Combination Immunotherapy for Glioblastoma Multiforme With the Combination of GVAX and PD-1 Blockade, Using Anti-PD1 Antibody

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Combination Immunotherapy for Glioblastoma Multiforme with the Combination of GVAX and PD-1 Blockade, using Anti-PD1 Antibody

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A Thesis in the Field of Biology
for the Degree of Master of Liberal Arts in Extension Studies

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Abstract

Glioblastoma Multiforme (GBM), also known as grade IV astrocytoma, is generally found in the cerebral hemispheres but can be found anywhere else in the brain or the spinal cord. Even after surgery and chemotherapy, GBM has a high rate of recurrence. Median survival after diagnosis is 12 months, with less than 3 to 5% people surviving greater than 5 years. Without treatment, average survival is about 3 months. Immune checkpoint inhibition is a recent addition to the regimen of treatment of cancers and has shown some promising results in Melanoma and lung cancer.

The primary research goal of this thesis was to study if treatment with a combination of GVAX and PD-1 blockade leads to improved survival in comparison to PD-1 treatment alone in a mouse glioma model. If a combination treatment of GVAX and PD-1 leads to improved survival as compared to PD-1 treatment alone, a secondary goal of my research was to identify the underlying immune mechanisms that were responsible for this survival advantage. Finally, a third goal of my research was to examine survival advantage, if any, from treatment with GVAX and PD-1 can be enhanced further with the addition of a second immune checkpoint molecule.

Data from this study is presented which shows that combination immunotherapy with GVAX and Anti-PD1 does provide survival advantage by increasing tumor infiltration of Cd8+ T-cells into the tumor and by increasing Cd8+ T-cells to T_reg cell ratio. The survival advantages conferred by the combination of GVAX and Anti-PD1 was further enhanced by the addition of second immune checkpoint molecule OX40. Triple combination treated mice also developed immune memory towards the tumor and long-term survivors rejected a tumor challenge with three times more cells than the initial tumor challenge. These results confirm the initial hypothesis that combination immunotherapy with GVAX and immune checkpoint molecules can be an efficacious treatment for patients with GBM.
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Chapter I
Introduction

Glioblastoma Multiforme

Glioblastoma Multiforme (GBM), also known as grade IV astrocytoma, is generally found in the cerebral hemispheres, but can be found anywhere else in the brain or the spinal cord. They usually contain a mixture of cell types, including cystic minerals, calcium deposits and blood vessels. Because these tumors originate from normal brain cells, it is easier for them to invade and live within normal brain tissue. However, glioblastomas rarely spread elsewhere in the body. Glioblastomas account for about 17% of all primary brain tumors and about 65-70% of all astrocytoma’s. It is most common in adults between the ages of 45 to 70 years, and only 3% of childhood brain tumors are GBM (Urbanska, et. al, 2014).

Males are at a higher risk for GBM as compared to females. Most GBM tumors appear to be sporadic, without any genetic predisposition. No link has been found between GBM and smoking but alcohol consumption may be a possible risk factor. GBM has been associated with SV40, HHV-6 and Cytomegalovirus. Male Caucasians, Asians and Hispanics over 50 years of age have the greatest risk of developing GBM. Having a low-grade astrocytoma also increases one’s chance to develop GBM. (Kerkhof and Vecht, 2013). There is no clear way to prevent GBM and the standard of care includes, surgery, radiation, and chemotherapy.

Even after maximal surgery and chemoradiation, GBM has a high rate of recurrence. Median survival after diagnosis is 12 months, with less than 3 to 5% of patients surviving greater
than 5 years. Without treatment, average survival is about 3 months. Despite many clinical trials, including molecularly targeted therapies and some immunotherapy, the only intervention that has shown efficacy since 2005 in a randomized study has been the use of tumor treating field as shown by the EF-14 clinical trial, but the survival impact of the treatment was very modest. (Effect of Tumor Treating Field, 2017)

Cancer Immunotherapy

Cancer immunotherapy refers to clinical therapies that manipulate the body’s immune system to fight cancer. Broadly speaking, immunotherapy can be categorized into three subcategories; which are active immunotherapy, passive immunotherapy and immunomodulatory therapy. Immunomodulatory therapy is the use of endogenous chemokines and cytokines o activate the immune system to target cancer cells. An example of immunomodulatory therapy is the use of IL-2 in combination with Chemotherapy against melanoma (Farkona et. al, 2016).

Passive immunotherapy on the other hand is the use of monoclonal antibodies that target an antigen specifically expressed on the tumor cells. This type of immunotherapy is considered to be passive because it employs the use of a monoclonal antibody directly acting on the tumor antigen and therefore doesn’t require a broader activation of the immune system.

In contrast to passive immunotherapies, active immunotherapies drive the activation of the body’s immune system to recognize and fight the tumor. Cancer vaccines exemplify active immunotherapy (Farkona et. al, 2016).
Cancer Vaccines

Cancer vaccines, unlike prophylactic vaccines, are administered to cancer patients and are designed to eradicate cancer cells by strengthening the patient’s own immune response. The benefit of these cancer vaccines is that they activate the immune system to specifically attack cancer cells and spare the normal cells. Two cancer vaccines are currently approved by FDA. One is Sipleucel-T, which is an autologous Dendritic cell (D.C) based vaccine used for the treatment of Prostate cancer (Plosker, 2012). The second approved cancer vaccine is Gardasil, a prophylactic against HPV and targets HPV types 16 and 18 responsible for 70% of cervical cancer cases (Harper and DeMars, 2017). Several other cancer vaccines are currently under development or being tested (Guo et al. 2013).

There are many distinct types of Cancer vaccines. Some of them are Tumor cell vaccines, Dendritic cell (D.C vaccines), Peptide based vaccines and Genetic vaccines. Tumor vaccines are derived from patient tumor cells that are combined with an immune-stimulatory adjuvant and injected back into patients. Tumor cell vaccines can be autologous or allogenic, the difference being that allogenic whole tumor cell vaccines is that they contain cells from a source other than the patient’s own cells whereas Autologous vaccines contain cells from the patient themselves. Allogenic cell lines therefore provide a greater source of tumor antigens in comparison to an autologous vaccine (Guo et al. 2013).

Dendritic cell vaccines exploit the antigen presenting capabilities of these cells. Dendritic cells (DCs) can be used for vaccination by ex vivo processing. First, the cells of the patients are
harvested in a leukapheresis procedure. Second, these cells are stimulated with specific cytokines such as GM-CSF and then loaded with antigen material which can be tumor peptides or lysate. These primed cells are then reinfused into the patient. (Guo et al. 2013).

Viral based cancer vaccines are another class of cancer vaccines that have gained significant interest recently. The reason for using viruses as vaccines is based on the idea that infected cells tend to express viral associated antigens. Cancer vaccines which have been modified to not code for their disease-causing properties and selectively replicating in tumor cells serve as vehicles to label the cancer cells for the immune system. Furthermore, viruses can be engineered to secrete immune-stimulatory cytokines that can further enhance the immune system activation aiding in the immune response against the tumor (Farkona et al., 2016).

