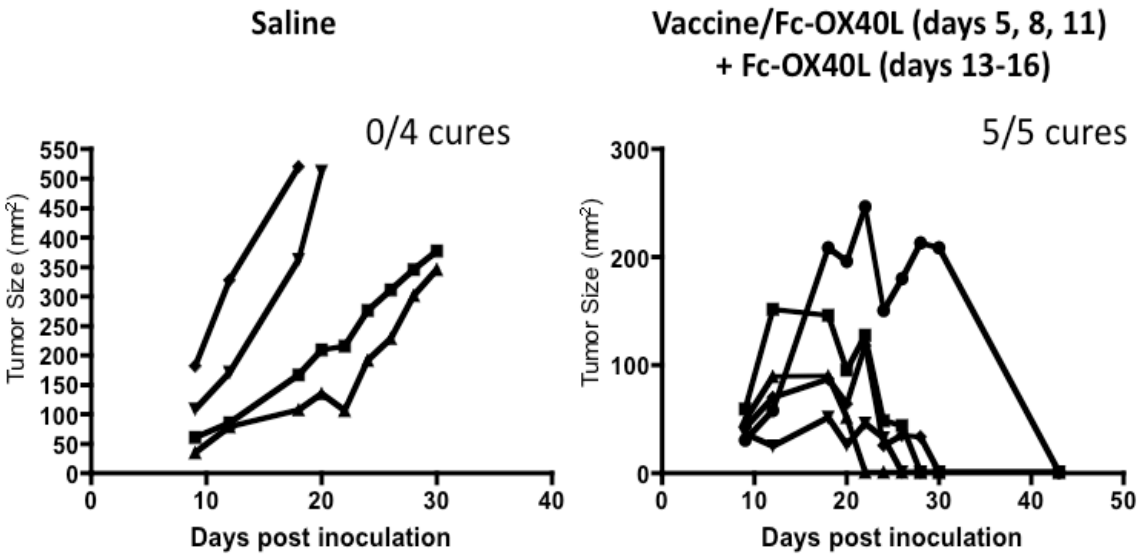


Figure 2-8. Efficacy of vaccine/Fc-OX40L against orthotopic breast carcinoma. EMT6 tumor bearing mice were treated with vaccine/Fc-OX40L (days 5, 8, and 11) and Fc-OX40L (days 13-17). Caliper measurements were taken to track tumor progression and plotted over time.

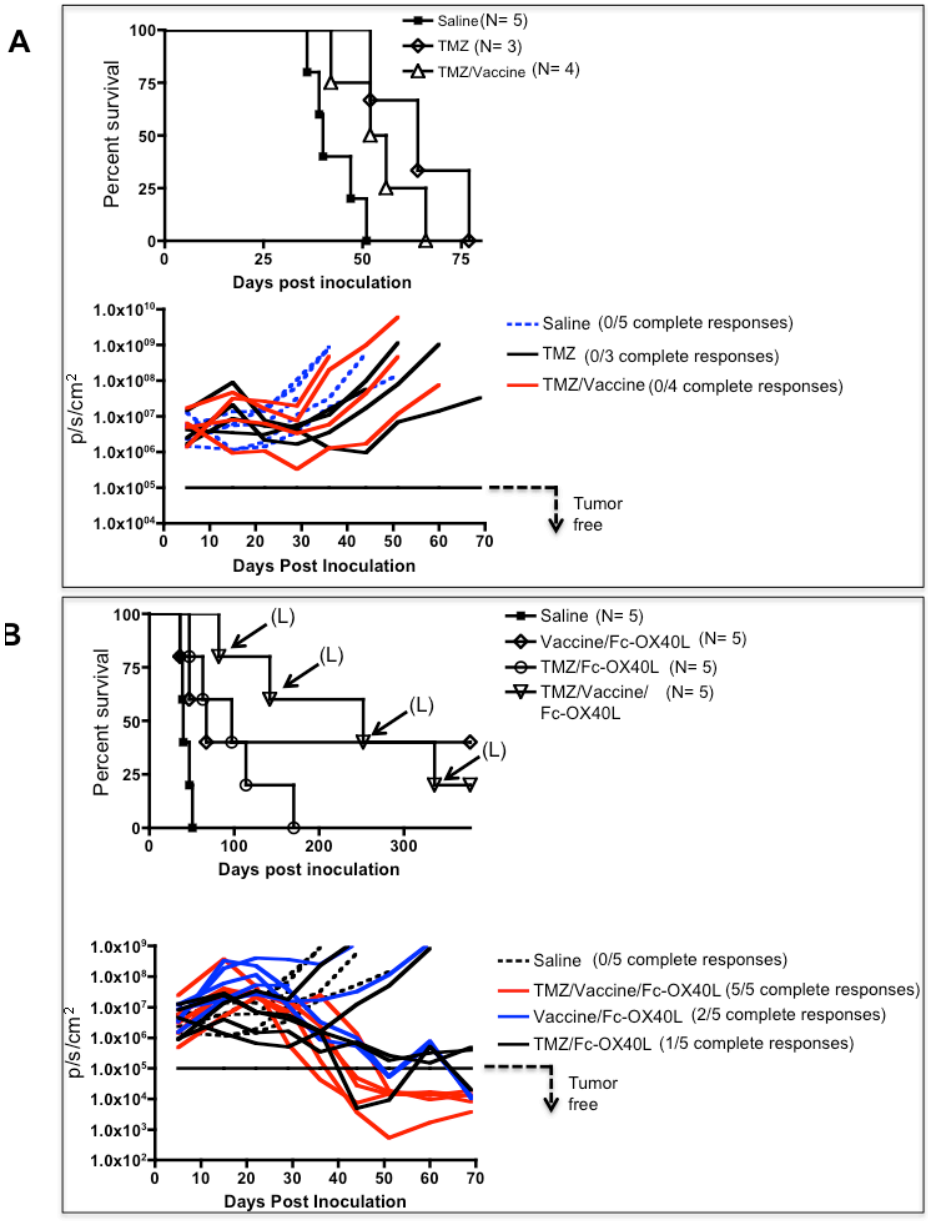


Figure 2-9. Combination therapy with TMZ is efficacious but associated with secondary malignancy. TMZ was administered via oral gavage for 5 days beginning on day 7 post GL261 inoculation. Vaccinations occurred on days 12 and 19, followed by 5 daily doses of Fc-OX40L beginning on day 19. BLI plots are shown below corresponding survival data in each panel, whereby each line represents the tumor-burden of an individual mouse over time. (A) TMZ given alone was compared to saline and TMZ with vaccination. (B) The survival effect of the combinations of Fc-OX40L with vaccine, TMZ, and vaccine/TMZ were compared in glioma-bearing animals. "L" indicates death due to lymphoid neoplasia rather than glioma burden.

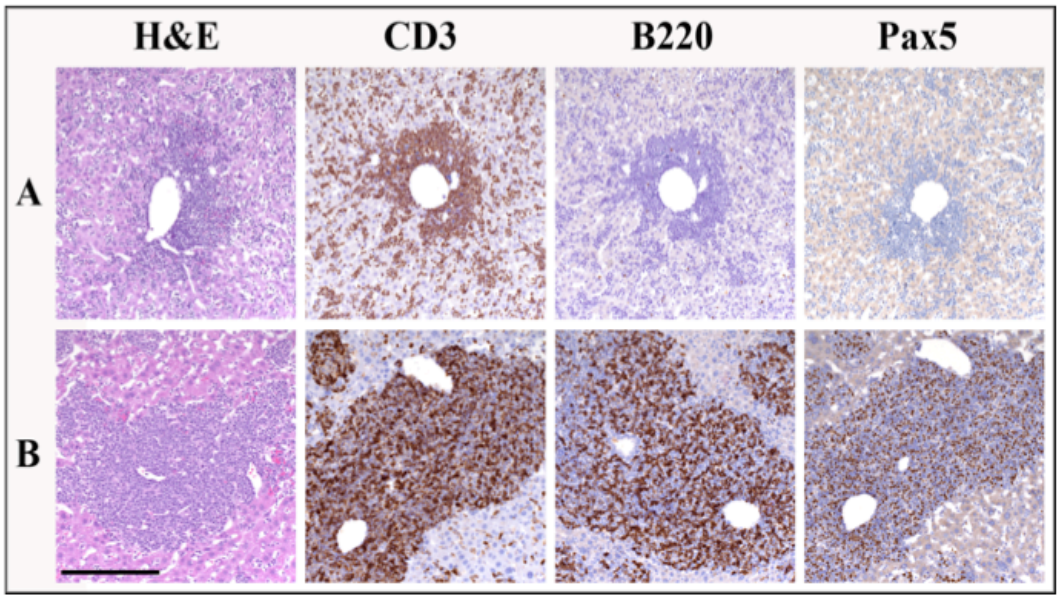


Figure 2-10. Histological Analysis of TMZ/Vaccine/Fc-OX40L treated animals. Hematoxylin and eosin staining, and immunohistochemical labeling of lymphoid neoplasia involving the liver of TMZ/vaccine/Fc-OX40L-treated animals. Neoplastic cells are CD3 positive, but B220 and Pax5 negative, in one animal consistent with a T cell lineage (Panel A), whereas neoplastic cells are CD3, B220 and Pax5 positive in another animal consistent with both T and B cell lineages (Panel B).

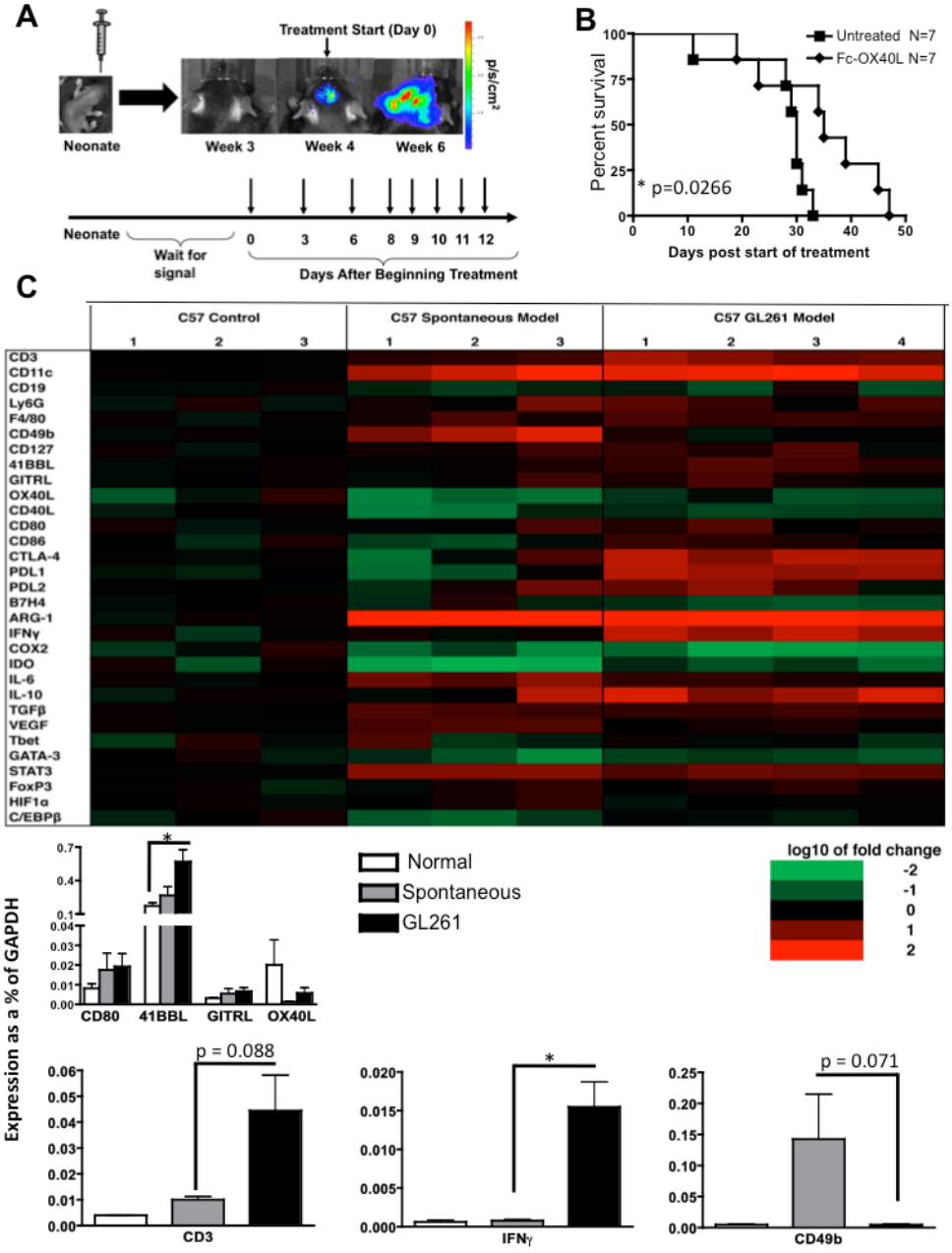


Figure 2-11. Differential survival and inflammatory gene expression patterns between implanted and spontaneous glioma models. (A) Schematic illustrating spontaneous tumor model and treatment schedule. (B) Survival of glioma-bearing mice following Fc-OX40L monotherapy (C) qRT-PCR analysis was performed on tumors collected from GL261 implanted and plasmid oncogene induced (spontaneous) animals. Gene expression was normalized to the housekeeping gene GAPDH. 3-4 animals per group were analyzed in triplicate; statistical significance is indicated as $p < 0.05$ (*), mean values are shown -/+ SEM.

