THE DYSREGULATION OF T FOLLICULAR REGULATORY CELLS IN A MOUSE MODEL OF GLIOBLASTOMA MULTIFORME LEADS TO HUMORAL IMMUNE DYSFUNCTION

A Dissertation

by

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ABSTRACT

Glioblastoma multiforme (GBM) is a highly lethal cancer arising from mutations in glial cells. Many therapies currently in development for this disease focus on the role of the immune system and its inability to interact with the tumor. One of the reasons for its poor prognosis is its ability to evade the immune system. This is accomplished by the upregulation of $Foxp3^+$ T regulatory cells (T_{regs}), a T cell subset that is capable of potent immunosuppression. This subset of cells has long been characterized in glioblastoma and many other cancers.

In this dissertation, we outline how a newer subset of T_{regs}, T follicular regulatory cells (T_{fr} cells) are capable of controlling the humoral immune response within GBM. In the healthy body, this subset of cells is responsible for terminating germinal center (GC) responses following infection. In particular, T_{fr} cells function by limiting the number of available $CXCR5^+$ T follicular helper cells (T_{fh} cells) and GC B cells in vivo. We demonstrate that the amount of T_{fr} cells that contribute to the total pool of $CXCR5^+$ cells is increased in the dural lymphatic vessels, a lymphatic vessel that remains uncharacterized in GBM, of immunocompetent GBM tumor-bearing mice following infection with Complete Freund's Adjuvant. We additionally show that T_{fh} function is inhibited in these GBM model mice, as they are impaired in their ability to display CD40L, a surface marker that is crucial to the formation of GCs and, thus, antibody-mediated immune responses. We further show that the downstream generation of antibody-producing plasma cells is inhibited in these tumor-bearing mice. We also show

that these T_{reg} cells in GBM under inflammatory conditions appear to be of extrathymic rather than thymic origins.

We additionally show that treatment with PD-L1 monoclonal antibody (mAb) is capable of preventing the expansion of T_{fr} cells and is capable of rescuing the development of plasmablasts and plasma cells in tumor-bearing mice. Finally, we show that addition of PD-L1 mAb is capable of slightly extending the survival time of tumor bearing mice when paired with CFA treatment. When taken together, these results show that a profound dysfunction exists in the humoral immune system of this GBM mouse model and can be rescued with PD-L1 blockade.

DEDICATION

This dissertation is dedicated to my parents and the tremendous sacrifices they have made to allow me to live a better life than they have. Their past has been tense so my future can be perfect. It is also dedicated to my brother and sisters, who have shaped the person I have become.

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I conducted the initial surgeries in this study with the assistance of Dr. Sanjib Mukherjee and later independently. All other work in this study was conducted independently by myself. The work in this dissertation was supported by the Raleigh R. White III Endowment from the Department of Surgery, Baylor Scott & White Health, to Dr. M. Karen Newell Rogers as well as salary savings support from the Texas A&M Health Science Center.

NOMENCLATURE

1-MT	1 Methyltryptophan
AhR	Aryl Hydrocarbon Receptor
APC	Allophycocyanin
BBB	Blood Brain Barrier
CFA	Complete Freund's Adjuvant
CSC	Cancer Stem Cell
Cy7	Cyanine7
DSCM	Defined Stem Cell Medium
EGFR	Epidermal Growth Factor Receptor
F_0B	Follicular B Cell
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead Box P3
GBM	Glioblastoma Multiforme
GC	Germinal Center
GFAP	Glial Fibrillary Acidic Protein
HIF1α	Hypoxia Inducible Factor 1 Alpha
ICOS	Inducible T-cell Co-Stimulator
ICOSL	Inducible T-cell Co-Stimulator Ligand
IDO	Indoleamine 2,3-dioxygenase
IL-21	Interleukin 21

iT_{reg}	Inducible T Regulatory
mAb	Monoclonal Antibody
MFI	Mean Fluorescence Intensity
MHCII	Major Histocompatibility Complex II
MIF	Macrophage Migration Inhibitory Factor
NBCS	Newborn Calf Serum
NK	Natural Killer
Nrp	Neuropilin-1
PBS	Phosphate Buffered Saline
PD-1	Programmed Cell Death Protein 1
PD-L1	Programmed Death Ligand 1
PE	Phycoerythrin
pT_{reg}	Peripheral T Regulatory l
T_{fh}	T Follicular Helper
T_{fr}	T Follicular Regulatory
T_H	T Helper
T_{reg}	T Regulatory
tT_{reg}	Thymic T Regulatory
TMZ	Temozolomide
VEGFR	Vascular Endothelial Growth Factor Receptor

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CHAPTER I

OVERVIEW AND INTRODUCTION

Immune Tolerance

The purpose of the immune system in all creatures is to defend the self against foreign pathogens and internal dangers. The key question that follows is, "What is self and what is non-self?" Furthermore, how does the immune system know the difference? Self can be defined as the immune system's host body self. Non-self can be defined as anything else. In a healthy individual, the immune system is adept at recognizing the differences between the two. This concept is known as tolerance. The immune system is tolerant of the cells of its host and intolerant of foreign pathogens. Tolerance can be broken down into central and peripheral tolerance.

Antigens are molecules that can be recognized by the immune system. For the immune system to recognize non-self, two processes must take place during immune development. The first process that must take place in the body for the body to begin discriminating between self and non-self is clonal deletion. During clonal deletion, B cells and T cells that display receptor for self-antigens are selectively deleted by apoptosis in the bone marrow and thymus, respectively. This can be seen in figure 1. If an immune cell that displays receptor for self-antigen passes through the bone marrow or thymus, autoimmune disorders are likely to develop in the host.

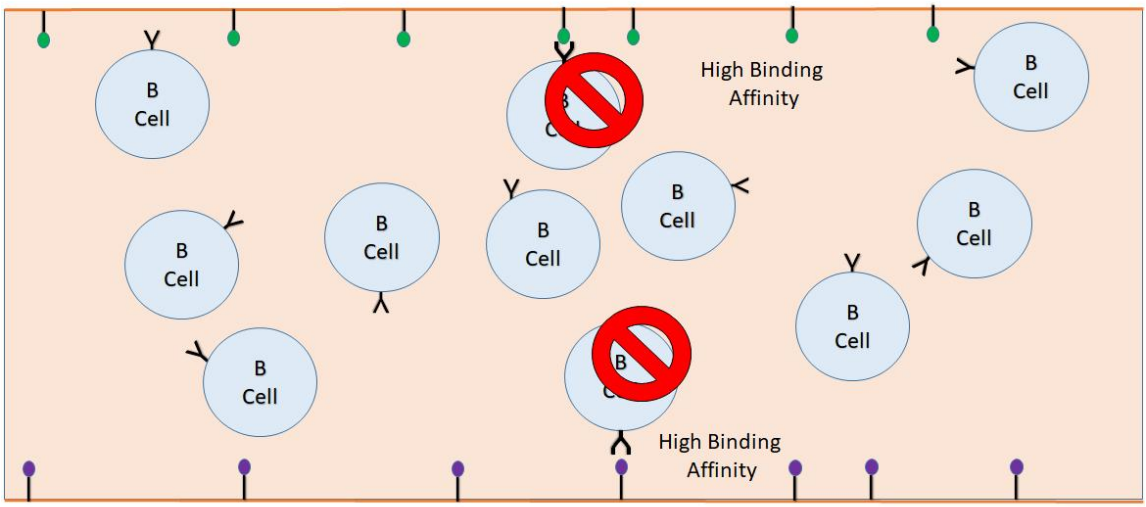


Figure 1. Clonal deletion. During clonal deletion, B cells that bind with high affinity towards self-antigen in the bone marrow are selected against and deleted by apoptosis.

The second process is clonal expansion (figure 2). Clonal expansion is the converse of clonal deletion. During clonal selection, B cells and T cells compete for survival signals. Those which are unable to receive survival signals are subject to apoptosis. Those that do receive these survival signals, however, are able to expand and acquire various effector and memory cell phenotypes. In this manner, the immune system is able to become competent and protect the host against foreign antigens.

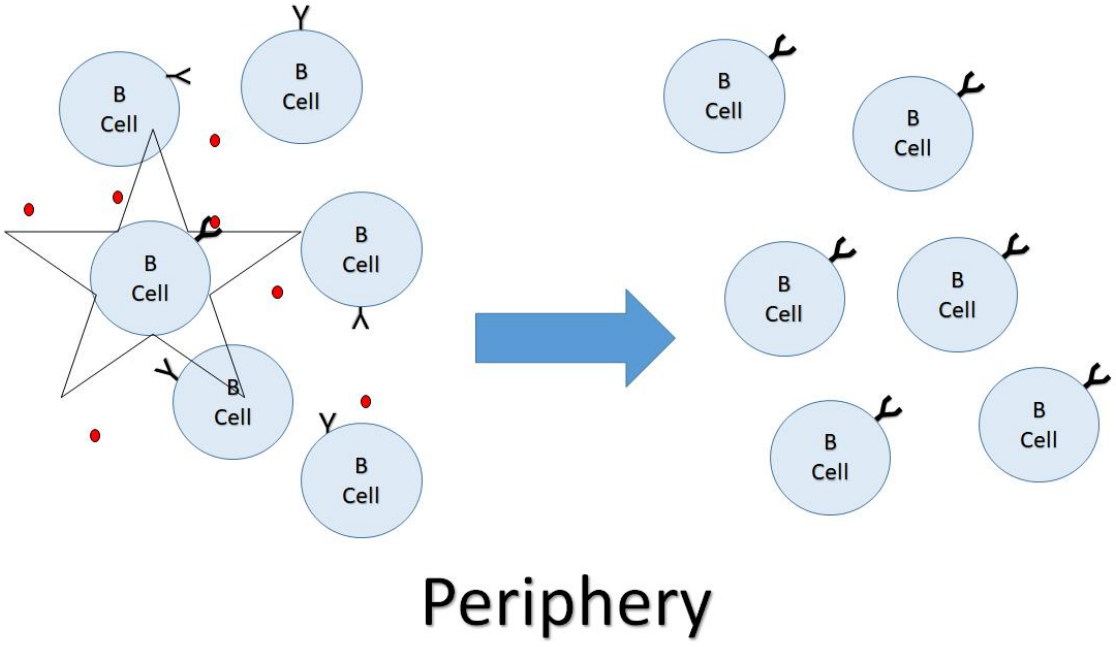


Figure 2. Clonal expansion. When B cells are exposed to antigen, the ones that are capable of binding antigen with high affinity, in this case the B cell within the star, rapidly divide.