Peptide based cancer vaccines are vaccines developed from peptides associated with different tumor associated antigens and are usually administered with an adjuvant or an immune modulator. MAGE-1 was the first antigen that was reported to encode a tumor associated antigen recognized by t-cells. These tumors associated antigens can be classified into multiple categories. Cancer Testis antigens such as MAGE are inactivated in adult tissue but activated in tumor tissue. Other classes of tumor associated antigens include differentiation associated antigens, tumor suppressor gene antigens and certain apoptotic antigens. Recognizing these specific antigens helps develop very targeted peptide vaccines towards these antigens (Guo et al., 2013).
Glioma Vaccination

Vaccines have shown limited efficacy against glioma. Some of the ways vaccines have been used to target glioma is by modifying glioma cell lines ex-vivo and using the modified cell line as vaccine against the parent tumor such as in the case of SMA-560 glioma cell lines engineered to MICA, which is a receptor on NK and T-cells which stimulates the immune response (Taemin et. al, 2016). Another example is GVAX in which the cell line is engineered to secrete GMCSF an immune-stimulatory cytokine. Peptide based vaccines have also shown efficacy in glioma as in the case with an antibody targeting transforming growth factor (TGF-β). These vaccines have been shown to be more effective when given in combination with molecularly targeted therapies. GVAX in combination with CTLA-4 has been shown to improve survival in mice bearing glioma (Agarwalla, et. al, 2012).

Another class of vaccines targeting EGFR have been developed. The EGFR gene is amplified in approximately 40% IDH wildtype glioblastomas. More than half of EGFR mutated tumors exhibit a deletion mutation that results in expression of a truncated protein that results in expression of protein known as EGFRvIII. This protein results in the loss of ligand binding domain which results in constant kinase activity. This truncated protein expresses a peptide that has been found to be immunogenic (Oh, et. al, 2016).

Heat shock protein vaccines are another class of vaccines that have been used to treat glioma. Heat shock proteins (HSP’s) are proteins that are primary involved with protein folding and chaperonning. Heat shock protein vaccines capitalize on the interaction between antigen presenting cells and heat shock proteins. Antigen presenting cells, treat HSP’s as any other
antigen, internalize it and then present these HSP proteins on MHC complexes (Oh, et. al, 2016). Because of this interaction HSP vaccines are designed as tumor associated antigens that are conjugated to heat shock proteins. When antigen presenting cells come in contact with these HSP conjugated tumor associated antigens, they internalize tumor antigens and present them on MHC complexes resulting in immune activation and response against the tumor. Heat shock protein glioma vaccines are currently being tested in clinical trials (Gou et. al, 2013).

**Whole Tumor Cell Vaccines**

Modified whole tumor cell vaccines utilize modified whole tumor cells as a source of tumor antigens. The advantage of whole tumor cell vaccines rather than a specific peptide or tumor antigen vaccine is that the whole tumor cells provide a source of all possible tumor antigens which eliminates the need to find and target the most optimal antigen. Furthermore, whole tumor cell vaccines provide the ability to target more than one tumor antigen at the same time which avoiding the issues caused by tumor antigen loss (Taemin et. al, 2016).

Whole cell vaccines are genetically altered to express certain cytokines or chemokines to stimulate the immune response towards the injected irradiated tumor cells. Many phase I and II clinical trials have demonstrated this approach to be safe in patients. Vaccine induced immune response can be checked with a delayed type hyper sensitivity response (DTH). DTH responses have been shown to correlate with patients given modified tumor cell vaccines (Taemin et. al, 2016). Granulocyte Macrophage Colony Stimulating factor (GMCSF) has been found to be more efficacious in comparison to multiple other immune-stimulatory cytokines such as IFN-γ when transduced into irradiated melanoma cells in pre-clinical studies. It has been shown that
administration of GMCSF transduced whole tumor cell vaccination results in massive influx of Dendritic cells, macrophages, eosinophils and T-cells at the vaccination site. The influx of Dendritic cells to the site of vaccination leads to CD8 t-cell priming leading to a better immune response against the tumor. In the case of developed cancers that have already evaded the immune system, the combination of whole cell based modified vaccines with immune checkpoint molecules can further enhance the activity of whole tumor cell vaccines and further improve their efficacy (Taemin et. al, 2016).

The Immune System and Immune Checkpoints

The immune system acts as the body’s first line of defense against multiple kinds of threats including bacterial, viral and uncontrollably dividing cells. The immune system is itself divided into parts that allow for both the speed and specificity of the immune reaction. These two parts of the immune system are the innate immune system and the adaptive immune system (Parkin and Cohen, 2001).

The innate immune system serves as the first line of defense against many common microorganisms. The cells of the innate immune system are called leukocytes. When microorganisms interact with macrophages, they engulf the bacteria and start the release of inflammatory cytokines, recruiting other cells to the location of the infection (Getz, 2005).

The initiation of an adaptive immune system begins when a pathogen is ingested by an antigen-presenting cell, the most professional of which is the DC. Immature D.C have receptors on their surface that help them recognize a pathogen. Once these receptors encounter a pathogen, they engulf them. The engulfing of a pathogen by a dendritic cell results in it being activated.
which transforms it into an effective antigen presenting cell. This activated antigen presenting cells travels to the lymph node and displays these antigens to naïve t-lymphocytes (Getz, 2005) via major histocompatibility complex (MHC) – T cell receptor (TCR) interaction. (Getz, 2005)

The presentation of the antigen to the naïve T lymphocytes by the antigen presenting cells begins the adaptive immunity part of the immune system. The adaptive immune responses are carried out by cells called lymphocytes. There are two broad categories of the adaptive immune response, which are the humoral response and the cell-mediated immune response. The humoral response is carried out by the b cells and the cell-mediated immune response is carried out by the t-cells. In a humoral response, activated b-cells secrete antibodies which attack the antigen in response to which they were secreted. In the cell mediated response, activated T-cells directly act on the cell expressing their “cognate” antigen. (Parkin and Cohen, 2001).

T-cells require two signals to activate and mount an immune response. The first signal comes from the T-cell receptor upon recognition of an antigen which is the result of the T-cell receptor and MHC binding. The second signal comes from the interaction between the receptors and the ligands of the co-stimulatory and/or co-inhibitory signals, the T-cell receptor in this case is the CD28 and the costimulatory molecules on the Antigen presenting cells are B7-1 and B7-2. Under normal conditions, there is a balance between co-stimulatory and co-inhibitory signals which is necessary for self-tolerance and immune homeostasis. This protects the normal cells and tissues from damage from the immune system (Parkin and Cohen, 2001). Dysregulation in this process is linked to the development of auto-immunity. Tumor cells exploit this process and express immune-checkpoint proteins which helps them evade the immune system (Yang, 2015).
Inhibitory ligands and receptors (parts of the immune checkpoint) that regulate T-cell effector function in tissues are commonly over expressed in the tumor cells and non-transformed cells in the tumor microenvironment. This over expression of inhibitory ligands is one of the reasons why tumors have evolved to avoid immune detection and destruction. Since these ligands and receptors are over expressed on the tumor, immune checkpoint inhibitors that target and knockout these specific receptors and ligands are effective at restoring T-cell effector response against the tumor. Effector T-cell response against the tumor is associated with better survival. One of these receptors is Programmed cell death protein 1 (PD-1) (Chen and Han, 2015), (Yang, 2015).