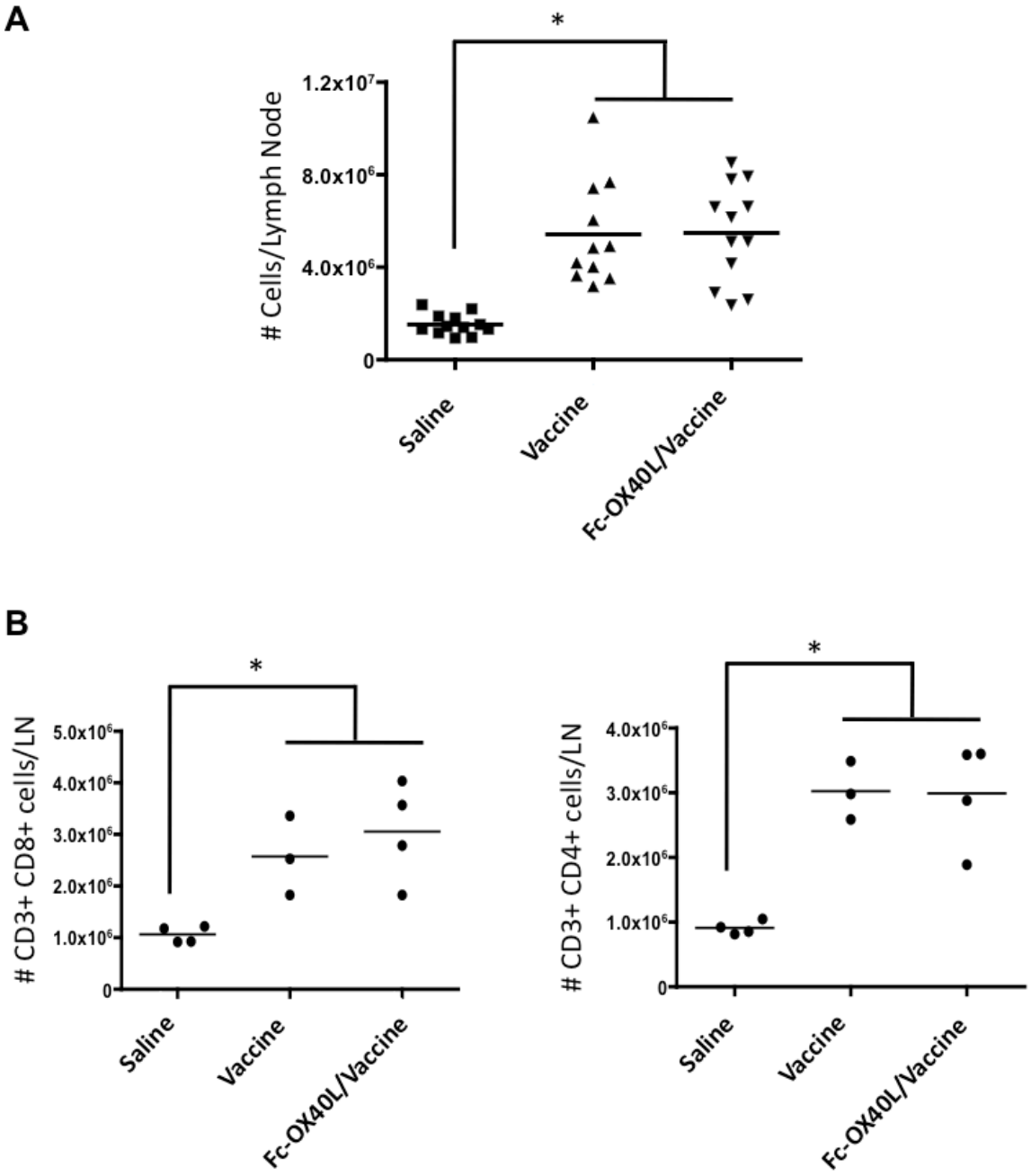


Figure 2-12. No difference in T cell expansion between vaccine and vaccine/Fc-OX40L groups at early time points. Glioma-bearing animals were treated with vaccine/Fc-OX40L on days 7, 10, and 13 post inoculation. On day 15 animals were euthanized and cervical lymph nodes were collected. (A) Isolated cells were counted as the number per lymph node collected. (B) Flow cytometry analysis was used to determine the number of CD4 and CD8 T cells isolated per lymph node. Statistical significance is indicated as $p < 0.05$ (*).

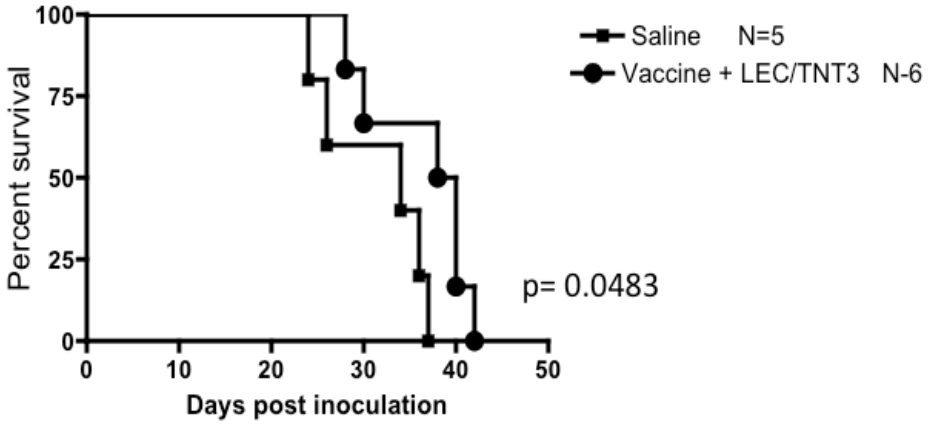


Figure 2-13 Combination vaccination with LEC/chTNT3 treatment. Animals were inoculated with 15,000 GL261-Fluc cells on day 0. Vaccine, lysate/CpG, was administered i.d. on days 3, 7,and 10. On day 17 animals received LEC/chTNT3 or PBS control IP for five consecutive days. Overall survival compared to saline controls was documented.

Chapter 3

Insights into the mechanism of immune-based tumor regression induced by Fc-OX40L in a murine model of glioma

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Despite the growing number of pre-clinical and clinical trials focused on immunotherapy for the treatment of malignant gliomas the prognosis for this disease remains grim. Although some promising advances have been made, the immune responses stimulated have been inefficient at complete tumor elimination. The lack of understanding of the necessary effector functions of the immune system has contributed to this stalemate. The efficacy of a tumor lysate vaccine/Fc-OX40L treatment therapy has been shown in a mouse model of glioma. The following experiments aim to shed light on the mechanism of action of this therapy that has shown great potency. The hope is that understanding which components of the immune system are being targeted and actively contributing to tumor clearance will guide the design of future treatments for human disease. The evidence subsequently outlined indicates a CD8 T cell independent and CD4 T cell, NK cell, and B cell dependent means of prolonged survival. Ensuing experiments have sought to further define the role that these cells play in tumor elimination. CD8 T cell independent tumor clearance is not completely novel, yet not fully accepted by tumor immunologists. Hopefully these results further provide proof for this means of anti-tumor response and lead to additional examination of the potential manipulation of this mechanism for future treatment strategies.

Introduction

Many researchers and clinicians are attempting to manipulate the immune system to eliminate cancer cells. Despite the numerous attempts in laboratory models and clinical trials, success has been limited. One problem is the lack of understanding of the mechanism of immune clearance of tumors. Understanding the necessary effector cells and mechanisms that yield efficient tumor clearance will guide therapeutic approaches for clinical application. The cytotoxic activity of CD8 T cells has long been presumed to be the effector function necessary for tumor regression. However, some studies suggest other immune functions are also at play (93). Many studies have shown that CD8 T cell responses can be elicited by a variety of immunotherapy approaches taken (28, 94-96). However, these cells are not able to efficiently eradicate tumors in many instances, leading some investigators to study the role of CD4 T cells in the anti-tumor immune response (93, 97). CD4 T cells are often ignored in tumor immunotherapy, despite the central role they play in initiating and maintaining an effective immune response.

Previous research has provided some clues as to how CD4 T cells may be contributing to an anti-tumor response. One recent publication described CD4 T cells as being more efficient at tumor rejection, than CD8 T cell, in an NK cell dependent manner (93). The authors proposed several explanations for this result, cross talk between NK cells and CD4 T cells, release of tumor inhibiting

cytokines by CD4 T cells, but did not show any direct killing of tumor cells induced by CD4 T cells.

Although the previous report suggests an indirect method of CD4 T cell mediated killing, CD4 T cells have been shown to act as efficient cytolytic cells, particularly in viral infection models (98). Despite this growing evidence for CD4 based mechanisms of killing that are independent of CD8 T cells the field of tumor immunotherapy has not yet addressed these important findings.

There are many components of the immune system, any of which could be responsible for the response to immunotherapy. In addition to T cells, B cells and NK cells can also induce an anti-tumor response. Currently, most of the attention has been given to the role of T cells, while B cells have largely been ignored. When B cells encounter antigen they can differentiate into antibody-secreting plasma cells or memory B cell, which confer protection upon secondary encounter with antigen (99). B cells can also present antigen to T cells, contributing to T cell activation (99). The BBB restricts the passive diffusion of large proteins, including immunoglobulins, into the brain and thus the role of an antibody response in the efficacy of immunotherapy has largely been ignored (43). Paradoxically, one study has shown a correlation between the presence of tumor-reactive antibodies in GBM patients and survival (100).

Members of the innate immune system are also important players that are often overlooked in tumor clearance. The recent influx of costimulatory molecules into pre-clinical and clinical trials for cancers has led to interesting findings. Of particular note was the characterization of the anti-tumor response induced by CD40 ligation through administration of a CD40 agonist antibody. This costimulatory molecule targets dendritic cells; presumably activation of this population would result in more robust antigen presentation to T cells and increased cytolytic T lymphocyte (CTL) activity. However, the findings reported an influx of activated macrophages with tumoricidal function into tumors in humans and a mouse model of pancreatic cancer (101). The observed tumor regression induced by CD40 agonist antibody treatment was further shown to be dependent on macrophages and independent of a CD4 or CD8 T cells (101).

Previous research has suggested alternative means of tumor eradication to the canonical CD8 killer T cell mechanism; which may shed light on routes of immune modulation that result in tumor clearance and lend insight into the reason for the current lack of effective immunotherapies despite the mounting clinical trials. The work described subsequently aims to dissect the mechanisms at work in a potent anti-tumor therapy model, utilizing vaccine/Fc-OX40L treatment in glioma-bearing animals. Our results indicate a CD4, B cell, and NK cell dependent means of tumor eradication, whereas CD8 T cells appear to be dispensable for enhanced survival. The following work should aid in

understanding the mechanisms at play in an effective anti-tumor response and guide future therapeutic designs.

Materials and Methods

Animal Models and Cell Lines. GL261-Luc and EMT6 culture conditions have been described previously (73, 75). Animals were maintained in a specific pathogen free facility according to the University of Minnesota Institutional Animal Care and Use Committee (IACUC) guidelines. Seven-week-old C57BL/6 (B6), B6.129S2-Cd8a^{tm1Mak}/J (CD8a$^{-/-}$), and B6.129S2-*Ighm*^{tm1Cgn}/J (μMT) mice were purchased from Jackson Laboratory. Five to seven week old B6.129P2-*Fcer1g*^{tm1Rav} N12 (FcRγ$^{-/-}$) mice were purchased from Taconic. Tumors were established by intracranial inoculation of 15,000 GL261-Luc glioma cells in 1 μL of Hank's balanced salt solution (HBSS) (Gibco) to animals anesthetized with a ketamine/xylazine cocktail (54.7 mg/mL ketamine and 9.26 mg/mL xylazine). Cells were implanted into the right striatum at coordinates 2.5 mm lateral, 0.5 mm anterior from the bregma, and 3 mm ventral from the surface of the brain and delivered at a rate of 0.2 μL/min over 5 minutes (73). Bioluminescence imaging 3 days following inoculation confirmed tumor implantation. Animals received 100 μL Luciferin (Gold Biotechnology) by intraperitoneal (IP) injection and were imaged with an IVIS50 system (Caliper Life Sciences). Living Image software (Caliper Life Sciences) was used to determine tumor burden in animals as a

measure of photons/second/cm^2 ($p/s/cm^2$); periodic bioluminescence imaging tracked tumor progression over time.

Breast carcinoma was induced in BALB/c and JHD (B cell deficient) animals purchased from Taconic by injecting 4 $x10^6$ EMT6 cells in 100 μL of HBSS cells into the upper right mammary fat pad while under sedation from isoflurane gas (75). Animals were humanely euthanized when a measurement of 20mm was reached for either the greatest longitudinal diameter (length) or greatest transverse diameter (width) by caliper measurements.

Vaccine Production and Delivery. Tumor cells were washed 3 times with phosphate buffered saline (PBS), resuspended in PBS, and flash frozen with liquid nitrogen. Cell were subjected to 5 cycles of freezing in liquid nitrogen and thawing in a 37°C water bath, vortexing after each round, to induce cell lysis and stored at -80°C until use. Trypan blue dye exclusion was used to verify complete cell death. A Pierce BCA Assay kit (Thermo Scientific) was used to determine protein concentration of the lysates. Purified, endotoxin free, CpG 1826, an unmethylated oligodeoxynucleotide (ODN) sequence (5'-tccatgacgttcctgacgtt-3') with a full phosphorothioate backbone was obtained from Integrated DNA Technologies (IDT, Coralville, IA), resuspended in 1x TE at a concentration of 10 mg/mL and stored at -80°C until use. CpG 685 (5'-tcgtcgacgtcgttcgttctc-3') was a kind gift from Dr. Wei Chen. Vaccine in Regimen

A consisted of lysate generated from 4 x 10^6 cells and 50 μg CpG 685, while Regimen B/C vaccine contained 65μg lysate and 50 μg CpG 1826. Animals received 100 μL of vaccine or saline by intradermal (i.d.) injection above the shoulders.

Costimulatory Fusion Protein Production and Delivery. Fc-OX40L was genetically engineered, biochemically characterized, and functionally validated previously (71). Fc-OX40L was given at 50 μg/dose, brought to a final volume of 100 μL per dose with PBS and delivered by IP injection.

Lymphocyte Depletion. Specific lymphocyte populations were depleted by administering depleting antibodies by IP injection. 100μg of anti-NK1.1 (clone PK136, ebioscience) or anti-CD4 (clone GK1.5, ebioscience) or 200μg of anti-CD8 (clone 53-6.7, ebioscience) delivered on days 1 and 2 before the first immunization, followed by injections one day before each additional vaccine (day 6 and 9) and before beginning Fc-OX40L treatment (day 16). Depletion was verified by euthanizing one animal from each group on day 3 before the first vaccination and analyzing splenocytes for presence of specified lymphocyte populations by flow cytometry, utilizing the same clones as described for the depletion.

Flow Cytometry. Brain-infiltrating lymphocytes (BIL) were harvested from animals on days 25, 35, and 45 post-inoculation. Mice were euthanized with a ketamine/xylazine cocktail and perfused with PBS to flush the capillaries. The brains were removed, minced with a razor, and passed over a 70 mm filter. Lymphocytes were collected from the interface of a two-layer Percoll gradient (70% and 30%) after centrifugation at 400xg for 30 minutes. Cell counts were obtained using trypan blue dye exclusion and a hemocytometer. Cells were stained with the following antibodies for phenotypic analysis CD3 (clone 17A2, ebioscience), CD4 (clone GK1.5, ebioscience), CD8 (clone 5H10, Invitrogen), NK1.1 (clone PK136, ebioscience), MHC II (clone M5.114.15.2, ebioscience), F4/80 (clone BM8, ebioscience), CD11b (clone M1/70, ebioscience), CD11c (clone N418, ebioscience), Foxp3 (clone FJK-16s, ebioscience), and Perforin (clone eBioOMAK-D, ebioscience). Intracellular staining of Foxp3 was achieved by utilizing the Foxp3/transcription factor intracellular staining kit according to the manufacturer's instructions (ebioscience). The BD Cytofix/Cytoperm plus kit (BD Biosciences) was used to gain intracellular staining of perforin. Cells were analyzed on a BD Bioscience FACSCanto and data were analyzed using Flowjo software (Tree Star). The percentage of stained cells was multiplied by the total number of viable cells, determined previously by trypan blue dye exclusion, to obtain the total number of stained cells, then divided by tumor burden ($p/s/cm^2$) to obtain the number of cells relative to tumor size.