One of the hallmarks of cancer and oncogenesis is the ability to escape immune surveillance. This is termed the immune escape paradigm, which will be discussed in this dissertation. It is important to make the distinction between self, which is the healthy cell in the human body, and non-self, which is the foreign pathogen that causes harm to the body. The subject of tolerance is mentioned here because the immune system is, in effect, tolerant of the tumor. Although the tumor cell does arise from healthy cells that are considered self, tumorigenesis leads to the creation of the tumor cell, a cell with genetic mutations that exhibits antigens that are distinct from the host,

and should not be considered self. The tumor should therefore be visible to the immune system.

Affinity Maturation

Antibodies are molecules with the ability to bind antigens with a high degree of specificity. The purpose of antibodies is to neutralize bacteria and other pathogens or toxins. The process of coating and internalizing bacteria is termed opsonization. After the antibodies bind the foreign cell or antigen, the opsonized cell is then targeted for destruction by phagocytes. Broadly speaking, affinity maturation is the process through which naïve B cells become able to produce antigen-specific antibodies. This process occurs in germinal centers (GCs), which are located in lymph nodes. This is a two-step process.

The first step is somatic hypermutation. During this somatic hypermutation, follicular dendritic cells display antigen to follicular B cells, which leads to the acquisition of antigen by follicular B cells. Once this criterion is met, follicular B cells then display antigen to $CD4^+$ T follicular helper (T_{fh}) cells. This causes the follicular B cells to undergo class switching and monoclonal expansion, where they, now termed centroblasts, then make up the dark zone of the GC. During this stage, B cells rapidly mutate their B cell receptors so that they are able to bind to the antigen of interest with high affinity. This is accomplished through V(D)J recombination in the hypervariable regions in DNA.

The next step in affinity maturation is clonal selection. After B cells undergo somatic hypermutation, they then compete for resources and signals. The B cells at this

stage are in a state of activated apoptosis and need a survival signal from T_{fh} cells to avoid death. B cells that have higher affinity to antigen are able to migrate to the light zone of the GC. Here, the B cell surface antibody binds to the antigen presented by the T_{fh} cell. A second signal of CD40L on the T_{fh} cell interacts with CD40 on the B cell conferring survival on the B cell. The B cell then undergoes isotype class switching to produce soluble antibody as well as clonal expansion to increase the total number of antibody-producing B cells.

T Follicular Helper Cells

T_{fh} cells play a crucial role in affinity maturation and the development of antibody production from GCs. Traditionally, these cells were defined as $CD4^+CD19^-CXCR5^+ICOS^+$, but due to the recognition of T follicular regulatory (T_{fr}) cells, the additional marker of $Foxp3^-$ has also been added. As stated in the previous section, Tfh cells provide CD40L to GC B cells to confer survival.

One of the critical roles of T_{fh} cells is the expression of IL-21. IL-21 is a pleiotropic cytokine that is produced mainly by natural killer (NK) T cells, T_H17 cells and T_{fh} cells. It is also produced in low levels by other cell types.(Spolski and Leonard, 2014) IL-21 is critical in the development and proliferation of Tfh cells, as well as the functionality of GCs. Additionally, this cytokine is necessary for plasma B cell differentiation and development, as well as the production of antibodies.(Ozaki, 2002) This can be seen in figure 3. Because of its ability to modulate the immune system, IL-21 is being treated as an oncological therapeutic. In particular, it has been shown that application of IL-21 can cause the rejection of GBM xenografts in an immunocompetent

mouse model.(Daga et al., 2007) In this study, Daga et al. transduced a slow growing glioma cell line to produce IL-21, among other cytokines in the same glioma cell line. They found that this cell line was rejected every time it was injected. To further examine the role of this cytokine, the researchers injected 1 µg of recombinant IL-21 directly into established tumors and found that the tumor was rejected with high frequency. It was discovered that this tumor rejection was mediated through antibody-dependent cell lysis.

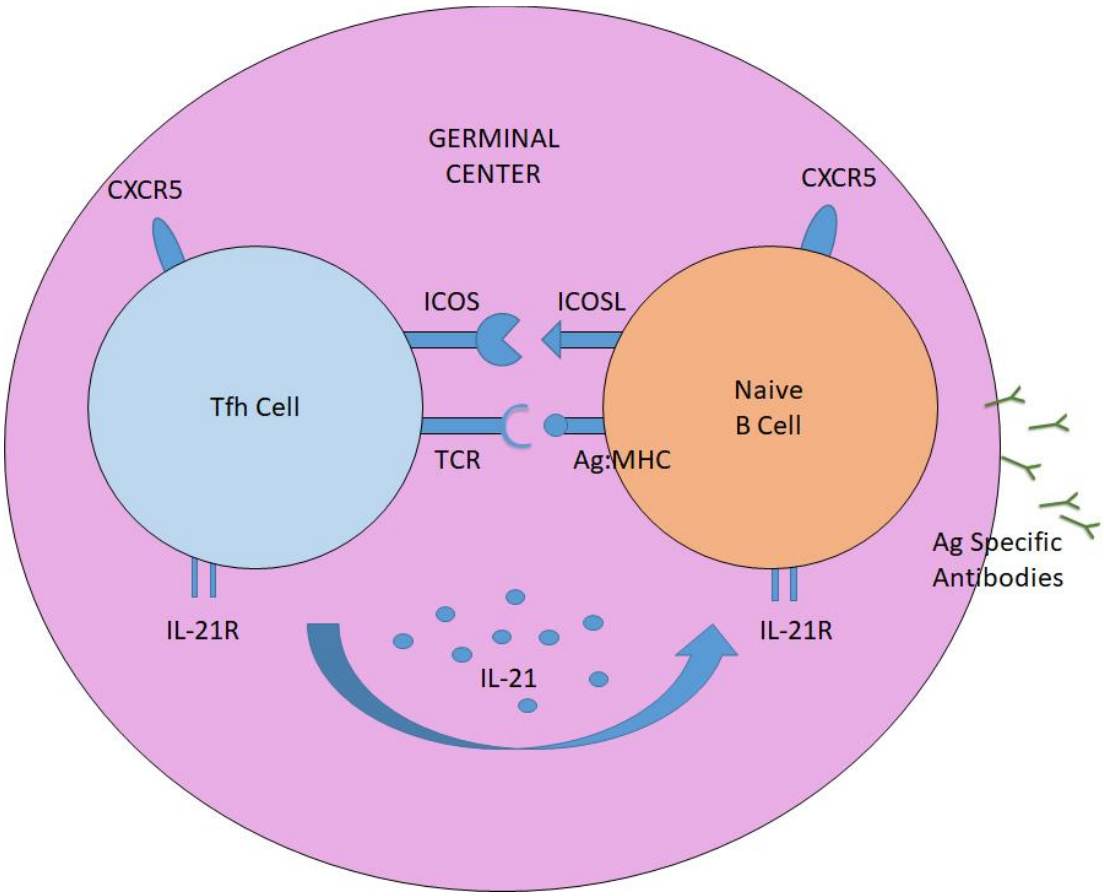


Figure 3. IL-21 signaling. IL-21 signaling is necessary for the development of germinal centers and isotype switching.

T Regulatory Cells

T regulatory (T_{regs}) cells are a subset of $CD4^+$ T cells that are responsible for the suppression of various lymphocyte cell types, especially T helper (T_h) subsets.(Chaudhry et al., 2009) T_{regs} are commonly identified as $CD4^+CD25^+Foxp3^+$. Foxp3 is a transcription factor that has been shown to be crucial to the development and maintenance of T_{regs}.(Ohkura et al., 2013) It has been demonstrated that mutations in Foxp3 results in the impairment of T_{regs}, and subsequently, immunodysregulation and the development of autoimmune disorders.(Bennett et al., 2001)

The purpose of T_{regs} in healthy individuals is to maintain tolerance to self and to prevent the development of deleterious immune effects. To this end, T_{regs} induce clonal anergy at sites of inflammation. Clonal anergy is a process through which previously activated immune cells become functionally quiescent. As a result, the anergic cells cannot damage the tissue by producing reactive oxygen species. In this manner, the immune response is terminated when the antigen is no longer present. This process is outlined in figure 4.