Programmed Cell Death Protein-1 (PD-1)

PD-1 serves an immunosuppressive function as its major role during an inflammatory response is to limit the activity of T-cells in the peripheral tissue to the infection site and limit auto-immunity. Tumor cells can use PD-1 to their advantage by over expressing its ligand, PD-L1, when T-cells become activated. When activated T-cells encounter PD-L1, the effect is the intracellular inhibition of kinases that are involved in T-cell activation through the phosphatase SHP2.

PD1 is highly upregulated on tumor infiltrating lymphocytes (TILs) and its ligand PD-L1 is highly expressed on tumors. Based on this, monoclonal antibodies that target PD-1 or its ligand PD-L1 have been developed, studied in preclinical cancer models, and used with efficacy in cancer clinical trials.(Alsaab et. al, 2017) Nivolumab is one such anti-PD1 antibody that is
FDA approved for use against multiple cancers including metastatic melanoma, Non-small cell lung cancer, classical Hodgkin lymphoma, metastatic colorectal cancer, urothelial cancer, squamous cell carcinoma and hepatocellular carcinoma. Even though treatment with anti-PD1 based therapies have increased survival in patients with different types of cancer, there is still a need for improvement in immune checkpoint inhibitor based treatments to fully benefit from them. In fact, in a randomized study in patients with recurrent glioblastoma, anti-PD1 immunotherapy with Nivolumab had no survival benefit. A current approach to improving outcomes for cancer patients is to combine anti-PD1 therapy with other checkpoint inhibitors or with other immunoactive approaches, aiming for synergy. (Chen and Han, 2015).

**GVAX**

GVAX is a cancer vaccine that is prepared by genetic modification of whole tumor cells to secrete the immunostimulatory cytokine granulocyte macrophage colony stimulating factor (GM-CSF) which are then irradiated to prevent further cell division. The cells are then implanted subcutaneously and intradermally into the subject. An advantage of GVAX is that it provides a polyvalent source of tumor antigens to the immune system and the secretion of GM-CSF by the modified tumor cells greatly enhances the immune response by recruitment and activation of dendritic cells, which serve as the first step in mounting an appropriate antitumor immune response. GVAX has been clinically tested in several trials and results have consistently demonstrated safety and the development of both humoral and cell-mediated antitumor immunity (Nemunaitis, 2005). GVAX, has also been shown to increase T-lymphocyte activation with significantly increased expression of PD-1 and OX40 on CD4+ cells and PD-1 on CD8+ T-cells in a phase 1 clinical trial in patients with GBM (Curry, et. al, 2016)
The rationale for combining GVAX with PD1 is that vaccination broadens the systemic antitumor immune response by providing a source of tumor antigens which are presented to T lymphocytes, which are then activated. Then, blocking PD-1 should allow a stronger and more durable adaptive immune response towards the tumor as the inhibitory action of PD-1 ligation is prevented. GVAX in combination with PD-1 blockade has been shown to facilitate effector T-cell infiltration into mouse pancreatic tumor models (Soares, et. al, 2015)

OX40

OX40, also known as CD134 T-cells, is a member of the TNFR superfamily. Costimulatory signal from OX40 helps to promote T-cell division and survival. Additionally, OX40 ligation is known to suppress the activity and differentiation of Treg cells, further aiding the activity of effector CD4 and CD8 T-cells. OX40 stimulation has shown to be advantageous in combination with GVAX (Jahan, et. al, 2017) in a syngeneic mouse glioma model.

The ligand for OX40, OX40L (CD252) is a member of the TNF superfamily. OX40L was first identified as gp34 on HTLV-1 transformed cells. OX40L, like OX40, is not expressed constitutively but is expressed on B-cells, Macrophages and Dendritic cells after recognition of an antigen for T-cell priming. T-cells also express OX40L that is functional during T cell – T cell interactions further amplifying T-cell responsiveness.

Though not in glioma, preclinical data suggests that combining PD-1 blockade and OX40 ligation is more effective than either therapy alone against murine cancers. OX40 impacts the antigen-specific activity of stimulated T cells via a different receptor than PD1 and OX40
ligation, furthermore, it would be expected to have strong anti-Treg effect. Triple combination therapy with vaccination, anti-PD1 monoclonal antibody, and agonizing anti-OX40 antibody, may then create a heightened response against a broader array of tumor-associated antigens. (Croft, et. al, 2009)

Tumor Immune Evasion

The ability of cancer cells to grow unchecked and disguise themselves from the immune system is one of the main reasons why it has been so hard to design an effective treatment. Advancements in molecular and cellular biology have led to a better understanding of how a tumor can grow and evade the immune system.

The development of a tumor and its growth is a complex process which is dependent on many factors. Some of these include the interplay between cancer cells, the normal cells and host defense mechanisms including the immune system. Under normal conditions, CD4 and CD8 cells, stop tumor development and growth through the action of Interferon Gamma and cytotoxins, but research has shown that certain other factors such as chronic inflammation might interfere with the function of CD4 and Cd8 cells and aid tumor development. Chronic inflammation has been shown to play an important role in the development of colon and pancreatic cancers (Vinay, et. al, 2015).

Recent discoveries in the last two decades have shown that the immune system plays a critical role in maintaining an equilibrium between immune recognition and tumor development, with the capacity to both promote and suppress tumor growth. These findings have given rise to the concept of immunoediting. Immunoediting explains why tumors can lay dormant in patients
for years before re-emerging. Immunoediting also helps us explain why tumors still grow, even when the immune system is fully functioning (Mittal, et. al, 2014).

During cancer immune editing, cancer cells, that are most vulnerable to the immune system are eradicated by the immune system. At the same time, due to high division and mutation rate of the cancer cells, new cells arise that are able to evade the immune system. This state in which tumor cells are eliminated by the immune system and the generation of new ones that can evade the immune system is called equilibrium. During equilibrium, cancer cells continue to divide gaining mutations by chance or because of immune-induced inflammation. This leads to a balance between immune control and tumor growth which appears as tumor dormancy (Mittal, et. al, 2014).

Long lasting equilibrium leads to a loss of the ability of the immune system to control the tumor usually by the loss of target antigen expression or by immune suppressive effects. This is the stage at which the tumor evades the immune system and clinical cancer manifests (Mittal, et. al, 2014).

Exploiting the regulatory T cells is another method a tumor uses to evade the immune system. Regulatory T cells are a subpopulation of T-cells that modulate the immune system to maintain tolerance to self-antigens. Tolerance to self-antigens is very important to avoid autoimmune diseases. The tumor exploits this mechanism of self-tolerance to avoid the immune system (Zindl and Chaplin, 2010).