To detect tumor reactive serum antibodies GL261 tumor cells were harvested and incubated with serum (1:100, by volume), washed thoroughly, and stained with a fluorescently labeled rat anti-mouse IgG (Jackson ImmunoResearch Laboratories) or IgM (clone II/41, ebioscience) antibody. Samples were analyzed on a BD Bioscience FACSCanto and mean fluorescence intensities were determined using Flowjo software (Tree Star).

IHC. Immunohistochemistry (IHC) was performed on brains isolated from tumor bearing animals. Animals were euthanized and perfused with phosphate-buffered water and 4% paraformaldehyde. Isolated brains were fixed in 10% formalin. Fixed brains were paraffin embedded; 5 μm sections were cut and mounted on slides and rehydrated. Tissues were stained with biotinylated anti-mouse secondary (Covance) reactive to heavy and light chains of IgA, IgD, IgE, IgG and IgM. Peroxidase-labeled streptavidin and DAB chromogen were used and hematoxylin counterstain provided cytological detail.

Western Blots. Tumor tissue was sonicated in RIPA buffer (25mM Tris-HCl,0.1% SDS, 1% Triton X-100, 1% sodiumdeoxycholate, 0.15M NaCl, 1mM EDTA) with protease and phosphatase inhibitors (1:100) (Calbiochem). Protein concentrations were determined using the Pierce BCA Assay kit (Thermo Scientific). For SDS PAGE, lysates were made 2mg/ml with laemmli reducing sample buffer. Protein standards (BioRad) were loaded next to each 40ug lysate

and resolved on NuPAGE 4-12% Bis/Tris gels (Invitrogen). Gels were equilibrated for 30 minutes in Towbins transfer buffer and proteins were then transferred to nitrocellulose (Amersham) at 5v constant voltage overnight using semi-dry transfer (BioRad). The membranes were blocked in 5% non-fat dry milk/ Tris-Tween buffered saline (NFDM/TTBS) at room temperature for one hour. For the detection of tumor reactive antibody each membrane was incubated at room temperature for one hour with serum diluted 1:1000 in 5%NFDM/TTBS. Membranes were washed six times for 10 minutes each in TTBS. Membranes were then incubated at room temperature for one hour in HRP conjugated secondary antibody (Jackson Immunoresearch) at 1:50,000 in 5% NFDM/TTBS and washed six times for 10 minutes each in TTBS. Immunoreactive bands were then detected using ECL Plus Western Blotting Detection System (Amersham) exposing membranes to HyBlot CL autoradiography film (Denville).

Statistical Analyses. A Mann-Whitney U test was used for the statistical comparison of lymphocyte counts and mean fluorescence intensities. Animal survival was evaluated by log-rank test. All tests were performed with Prism 4 software (Graph Pad Software, Inc). P values <0.05 were considered statistically significant.

Results

Multiple Treatment Strategies Utilized

Previously, it was shown that addition of the costimulatory molecule Fc-OX40L to vaccinations with tumor antigen and CpG adjuvant significantly enhanced the anti-tumor response in glioma-bearing animals (Chapter 2). Further insight into the mechanisms behind this response is crucial for designing future treatments. In the course of experimenting with the efficacy of treatment strategies, a variety of approaches have been utilized. To clarify the details of these therapeutic strategies the description of the treatment regimens used are modeled in **Figure 3-1**. Regimen A describes a vaccine only approach that utilized lysate generated from $4x10^6$ GL261 cells and 50μg of CpG 685. Regimen B outlines the first approach taken combining vaccination with Fc-OX40L. Vaccinations delivered on days 3, 7, and 10 consisted of 65μg of GL261 lysate and 50μg of CpG 1826 followed by 5 daily doses of Fc-OX40L (50μg) starting on day 17. Regimen B summarizes the optimized treatment strategy determine previously in Chapter 2, in which vaccination, same consistency as Regimen B, is delivered concurrently with Fc-OX40L on days 7, 10, and 13, followed by five daily doses of Fc-OX40L beginning on day 15.

In Vivo depletion of Lymphocytes

To determine the role of specific lymphocyte subsets contributing to the ability of vaccine/Fc-OX40L treatment to eradicate tumors, we depleted CD8, CD4, and NK cells using monoclonal antibodies in animals treated as described in Regimen B (**Fig. 3-1**). Antibodies were administered IP on day 1 and 2 post-inoculation and subsequently one day before each vaccination (day 6 and 9) and the day before the first Fc-OX40L administration (day 16). On day 3 before vaccination one animal from each depletion group was sacrificed and flow analysis was performed on isolated splenocytes to verify depletion (**Fig. 3-2A**). Survival of the depleted animals was compared to tumor bearing animals that were not depleted. Interestingly, CD4 depletion completely abrogated the survival benefit conferred from treatment (**Fig. 3-2B**). NK depletion also had an effect on survival in treated animals although it was not as severe as CD4 depletion. NK depleted animals survived significantly longer than saline, non-depleted animals, although, survival never reached the magnitude of animals that received vaccine/Fc-OX40L treatment in non-depleted animals. Surprisingly, CD8 depletion did not negatively affect overall survival (**Fig. 3-2B**), which suggests that the mechanism of action is CD8 independent.

The unexpected CD8 T cell independent finding raised the possibility that antibody depletion of CD8 T cells was not complete and thus an effect on survival was not observed. To address this concern, CD8 deficient ($CD8^{-/-}$) mice were challenged with GL261 and treated with vaccine/Fc-OX40L (Regimen B

and C, **Fig. 3-1**). The results of this experiment upheld the previous findings that CD8 deficiency did not negatively affect treatment efficacy (**Fig. 3-3A,B**). This was the case for both treatment regimens, and therefore suggests a CD8 T cell independent immune response responsible for increased survival and tumor clearance.

This is a surprising result considering that most tumor immunologists believe tumor eradication to be CTL mediated. This assumption is not unfounded, considering the mechanism of action CTL would seem to be the most efficient means of tumor killing. However, more recent work has demonstrated alternative mechanisms of killing, which may be highly dependent on tumor type, location, and treatment regimen. We aimed to determine the alternative means of tumor eradication induced by this treatment method.

Antibody response following vaccination

Previous work in the lab utilizing vaccine-based treatments (in the absence of Fc-OX40L) yielded results that shed light on the mechanism of action. Priming tumor-bearing animals with lysate/CpG vaccines alone had shown to be sufficient to produce an antibody response to the tumor. Tumor-bearing animals that had been treated as described for Regimen A (**Fig. 3-1**) were euthanized when they became moribund and their brains and serum analyzed for the presence of antibody. IHC of tumor sections with an anti-mouse Ig antibody revealed diffuse

staining throughout the tumor region, but absent in the normal surrounding brain (**Fig. 3-4A**). This staining was completely lost in μMT animals, which do not secrete antibodies, suggesting that this is not an artifact of the staining process (**Fig. 3-4B**). This staining, which was present in both saline and vaccine treated animals, suggests that antibodies are being deposited within the tumor core of animals. This same staining revealed the presence of plasma cells, which are antibody-secreting cells (**Fig. 3-4A**). Plasma cells that had infiltrated the tumor region were counted per tumor section and revealed an increase in the number of tumor infiltrating plasma cells in vaccinated animals compared to saline treated animals, plasma cells were not observed in μMT animals, as expected (**Fig 3-4C**). This infiltration of plasma cells into the tumor region may play an important role in the deposition of antibody at the tumor site. To verify the presence of antibody in the tumors of these mice, brains were harvested at a moribund stage and tumors were separated from the contralateral hemisphere (non-tumor bearing). Western blot analysis performed on these samples indicated that IgM and IgG are both present in the tumors of these animals, but absent in the contralateral hemisphere, consistent with the IHC findings (**Fig. 3-4D**). We did not observe any Ig in samples of normal brain, which is to be expected (**Fig. 3-4D**). Serum samples taken from these animals were analyzed for the presence of tumor-reactive antibodies. Lysate from GL261 cells was run on SDS-PAGE and blotted against serum from treated and untreated animals. A secondary anti-mouse antibody detected antibodies that had bound to tumor-associated

antigens. This analysis revealed the presence of tumor reactive antibodies in the serum of vaccinated animals, that were not detected in the serum of saline treated, μMT, or normal (non-tumor bearing) animals (**Fig. 3-4E**).

In this treatment strategy an incremental increase in survival was observed that was B cell dependent, as this survival benefit was lost in B cell deficient animals (μMT) (**Fig. 3-4F**). This suggests that B cells, and likely antibody, are necessary for the efficacy of treatment. Due to the incremental survival results and variability of lysates, the survival benefit induced by vaccination alone was inconsistent and therefore abandoned for the more robust and consistent treatment regimen including Fc-OX40L (discussed in Chapter 2).

B cell dependent mechanism of vaccine/Fc-OX40L treatment

Based on these previously findings it is reasonable to investigate the role that B cells were playing in the Fc-OX40L treatment. To determine if the B cell dependent mechanism was upheld with the Fc-OX40L treatment, WT and μMT (B cell deficient) animals were inoculated with GL261 tumor cells. Glioma bearing animals were treated as described for Regimen B (with and without vaccine) and Regimen C. In all of the treatment regimens tested treatment benefit was lost in the μMT animals (**Fig. 3-5A,B,C**). This suggests that B cells are necessary for proper immune response and eradication of tumor. Due to the

superior efficacy of Regimen C treatment (discussed in Chapter 2) all subsequent experiments utilize this treatment strategy.

A previously published report observed that ligation of GITR or OX40, in this case with agonist antibodies, resulted in tumor regression and prolonged survival in a breast tumor model (102). They also observed that the mechanism of action was B cell dependent for GITR stimulation but B cell independent in the case of OX40 ligation (102). This result is profoundly different from that seen in the glioma model, leading to speculation that the B cell dependency may be location specific. As previously mentioned, the CNS is immunologically specialized and biased to a Th2 response. Therefore it is reasonable to assume that the differences in the outcomes may be microenvironment specific. To test this, BALB/c mice were challenged with EMT6 breast carcinoma cell line and treated, as described for glioma bearing animals Regimen C, with vaccine (made with EMT6 cell lysate) and Fc-OX40L. The treatment regimen was enhanced slightly to account for the difference in kinetics of the tumor models; EMT6 tumor-bearing animals were treated with vaccine/Fc-OX40L on days 5, 8, and 11 and Fc-OX40L on days 13-17. This treatment was tested in WT BALB/c mice and a B cell deficient strain on a BALB/c background (JHD). As expected, vaccine/Fc-OX40L treatment resulted in tumor regression and prolonged survival in WT animals. However, this treatment efficacy was lost in JHD mice, suggesting that the B cell dependency is less a factor of environment and more of a treatment factor (**Fig.**

3-6). However, we did not test Fc-OX40L in the absence of vaccine in this model, the potential exists that the vaccine contains factors influencing the dependency on B cells.

Antibody response to vaccine/Fc-OX40L treatment

The B cell dependency noted for treatment efficacy could indicate several different mechanisms of B cell action. B cells are able to act as antigen presenting cells, although this role is less well described for B cells than for dendritic cells. B cells are also able to differentiate into antibody secreting cells (plasma cells) and exert effector function through antibody-mediated mechanisms. The induction of an antibody response, seen previously following vaccination (**Fig. 3-4**), led to the investigation of the antibody response in the combination vaccine/Fc-OX40L treatment animals.

Flow cytometry can be used to detect antibodies that bind to intact tumor cells and offers a way to quantify the tumor reactive antibody response. Serum was collected from saline, vaccine, Fc-OX40L, and vaccine/Fc-OX40L treated animals at day 25 post- inoculation. Previous survival experiments had indicated that untreated animals generally began to die around day 28. The day 25 timepoint was chosen because this timepoint was after the treatment regimen had been completed, but prior to the health decline of the untreated animals. Serum was also collected at day 35 and 45 for vaccine/Fc-OX40L treated

animals to assess the later timepoints when the tumors begin to regress. Antibody binding to tumor cells measured through flow cytometry did not demonstrate significant differences in the presence of tumor reactive IgM antibody between the treatment groups (**Fig. 3-7**). For tumor reactive serum IgG, all groups had elevated levels of tumor reactive antibodies relative to serum from a normal (non-tumor bearing) animal (**Fig. 3-7**). Comparing between treatment groups the levels at day 25 were similar between groups, while levels at the later timepoints showed a trend to increasing IgG levels at days 35 and 45 in vaccine/Fc-OX40L treated animals, although this did not reach statistical significance. This is indicative of an endogenous antibody response in tumor bearing animals, which may indicate that Fc-OX40L is not responsible for the induction of antibody secretion. The trend toward increasing levels of IgG over time in the vaccine/Fc-OX40L treated animals indicates that Fc-OX40L may aid in the maintenance or help promote the antibody response over time.

Antibody deposition in tumor

Previously, antibody deposition was observed in the brains of vaccinated mice by western blot and IHC (**Fig. 3-4**). To determine if this deposition occurred in the vaccine/Fc-OX40L treated animals and to gain insight into the kinetics of this occurrence, animals were inoculated with GL261 tumor, treated with vaccine/Fc-OX40L, and euthanized on days 25, 35, and 45. Saline and vaccine alone controls were also collected on day 25, but not at later timepoints, as most

animals in these control groups do not survive that long. Tumors were isolated and analyzed by western blot.

Western blot analysis revealed the presence of IgG in the tumors of animals treated with vaccine/Fc-OX40L (**Fig. 3-8A**). Bands consistent with the heavy and light chain (75 kDa and 25 kDa, respectively) of IgG were detected in the tumors of animals treated with vaccine/Fc-OX40L. The 50kDa band consistent with the heavy chain of IgG was not detected in the tumors of saline treated animals. The presence of a 25kDa band in these tumors is likely due to the cross reactivity of the antibody with the light chain of the other immunoglobulins. IgG is also detected in the majority of the vaccine treated tumors as well. Interestingly, while all of the tumors analyzed for vaccine/Fc-OX40L at day 25 contain IgG, the number of animals with detectable IgG at the later timepoints decreases at day 35 (4/5) and day 45 (3/5). This may be due in part to the decreasing tumor burden at these later timepoints due to tumor clearance.