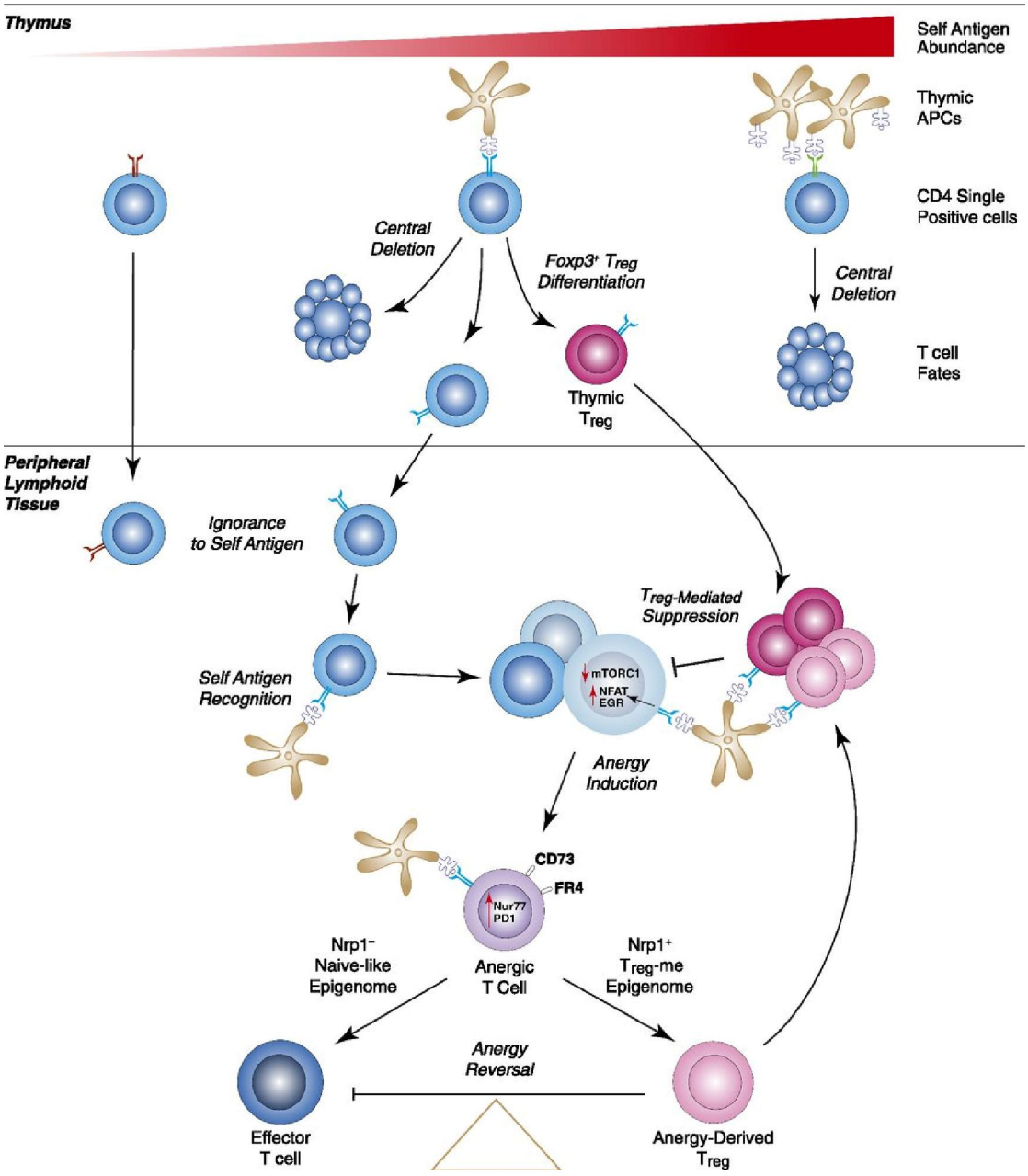


Figure 4. Development of T_{regs} and subsequent clonal anergy. This figure shows the differentiation of $_{Tregs}$ and how they suppress inflammatory processes. (Reprinted with permission from Kalekar and Mueller, 2017. Copyright 2017. The American Association of Immunologists, Inc.)

In vivo, T_{regs} can have one of two origins, the periphery and the thymus. These T_{regs} are termed pT_{regs} and tT_{regs}, respectively. Additionally, a third set of T_{regs}, those generated in vitro through the application of TGFβ, termed inducible T_{regs} (iT_{regs}), also exist, but are not relevant to this project.(Shevach and Thornton, 2014) Although pT_{regs} and tT_{regs} have the same general function, the suppression of inflammatory processes, the specifics of how each of these subsets are distinct remains poorly understood. Furthermore, pT_{regs} and tT_{regs} develop differently in the presence of Programmed Cell Death Protein 1 (PD-1)/Programed Death Ligand-1 (PD-L1) interaction. This interaction is crucial to the development of extrathymic pT_{regs}. In stark contrast, the absence of PD-1 causes enhanced tT_{reg} development.(Chen et al., 2014)

The tT_{regs} function by inhibiting T-effector cell trafficking to sites of inflammation. This population of cells, when polyclonal as opposed to antigen-specific, is also capable of preventing the differentiation of naïve $CD4^+$ T cells into pathogenic Th1 cells in autoimmune pathologies. The tT_{regs} are neuropilin-1^{hi} ($Nrp1^{hi}$).

The pT_{regs} function by acting on antigen presenting dendritic cells. Previous studies have shown that in an autoimmune gastritis model, iT_{regs} served as a substitute for pT_{regs}. Introduction of these cells led to an impairment in the ability of dendritic cells to present antigen, and thus, cause a specific immune response. The pT_{regs} are $Nrp1^{lo}$.

T_{regs} remain an attractive target for glioblastoma researchers.(Liu et al., 2016; Wainwright et al., 2013) Certain combinations of immunotherapeutics targeting T_{regs} have proven successful in rejecting xenografts.(Wainwright et al., 2014) The Lesniak group has shown that treatment with PD-L1 monoclonal antibodies (mAb), IDO

inhibitors (e.g., 1-MT) and CTLA-4 mAb is successful in preventing tumor-infiltrating lymphocytes in a mouse model of established glioblastoma. They further went on to show that mice that received this combinatorial treatment were long-term survivors and lived at least 150 days with the disease and associated this better prognosis with decreased number of tumor infiltrating lymphocytes.

T Follicular Regulatory Cells

A specialized class of T_{regs} are essential in the control of the GC and the development of antibody production. These cells are termed T_{fr} cells and can be identified on a flow cytometer as $CD4^+CD19^-CXCR5^+ICOS^+Foxp3^+$ cells.(Linterman et al., 2011) These cells were discovered by Lintermann et al. in 2011.(Linterman et al., 2011) Like conventional T_{regs}, these cells are $Foxp3^+$ and can have either a thymic or peripheral origin. As this class of immune cell has been discovered very recently, these cells are not very well-characterized. In contrast to T_{fh} cells, T_{fr} cells are believed to arise from natural T_{regs}, as opposed to $CD4^+$ naïve T cell precursors, from which T_{fh} cells arise.(Sage et al., 2012),(Chung et al., 2011)

It is believed these cells function by tightly limiting the expansion of T_{fh} cells and, therefore, by limiting the formation of GCs. They depend on Bcl-6, CD28 and B cells to proliferate. T_{fr} cells are, in turn, inhibited by PD-1/PD-L1 interactions. Additionally, these cells may be specific for antigen, i.e., shut down GCs that produce an antibody to a specific antigen. One other difference between T_{fr} and T_{fh} cells is based on the observation that T_{fh} cells produce IL-21 liberally to cause the formation of GCs

whereas T_{fr} cells are restricted by IL-21 through irresponsiveness of the IL-2R.(Jandl et al., 2017; Spolski and Leonard, 2010)

PD-L1

PD-L1, also known as CD274, is a cell surface ligand that interacts with PD-1, also known as CD279. This interaction is crucial for the recruitment of certain subsets of T_{regs} and the maintenance of self-tolerance through clonal anergy. In particular the interaction of PD-1 with PD-L1 is responsible for the maintenance of FoxP3 in iT_{reg} subsets during their suppressive activity. This can be seen in figure 5.

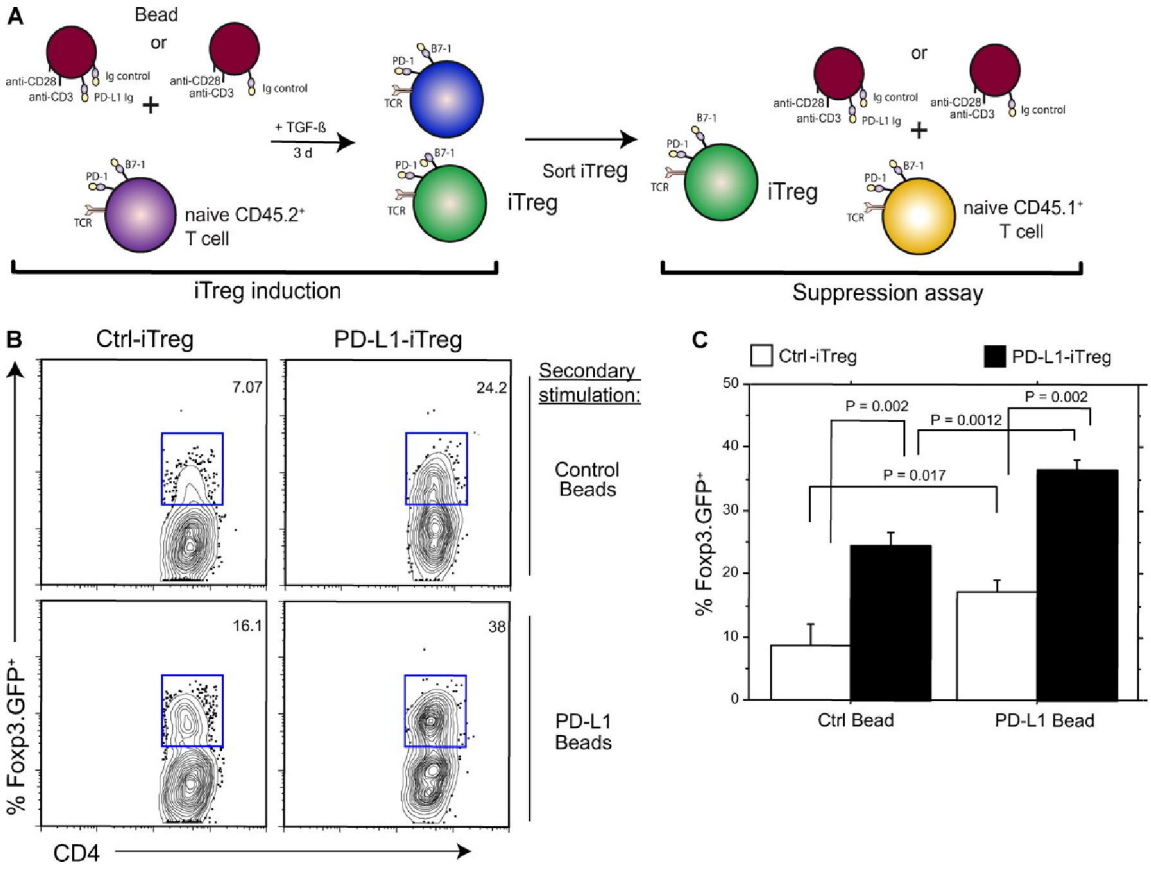


Figure 5. PD-L1 maintains Foxp3 expression by iT_{reg} cells during suppression of effector cell function (Francisco et al., 2009). Figure reproduced with permission from The Rockefeller University Press.

It has been shown that inhibition of PD-L1, among other immune checkpoint molecules, is able to extend survival times in a mouse model of GBM.(Wainwright et al., 2014) In other forms of cancer including melanoma, which is similar to GBM in regards to their neuroectodermal origins, inhibition of the PD-1/PD-L1 pathway leads to regression of the tumor and convalescence in patients.(Bertrand et al., 2017; Liao et al., 1981)

Conversely, the absence of PD-1 enhances tT_{reg} development,(Chen et al., 2014) Wainwright et al. suggest that the predominant subset of active T_{regs} in GBM are of thymic origin rather than induced origin.(Wainwright et al., 2011) Further complicating the role of PD-L1 in regards to immunosuppression is the fact that PD-L1 on neurons near the tumor are beneficial for the survival of the patient.(Liu et al., 2013) In this study, researchers found that neurons near the tumor that expressed PD-L1 were capable of targeting and eliminating glioblastoma, leading to a more favorable prognosis. The researchers did not link an immune component to this study, however. Nonetheless, the possibility that this beneficial PD-L1 may also inhibit the expansion of thymic T_{regs} does exist.

Immunosenescence

Immunosenescence is a term used to describe the changes in the immune system that are acquired as the result of natural aging. Immunosenescence is also seen in diseases that target the immune system, most notably human immunodeficiency virus infection. Immunosenescence is characterized by the loss of hematopoietic stem cells

within the bone marrow. This results in a decreased total pool from which immune cells can differentiate. This is a global rather than localized event in the immune system.