Immune suppression in the tumor microenvironment, mediated by CD4+CD25+ FoxP3+ regulatory T cells (Tregs), or other types of suppressive cells, seems to be a major mechanism of
tumor immune escape and can be a crucial hurdle for tumor immunotherapy. Studies have shown that Tregs obtained from a tumor have a much higher tumor suppressive activity than Tregs obtained from other sources. The tumor recruits Tregs by Tumor cells mediated chemokine production. The production of Transforming growth factor (TGF)–β by the tumor cells helps in transforming CD4⁺ T cells into suppressive Tregs (Zindl and Chaplin, 2010). Overcoming the immune suppressive effects in the tumor microenvironment is one of the key goals of cancer immune therapy.

**Cd8 to T\textsubscript{reg} Cell Ratio**

The engagement between the host immune system and the tumor is a complex process and involves multiple complex pathways. However, most anti-tumor effects from the host immune system can be attributed to the effector CD8 t-cells. Recent evidence has shown that tumors with a greater infiltration of CD8 t-cells have a better prognosis as compared to the ones that do not (Sideras, et. al, 2017).

The antitumor activity of CD8 T-cells is opposed by a population of CD4⁺ T-cells that express the forkhead box P3 (FoxP3) transcription factor. These cells have a regulatory activity on the immune system and protect normal cells from the over activation of the immune system. However, in the case of a tumor that is infiltrated with regulatory t-cells is associated with a poor prognosis in multiple malignancies, including glioblastoma. Furthermore, lower population of CD8⁺ effector T-cells in comparison to T\textsubscript{regs} is associated with a less favorable outcome in multiple tumors (Sideras, et. al, 2017).
GVAX, has been shown to increase the population of tumor infiltrating CD8 effector t-cells in mouse pancreatic tumors. (Soares, et. al, 2015). Addition of Anti-PD1 in combination with GVAX can further enhance the number of tumor infiltrating CD8 cells and provide a survival advantage in some tumor models.

Current Immunotherapy Clinical Trials Targeting GBM

Many different types of immunotherapeutics are currently under clinical testing. These immunotherapy approaches include antibody-drug conjugates, autologous infusions of modified chimeric antigen receptor expressing T cells, peptide vaccines, autologous dendritic cell vaccines, immunostimulatory viruses, oncolytic viruses, checkpoint blockade inhibitors, and drugs which alter the behavior of innate immune cells.

Rindopepimut, a vaccine targeting the EGFRvIII truncated peptide protein was tested in a phase three clinical study. The trial was terminated because it failed to meet the improved endpoint overall survival benefit versus the standard treatment (temozolomide). The failure of this clinical trial to achieve its endpoint goals highlighted the limitation of peptide vaccines in that they target very limited portion of the tumor associated antigens. Whole tumor cell vaccinations on the other hand don’t have this same limitation as compared to peptide vaccines (Xu, et. al, 2014).

Checkmate 143 was another clinical trial that tested the efficacy and safety of nivolumab (anti-PD1 monoclonal antibody), in comparison to bevacizumab, the current standard of treatment for patients who have recurred following radiation and temozolomide treatment. The
results from phase III showed that nivolumab failed to improve survival in patients as compared to bevacizumab (Xu, et. al, 2014).

Chimeric Antigen Receptor modified T-Cells (CAR-T cells) have shown promising results in Leukemia and Lymphoma. CAR-T cells targeted to the EGFRvIII receptor were tested in a phase I clinical trial, in 10 patients. Treatment was well tolerated and 9 out of 10 patients had stable disease at day 28. However, no survival impact was observed. (O’Rourke et. al, 2017) CAR-T cell therapy was recently approved for B-cell acute lymphoblastic leukemia (Xu, et. al, 2014), as well as for certain types of non-Hodgkin’s lymphoma. Heat shock protein vaccines have also been tested recently against GBM in clinical trials. Most clinical trials that have used HSP vaccines have used tumor associated antigens bound to a 96 KD chaperone heat shock protein. The first heat shock protein vaccine to be tested in GBM was called the HSP protein complex-96 vaccine (HSPPC-96; prophage). 42 patients were treated in a phase II clinical trial and treatment with HSPPC-96 increased median overall survival from 14.6 week to 42.6 weeks. Tumor biopsies post treatment with HSPPC-96 showed marked tumor infiltration of CD4⁺, CD8⁺ and CD56⁺ T-cells (Xu, et. al, 2014). However, a phase 3 multicenter study in patients with recurrent glioblastoma showed no improvement in survival for patients that were treated with HSP vaccination versus those that received bevacizumab either alone or in combination with HSP vaccination.

Combination of GVAX and Immune Checkpoint Molecules as a Treatment Strategy for Glioma

GVAX modestly improves survival in pre-clinical glioma models, however vaccination with GVAX does not cure or provide long term survivors in mice bearing GBM tumors
(Herrlinger et al, 1997). It has been shown that combination immunotherapy with GVAX and immune checkpoint inhibitory molecule CTLA-4 is associated with a much better survival outcome (Agarwalla et al, 2012). This is consistent with the observation that vaccination with GVAX increases overall number of tumor infiltrating lymphocytes, however further immune stimulation is needed to overcome the effects of negative immune regulation. In a phase I study in patients with recurrent glioblastoma, GVAX led to consistent stimulation of peripheral T lymphocytes, demonstrated by increased expression of co-stimulatory molecules, including CTLA-4, PD1, and OX40 (Curry et al, 2016).

The ligand for Programmed cell death protein 1 (PD-1 or CD279), PD-L1, is highly expressed in glioblastoma, and helps in the immune evasion by glioblastoma cells because the interaction between PD-1 and PD-L1 serves an immunosuppressive function. PD-1 ligation by monoclonal antibodies stimulates CD4+ and CD8+ T lymphocytes and has shown antitumor activity in osteosarcoma (Alsaab et al, 2017).

Since GVAX is an immune stimulatory vaccine and blocking PD-1 and PD-L1 interaction boosts anti-tumor immune response, a treatment approach that uses both GVAX and PD-1 blockade can further enhance immune activation and improve survival in mice bearing GBM tumors.

Research Aims, Goals and Hypothesis

The primary research goal of this thesis was to study if treatment with a combination of GVAX and PD-1 blockade leads to improved survival in comparison to PD-1 treatment alone in
a mouse glioma model. If a combination treatment of GVAX and PD-1 leads to improved survival as compared to PD-1 treatment alone, a secondary goal of my research would be to identify the underlying immune mechanisms that are responsible for this survival advantage. Finally, a third goal of my research would be to examine survival advantage, if any, from treatment with GVAX and PD-1 can be enhanced further with the addition of a second immune checkpoint molecule.

Primary objective: To show that combination immunotherapy with the combination of GVAX and Anti-PD1 is synergistic and leads to an improvement in survival in an orthotopic mouse GBM model.

Specific Aim 1: To show that combination treatment of GVAX and PD-1 leads to improved survival as compared to PD-1 treatment alone in an orthotopic mouse GBM model.

Methods: Treat mice implanted with orthotopic GBM with a combination of GVAX and Anti-PD1.