IgM was detected in the tumors of animals of all treatment groups, although with varying penetrance (**Fig. 3-8B**). The heavy chain of IgM was detectable and appears to be most penetrant at day 35 in vaccine/Fc-OX40L treated animals. It is important to note that this analysis is not quantifiable as multiple gels were used to run these samples. It is only accurate to determine the presence or

absence of Ig, but not to make conclusions about the relative levels of Ig detected.

Based on the previous analysis of serum antibodies saline treated animals appear to make IgG tumor reactive antibodies (**Fig. 3-7**), however we are not detecting IgG in the tumors. The presence of IgM in the tumors of saline treated mice, but not IgG, suggests that perhaps infiltration of class-switched immunoglobulin is impaired.

Involvement of the Fc Receptor

The indication that antibody production may play a significant role in tumor elimination, combined with the loss of treatment efficacy observed when NK cells were depleted (**Fig. 2B**) led to the investigation of the role that antibody-dependent cell-mediated cytotoxicity (ADCC) may play in treatment induced tumor regression. ADCC requires the Fc portion of immunoglobulins, bound to target antigen, to bind to Fc receptors (FcR) on the surface of effector cells, most commonly NK cells. This binding induces lysis of the target cell by the effector cell, generally through the production of perforin and granzymes.

To test if this mechanism was the link between the NK cell and antibody results, treatment efficacy was tested in mice deficient for the gamma chain of the FcR (FcRγ-/-). These mice lack the Fc receptors capable of binding IgG and IgE and

therefore ADCC action mediated through these immunoglobulins would be inhibited. The tumor burden of these animals was tracked over time through bioluminescence imaging. This analysis revealed that while saline treated animals of either genotype were not able to control tumor growth, the tumors of WT vaccine/Fc-OX40L treated animals grew at a much slower rate. The tumor growth of FcR$\gamma^{-/-}$ animals was intermediate between saline and WT vaccine/Fc-OX40L (**Fig. 3-9 A,B**). This indicates that FcR mediated mechanisms are required for complete tumor clearance. However because the tumor progression in FcR$\gamma^{-/-}$ animals is slower than in saline animals there are likely other, FcR independent mechanisms, which are capable of slowing tumor growth. Analysis of the overall survival of these animals revealed that WT and FcR$\gamma^{-/-}$ animals that received vaccine/Fc-OX40L treatment did not differ significantly (**Fig. 3-9 C**). However there was a significant difference in the overall survival of saline treated WT and FcR$\gamma^{-/-}$ animals, suggestive of an endogenous response involving the FcR (**Fig. 3-9 C**).

Analysis of brain infiltrating cells

To further understand the mechanism involved in tumor clearance, flow cytometry analysis was performed on lymphocytes isolated from the brains of tumor bearing animals. Glioma-bearing animals treated with vaccine/Fc-OX40L (Regimen C) were euthanized at days 25 and 35. Glioma-bearing animals treated with saline, Fc-OX40L alone, and μMT animals treated with vaccine/Fc-

OX40L were euthanized on day 25 only as health deterioration became limiting at later timepoints. One day prior to euthanasia the tumor burden of the animals was assessed through bioluminescence imaging. This revealed a significant difference in the tumor size amongst the groups; animals receiving Fc-OX40L as part of the treatment, with the exception of μMT animals, showed significantly less tumor burden (**Fig. 3-10A**). Lymphocytes harvested from the brains of these animals were counted and plotted as absolute numbers. An increase in the number of brain infiltrating cells was observed in the WT animals receiving vaccine/Fc-OX40L treatment at day 25. This only reached statistical significance when compared to μMT animals (**Fig. 3-10B**). The large variation in tumor size may distort the true value of infiltrating cells present in the brain, as larger tumors may contain more infiltrating cells simply due to volume. To compensate for this difference, the number of brain infiltrating cells was normalized to the tumor burden determined by bioluminescence imaging ($p/s/cm^2$). This analysis revealed that based on tumor burden there were more lymphocytes infiltrating the tumors of Fc-OX40L treated animals, with the exception of μMT animals (**Fig. 3-10C**).

Phenotypic staining assessed the composition of the lymphocyte population harvested from the brains of glioma-bearing animals. The T cell population was determined by analyzing the CD3+CD4+Foxp3- (Th) and CD3+CD8+ populations. An increase in the infiltration of CD4 T cells was observed for the

WT animals treated with Fc-OX40L (**Fig. 3-11A**), consistent with the earlier finding that CD4 T cells are necessary for efficacy of treatment (**Fig. 3-2B**). Interestingly, when the number of CD8 T cells was normalized to tumor burden, an increase in infiltration was observed in the Fc-OX40L treated animals (**Fig. 3-11B**), despite the fact that these cells are not necessary for effector function (**Fig. 3-2B**). Although these cells are present, this analysis does not indicate if this population is active. Analysis for the presence of perforin, used in the cytolytic function of CD8 cells, revealed that this population does not express perforin, which may suggest that they are not actively functioning as killer cells (data not shown). Importantly, CD4 T cells also did not demonstrate expression of perforin, indicating a potential indirect mechanism of killing by these cells. Ligation of OX40 on the surface of Tregs has been shown to decrease the suppressive function of this population (103). It is reasonable to assume that one mechanism of Fc-OX40L in tumor eradication is to suppress the regulatory function of these cells, thus allowing the effector cells to kill. Analysis of the CD3+ CD4+ Foxp3+ (Treg) population in the brains demonstrates an increase in the presence of Tregs in the tumors of animals treated with Fc-OX40L (**Fig. 3-11C**). This is contrary to what was expected, however, it is possible that the increased immune infiltration observed triggers an influx of Tregs to balance the inflammatory response. This analysis also does not indicate if this population is actively suppressing effector cells; functional analysis on isolated cells would be required to fully understand the effect on Tregs. Interestingly, the Treg

population was consistently decreased in the μMT vaccine/Fc-OX40L animals. These animals are known to have defects in the development of CD4 T cell, particularly Tregs, as normal B cell helper function is absent during key developmental times (104). Despite this apparent advantage it did not prove beneficial as all these animals succumb to tumor burden.

The NK cell population was of particular interest as loss of the NK cell population resulted in loss of treatment efficacy (**Fig. 3-2B**). The total CD3-NK1.1+ population was assessed, to rule out NK T cells, and observed to be increased in Fc-OX40L treated animals when normalized to tumor burden (**Fig. 3-12A**). Impressively, the CD3-NK1.1+perforin+ population was increased in the Fc-OX40L treated groups (**Fig. 3-12B**). This was exciting as it indicates that NK cells are not just present but are functional. Particularly interesting is the fact that perforin mediated killing by NK cells is a primary mechanism of ADCC mediated killing providing a possible link between the antibody observation and functional mechanism.

An intriguing observation was the presence of a distinct CD3+NK1.1+ population, which is indicative of NK T cells in the brains of Fc-OX40L treated animals (**Fig. 3-12C**). More surprisingly was the significant number of perforin expressing NK T cells in the Fc-OX40L treated brains (**Fig. 3-12D**).

The most noticeable difference between the treatment groups was the presence of a granular population present only in the vaccine/Fc-OX40L treatment groups that was absent in the saline and the μMT vaccine/OX40L treatment groups (**Fig 3-13**). Phenotypic analysis revealed that the majority of cells in this population are CD11b+ MHC II-. This phenotype and its granularity are indicative of a neutrophil population, which like NK cells are able to kill antibody coated cells through ADCC. It is important to note that this population is also consistent with the myeloid derived suppressor cell (MDSC) population, which are generally only distinguishable from neutrophils by functional assays. This granular population expresses perforin, which is consistent with neutrophil mechanism of killing, whereas MDSCs are generally suppressive through other means.

Discussion

Despite the increasing interest in utilizing immunotherapeutic approaches in cancer treatment, the prognosis for patients diagnosed with glioma is dismal. Advances in the field have been incremental and an inherent hurdle to progress is the lack of a basic understanding of the mechanism needed for an effective immune based anti-tumor response. One route of gaining insight into these mechanisms is examining the immune players at work during an effective anti-tumor response. A potent anti-tumor treatment was demonstrated previously in a murine model of glioma (Chapter 2). We aim to gain insight into the mechanisms

at work during tumor clearance to aid in the development of more robust clinical therapies in the future.

We have shown that this response is dependent on a CD4 and NK cell response as efficacy is lost upon depletion of these cells. Surprisingly, we did not observe a dependency of CD8+ T cells, as CD8 depletion did not alter survival outcome. We have shown that contrary to common belief the CTL response induced by CD8+ T cells is not as important as the CD4+ T cell response in this model. Many cancer vaccines have been designed to bias toward a CD8 response, however this may not be optimal. Further work remains to be done to identify the mechanism of CD4 action. It has been reported that CD4 T cells are capable of cytolytic function through direct and indirect killing in viral and tumor models (93, 98, 105). It may be possible that our model may be reproducing the results observed in these previous publications. It is also possible that in an attempt to prevent tissue damage from an inflammatory response the CD8+ T cells may be suppressive. Previous labs have shown that CD8 T cells express the suppressive cytokine IL-10 in response to viral infections in the lung and brain, where prolonged activation may cause deleterious bystander effects (24, 106). These remain attractive theories that need to be tested and utilizing the optimized strategy of vaccine/Fc-OX40L in the GL261 glioma model presents as a robust model to pursue these questions in the future.

Additionally, B cells are required for the efficacy of treatment. B cells may be necessary to act as APCs for CD4 activity or they may be differentiating into plasma cells and secreting tumor reactive antibodies. The observation that tumor-bearing animals generate tumor reactive antibodies and the deposition of antibody in the tumors of vaccine/Fc-OX40L treated animals is indicative of an antibody mediated mechanism. The lack of control of tumor growth in $FcR\gamma^{-/-}$ mice indicates that antibody binding to Fc receptors is necessary for slowing tumor growth, however these animals are able to control tumor growth better than saline treated animals and therefore indicates that multiple mechanisms are at play.

The infiltration of tumor by immune cells is key in understanding the mechanism of tumor clearance. The most notable observation was the increase in NK, NK T cells, and the large granular population, all expressing perforin. These populations are of particular interest due to the perforin expression because this indicates a functional capacity of these cells. Recruitment and activation of NK cells, NK T cells, and neutrophils may be mediated by cytokine secretion by CD4 T cells activated through OX40 ligation. Another explanation could involve direct activation by OX40L. Although the majority of research conducted on OX40:OX40L interactions has focused on activated T cells, there have been some reports indicating that NK cell (107), NK T cells (108), and neutrophils (109) may express the OX40 receptor. It remains a possibility that Fc-OX40L

administration could be acting directly on the innate immune cells to induce a productive anti-tumor response.

The observed infiltration of perforin positive, innate immune cells is particularly interesting in light of the antibody data, due to the known link between the NK cells, neutrophils and ADCC. It is possible that the perforin positive populations of these cells are actively killing antibody coated tumor cells and inhibiting growth in an FcR dependent mechanism. To truly answer if antibodies are necessary for tumor clearance inhibiting antibody secretion will be necessary. Mouse models exist that, unlike the μMT model, generate normal mature B cells, however fail to differentiate into plasma cells or secrete antibody. Utilizing these animals in future survival studies will be key to closing the link between these observations. It is also important to note that there remains an FcR independent mechanism of tumor clearance, as the FcR$\gamma^{-/-}$ phenotype was only intermediate. These mechanisms still need to be elucidated in order to fully understand the mechanisms at work.

It is not surprising that there are multiple mechanisms of tumor clearance occurring within the animal. Too often in the design of immunotherapeutics particular immune subsets are targeted while ignoring the remainder of the immune system. This is particularly noted for CD8 T cell biased therapies. Many vaccines have focused on CD8 restricted antigens or adoptive transfer

exclusively (95). Even in the case of CD8-mediated tumor clearance recruitment of additional immune cells is necessary, particularly CD4+ Th cells that are needed for maintenance of the CD8 response. Our data indicate that vaccine/Fc-OX40L treatment efficacy involves a CD8 independent mechanism. The observation of an increase in the influx of innate cells to the tumor site upon treatment and the presence of a tumor reactive antibody response appears to be linked through FcR dependent mechanism. The current working model of tumor clearance appears to be, at least in part, due to ADCC. Despite the evidence in favor of this model more needs to be done to fully describe the mechanisms involved. A deeper understanding of immune mediated mechanism of tumor clearance will certainly drive the design of immunotherapeutics in the clinic with the hope of fully harnessing the power of the immune system to mediate tumor clearance.