A common thread that links glioblastoma patients is advanced age. Although it is not unheard of for younger people to be diagnosed, peak incidence occurs around 60-84 years of age and the average age at diagnosis is 64 years. Advanced age is associated with immunosenescence, the gradual deterioration of the immune system. Immunosenescence is marked by loss of hematopoietic stem cells (leukocyte precursors), NK cells, and a diminished humoral immune response. It is feasible, then, that immunosenescence facilitates more precancerous cells to reach equilibrium and escape. Another way in which immunosenescence affects the immune system is the reduction of IL-7 expression.(Tsuboi et al., 2004) Chiodi et al. have recently suggested that IL-7 plays a crucial role in the interaction of B costimulatory molecules with T_{fh} cells.(Chiodi et al., 2017) In particular they point to one of their experiments in which T_{fh} cells were incubated with IL-7. They found that upon incubation with this cytokine, T_{fh} cells were able to express higher levels of PD-1. PD-1 in this context is crucial for plasma cell differentiation.

Glioblastoma Multiforme

Glioblastoma multiforme (GBM) is a cancer that is caused by mutations in glial cells. It is classified by the World Health Organization as grade IV, due to its high level of malignancy.(Louis et al., 2016) This is evidenced in its poor prognosis; only 30% of patients survive past two years. As is seen in most types of cancers, the primary driver of disease is uncontrolled tumor growth that leads to inflammation and other deleterious

effects. In GBM, this uncontrolled growth leads to increased intracranial pressure, edema, necrosis and, eventually, death.(Louis et al., 2016)

Tumorigenesis

The predominant paradigm that attempts to explain glioma and tumor progression is the immunoediting paradigm. This model seeks to explain the role of the immune system in oncogenesis. At its core are three stages: elimination/immunosurveillance, equilibrium, and escape. During the immunosurveillance/elimination stage, cells that have acquired mutations and have become precancerous are selected against by immune effector cells, including CD4+ and CD8+ T lymphocytes, macrophages and B lymphocytes. A key development in about 70% of cases of GBM is the development of mutations in the PI3K pathway, whether it be mutations in EGFR, VEGFR or PTEN.(Mao et al., 2012) This process is not always perfect, however, and some tumor cells escape surveillance. The stage in which tumor cells escape and survive, yet do not elicit an immune response nor reproduce rapidly is called equilibrium. In this extended stage, precancerous cells that have acquired mutations rendering them invisible to the immune system are selected for and are able to reproduce. Eventually these cells reach the escape phase in which the precancerous cells are able to proliferate rapidly and form a tumor.

The blood brain barrier (BBB), which will later be discussed at length in this dissertation, complicates the immunoediting paradigm in GBM. In healthy individuals, the BBB prevents immune activity inside the brain. Often, as the tumor grows, the BBB becomes disrupted and "leaky." This results in the loss of immune privilege and the

invasion of immune cells into the brain.(Dubois et al., 2014) However, before the BBB becomes permeable, the tumor should still be under the immune supervision of microglia, the brain's resident immune cell.(Hambardzumyan et al., 2015)

Tumor Heterogeneity

Glioblastoma is a highly heterogeneous tumor, both intertumorally and intratumorally. This can be seen in figure 6. There is an array of cell types that compose the tumor. The tumor is made of cells with distinct genotypes, phenotypes and epigenetic states and, thus, a hierarchy of cell types. Reya et al. believe that as there are clearly defined cellular hierarchies in both the tumor and the brain itself, GBM development replicates ontogeny.(Reya et al., 2001) Because of this, there must be some progenitor cell responsible for the development of this hierarchy. The cancer stem cell (CSC) is central to this idea, because it is capable of generating the various cell types that compose the tumor. The parallel drawn in healthy brain development is the neural pluripotent stem cell.

CSCs are defined by their functional characteristics, which include the ability for self-renewal, persistent proliferation and the ability to form tumors upon xenograft or injection. They are regulated by six main mechanisms. These are microenvironmental factors, the immune system, genetic and epigenetic changes, metabolism and niche factors.(Lathia et al., 2015) This dissertation will focus on the microenvironment and the immune system.

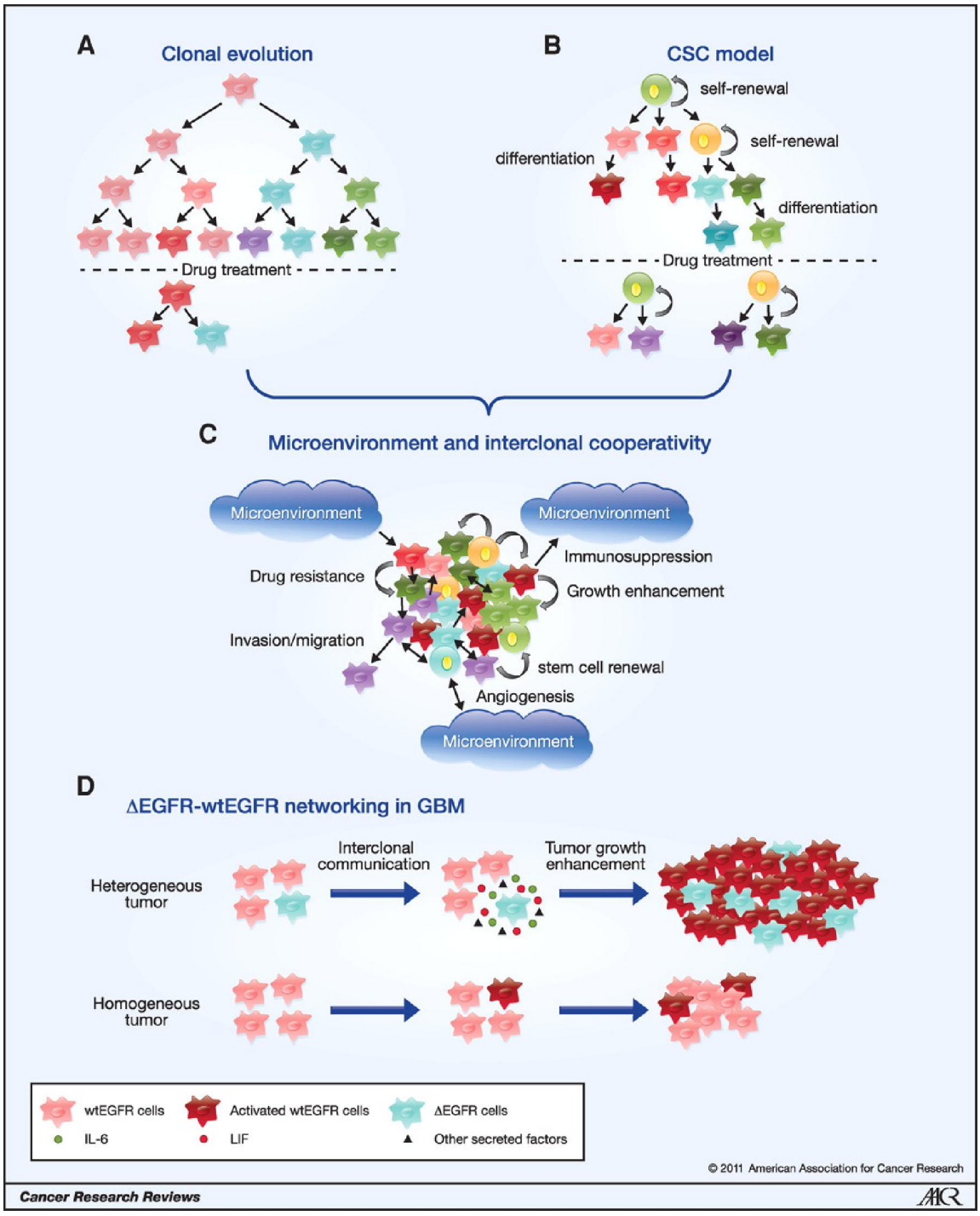


Figure 6. Heterogeneity origins and maintenance (Bonavia et al., 2011). Various paradigms that attempt to explain GBM heterogeneity. Figure reproduced with permission from the American Association for Cancer Research.

In addition to intratumoral heterogeneity, GBM also displays intertumoral heterogeneity. Soeda et al. have shown that CSCs taken from one patient's tumor are able to develop into heterogeneous subclones with varied phenotypes.(Soeda et al., 2015) Thus, while each tumor shows similar facets and features, e.g., angiogenesis and preferential glucose metabolism, each individual tumor is different. The obvious consequence of this diversity is that each GBM tumor may require specialized tailored treatment. As more immunotherapeutics are developed for GBM, targeting the tumor still remains a difficult prospect. Elucidating the mechanisms by which the tumor disguises itself may be a path forward.

Tumor Microenvironment

In the past, research has focused on the tumor itself, but current research has shifted towards the tumor microenvironment and its interactions with the host immune system. The tumor environment is particularly hypoxic. This leads to the upregulation of the transcription factor hypoxia inducible factor-1alpha (HIF1α). This protein has many immunosuppressive downstream effects.(Huang et al., 2016) One of the direct downstream targets of HIF1α is PD-L1, a previously mentioned immunosuppressive molecule.(Noman et al., 2014) Macrophage migration inhibitory factor (MIF), in turn, regulates HIF1α.(Winner et al., 2007)

MIF is a protein encoded under hypoxic conditions. It directly inhibits p53, allowing tumor initiating cells to continue to propagate.(Fukaya et al., 2016) MIF additionally contributes to the metabolic shift to autophagy that allows the tumor to grow.(Chuang et al., 2012; Gammoh et al., 2016) Furthermore, it allows the tumor to

escape surveillance by antigen-presenting dendritic cells in the brain.(Xu et al., 2016) Upon binding to CD74 and CD44, MIF is able to send antiapoptotic signals to a variety of cell subtypes, most relevantly, monocytes and T cells.(Bernhagen et al., 2007) Studies have shown that therapeutic targeting of MIF is beneficial in a variety of cancers.(Pasupuleti et al., 2014)

Tumor infiltrating lymphocytes are present in GBM subsets, indicating an active adaptive immune response. This immune response is thought instead to target the tumor, though a change in the prognosis is debated.

Recent studies show that the tumor is able to coopt hematopoietic stem cells, lymphocyte and monocyte precursors, and convert them into myeloid-derived suppressor cells.(Huang et al., 2017) This is accomplished by the expression of various growth factors and cytokines, including TGF-β, GM-CSF, VEGFR2 and EGFR and its mutants. The buildup of myeloid-derived suppressor cells around the tumor allows angiogenesis and tumor growth to progress undisturbed.