Expected results: Mice treated with the combination of GVAX and Anti-PD1 will have a better survival versus mice only treated with Anti-PD1.

Specific Aim 2: To identify the underlying immune mechanisms that are responsible for the survival advantage resulting from the combination treatment of GVAX and Anti-PD1.

Methods: Perform flow cytometry and Immunohistochemistry analysis to understand the immune mechanisms.
Expected results: Mice treated with GVAX and PD1 combination will show increased T-cell function and a decrease in Treg cell activity.

Specific Aim 3: To examine survival advantage, if any from treatment with GVAX and Anti-PD-1 can be enhanced further with the addition of a second immune checkpoint molecule.

Methods: Treat mice with orthotopic GBM with the combination of GVAX, Anti-PD1 and Anti-OX40.

Expected results: Mice treated with the combination of GVAX, Anti-PD1 and Anti-OX40 will show the greatest improvement in survival.
Materials and Methods

C57/Bl6 Mice: C57/Bl6 mice will be obtained from Charles river laboratories. Mice will be handled per the MGH IACUC regulations.

Gl261 Cell line: Gl261 is murine glioma cell line which has been widely used in glioma research in mice. It will be obtained from the Curry lab at MGH. Cultured in DMEM supplemented by 10% FBS in 5% CO\textsubscript{2} and at 37\textdegree C.

GVAX treatment: GL261 cells engineered to secrete GMCSF will be grown in similar conditions to the GL261 cell line. These cells will be irradiated at 35gy. Then a million cells per mouse in 100ul PBS will be injected subcutaneously into the right flanks of these mice on days 3, 6 and 9 post tumor implantations.

Anti-PD-1 treatment: Anti-Mouse PD-1 antibody was obtained from bio-excel (Lebanon, NH). 200ug of the antibody in 100ul PBS was injected into the intra-peritoneal cavity on days 3, 6 and 9 post tumor implantation.

Anti-OX40 treatment: Anti-Mouse OX40 antibody was obtained from bio-excel (Lebanon,NH). 250ug of the antibody in 100ul PBS was injected into the intra-peritoneal cavity on days 3, 6 and 9 post tumor implantation.
In-vivo studies:

Survival Study: Mice were anesthetized using pentobarbital and then implanted with 75,000 Gl261 cells in 3µl DMEM in their right frontal lobes, 2.5mm from the bregma and 2.5mm deep, using a Hamilton 1701N gastight syringe (Hamilton Co., Reno, NV) and a stereotactic mouse frame (Kopf Instruments, Tujunga, CA) (Agarwalla, et. al, 2013). They were then randomly divided into four groups: Control, Control + PD-1, Control + GVAX, GVAX + PD-1. In the case of triple combination experiment mice were divided into eight groups: Control, Control + PD-1, Control + GVAX, GVAX + PD-1, Control + OX40, GVAX + OX40, Control + PD1 + OX40 and GVAX + PD1 + OX40.

On days 3, 6 and 9 post-tumor implantations, appropriate mice were subcutaneously injected with $1 \times 10^6$ irradiated (35 Gy) GL261-GM-CSF cells, suspended in 100 microliters of PBS. At the same time, depending upon their study group, mice were administered 200 micrograms of either anti-PD-1 (clone: RMP1-14, BioXCell, West Lebanon, NH) or isotype control (Rat IgG2a) prepared in 100 microliters of PBS on days 3, 6 and 9 post-tumor implantations intraperitoneally. In triple combination experiments, depending on their groups, mice were also treated with 250 micrograms anti-OX40 (clone: OX-86, BioXcell, West Lebanon, NH) or isotype control (Rat IgG1). Mice were then followed for survival and sacrificed when neurological symptoms became evident, per the IACUC protocol (Agarwalla, et. al, 2013).

Flow Cytometry analysis: Tumors were implanted, and mice were divided into four groups: Control, Control + PD-1, Control + GVAX, GVAX + PD-1. In the case of triple combination
experiment mice were divided into eight groups: Control, Control + PD-1, Control + GVAX, GVAX + PD-1, Control + OX40, GVAX + OX40, Control + PD1 + OX40 and GVAX + PD1 + OX40. Mice were then treated based on their groups on days 3, 6 and 9 post tumor implantations. On day 18 following tumor implantation mice were sacrificed. The tumor quadrant of the brain was harvested and a single cell suspension was made. Fluorescently labeled antibodies used for flow cytometry analyses were purchased from Biolegend (BV 605-CD3, PerCP/Cy5.5-CD4, BV 510-CD8a, Alexa Fluor 647-FOXP3, PE/Cy7-CD279, BV 421-CD134, PE-CD366, BV 785-CD223). Dead cells were excluded using the Zombie UV™ Fixable Viability kit (Biolegend). Flow cytometry data was acquired using LSRii flow cytometry machine and analyzed using Flowjo software.

Immunohistochemistry: Tumors will be implanted in mice and they were divided into four groups: Control, Control + PD-1, Control + GVAX, GVAX + PD-1. Mice were then treated based on their groups on days 3, 6 and 9 post tumor implantations. On day 18, post tumor implantations, mice were sacrificed and their brains were collected. The harvested brains were then fixed in 10% formalin followed by 70% ethanol. The brains were subsequently paraffin embedded and cut into 5-μm sections. Sections were then deparaffinized in xylene and dehydrated in ethanol followed by microwave treatment in 10 mM sodium citrate buffer (pH 6.0) for 15 min for antigen retrieval. The sections were next treated with 3% H2O2 to block endogenous peroxidase. Bovine serum albumin and antibody specific protein blocking buffer were then used to block non-specific binding for 20 and 30 minutes respectively. The sections were then incubated overnight with anti-CD3 (ab5690, abcam), anti-CD4 (4SM95, eBioscience), and anti-
CD8a (4SM15, affymetrix) antibodies respectively. Blocking buffer was used instead of the primary antibody for negative controls. Next morning the sections were incubated for 30 mins at room temperature with the peroxidase labelled secondary antibody dependent on the primary antibody provided by the manufacturer in the kit (Vector Lab ImmPRESS polymer detection kit, Vector labs, Burlingame, CA). Diaminobenzidine (DAB) was used for color development and the sections were counter stained with hematoxylin. Finally, the sections were mounted with Cytoseal-XYL (8312-4, Thermo scientific, Waltham, MA) and photographed under a light microscope (Nikon optiphot 2) using spot software.