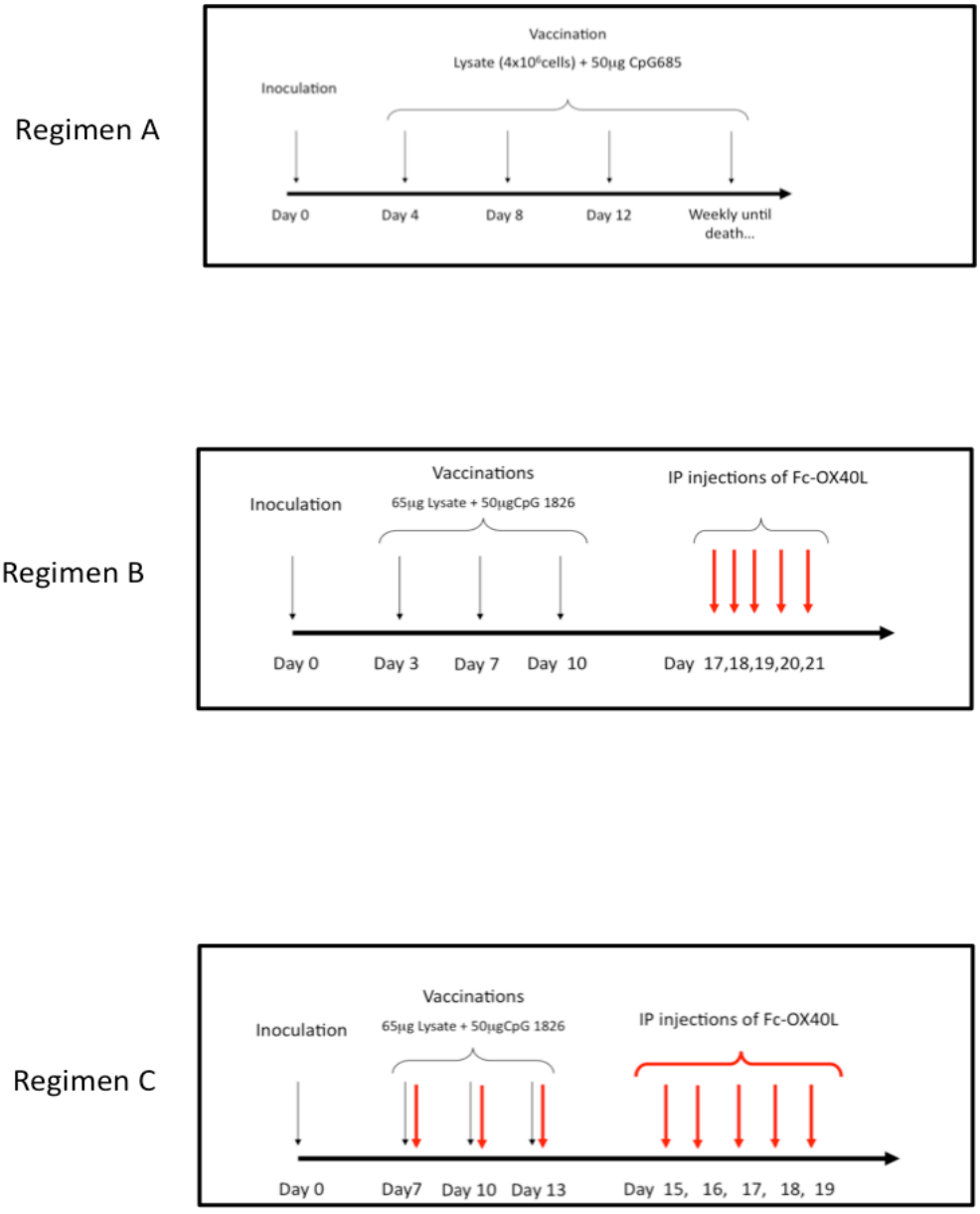
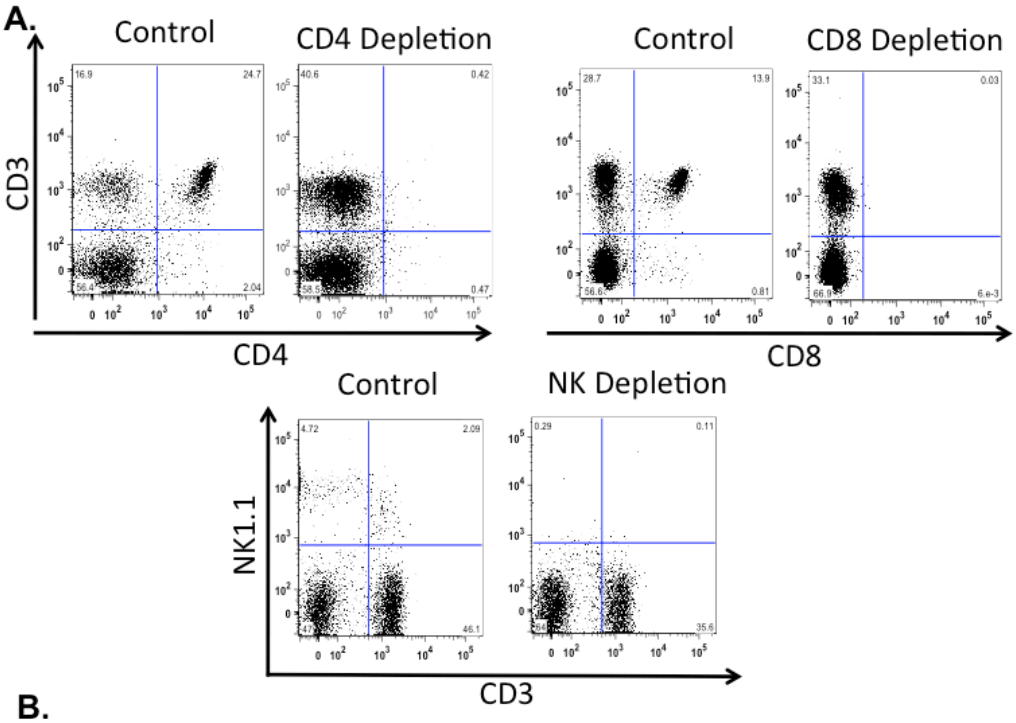
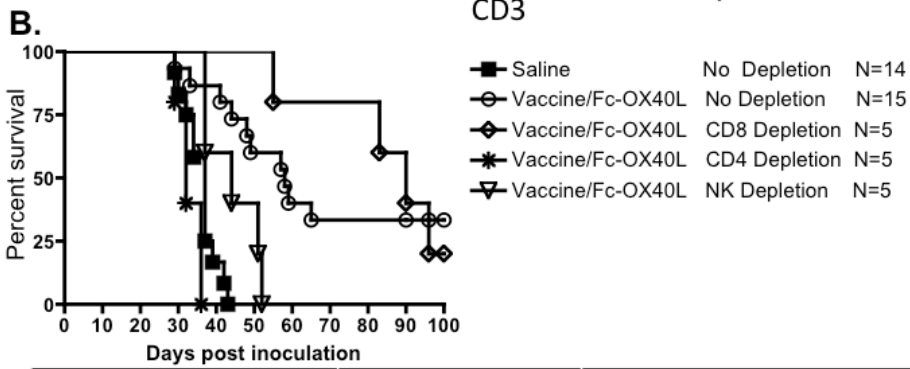


Figure 3-1. Description of treatment regimens used. Several different treatment strategies were employed throughout the numerous experiments. Each treatment regimen is outlined and referenced through the text. Regimen A describes vaccination alone consisting of lysate from $4x10^6$ cells and 50 μg CpG 685. Regimen B utilizes a vaccination of 65 μg of lysate and 50 μg CpG 1826 on days 3, 7, and 10 followed by five daily doses of Fc-OX40L (50μg) starting on day 17. Regimen C involves vaccination with 65 μg of lysate and 50 μg CpG 1826 on days 7,10, and 13, concurrent with 50 μg of Fc-OX40L followed by five daily doses of Fc-OX40L starting on day 15.

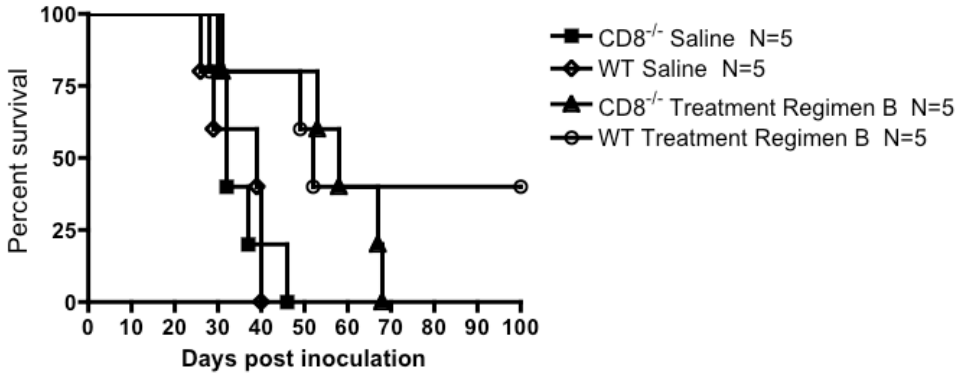




Group	Median Survival	P value (vs Vaccine/Fc-OX40L No Depletion)
Saline No Depletion	37	<0.0001
Vaccine/Fc-OX40L CD8 Depletion	90	N.S.
Vaccine/Fc-OX40L CD4 Depletion	32	0.0003
Vaccine/Fc-OX40L NK Depletion	44	0.0364

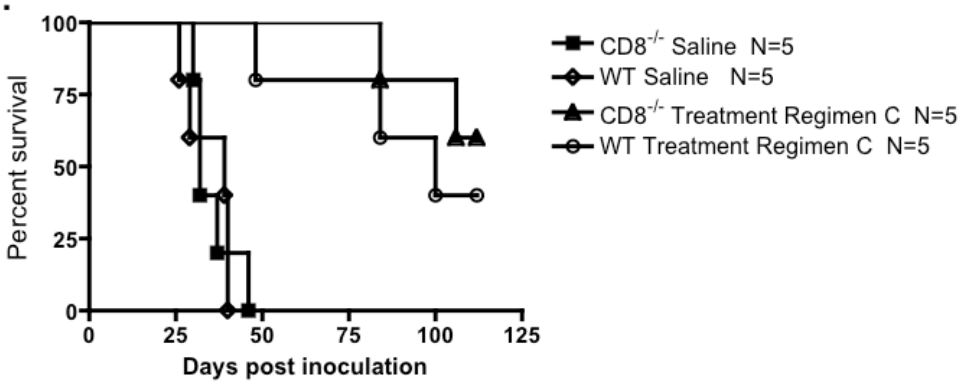
Figure 3-2. In vivo depletion of lymphocytes. Necessity of lymphocyte populations for treatment efficacy was determined through *in vivo* depletion of CD4, CD8, and NK cells. (A) Verification of depletion by flow analysis of splenocytes following two days of depleting antibodies. (B) Glioma-bearing animals were treated (Regimen B) and survival of animals depleted of lymphocyte populations was compared to non-depleted saline and vaccine/Fc-OX40L treated animals.

A.



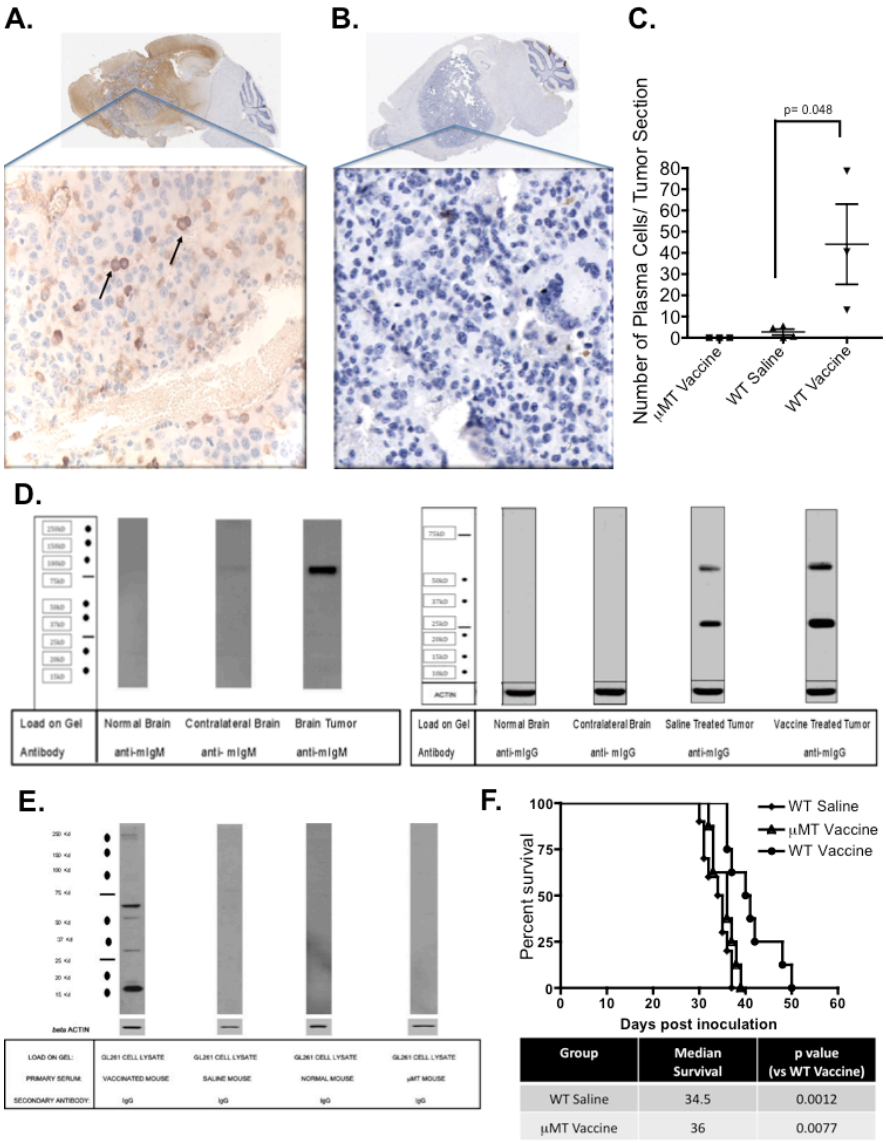
Group	Median Survival	P value (vs WT Vaccine/Fc-OX40L) Regimen B
WT Saline	39	0.034
CD8-/- Saline	32	0.035
CD8-/- Vaccine/Fc-OX40L	58	N.S.

B.



Group	Median Survival	P value (vs WT Vaccine/Fc-OX40L) Regimen C
WT Saline	39	0.0027
CD8-/- Saline	32	0.0018
CD8-/- Vaccine/Fc-OX40L	undefined	N.S.

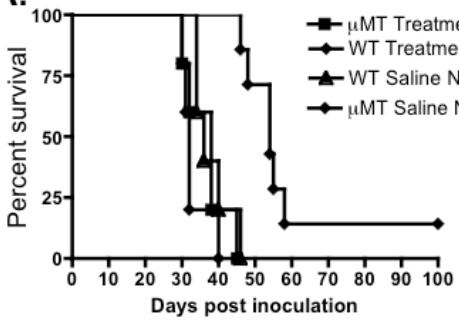
Figure 3-3. Survival in CD8 deficient animals. To verify the depletion results the survival of glioma-bearing animals was assessed in WT animals compared to $CD8^{-/-}$ animals with treatment from (A) Regimen B and (B) Regimen C.



Group	Median Survival	p value (vs WT Vaccine)
WT Saline	34.5	0.0012
μMT Vaccine	36	0.0077

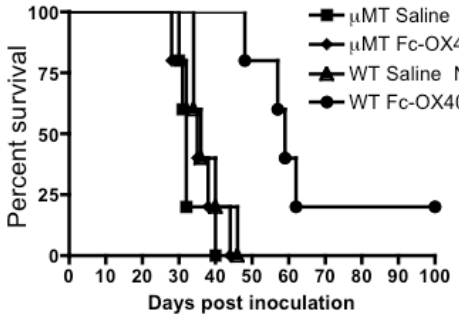
Figure 3-4. Antibody response induced by vaccination. Glioma-bearing animals vaccinated according to Regimen A were analyzed for the induction of an antibody response at a moribund stage. Brains were stained for anti-mouse Ig in (A) WT and (B) μMT animals, with magnification of tumor region, arrows indicating plasma cells. (C) The number of tumor infiltrating plasma cells was plotted for WT saline treated, and vaccinated WT and μMT animals. (D) Western blot analysis for the presence of IgM and IgG in tumors compared to normal brain. (E) Western blot analysis of tumor reactive serum antibodies against GL261 tumor cell lysate, comparing serum from vaccinated animals to saline treated and normal sera. Serum was collected from μMT animals as a negative control. (F) Survival of WT animals treated with saline or vaccine compared to μMT vaccinated animals.