Cancer Antigens

Cancer antigens are biomolecules produced by the tumor that should illicit an immune response. EGFR mutants, in particular EGFRvIII, are overexpressed in about 60% of primary GBMs. Miao et al. have recently demonstrated that T cells with chimeric antigen receptors specific for EGFRvIII are capable of migrating to the tumor site and inducing tumor cell death when injected into the tail veins of tumor-bearing mice.(Miao et al., 2014) This indicates that the tumor is molecularly different from healthy tissue and is capable of being recognized by the immune system.

Because there are antigens presented by the tumor and because these antigens can be recognized and killed by immune cells, the topic turns to how the tumor evades or subverts the immune system. For these antigens to trigger an immune response, the antigen must be displayed in the context of the major histocompatibility complex II (MHCII), a process known as "antigen presentation". Antigen presentation allows the tumor to be "seen" by the immune system, which will then cause the tumor to be targeted. One possible way the tumor subverts the immune system is by preventing the expression of the antigen on MHCII or by downregulating the expression of MHC class II.

Tumor Immunology

It has been previously shown that GBM is very capable of evading the immune system. Although in the healthy individual, the brain is acknowledged as an "immune privileged" area, this privilege can become compromised in GBM. Previous studies show that the BBB becomes "leaky" in patients with GBM.(Dubois et al., 2014) This effect is mediated by aquaporins, integral membrane proteins responsible for the transfer of water across channels. As a result, the brain and tumor then become subject to immune scrutiny and are no longer immune privileged. Immune effector cells are then allowed to infiltrate the brain and tumor areas. One of the reasons infiltration occurs is because of the previously mentioned hypoxic tumor microenvironment.

Tryptophan is metabolized via the kynurenine pathway. This pathway is outlined in figure 7. As can be seen, the first step is the metabolism of tryptophan to kynurenine by either indoleamine 2,3-dioxygenase (IDO) or tryptophan dioxygenase

(TDO).(Thackray et al., 2008) TDO is expressed predominantly in the liver, but can be expressed in the brain under certain stimuli, whereas IDO is expressed globally. The upregulation of IDO is correlated with worse prognosis in GBM patients.(Wainwright et al., 2012) Previous research has shown that kynurenine interacts with the aryl hydrocarbon receptor (AhR). When kynurenine binds to the AhR on naïve T cells, the T cells then differentiate into immunosuppressive $Foxp3^{+}$ T_{regs}.(Mezrich et al., 2010) Recent publications have shown that blockade of various immune checkpoint proteins extend the survival times of GBM model mice.(Wainwright et al., 2014),(Allen et al., 2017),(Reardon et al., 2016)

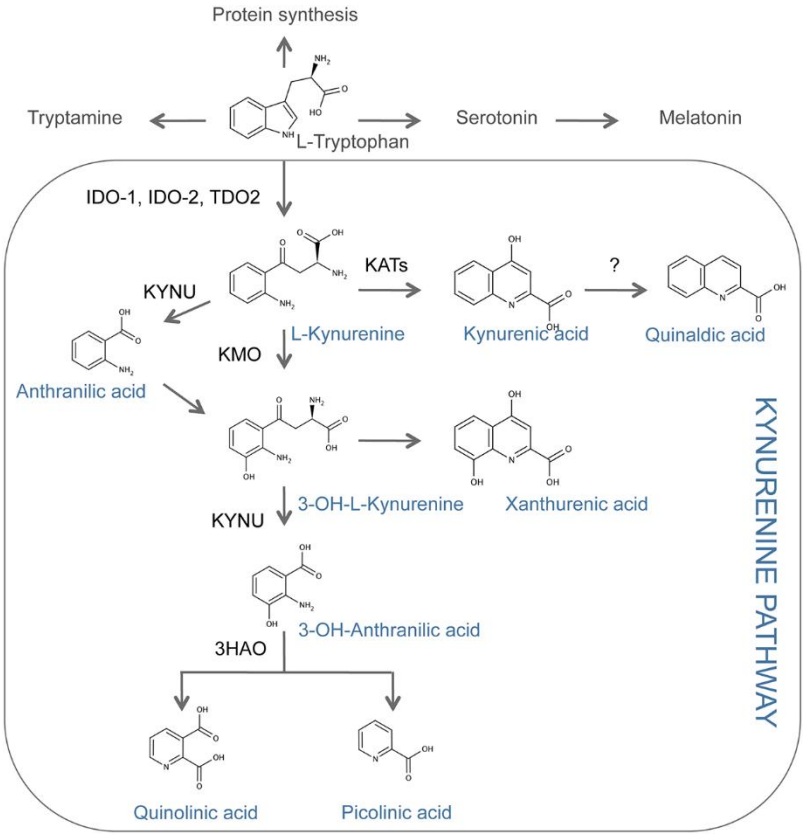


Figure 7. The kynurenine pathway (Puccetti et al., 2015). Figure reproduced with permission from PLOS ONE.

The Blood Brain Barrier

One of the more confounding aspects in GBM therapy is its location. Because GBM is a cancer that occurs in the brain, treatment of the tumor must account for the BBB. The BBB is a selective, semi-permeable membrane through which blood is kept apart from the extracellular fluids of the brain. A diagram can be seen in figure 8. The BBB in healthy individuals is highly selective. However, in GBM patients, the BBB is compromised and has increased permeability. One of the consequences of this effect is the influx of immune cells into the tumor microenvironment. Specifically, T_{regs} are able to infiltrate into this space in an immunocompetent intracranial injection mouse model of GBM, due to increased expression of IDO and tryptophan catabolism by the tumor. The end result is immunosilence. Recent studies have shown that macromolecules and interstitial fluid are able to circulate using the recently characterized dural lymphatic vessel. It may be through this vessel that the tumor is able to disseminate immunosuppressive molecules.(Aspelund et al., 2015)

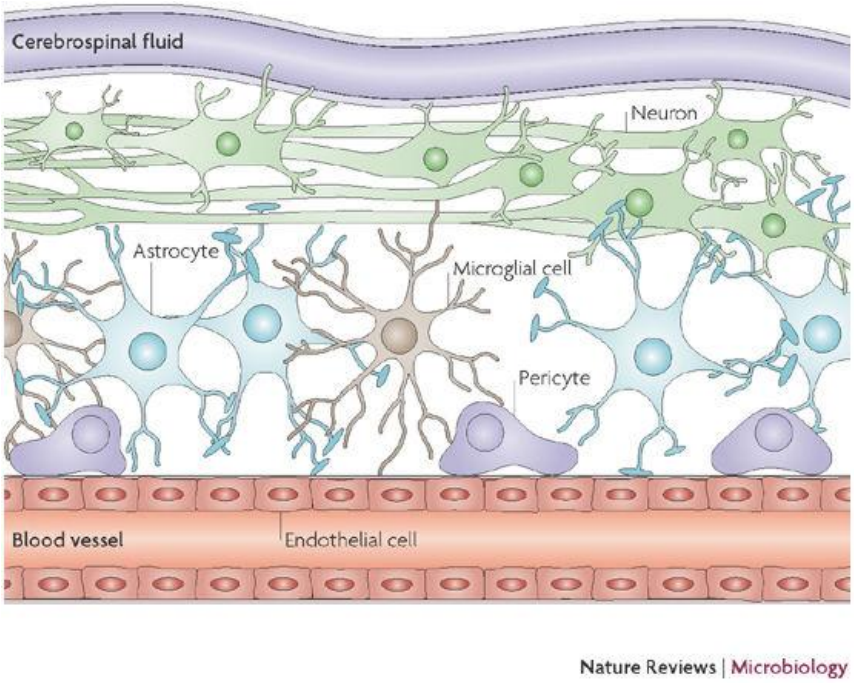


Figure 8. The blood brain barrier (Kim, 2008). Figure reproduced with permission from The Nature Publishing group

Rationale

The current standard of care for GBM is surgical resection combined with temozolomide (TMZ) treatment. TMZ is an alkylating agent that is able to cross the BBB. It induces cell death by causing damage to DNA of rapidly dividing cells, leading to apoptosis. However, drug resistance can be a problem. Drug resistance is often accompanied by demethylation of the O^6-alkylguanine DNA alkyltransferase promoter.(Morandi et al., 2010) O^6 alkylguanine DNA alkyltransferase is able to reverse DNA damage caused by alkylating agents, in this case, TMZ, by dealkylating O^6 alkylguanine through an S_N2 reaction.(Kaina et al., 2007) Furthermore, as TMZ

damages all rapidly dividing cells, it may damage white blood cells, which may inhibit the immune response against GBM.

The current standards of care for GBM are insufficient and new treatment modalities must be developed to treat this illness. As can be inferred in the presented information, developing therapeutics for GBM will require the synthesis of many different ideas.

IL-21 is able to induce the rejection of tumor xenografts in mice. It also plays a central role in affinity maturation and the development of naïve B-cells. It has also been observed that the presence of allergies in youth, is correlated with a decreased likelihood for GBM in later life.(Schoemaker et al., 2006). Allergic rhinitis is also associated with the overexpression of IgE in the serum.(Jen et al., 2015) Increased levels of serum IgE are in turn associated with increased levels of IL-21. Paradoxically, IL-21 inhibits the secretion of IgE in mice by blocking class switching towards this antibody class type in the germinal centers.(Ozaki, 2002) A possible explanation for this may be that a high level of serum IgE necessitates regulation via increased expression of IL-21. Daga et al. have shown that mice that receive IL-21 immunotherapy are capable of rejecting cellular glioma tumor implants.(Daga et al., 2007) For these reasons, we believe that the humoral immune response has a pivotal role in GBM. Additionally, it has been observed that Tregs are elevated in the blood of GBM patients, suppressing immune responses. This leads us to believe that subsets of cells that have $FoxP3^{+}$ Tregs as precursors are then also elevated. In addition, it has been found that PD-L1 expressed on neurons near the tumor bulk is beneficial in GBM patients. PD-L1 is necessary for the

development of Tfr cells. This, taken together with our assumption that the humoral immune response plays a critical role in GBM, leads us to our central hypothesis that the ratio of Tfr:Tfh cells is increased in GBM. We assume it is the ratio, rather than the total number that is affected, because it is the ratio that determines the magnitude of the humoral immune response.