IFN-γ ELISPOT assay: Tumors were implanted in mice and they were divided into four groups: Control, Control + PD-1, Control + GVAX, GVAX + PD-1. In the case of triple combination experiment mice were divided into eight groups: Control, Control + PD-1, Control + GVAX, GVAX + PD-1, Control + OX40, GVAX + OX40, Control + PD1 + OX40 and GVAX + PD1 + OX40. Mice were then treated based on their groups on days 3, 6 and 9 post tumor implantation. On day 18 after tumor implantations, splenocytes were harvested from mouse spleens. 1×10^6 splenocytes were stimulated for 48 hours in vitro with 1×10^5 irradiated (35Gy) GL261 cells or in RPMI 1640 medium, supplemented with 10% IFCS, 2 mM glutamine, 20 mM HEPES, penicillin-streptomycin in 6 well tissue culture plates (BD Falcon, San Jose, CA). 1×10^5 splenocytes from mice in each treatment and control group were then loaded in duplicates onto 96-well PVDF-backed microplates coated with anti-IFN-γ mAb (R & D Systems, Minneapolis, MN). These plates were then incubated at 5% CO2 and 37°C for 24 hours. After
incubation, the plates were washed four times with the wash buffer provided by the manufacturer and incubated with biotinylated anti-IFN-γ monoclonal antibody overnight at 4°C. Next morning the plates were washed four times again and Streptavidin-AP was added into each well and the plates were incubated at room temperature for two hours. Plates were then washed again and BCIP/NBT chromogen was added to each well and incubated for an hour at room temperature. The contents of the plates were then discarded and they were washed with distilled water, dried and read using an elispot reader (Agarwalla, et. al, 2013).

Memory Re-challenge experiment: Mice that survived the initial tumor challenge were followed for a 120 day from the initial challenge and then re-challenged with the tumor with 300000 cells, not treated and followed for survival.

H&E stain: On day 11, following tumor re-challenge, mice were sacrificed, their brains collected and fixed in 10% formalin and then paraffin embedded. 5µm sections were then made from paraffin embedded brains. The sections were then deparaffinized in Xylene for 10 mins, followed by series of 100%, 95% and 70% EtOH dehydration for 5 minutes each. The slides were then stained with 100% Hematoxylin for 1 minute and then rinsed with distilled water followed by wash in running tap water for 3 minutes. The slides were then counter stained with Eosin for 45 seconds, followed by dehydration in 95% and 100% EtOH for 5 minutes each. The sections were then dip in Xylene for 10 minutes followed by mounting with Cytoseal XYL.

Memory Response Flow Cytometry study: On day 11 following tumor re-challenge, mice were sacrificed. The tumor quadrant of the brain was harvested and a single cell suspension was made. Fluorescently labeled antibodies used for flow cytometry analyses were purchased from
Biolegend (CD62L anti-mouse and CD44 Anti mouse). Dead cells were excluded using the Zombie UV™ Fixable Viability kit (Biolegend).

Statistics: Graphpad prism was used to perform the statistical analysis on the data collected. Data were expressed as mean ± SD, and differences were considered significant at P < 0.05. Individual data sets were compared using students t-test when comparing two groups. Kaplan-Meir survival was used for mouse survival studies and the groups were compared using log rank test.
Chapter III

Results

Survival Study # 1

GVAX treatment alone, significantly increased survival versus control treatment ($P \leq 0.001$). Anti-PD-1 treatment alone significantly improved survival versus GVAX treatment alone ($P \leq 0.05$). Furthermore, combination treatment with GVAX + Anti-PD1 was most successful and significantly improved survival, versus Anti-PD1 treatment group alone ($P \leq 0.05$). The double treatment group also had the most long-term survivors at 120 days post tumor implantation versus any other group (n=4/8 versus n=2/10 from the Anti-PD-1 alone treatment group, the only other group with long term survivors). Figure 1

immune mechanism behind improved survival

Cd8$^+$/Foxp3$^+$ Ratio

GVAX treatment alone significantly improved the ratio of cytotoxic CD8$^+$ T-cells versus regulatory FoxP3$^+$ cells in comparison to all other treatment groups ($P \leq 0.05$). Combination treatment with GVAX + Anti-PD1 also significantly improved the ratio of cytotoxic CD8$^+$ T-cells versus regulatory FoxP3$^+$ cells in comparison to the Anti-PD1 alone treatment group and the control group ($P \leq 0.05$). Figure 2
Immunohistochemistry Analysis on Brain Tissue

Immunohistochemistry analysis on brain tissue for Cd3, Cd4 and Cd8 showed a significant increase in Cd3\(^+\) T-cell infiltration into the tumor with GVAX + Anti-PD1 treatment versus control mice. Cd4\(^+\) t-cell infiltration also increased the most in mice treated with GVAX + Anti-PD1 combination but it was only significantly different in mice treated with GVAX alone versus the control group (P \(\leq 0.01\)), even though as mentioned before double treatment was found to have the greatest number of tumor infiltrating lymphocytes. Cd8\(^+\) t-cell infiltration into the tumor increased significantly with GVAX + PD1 treatment versus Anti-PD1 alone treated mice (P \(\leq 0.01\)), GVAX alone treated mice (P \(\leq 0.001\)) and Control mice (P \(\leq 0.01\)). Figure 3

Addition of a second checkpoint molecule

Survival Study # 2

Addition of a second checkpoint molecule, Anti-Ox40 increases survival versus Anti-PD1 alone or Anti-OX40 combination treatment (Figure 4), however the long-term survival is similar to GVAX and Anti-PD1 combination (Figure 1). Treatment with the combination of Anti-PD1 and Anti-OX40 also increases the ratio of Cd8\(^+\) to T\(_{reg}\) cells (Figure 5). GVAX + Anti-PD1 + Anti-OX40 treatment significantly increases survival versus Anti-PD1 and Anti-OX40 treatment (P \(\leq 0.01\)), and GVAX + Anti-OX40 treatment as well as GVAX + PD1 treatment (P \(\leq 0.01\) and P \(\leq 0.05\) respectively). (Figure 6)

Elispot analysis of systemic Immune response with Triple combination treatment
Elispot analysis of Splenocytes showed a significant increase in IFN-Gamma production with triple combination treatment (GVAX + Anti-PD1 + Anti-OX40) versus GVAX + Anti-PD1 (P ≤ 0.01), GVAX + Anti-OX40 (P ≤ 0.05) and Anti-PD1 + Anti-OX40 (P ≤ 0.01). IL-2 production also increased significantly with the triple combination treatment versus GVAX + Anti-PD1 treatment (P ≤ 0.01), GVAX + Anti-OX40 treatment (P ≤ 0.05) and Anti-PD1 and Anti-OX40 treatment (P ≤ 0.01). Triple combination treatment also significantly increased TNF-Alpha production versus GVAX treatment alone (P ≤ 0.05) and Control group (P ≤ 0.01). (Figure 7)

Flow Cytometry analysis on Tumor infiltrating lymphocytes

Flow cytometry analysis on tumor infiltrating lymphocytes (TILs) showed that the number of CD4\(^+\) cells increased in TILs upon triple combination treatment, the percentage of these CD4\(^+\) t-cells that were T-regulatory cells was the lowest in triple combination treated group and significantly lower than GVAX + Anti-PD1, PBS + Anti-PD1 and Control groups (P ≤ 0.05). (Figure 8)

Tumor Re-challenge in long term survivors of Triple combination treated with GAVX + Anti-PD1 + Anti-Ox40 combination

Tumor re-challenge with five times more cells versus initial tumor in long term survivors of triple combination treatment showed that 100% of these mice were able to reject the tumor challenge. Age matched controls on the other hand were all dead around day 25. (Figure 9)
Qualitative analysis of mouse brains with Tumor Re-challenge using H&E staining