A.



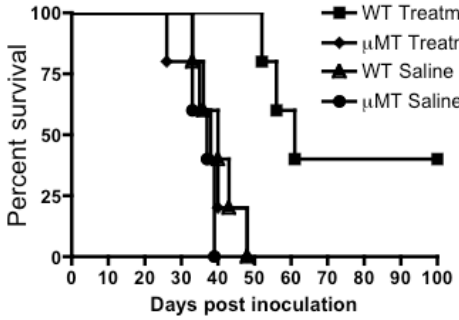
Group	Median Survival	p value (vs WT vaccine/ Fc-OX40L)
WT Saline	36	0.0007
μMT Saline	32	0.0004
μMT Vaccine/Fc-OX40L	38	0.0004

B.



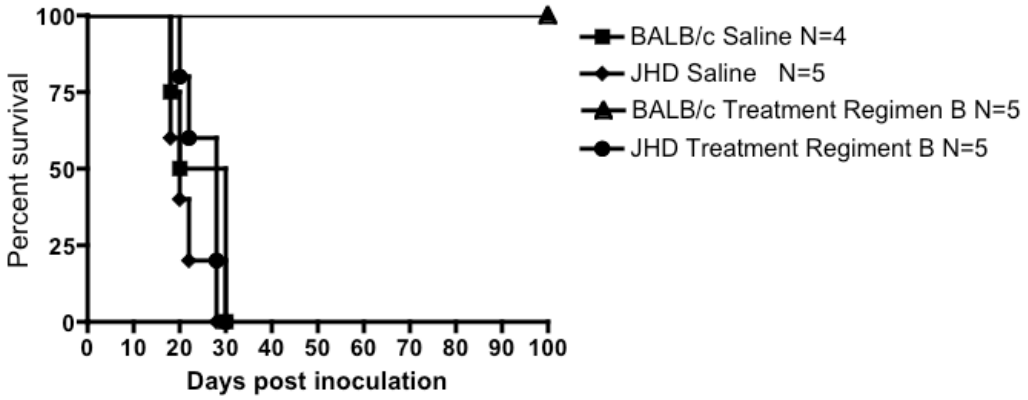
Group	Median Survival	p value (vs WT Fc-OX40L)
WT Saline	36	0.0017
μMT Saline	32	0.0021
μMT Fc-OX40L	35	0.0018

C.



Group	Median Survival	p value (vs WT vaccine/ Fc-OX40L)
WT Saline	40	0.0018
μMT Saline	37	0.0025
μMT Vaccine/ Fc-OX40L	38	0.0018

Figure 3-5. B cell dependent mechanism of treatment efficacy. To assess the role of B cells in treatment efficacy the survival of WT animals was compared to μMT animals receiving treatment as described for (A) Regimen B, (B) Regimen B, without vaccination, and (C) Regimen C.



Group	Median Survival	p value (vs BALB/c vaccine/Fc-OX40L)
BALB/c Saline	25	0.0040
JHD Saline	20	0.0017
JHD Vaccine/Fc-OX40L	28	0.0021

Figure 3-6. B cell dependent mechanism in breast carcinoma model. To determine if the B cell dependency described in Figure 3-5 was tumor location specific, treatment efficacy was assessed in an EMT6 breast carcinoma model in a BALB/c strain. WT BALB/c and JHD (B cell deficient) animals were treated with vaccine/Fc-OX40L on days 5, 8, and 11 and Fc-OX40L on days 13-17 and survival was monitored.

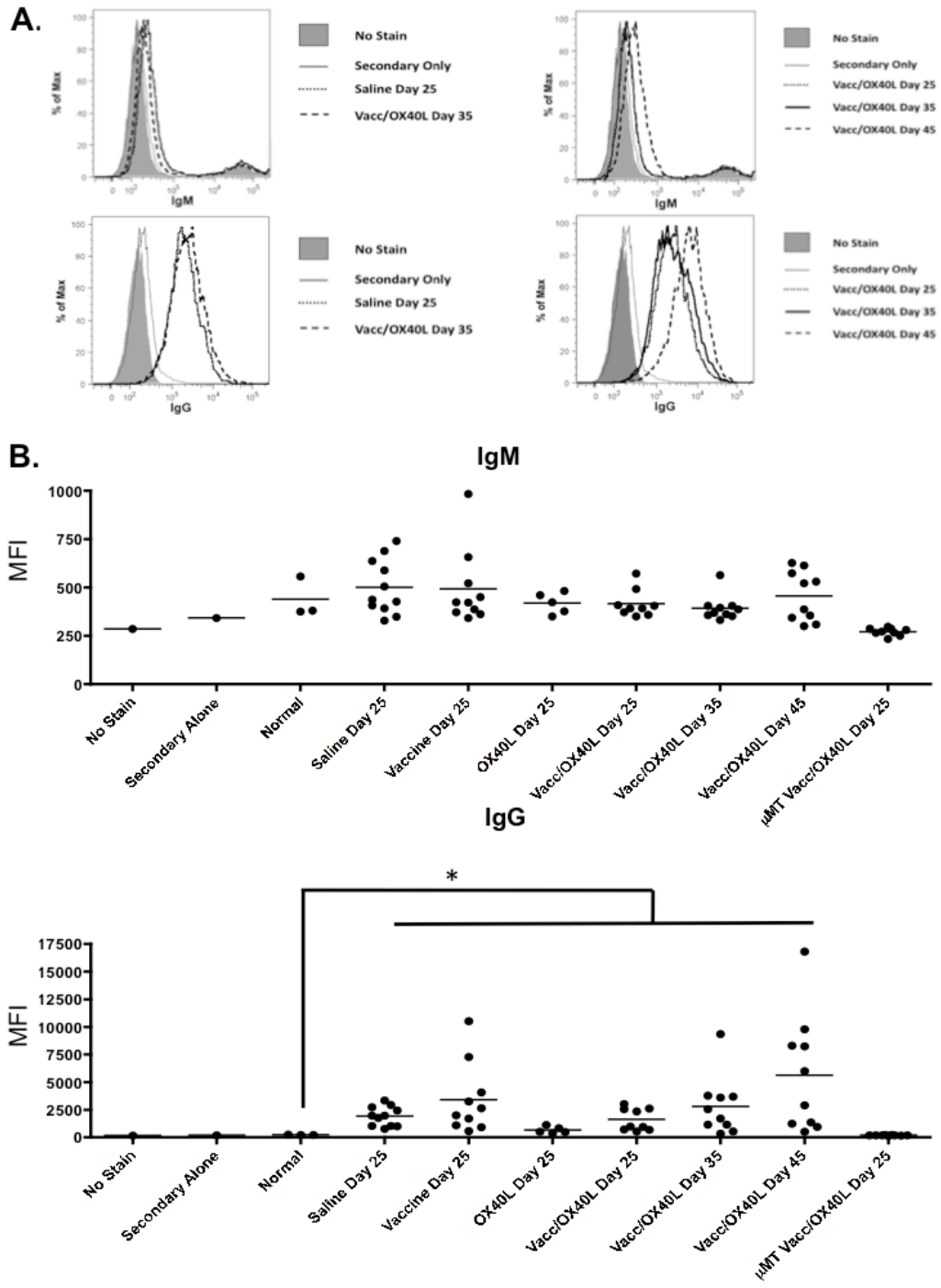


Figure 3-7. Antibody response to vaccine/Fc-OX40L treatment. The presence of tumor-reactive antibodies in the serum of animals was determined through flow cytometry analysis. Glioma-bearing animals were treated (Regimen C), euthanized at noted timepoints, and sera collected. Tumor reactive IgM and IgG antibodies were plotted based on mean fluorescence intensities. Statistical significance is indicated as $p < 0.05$ (*); n=5-11/treatment group.

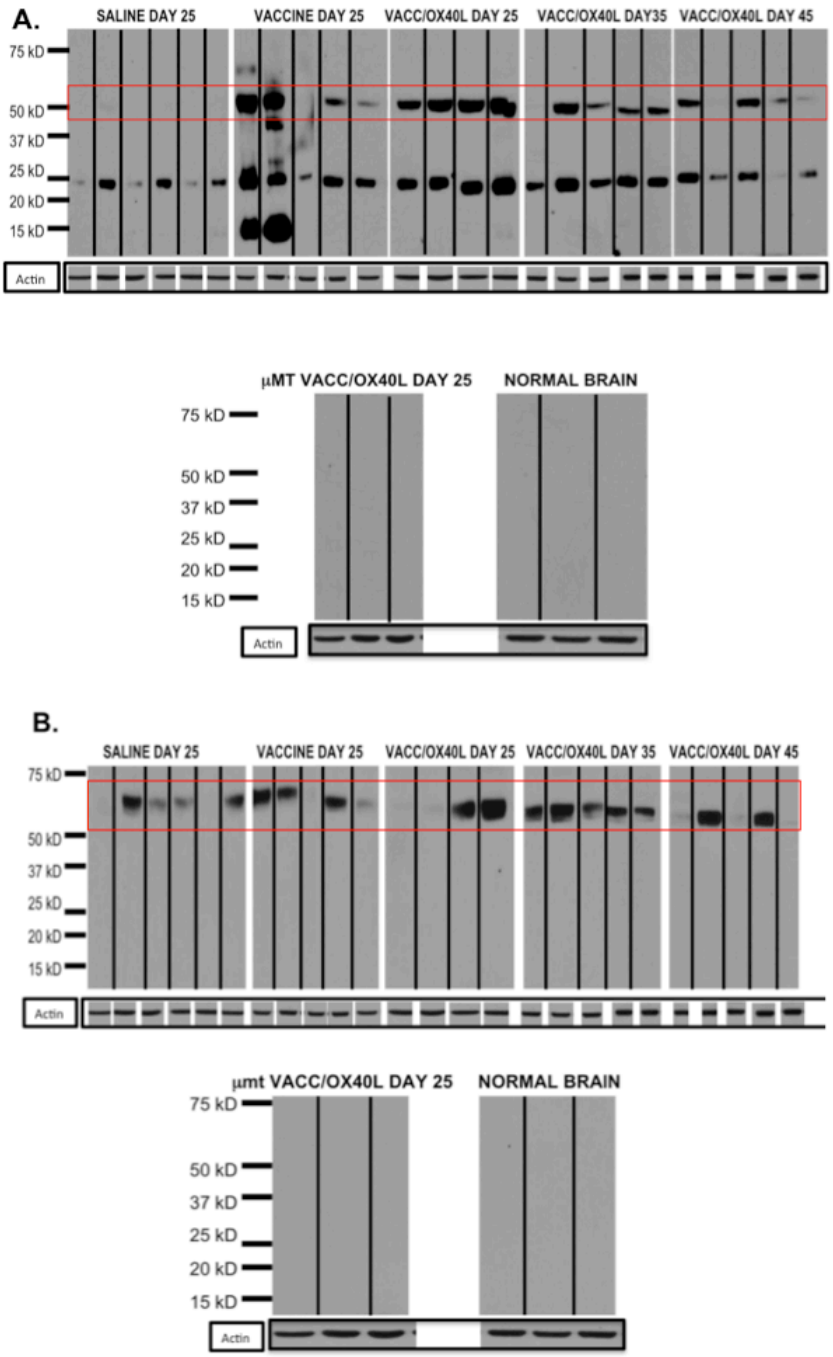


Figure 3-8. Western analysis of immunoglobulin in tumors. The presence of immunoglobulins was detected in tumors harvested from brains of glioma-bearing animals at noted timepoints. Tumors were run on SDS-PAGE and probed for (A) IgG and (B) IgM to detect the presence of these immunoglobulins. Tumors from µMT animals and normal (non-tumor bearing brains) were used as negative controls.

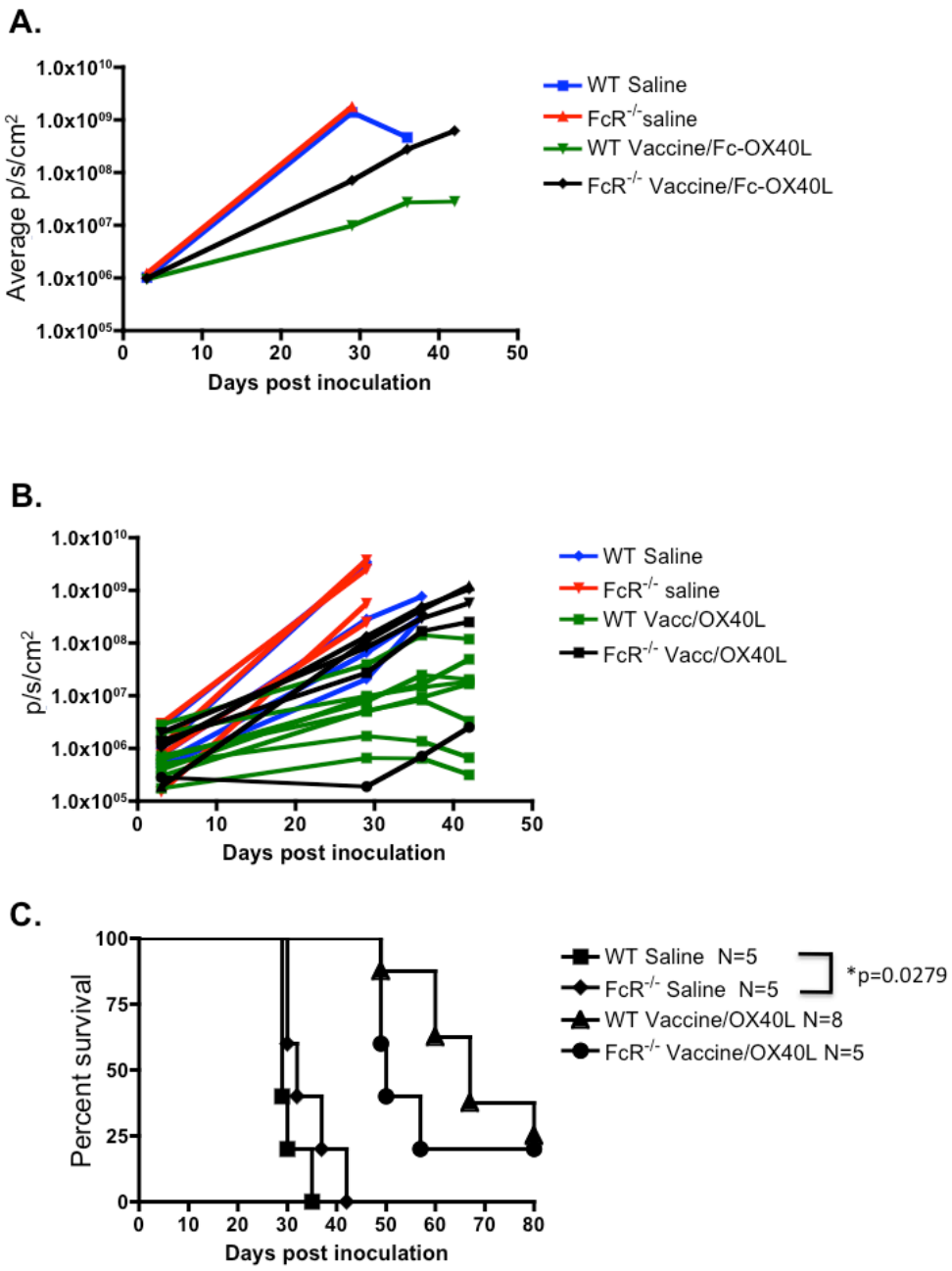


Figure 3-9. Involvement of the Fc Receptor. To determine the necessity of antibody dependent mechanisms in tumor clearance the tumor growth of FcRγ$^{-/-}$ mice was compared to WT mice. Glioma-bearing animals were treated with saline or vaccine/Fc-OX40L (Regimen C). Bioluminescence imaging was utilized to track tumor progression over time. (A) Averaged signal per group was plotted over time. (B) Signal from individual animals plotted over time. (C) Overall survival of glioma-bearing WT and FcRγ$^{-/-}$ animals.