This hypothesis was tested in three aims. The first aim was to demonstrate that the amount of T_{fr} cells that contributes to the total pool of $CXCR5^{+}$ cells is elevated in tumor-bearing mice. In particular, we believe that this is caused by the expansion of PD-L1. To prove this, we examined the expression of PD-L1 in the brain near the tumor site. We also blocked PD-L1 with monoclonal antibodies intraperitoneally injected into the experimental model. The second aim was to show that this upregulation of T_{fr} cells is localized near the tumor site. Specifically, we believe that T_{fr} ratios are upregulated in the dural lymphatics of tumor-bearing mice. Our final aim was to demonstrate that T_{fh} cells in GBM are defective in terms of their ability to generate the formation of plasma cells and plasma centers using CD40L.

CHAPTER II

MATERIALS AND METHODS

Reagents

DMEM/F12 cell culture medium, RPMI 1640 cell culture medium, heat inactivated fetal bovine serum (FBS), 50x B27 supplement without Vitamin A, 200mM L-glutamine, 1M HEPES buffer, ACK lysis buffer, gentamicin sulfate, 100mM sodium pyruvate, phosphate buffered saline (PBS), and LIVE/DEAD® Fixable Aqua Dead Cell Stain Kits were purchased from ThermoFisher Scientific Inc. (Waltham, MA); penicillin – streptomycin (concentration of 10,000 units/mL and 10 mg/mL, respectively), epidermal growth factor, and basic fibroblast growth factor were purchased from Peprotech (Rocky Hill, NJ); and 40μm nylon cell strainers were purchased from ThermoFisher (Waltham, MA). We purchased a Foxp3/Transcription Factor Staining Buffer Set from eBioscience (San Diego, Ca) to facilitate staining of Foxp3. Incomplete Freund's adjuvant was purchased from Sigma-Aldrich Inc. and made complete with the addition of inactivated *Mycobacterium tuberculosis* H37 Ra from BD (Franklin Lakes, NJ) at a concentration of 10 mg/mL.

Antibodies

For *in vivo* usage, we purchased *InVivo* MAb anti-mouse PD-L1 from BioXCell (West Lebanon, NH). For analysis using flow cytometry, we purchased 2 panels of antibody stains. The plasma cell panel consisted of rat anti-mouse CD19 conjugated to fluorescein isothiocyanate (FITC, clone 6D5), rat anti-mouse conjugated to phycoerythrin (PE, Clone 17A2), and rat anti-mouse CD138 conjugated to PE-CY7

(Clone 281-2), in addition to aforementioned Aqua, all purchased from Biolegend Inc. (San Diego, CA). The Tfr panel consisted of rat anti-mouse CD4 conjugated to Pacific Blue (clone GK1.5), rat anti-mouse CD19 conjugated to allophycocyanin (APC)-CY7 (clone 6D5) all purchased from Biolegend Inc. Additionally, rat anti-mouse CXCR5 conjugated to FITC (clone 614641) was purchased from R&D Biosciences (Minneapolis, MN). Hamster anti-mouse ICOS conjugated to APC (clone C3984A) was also purchased from Invitrogen (San Diego, CA). This panel was then supplemented with either rat anti-mouse neuropilin-1 conjugated to PE-CY7 (clone 3E12) or rat anti-mouse CD40L (clone MR1) both from Biolegend Inc. Chicken anti-mouse glial fibrillary acidic protein (GFAP) was purchased from Novus (Littleton, CO). Goat anti-mouse mB7-H1 (PD-L1) was purchased from R&D. Donkey anti-goat antibody conjugated to APC was purchased from Jackson ImmunoResearch (Westgrove, PA). Rabbit anti-chicken antibody conjugated to FITC was purchased from Invitrogen.

Cell Lines

The GL261 cell line was purchased from the National Institutes of Health's Department of Developmental Therapeutics Cell Repository, run under the management of Charles River Laboratory (Frederick, MD). Cells were maintained in RPMI 1640 cell culture medium supplemented with heat inactivated FBS at 10% final concentration (vol/vol %), 2mM L-glutamine, 10mM HEPES, 1mM sodium pyruvate, 100 IU/mL of penicillin and 50 milligrams/mL of streptomycin. The cells were incubated at 37°C in a water-jacketed CO2 incubator (ThermoFisher Scientific Inc., Waltham, MA) with 5% CO2 concentration and 95% room air.

Neurosphere Transformation

Neurosphere transplantation was performed as previously described.(Yi et al., 2013) Briefly, 261 cells were seeded into a solution of DMEM/12 supplemented with 10 mM HEPES, 1mM sodium pyruvate, 100 IU/mL of penicillin and 50 mg/mL of streptomycin, 20 ng/mL epidermal growth factor and 10 ng/mL basic fibroblast growth factor and an appropriate volume of B27 supplement, hereon referred to as defined stem cell medium (DSCM). Cells were seeded at a concentration of 1 x 10^4 cells/mL DSCM. The cells were incubated in a water-jacketed CO2 incubator (ThermoFisher Scientific Inc.) with 5% CO2 concentration and 95% room air. Complete transformation occurred 6 days after seeding. Cells were then collected by gentle pipetting.

Mice

Fully immunocompetent male C57/BL6 mice, aged 6-8 weeks were purchased from Jackson Laboratory Inc. (Bar Harbor, ME). All animals were housed in the Baylor Scott and White Hospital vivarium facility and all procedures were performed according to the Institutional Animal Care and Use Committee guidelines.

Neurosphere Implantation

Neurospheres were resuspended in DSCM at a concentration of 5 x 10^6 cells/mL. Cells were kept on ice when not in use. Mice were anesthetized with isofluorane and placed into a Stoelting stereotaxic apparatus (Wood Dale, IL) underneath a heat lamp. Neurospheres (5 uL) were then loaded into a Hamilton syringe (Boston, MA). A sagittal incision was made on the top of the mouse's head and bregma was located. The stereotax was then set for the caudate nucleus (AP=+.5, L=+2.0, H=3.5) and a hole was

drilled in that approximate spot. The syringe was slowly lowered to the set depth and the neurospheres were injected over the course of 15 minutes so as to be accepted into the cranium. The incision was then closed with 4-0 vicryl suture and the mice were allowed to warm before being returned to their cage. A similar procedure was performed with DSCM replacing neurospheres for control mice.

Splenocyte Isolation

Mice were euthanized using CO_2, followed by cardiac puncture and dissection of spleens from the animals. Spleens were collected, washed in PBS containing 3% newborn calf serum (NBCS, final vol/vol %) (NBCS/PBS) and passed through 40μm nylon cell strainers to make single cell suspensions. The red blood cells were lysed by incubating the cell pellet in ACK buffer for 6 minutes at room temperature (25^{o}C) in the dark and washed twice with 5 mL of 3% NBCS/PBS to remove traces of ACK buffer. The splenocytes were then resuspended in RPMI 1640 supplemented with 10% FBS mixed with freezing media (10% DMSO/FBS, vol/vol%). Splenocytes were then moved to cryovials and stored in Nalgene freezing containers containing isopropanol and kept frozen in a -80^{o} C freezer for up to 6 weeks. Frozen cells were thawed by chilling on ice and washing twice with 5 mL of 3% PBS/NBCS before being resuspended in PBS for analysis.

Brain Harvesting

Brains were harvested from mice following cardiac puncture. Brains were stored in 4% paraformaldehyde for 24 hours before being moved to a 0.1% solution of sodium azide in PBS (w/v).

Dural Isolation

The dura was collected from mice after CO_2 asphyxiation, cardiac puncture and decapitation. The procedure for collecting the dura was performed as described previously.(Louveau et al., 2015) After the dura was collected, the tissue was processed in the same manner as the splenocytes.

Cell Surface Staining and Flow Cytometry

Plasma cell subsets were evaluated by surface staining of the splenocytes with rat anti-mouse CD19 conjugated to FITC (clone 6D5), rat anti-mouse conjugated to PE (Clone 17A2), and rat anti-mouse CD138 conjugated to PE-CY7 (Clone 281-2), in addition to Aqua Live/Dead Stain. The cells were analyzed on a Becton Dickinson FACS Canto II flow cytometer (BD Biosciences Inc., San Jose, CA), consisting of a 3-laser 10-parameter system with FACS Diva software (BD Biosciences Inc., San Jose, CA). The flow data was analyzed using FlowJo® software (FlowJo, LLC, Ashland, OR)

Intracellular Staining

Intercellular staining was performed for T_{fr} and T_{fh} cells by first performing cell surface staining with rat anti-mouse CD4 conjugated to Pacific Blue (clone GK1.5), rat anti-mouse CD19 conjugated to APC-CY7 (clone 6D5), rat anti-mouse CXCR5 conjugated to FITC (clone 614641), and hamster anti-mouse ICOS conjugated to APC (clone C3984A). This panel was then supplemented with either rat anti-mouse Nrp-1 conjugated to PE-CY7 (clone 3E12) for T_{reg} origins or rat anti-mouse CD40L (clone MR1) for T_{fh} function. Intercellular staining was performed using an intercellular Foxp3 staining kit from eBioscience.

Immunohistochemistry

Fixed brains were sliced at a thickness of 25-30 μM on a Leica VT1000 vibratome (Wetzlar, DE). Tissue slices were then mounted on microscope slides from ThermoFisher. Tissue was permeabilized by incubation in 0.25% Triton X100 in PBS for 20 min. The tissue was then washed 3 times with 0.1% Triton X100 for 10 minutes each. The tissue was blocked with 2.5% goat serum in PBS for one hour and then incubated with the chicken anti-mouse GFAP at a dilution of 1:2500 and goat anti-mouse mB7-H1 at a concentration of 10 μg/mL overnight at 4° C. The tissues were then washed with PBS at least 5 times before being incubated in donkey anti-goat antibody conjugated to APC and rabbit anti-chicken antibody conjugated to FITC, both at dilutions of 1:500 in 2.5% goat serum in PBS. Tissues were then imaged on an Olympus IX-71 confocal microscope.