Qualitative analysis of long term survivors that were re-challenged with tumor and sacrificed on day 11 to perform H&E staining on the brains showed that triple combination treated animals had the smallest amount of brain tissue showing signs of tumor followed by Anit-PD1 + Anti-OX40 treated and the control had the biggest tumor size qualitatively. (Figure 10)

Flow cytometry Analysis of Immune Memory in Tumor Re-challenged Long Term Survivors

Naïve T-cells exhibit high levels of CD62L and low expression of CD44, whereas memory T cells are identified by high CD44 and low CD62L expression. Flow cytometry analysis of TILs of re-challenged mice showed that the GVAX + Anti-PD1 + Anti-OX40 treated mice had the greatest number of high CD44 and low CD62L Cd4+ T helper cells which was significantly more than Anti-PD1 + Anti-OX40 treated re-challenge mice (P ≤ 0.05). (Figure 11)
Chapter IV
Discussion

Significance of Results

GVAX is a cancer vaccine that has shown modest results in people treated with it in different types of cancer but it has failed to produce sustained response. PD1 blockade with the use of monoclonal antibodies has been shown to efficacious in different types of tumors, but in the case of brain tumors, the results fall short of a sustained survival response and need further enhancement. Here, I proposed using a combination of GVAX and Anti-PD1 treatment in mice bearing GL261 brain tumors.

The results of the combination treatment (GVAX + Anti-PD1) showed the greatest improvement in survival versus any of the other single treatments alone. The more notable part of the results is that treatment with GVAX alone increases the ratio of CD8$^+$ cells to T$_{reg}$ cells the most in the tumor environment versus any other treatment group. These results show that GVAX is important for increasing the ratio of CD8$^+$ cells to T$_{reg}$ cells in the tumor environment. The increase in the ratio of CD8$^+$ cells to T$_{reg}$ cells might set a more favorable stage for PD1 blockade and be the chief reason for synergistic impact.

OX40 is a member of the Tumor Necrosis Factor Receptor family and is presented on activated T-cells. Ligation is known to increase T-cell proliferation and survival. OX40 has also been shown to reduce the levels of T-cell exhaustion in the glioma microenvironment. The rationale for a combination treatment with Anti-PD1 and Anti-OX40 was that treatment with
Anti-PD1 would reverse the negative signal on T-cells from the PD-L1 ligand and the addition of Anti-OX40 would further help with T-cell proliferation and mounting a sustained immune response against the tumor.

Treatment with a combination of Anti-PD1 and Anti-OX40 also significantly improved survival but the results were similar to treatment with a combination of GVAX and Anti-PD1. Combination treatment with Anti-PD1 and Anti-OX40 also improved the ratio of CD8$^+$ cells to T$_{reg}$ cells. The results with the combination of GVAX and Anti-PD1 or Anti-PD1 and Anti-OX40 showed a positive treatment trend with combination treatment producing significant improvement in survival versus control treatment but still showed room for improvement.

Even though GVAX + PD1 or PD1 + OX40 showed significant improvement in survival, the overall number of long-term survivors (Survivors past day 120, post tumor challenge) with both treatments was under 50%. To further improve on this survival advantage, I decided to test a triple combination treatment of GVAX + PD1 + OX40.

Triple combination treatment with GVAX + PD1 + OX40, resulted in a 100% survival in triple combination treated mice. Overall the survival was also significantly better versus Anti-PD1 and Anti-OX40 treated mice, GVAX + OX40 treated mice and GVAX + PD1 treated mice. Mechanistically, triple combination treatment resulted in a significant increase in the amount of IFN-$\gamma$, TNF-$\alpha$ and IL-2 production by splenocytes, each of which are indicators of a Th1 immune response. Over all, the ratio of tumor infiltrating CD4$^+$ T-cells to Cd8$^+$ T-cells increased, but the overall number of those Cd4$^+$ t-cells that were T$_{reg}$ cells decreased with triple combination treatment indicating that most of those CD4$^+$ T-cells that increased with triple
combination treatment were T-helper cells. Triple combination treated mice also developed immune memory towards the tumor and long-term survivors rejected a tumor challenge with three times more cells than the initial tumor challenge. Furthermore, these mice also showed a stronger systemic development of both effector and central memory to the tumor challenge.

The results of triple combination treatment indicate that cancer immunotherapy can be a useful tool in cancer treatment. The first multiple immune checkpoint study to be tested in Glioblastoma patient used a combination of Anti-PD1 and Anti-CTLA-4. This combination treatment proved to be toxic in patients. My results with the GVAX + Anti-PD1 + Anti-OX40 treatment show that there are alternative combination immunotherapy treatment options that can be used clinically as an alternative approach.

Another important application from these results is actually looking at individual patient tumors and lymphocytes and their expression of immune checkpoint markers in order to treat them with a more personalized combination of immune-checkpoint targeting molecules. These results serve as a base line proof of concept that a combination treatment with checkpoint molecules is a viable approach for patients with glioblastoma.

The results from the triple combination treatment were tested in a different tumor model (CT2A) which is another glioblastoma model syngeneic with C57/Bl6 mice. CT2A is a more aggressive tumor and only 10000 cells result in a tumor that is potent and results in mortality. Though Triple treatment did result in an improvement in survival versus any other double or single treatment, the results were not significant. Even though the improvement in survival was not significantly different versus other treatments, the result that triple combination showed the
best results is indicative of the fact that the triple combination results are just not limited to one tumor model and may be generalizable

Overall, our results showed that double combination treatment (that is treatment with GVAX + Anti-PD1 or Anti-PD1 + Anti-OX40) in mice with GBM produce a significant improvement in survival with strong mechanistic correlates to the improvement in survival. These survival results are further improved with the addition of GVAX in combination with Anti-PD1 and Anti-OX40 resulting in a 100% survival.

Study Limitations

Triple combination treatment with GVAX + Anti-PD1 + Anti-OX40 resulted in a 100% survival in mice treated, it is important to note that this combination of immune checkpoint molecules is not exhaustive. Based on individual screening of each patient tumor or of circulating lymphocytes for expression level of certain immune checkpoint molecules, a different combination of immune checkpoint inhibitors or agonists might produce better results.

The effects of the triple combination treatment were looked at through a combination of activation markers on Cd4+ and Cd8+ T-cells, as well as the activity of certain cytokines. The role of other immune cell populations such as Dendritic cells, Macrophages and Natural killer cells was not explored during this study. Looking at the role these and other immune cell populations play during an anti-tumor immune response might provide a better understanding of the immune response and will help in creating even better treatment options.
The results of this project also look at a population that responds to checkpoint blockade immunotherapy. There is a population of patients that do not respond to checkpoint blockade immunotherapy or respond initially and then stop responding. The role of genes that make these patients not respond to immunotherapy should have been studied to make these results more applicable to a broader group of patients.