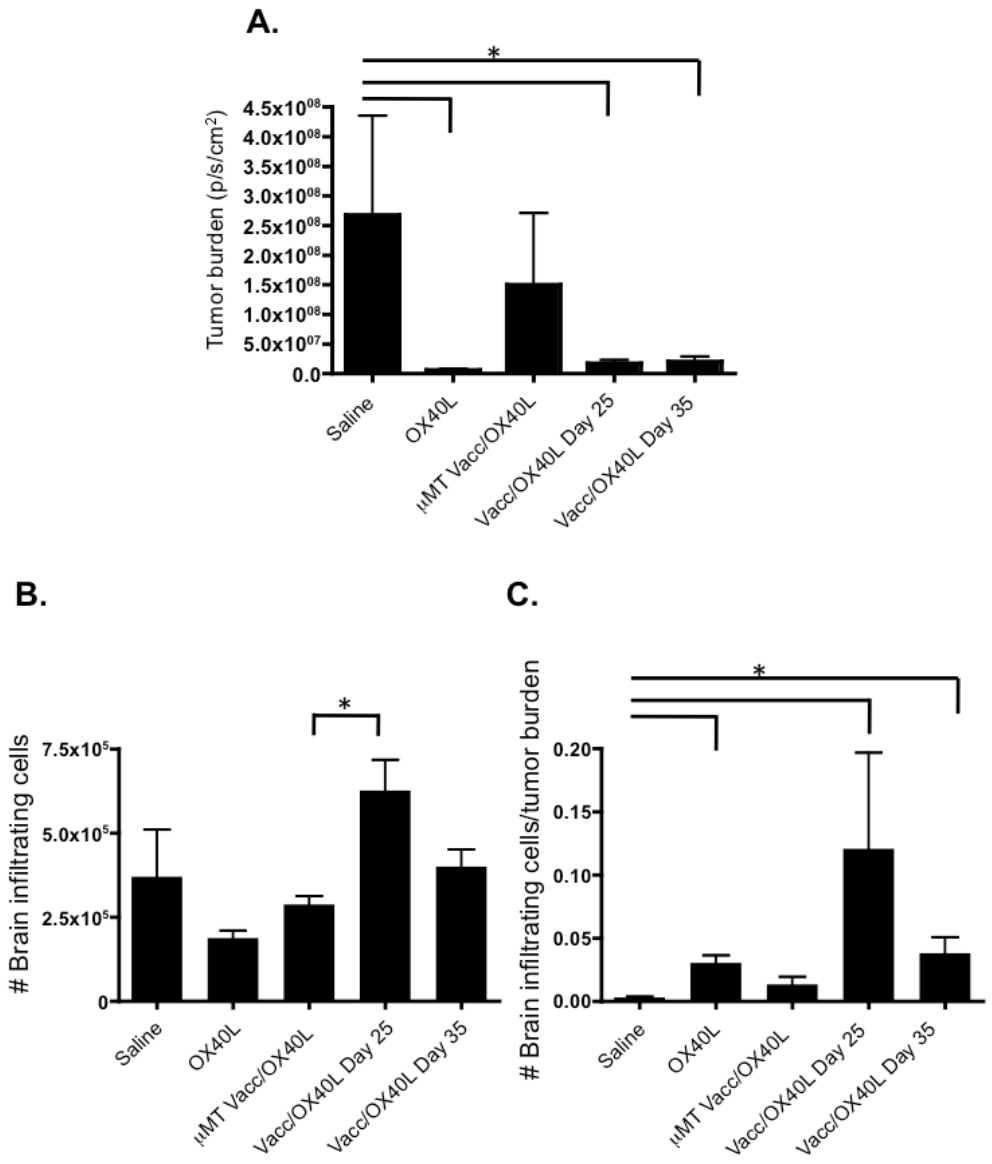


Figure 3-10. Analysis of brain infiltrating cells. (A) Tumor burden in glioma-bearing animals was determined by bioluminescence imaging one day prior to euthanasia at noted timepoint. (B) Absolute number of brain infiltrating lymphocytes for each treatment group and (C) number of brain infiltrating lymphocytes relative to tumor burden (p/s/cm^2). Statistical significance is indicated as $p < 0.05$ (*). Mean values are shown as +/- SEM; n=5-10/group.

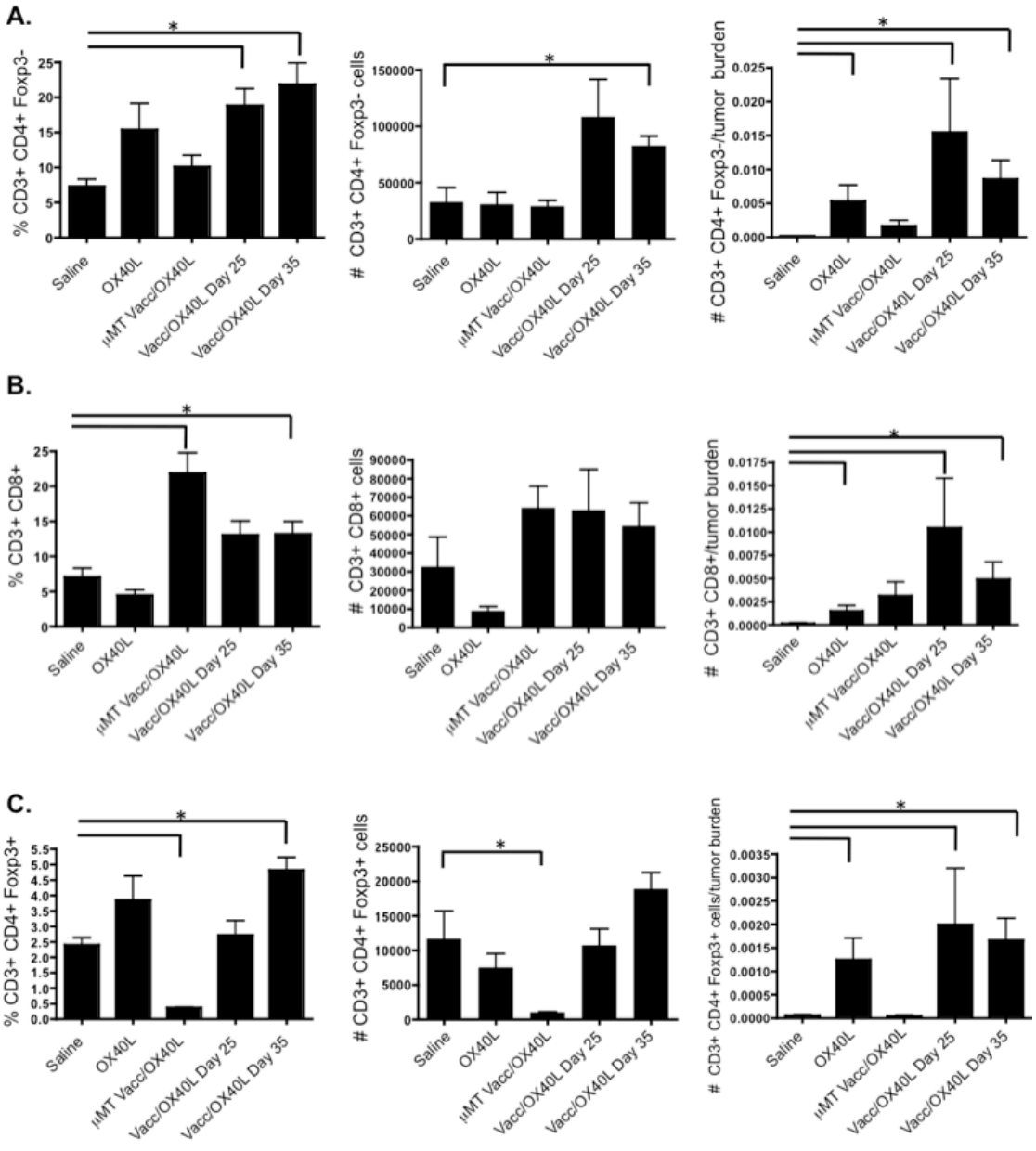


Figure 3-11. Analysis of brain infiltrating T cells. The presence of T lymphocytes was assessed through flow cytometry analysis. (A) A CD3+CD4+Foxp3- population was determined. (B) The CD8 T cell population was determine by CD3+CD8+ staining. (C) A Treg population was determined by CD3+ CD4+ Foxp3+ staining. Each population is represented as percent of total live cells, absolute number, and number of cells relative to tumor burden. Statistical significance is indicated as $p < 0.05$ (*).Mean values are shown as +/- SEM; n=5-10/group.

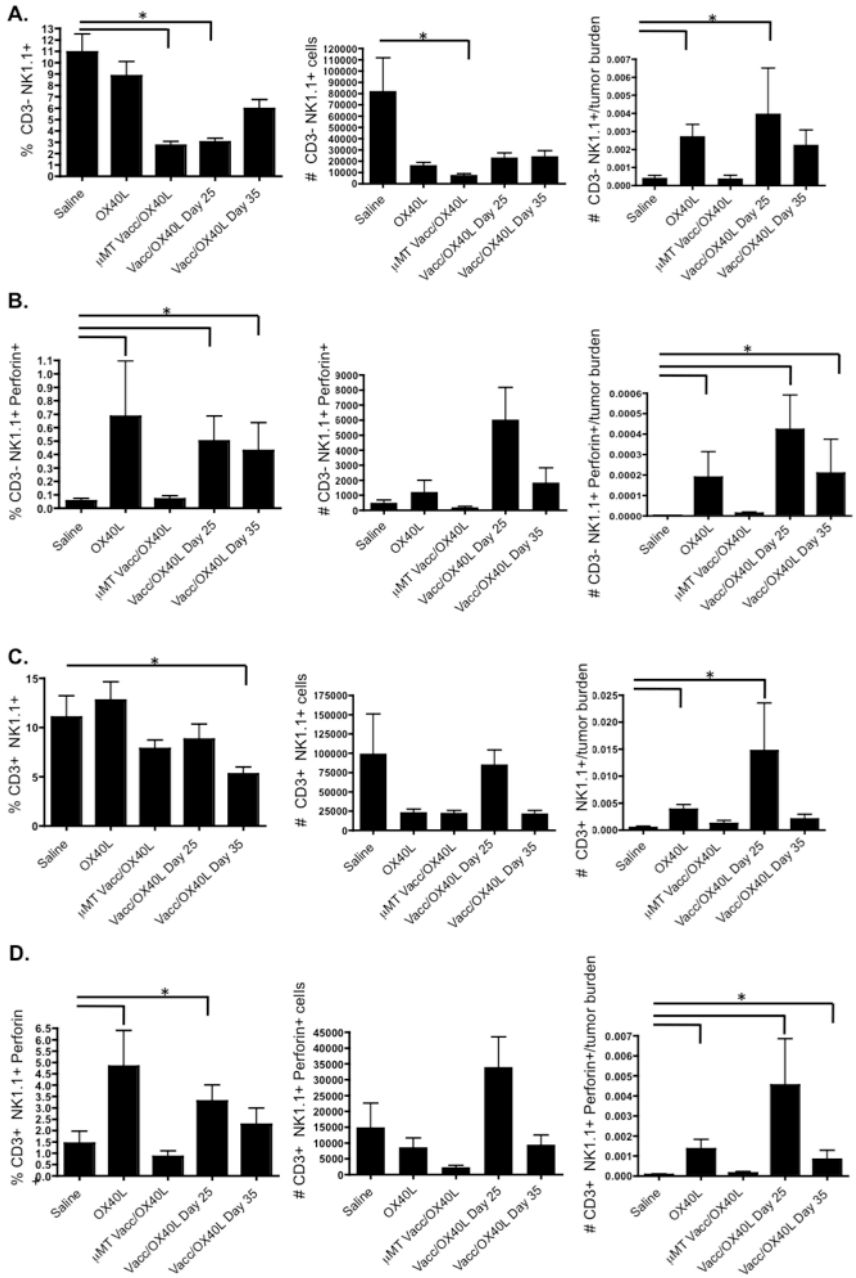


Figure 3-12. Analysis of brain infiltrating NK cell. The presence of an NK cell population was assessed through flow cytometry analysis. (A) NK cells were determined based on CD3-NK1.1+ staining. (B) The NK cell population was analyzed for expression of perforin. (C) A NK T cell population was determined by CD3+ NK1.1+ staining. (D) The NK T cell population was analyzed for expression of perforin. Each population is represented as percent of total live cells, absolute number, and number of cells relative to tumor burden. Statistical significance is indicated as $p < 0.05$ (*). Mean values are shown as +/- SEM; n=5-10/group.