Statistical Analysis

Data obtained from FlowJo was analyzed on Graphpad Prism 6.0. All comparisons were performed using student's *t* test. Significance is as follows:

* $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$

CHAPTER III

RESULTS

Basal Levels of T Follicular Regulatory Cells are Elevated in Tumor-bearing Mice

We first examined levels of immune regulation in both tumor-bearing and control mice prior to immunization. We examined this because although patients with GBM have been shown to have various immune impairments, their humoral immune systems are not necessarily always activated.(Fornara et al., 2015) To do this, we injected two sets of fully immunocompetent C57/BL6 mice, one set (n = 3) that had sham surgery in which mice received intracranial injections of 5 µL of DSCM and one set (n=5) that received intracranial injections of 261 neurospheres (5 µL containing 2.5 x 10^4 cells) in DSCM. The neurosphere technique was used due to its high rate of tumor acceptance in mice.(Yi et al., 2013) Mice were then sacrificed 28 days after injections. Spleens and inguinal lymph nodes were harvested and stained with antibodies for CD4, CD19, CXCR5, ICOS, FOXP3 and a live/dead stain. We harvested these tissues as they are sites of immune activity. These immune cells were then examined for differences in T_{fr} and T_{fh} cells using flow cytometry. The gating strategies used in this dissertation can be seen in figure 9. Figure 9A shows the gating strategy for T_{fh} cells, 9B shows the strategy for T_{fr} cells and 9C shows the strategy for the percentage of T_{fr} cells that comprise the total pool of $CXCR5^+$ cells. We utilized this strategy because it has been determined that this is what dictates the magnitude of the immune response. Although an immune response might cause an increase in T_{fh} cells, if there is also a corresponding increase in T_{fr} cells, the level of the humoral immune response in regards to cytokines and antigens

may be similar, thus proving the importance of this ratio.(Sage et al., 2012)

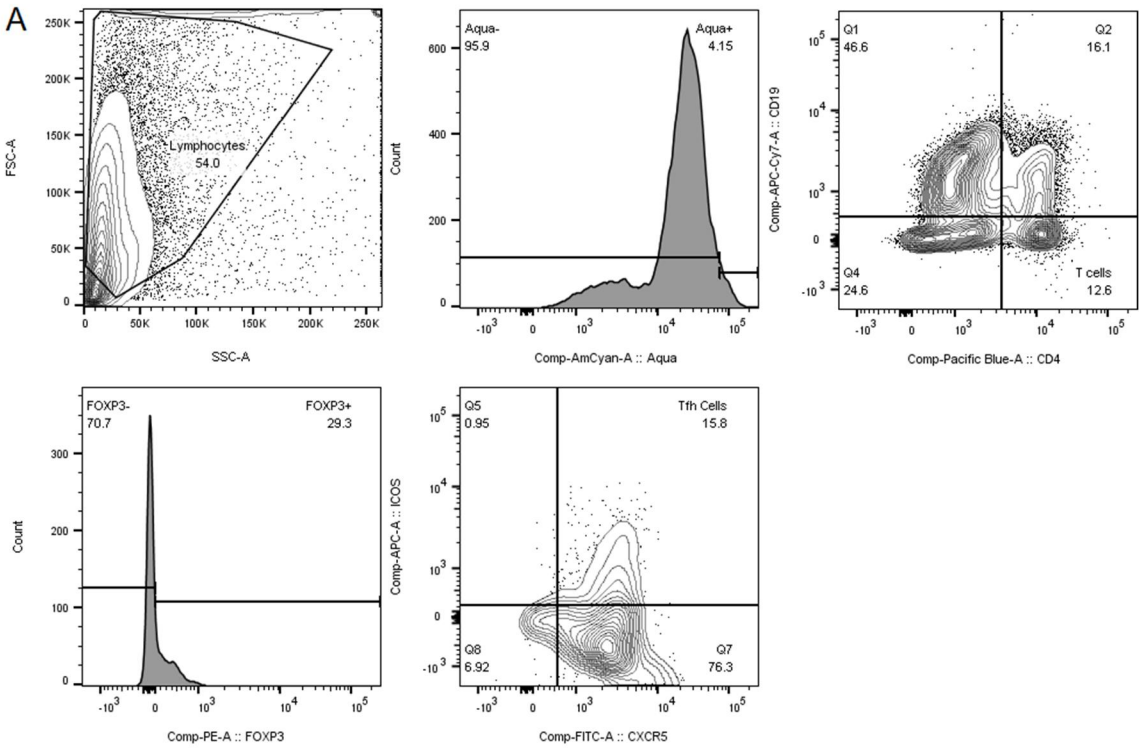
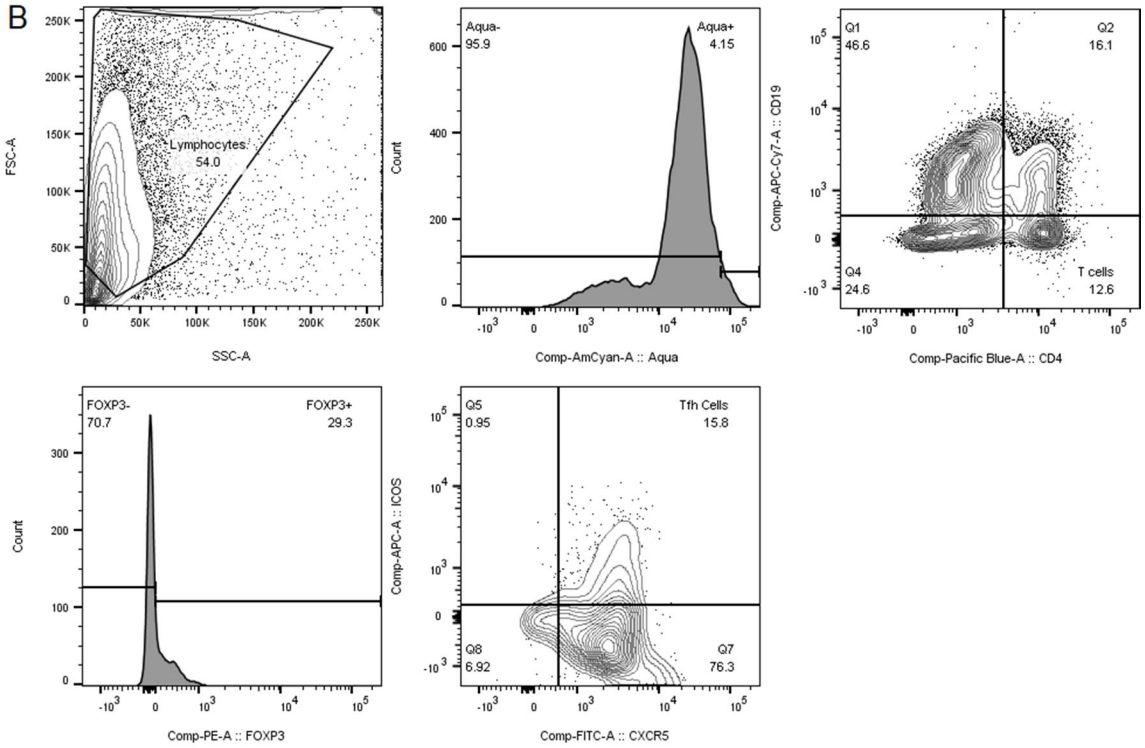


Figure 9. Gating strategies for T_{fh} and T_{fr} subsets. (A) T_{fh} cell gating strategy. Lymphocytes are gated on forward scatter (FSC) vs. side scatter (SSC). Following this, $Aqua^-$ live cells are chosen. $CD4^+CD19^-$ T cells are then gated followed by gating of $Foxp3^-$ cells. Finally, $ICOS^+CXCR5^+$ T_{fh} cells are gated. (B) T_{fr} cell gating strategy. Lymphocytes are gated on FSC vs SSC. Following this, $Aqua^-$ live cells are chosen. $CD4^+CD19^-$ T cells are then gated followed by taing of $Foxp3^+$ cells. Finally, $ICOS^+CXCR5^+$ T_{fh} cells are gated. (C) Gating strategy for determining the percentage of T_{fr} cells that contribute to the total pool of $CXCR5^+$ cells. Lymphocytes are gated on FSC vs SSC. Following this, $Aqua^-$ live cells are chosen. $CD4^+CD19^-$ T cells are then gated followed by gating of $CXCR5^+$ cells. Finally, $ICOS^+Foxp3^+$ T_{fr} cells are gated.



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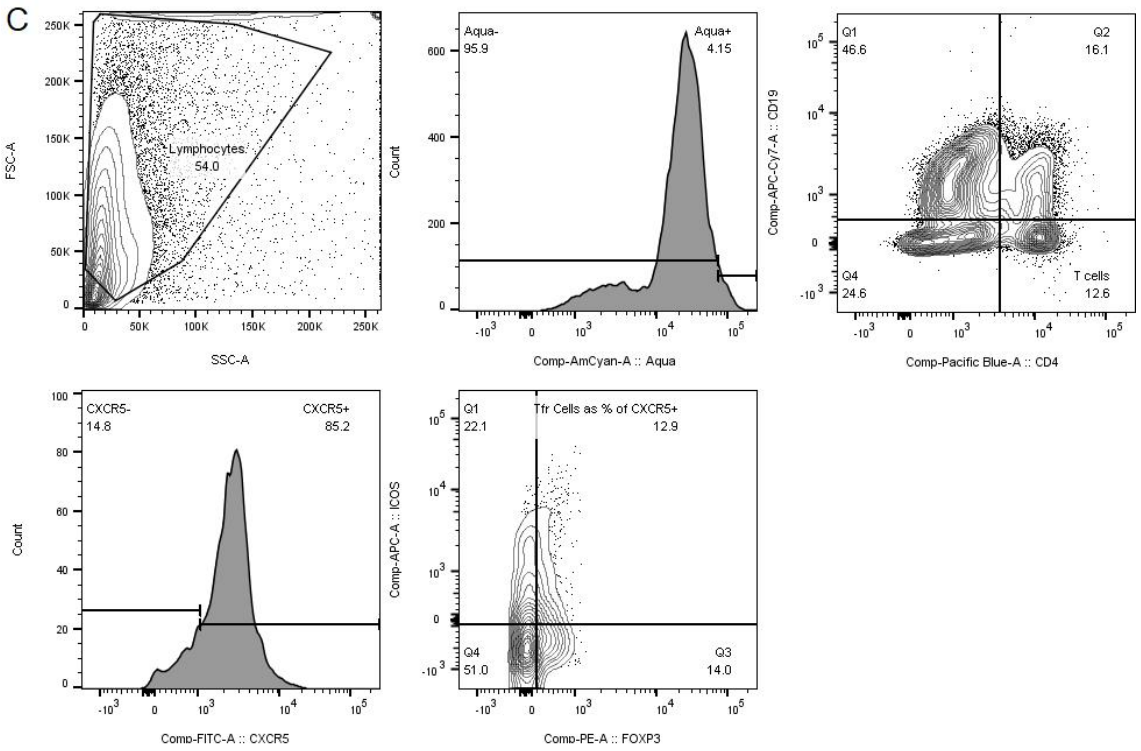

Figure 9. Continued.