Future Directions

This study looked at the effect of immunotherapy with the combination of GVAX and checkpoint inhibitors. The cell populations that were studied the most during this study were Cd4+ and Cd8+ t-cells. Even though these two cells are two necessary components of the adaptive immune response, other cells of the immune system are also at play during an immune response. A look into the role that other immune cells such as Dendritic cells, Macrophages and Natural killer cells play is an important area to be explored in regard to checkpoint blockade immunotherapy. Furthermore, these cell populations can be targeted themselves to produce an anti-tumor immune response. An approach that combines Cd4+ and Cd8+ t-cells to other immune cells such as Macrophages might further improve these results.

Another important aspect is understanding why certain patients or tumors do not respond to immunotherapy. An understanding of the genomic mechanisms that give rise to resistance to immunotherapy in patients need to be understood to improve the results from immunotherapy.

The results of this study show that combination immune checkpoint blockade and stimulation can produce efficacious results in GBM. The immune synapse has many other
checkpoint molecules. Screening individual tumors for their expression of these checkpoint molecules will help recognizing the best checkpoint molecules to be targeted in the case of a specific tumor.

Conclusion

I hypothesized that combination immunotherapy with GVAX and Anti-PD1 will provide survival advantage in mice bearing orthotopic GBM. Data from this study is presented which shows that combination immunotherapy with GVAX and Anti-PD1 does provide survival advantage by increasing tumor infiltration of Cd8\(^{+}\) T-cells into the tumor and by increasing Cd8\(^{+}\) T-cells to T\(_{reg}\) cell ratio.

The survival advantage conferred by the combination of GVAX and Anti-PD1 was further enhanced by the addition of second immune checkpoint molecule OX40. Triple combination treatment resulted in a significant increase in the amount of IFN-\(\gamma\), TNF-\(\alpha\) and IL-2 production, all of which are indicators of a Th1 immune response. Over all, the ratio of tumor infiltrating CD4\(^{+}\) T-cells to Cd8\(^{+}\) T-cells increased, but the overall number of those Cd4\(^{+}\) t-cells that were T\(_{reg}\) cells decreased with triple combination treatment indicating that most of those CD4\(^{+}\) T-cells that increased with triple combination treatment were T-helper cells. Triple combination treated mice also developed immune memory towards the tumor and long-term survivors rejected a tumor challenge with three times more cells than the initial tumor challenge. Furthermore, these mice also showed a systemic development of both effector and central memory to the tumor challenge. These results confirm the initial hypothesis that combination
immunotherapy with GVAX and immune checkpoint molecules can be an efficacious treatment for patients with GBM.
Figure 1: GVAX treatment alone, significantly increased survival versus control treatment ($P \leq 0.001$). Anti-PD-1 treatment alone significantly improved survival versus GVAX treatment alone ($P \leq 0.05$). Furthermore, double treatment with GVAX + Anti-PD1 was most successful and significantly improved survival, versus Anti-PD1 treatment group alone ($P \leq 0.05$). The double treatment group also had the most long-term survivors at 120 days post tumor implantation.
Figure 2: GVAX treatment alone significantly improved the ratio of cytotoxic CD8$^+$ T-cells versus regulatory FoxP3$^+$ cells in comparison to all other treatment groups (P ≤ 0.05). Combination treatment with GVAX + Anti-PD1 also significantly improved the ratio of cytotoxic CD8$^+$ T-cells versus regulatory FoxP3$^+$ cells in comparison to the Anti-PD1 alone treatment group and the control group (P ≤ 0.05)
Figure 3: Immunohistochemistry analysis on brain tissue for Cd3, Cd4 and Cd8 showed a significant increase in Cd3⁺ T-cell infiltration into the tumor with GVAX + Anti-PD1 treatment versus control mice. Cd4⁺ t-cell infiltration also increased the most in mice treated with GVAX + Anti-PD1 combination but it was only significantly different in mice treated with GVAX alone versus the control group (P ≤ 0.01), even though as mentioned before double treatment was found to have the greatest number of tumor infiltrating lymphocytes. Cd8⁺ t-cell infiltration into the tumor increased significantly with GVAX + PD1 treatment versus Anti-PD1 alone treated mice (P ≤ 0.01), GVAX alone treated mice (P ≤ 0.001) and Control mice (P ≤ 0.01).
Figure 4: Addition of a second checkpoint molecule, Anti-Ox40 increases survival versus Anti-PD1 alone or Anti-OX40 combination treatment, the results are not significant between double Checkpoint molecule, versus single checkpoint molecule combination.
Figure 5: Treatment with the combination of Anti-PD1 and Anti-OX40 increases the ratio of Cd8$^+$ to T$_{reg}$ cells.
Figure 6: GVAX + Anti-PD1 + Anti-OX40 treatment significantly increases survival versus Anti-PD1 and Anti-OX40 treatment (P ≤ 0.01), and GVAX + Anti-OX40 treatment as well as GVAX + PD1 treatment (P ≤ 0.01 and P ≤ 0.05 respectively).
Figure 7: Elispot analysis of Splenocytes showed a significant increase in IFN-Gamma production with triple combination treatment (GVAX + Anti-PD1 + Anti-OX40) versus GVAX + Anti-PD1 (P ≤ 0.01), GVAX + Anti-OX40 (P ≤ 0.05) and Anti-PD1 + Anti-OX40 (P ≤ 0.01). IL-2 production also increased significantly with the triple combination treatment versus GVAX + Anti-PD1 treatment (P ≤ 0.01), GVAX + Anti-OX40 treatment (P ≤ 0.05) and Anti-PD1 and Anti-OX40 treatment (P ≤ 0.01). Triple combination treatment also significantly increased TNF-Alpha production versus GVAX treatment alone (P ≤ 0.05) and Control group (P ≤ 0.01).
Figure 8: Flow cytometry analysis on tumor infiltrating lymphocytes (TILs) showed that the number of CD4$^+$ cells increased in TILs upon triple combination treatment, the percentage of these CD4$^+$ t-cells that were T-regulatory cells was the lowest in triple combination treated group and significantly lower than GVAX + Anti-PD1, PBS + Anti-PD1 and Control groups (P ≤ 0.05).
Figure 9: Tumor re-challenge with five times more cells versus initial tumor in long term survivors of triple combination treatment showed that 100% of these mice rejected the tumor challenge. Age matched controls on the other hand were all dead around day 25.
Figure 10: Qualitative analysis of long term survivors that were re-challenged with tumor and sacrificed on day 11 to perform H&E staining on the brains showed that triple combination treated animals had the smallest amount of brain tissue showing signs of tumor followed by Anit-PD1 + Anti-OX40 treated and the control had the biggest tumor size qualitatively.
Figure 11: Naïve T-cells exhibit high levels of CD62L and low expression of CD44, whereas memory T cells are identified by high CD44 and low CD62L expression. Flow cytometry analysis of TILs of re-challenged mice showed that the GVAX + Anti-PD1 + Anti-OX40 treated mice had the greatest number of high CD44 and low CD62L Cd4⁺ T helper cells which was significantly more than Anti-PD1 + Anti-OX40 treated re-challenge mice (P ≤ 0.05).
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