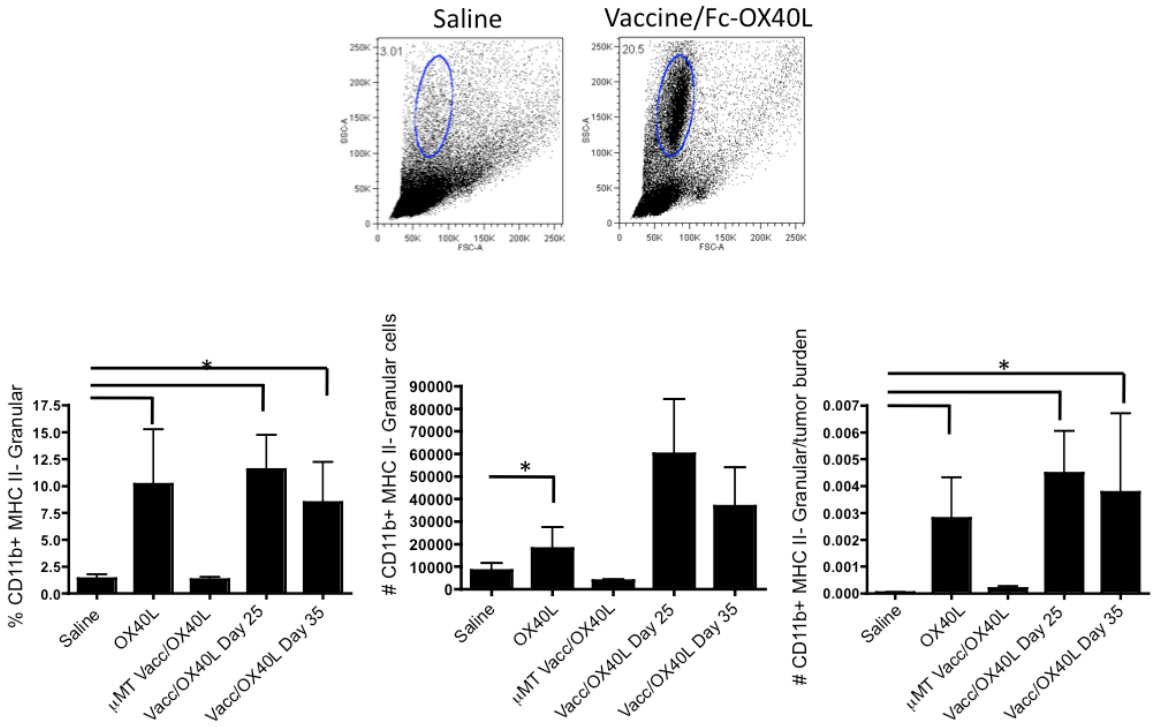


Figure 3-13. Observation of distinct granular population. Flow cytometry scatter plots of brain infiltrating cells revealed a distinct granular population. The presence of a CD11b+ MHC II- granular population was determined and represented as percent of total live cells, absolute number, and number of cells relative to tumor burden. Statistical significance is indicated as $p < 0.05$ (*). Mean values are shown as +/- SEM; n=5-10/group.

Chapter 4

Summary

Immunotherapy for malignant brain tumors is an attractive alternative to the current standard of care practices. Radiation and chemotherapy have offered very little evidence of significant enhancement of survival while the side effects of such treatments can be devastating. The collective evidence that tumor formation requires immune evasion and the ability of host immune cells to selectively target and eliminate malignant cells has generated interest in exploiting the immune system in a controlled manner to target tumors in patients whose tumors had previously escaped immune detection. There is accumulating evidence through *in vitro* studies, *in vivo* animal models, and clinical trials that it is possible to generate an immune response specific to tumors through manipulations including vaccines and cytokines. However, despite many exciting advances made in the laboratory, few treatments have translated to human patients with the same efficacy as experiments would have predicted. This is largely due to the multiple mechanisms in which tumors survive and evade the immune response, which cannot be replicated in simplistic models such as mice and cell culture. Understanding these mechanisms will be needed to fully harness the potential of the immune system for an effective anti-tumor response.

Vaccination with autologous tumor lysates has sought to overcome the inherent lack of adequate tumor antigen. These treatments have been successful in priming the immune cells as evidenced by increased proliferation and *ex vivo* cytotoxicity assays. However, these primed immune cells display poor

tumoricidal capacity *in vivo,* as tumor burden is still ultimately lethal. Anergy, the functional inactivity of immunologically primed cell, has been described as one cause of insufficient immune responses subsequent to antigen recognition. Anergy can be induced when T cells recognize cognate antigen presented by APCs without the proper level of costimulation. It is now realized that providing costimulatory signals systemically can prevent and even reverse the anergic state of lymphocytes. This awareness has led researches to pursue this branch of immune modulation in the therapeutic setting.

My research has aimed to generate more effective immunotherapies for the treatment of malignant gliomas utilizing mouse models of gliomas to test these treatment strategies. Lysate-based vaccines in glioma models had previously been shown to enhance survival, however when treatments were modified to doses that were consistent with feasibility limitations in the clinic this benefit was significantly reduced (73, 74). Vaccination was capable of priming the immune system as animals treated in the manner displayed increased numbers of T lymphocytes present in the draining lymph nodes within days of vaccination compared to saline treated animals (**Fig. 2-12**). The apparent functional inactivity of these cells instructed the addition of costimulatory molecules to our treatment protocol. The screen of costimulatory ligand-Fc fusion proteins identified Fc-OX40L as the most potent molecule in enhancing survival (**Fig. 2-1**). Impressively, this treatment strategy resulted not only in enhanced survival but

resulted in complete tumor regression in 20-50% of these animals (**Fig. 2-2**). Animals rechallenged with the identical tumor were capable of rapidly eliminating these tumors indicating the generation of immunological memory. This is important due to the nature of gliomas. A hallmark feature of gliomas is the diffuse nature of these tumors, individual cells are able to infiltrate into normal tissue. Surgical tumor resection is often able to remove nearly the entire bulk tumor, but inherently unable to eliminate infiltrating tumor cells. These remaining tumor cells are responsible for recurrence in patients, which is ultimately lethal. Therefore generation of immunological memory demonstrates the ability to eliminate recurrent tumors generated by these stray cells.

I have further sought to optimize the treatment strategy, testing multiple variations of treatment timing and component variables (**Figs. 2-6**). The optimized treatment strategy gave the most potent and consistent results observed in the model. The combination with vaccine, consisting of tumor lysate and TLR agonist and Fc-OX40L, demonstrated the best overall outcome when compared to Fc- OX40L alone (**Fig. 2-6)** or lysate/Fc-OX40L (in the absence of adjuvant) (**Fig.2-7**).

One of the most interesting finding has been the combined effect of TMZ with our immunotherapy. We originally conducted this experiment due to fears that lymphopenia induced by TMZ would negatively affect the efficacy of treatment.

We were happily surprised when we observed that indeed animals receiving the combination therapy were able to respond just as well (**Fig. 2-9**). The fact that these animals were able to reject their primary tumors and yet developed and eventually died from a secondary lymphoid malignancies (**Fig. 2-9B, 2-10**) suggests that the mutagenic properties of alkylating chemotherapies may have adverse effects in combination with immunotherapy. We believe that this is a direct effect of the mutagenesis induced by TMZ acting on rapidly proliferating T and B cells induced by the immunotherapy, as this result was never seen in the absence of chemotherapy. It is not unusual to observe secondary tumors in chemotherapy patients that have survived their primary cancers, however the alarming rate at which this occurred in a murine model should cause hesitation for combination therapies in the clinical setting (110). This phenomenon should be further developed to validate that secondary malignancy formation is dependent on the immunotherapy/chemotherapy combination and not an effect induced solely by TMZ or an artifact of strain dependent susceptibilities.

This treatment was tested in other tumor models in addition to the implanted glioma model and proved efficacious in a breast tumor model (**Fig. 2-8**); however treatment in a spontaneous model of glioma did not demonstrate the same impressive potency (**Fig. 2-11B**). The spontaneous tumor model has certain advantages compared to the implanted tumors in modeling human cancers; mainly the *de novo* pathway for tumor formation allows the tumor to develop with

the host immune system, which may better recapitulate tolerance to tumor antigen, and the infiltrative nature of the tumors of the *de novo* model is more consistent with clinical presentation, while implanted gliomas are more well circumscribed (76).

Gene expression analysis revealed that the two glioma models tested have very different immune signatures. The implanted glioma model is far more inflammatory than the spontaneous tumors (**Fig 2-11C**). This striking difference may likely explain the difference seen in treatment outcome. The differences observed between tumor models is reminiscent of the differences observed in human gliomas. Recent identification of molecular subtypes of glioma has revealed differences in the tumor microenvironment that may be predictive of treatment outcome. Previous work has shown that patients with the mesenchymal class respond best to DC vaccines when compared to proneural subclass, indicating that the pre-existing immune state of the tumor is important to treatment outcome (86). This observation suggests that subclass characterization could dictate patient treatment and our models are consistent with this observation. Future development in identifying the unique features of individual tumors will aid in the determination of the most effective treatment for that tumor, which is at its core individualized medicine. These advances may also expand our understanding of the barriers that face immunotherapy in these tumors, such as lack of T cell infiltration into the tumor site (**Fig. 2-11C**), and

instruct the selection of potential treatment to selectively overcome these constraints, such as addition of lymphocyte recruiting chemokines to the treatment regimen (discussed in Chapter 2).

The data described in Chapter 2 indicates that Fc-OX40L is potent at producing an anti-tumor response in mice and these results encourage hope for efficacy in human clinical trials. However, as mentioned previously, often times mouse models do not accurately predict clinical responses in humans. Ongoing experiments in the laboratory include introduction of the vaccine/Fc-OX40L treatment into a clinical trial involving pet canines with brain tumors. Moving into a different animal model may help more accurately predict responses in humans. Despite the reality that the survival outcomes observed for mice may not be recapitulated in humans this model provides a useful source for the characterization of an effective immune response. Employing such a potent treatment provides a way to describe the immune players at work in an effective anti-tumor immune response in comparison to the ineffective endogenous response in untreated animals.

Chapter 3 aims to shed light on the mechanisms behind the observed tumor regression in the hope that understanding how an effective immune response is capable of clearing tumor will guide the design of immunotherapeutics in the clinic. To gain an understanding into the immune cells involved in tumor

clearance we observed the survival outcomes of tumor-bearing animals deficient for specific subsets of immune cells. These results were surprising, as depletion of CD8 T cells from the immune system did not adversely affect treatment outcome (**Figs. 3-2 and 3-3**). This is in stark contrast with the current conventional wisdom that CD8 T cells are the main mediator of tumor killing. This assumption is logical since CD8 T cells have the capacity to directly induce killing, through perforin and Fas ligation. Additionally, CD8 T cells are able to recognize antigen presented by MHC I, which is present on the surface of all nucleated cells, and responsible for processing and presenting endogenous antigens, including potential tumor associated epitopes. Finally, CD8 T cells are part of the adaptive immune system, which indicates that these cells are able to recognize and respond to specific antigen and generate memory cells that are capable of rapid response to additional challenges. These observations argue in favor of CD8 T cells being crucial for the efficacy of any cancer immunotherapy. However, accumulating evidence suggest that CD8 T cells may not be as important in the anti-tumor immune response as previously thought (93, 101).

In light of these findings we sought to determine which immune cells were necessary for tumor clearance and determined that treatment efficacy was dependent on CD4 T cells, NK cells, and B cells (**Figs. 3-2 and 3-5**). These three key players raised an interesting hypothesis; CD4 Th2 cells can promote the humoral immune response, inducing antigen specific B cells to terminally

differentiate into antibody secreting plasma cells. Antibody binding of tumor cells can induce targeted killing by NK cells binding the immunoglobulin through the Fc receptor inducing death by production of perforin and granzymes, a process referred to as antibody-dependent cell-mediated cytotoxicity (ADCC). Consistent with this hypothesis we observed a tumor reactive antibody response in the serum of tumor bearing animals (**Fig. 3-7**) and deposition of IgG in the brains of vaccine treated animals (**Fig. 3-8**).

If indeed ADCC is the main effector function of this treatment it would be predicted that loss of the FcR would abrogate the treatment benefit and result in uncontrolled tumor growth. Tumor-bearing FcRγ-/- animals were treated with vaccine/Fc-OX40L and tumor growth was assessed. We found that while saline treated animals were unable to control tumor growth and rapidly progressed until death. WT vaccine/Fc-OX40L treated animals on the other hand demonstrated a much slower rate of tumor growth overall; while some animals showed progression that ultimately resulted in death the kinetics of tumor progression was reduced. Interestingly, the FcRγ-/- mice demonstrated an intermediate phenotype, while tumor growth continued to progress at a faster rate than for their WT counterparts the progression was not as rapid as for saline controls (**Fig. 3-9**). This intermediate effect on loss of the FcRγ suggests that while antibody mediated responses through FcR are important in slowing tumor progression it is certainly not the only mechanism at play.

To further dissect the immune components contributing to tumor eradication brain infiltrating cells were phenotypically analyzed. Flow cytometry analysis revealed an increase in total number of brain infiltrating cells relative to tumor size. As might be predicted based on the previous findings, the number of CD4 T cells were increased in animals receiving Fc-OX40L treatment. Interestingly, neither CD4 nor CD8 T cells express perforin indicating that these cells are not directly contributing to tumor killing through this mechanism, although additional mechanisms (Fas ligation) are still a possibility. Additionally, the numbers of perforin expressing NK and NK T cells were also increased in these animals. The presence of perforin expressing NK and NK T cells is suggestive of direct tumor killing.

One of the most striking observations was the presence of a distinct granular population that was generally only present in Fc-OX40L treated animals. This population is phenotypically and morphologically consistent with neutrophils. Neutrophils have been known to mediate tumor killing through phagocytosis of tumor cells, which can be opsonized by antibody, and through ADCC.
The identification of mechanisms contradictory to the conventional theories has been very exciting, however there are questions that remain to be answered. The neutrophil-like population needs to be validated, as this phenotype is also consistent with myeloid-derived suppressor cells, which may be recruited to the

tumor site to help control inflammation. Further characterization of these cells will need to be carried out. Additionally, linking the antibody observation to treatment efficacy would be enhanced by utilizing a mouse model that can generate mature B cells, but lacks the ability to secrete antibodies. Such models exist and should be utilized to directly tie these observations together.

The observations made in Chapter 3 highlight the need for future immunotherapies to take the whole immune system into account. This and other work has suggested mechanisms that previous researchers have neglected in the design of clinical therapies. The failure of many of these treatments may be due to the disregard for the majority of immune cells. It remains to be seen if these alternative mechanisms are tumor or location specific. The unique microenvironment of the brain could drive these non-conventional responses. As described in Chapter 1 evidence exists that supports a Th2 bias for brain related immune responses and could conceivably explain our observations.

These data should encourage scientist to think outside the box for future design of immunotherapies. The drive toward personalized medicine should take into account the state of the tumor microenvironment, understanding how tumor subtypes respond to therapies (and why they don't respond to some), as well as the immune bias of the organ in which they reside. All these variables should be considered when designing and implementing future treatments.

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