As can be seen in figure 10A, there was no significant difference in the total number of T_{fh} cells in the spleens of either set of mice. However, according to figure 10B, there is a significant increase in the total amount of T_{fr} cells in the spleens of tumor-bearing mice. This translates into a significantly increased amount of T_{fr} cells contributing to the total share of $CXCR5^+$ cells. Again, although we looked at both the number of T_{fh} cells that compose the $CD4^+Foxp3^-$ subset and the number of T_{fr} cells that comprise $CD4^+Foxp3^+$ subset, we emphasized the percentage of T_{fr} cells that comprise the $CXCR5^+$ subset of cells as it is describes the relationship between the T_{fr} and T_{fh} cells since both of these subsets share the unique CXCR5 marker, and it is this relationship

that describes the degree of the humoral immune response. Similar results can be found in the inguinal lymph nodes of tumor bearing mice (figure 10 D-F).

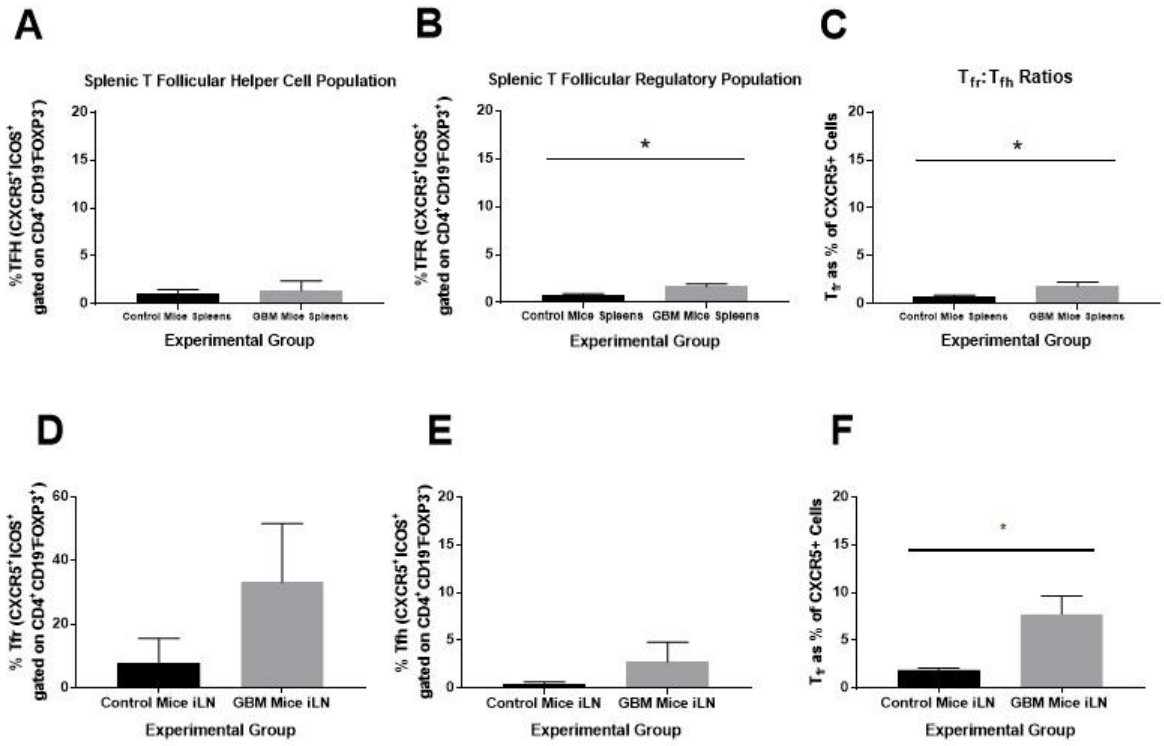

Figure 10. Basal levels of T follicular regulatory cells are elevated in the spleens of GBM-bearing mice. (A) Comparison of Tfh cells in the spleen of non-immunized control and tumor-bearing mice (B) Comparison of Tfr cells in the spleens of unimmunized control and tumor-bearing mice (C) Comparison of Tfr cell contribution to total CXCR5+ cells (Tfr:Tfh ratios) in the spleens of control and tumor-bearing mice (D) Comparison of Tfh cells in the inguinal lymph node (iLN) of unimmunized control and tumor bearing mice (B) Comparison of Tfr cells in the iLN of unimmunized control and tumor bearing mice (E) Comparison of Tfr cells to the contribution of total CXCR5+ cells (Tfr:Tfh ratios) in the iLN of control and tumor bearing mice. N = 5 GBM mice, N = 3 control mice.

As we saw that both the amount of T_{fr} cells and the percentage that the T_{fr} cells contribute to the total amount of $CXCR5^{+}$ cells is elevated prior to immunization in mice, we can assume that before there is any humoral insult, the immune systems of tumor-bearing mice are impaired at the spleens and inguinal lymph nodes when compared with healthy control mice.

T Follicular Regulatory Cells are Elevated in the Dura When Stimulated with Complete Freund's Adjuvant in Tumor-bearing Mice

We next examined the differences in the humoral immune responses in tumor-bearing mice as compared to healthy control mice after both the control and experimental groups were subjected to humoral insult. We deemed this necessary as it is traditionally during the humoral immune response that one would expect to find elevated levels of T_{fh} cells. Prior to immunization, there is no antigen that triggers the immune response. The sham surgery group (n=10) received an intracranial injection of DSCM and the experimental group (n=10) received an intracranial injection of GL261 neurospheres. To stimulate a humoral immune response, 100 uL of Complete Freund's Adjuvant (CFA) was subcutaneously injected into the middle dorsal skin of mice 14 days after intracranial injection. This schedule was chosen to maximize the impact of the humoral immune response while still allowing the tumor to establish itself. Previous studies as well as our own previous research have shown that injection of CFA may be capable of increasing the likelihood of rejection of some tumor cells. Thus, it is necessary to allow the tumor stem cells to differentiate and take hold before stimulating the immune response. As CFA releases a slow and constant stream of inactive

tuberculosis antigen into the mouse, the mouse then develops a sustained humoral immune reaction against the antigen. We decided to examine the dura as it is the location of immune activity closest to the tumor site. Furthermore, the dura is not a well characterized site in GBM, due to its status as recently discovered. We isolated the dura as previously described and processed the tissue in the same manner as spleens and lymph nodes.(Louveau et al., 2015) The resulting single cell suspension was then stained with antibodies for CD4, CD19, CXCR5, ICOS, FOXP3 and Nrp-1 as well as Aqua for viability.

As can be seen in figure 11B, there is an increase in T_{fr} cells in the dura of tumor-bearing mice. This appears to be caused by the upregulation of the surface marker Inducible T-cell Co-Stimulator (ICOS), as can be seen in the representative histogram, figure 12. As ICOS is a ligand that is important in maintaining T_{fh} cell phenotypes and as it is correlated with antigen specificity in T_{regs}, we decided to compare its expression in T_{fh} and T_{fr} cells in the two groups. To further investigate this, we examined the mean fluorescence intensity (MFI) of ICOS in both T_{fr} and T_{fh} cells (figure 13). We found that although there is no appreciable difference in MFI of ICOS in T_{fh} cells, there is a marked increase in the expression of ICOS in T_{fr} cells indicating that this protein is upregulated in this subset of cells.

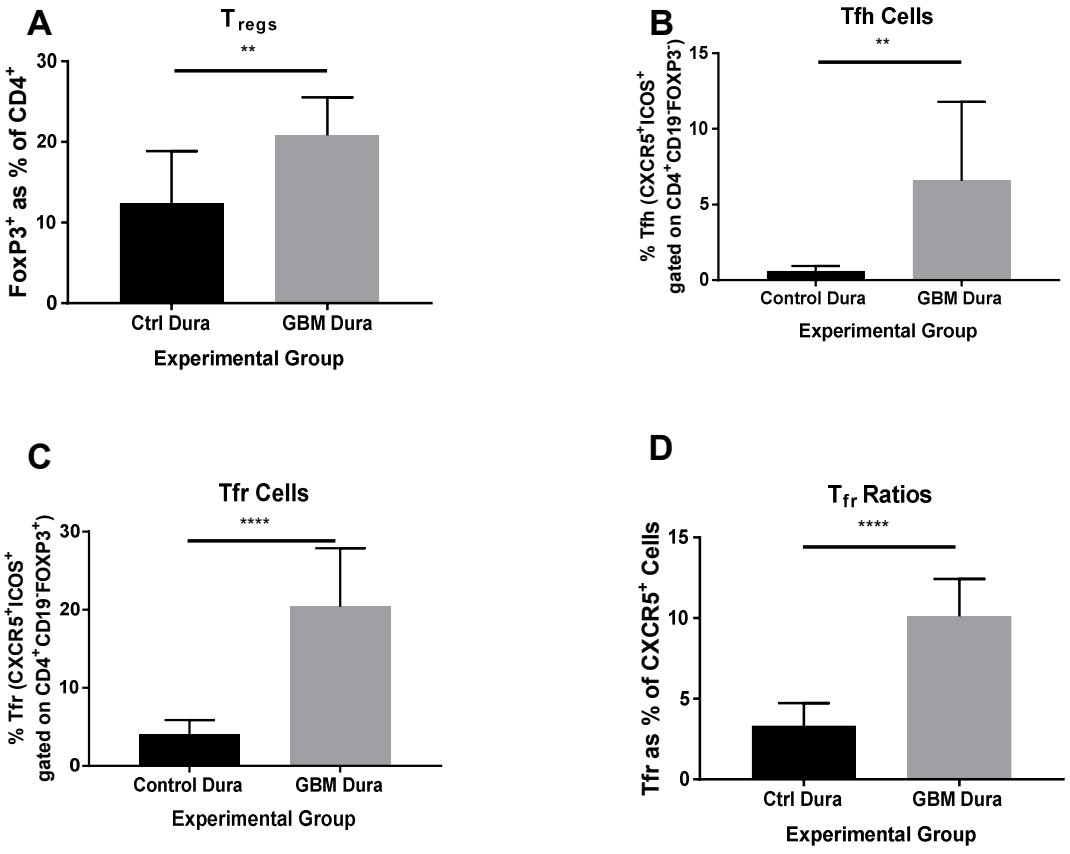

Figure 11. The ratio of Tfr cells is upregulated in the dura of GBM-bearing mice. (A) Comparison of $CD4^{+}Foxp3^{+}$ cells in control and tumor-bearing mice. (B) Comparison of Tfh cells between control and tumor-bearing mice, 14 days after immunization. (C) Comparison of Tfr cells between control and tumor-bearing mice, 14 days after immunization. (D) Comparison of the Tfr contribution to the pool of CXCR5+ cells (Tfr:Tfh ratio). (n = 10 animals for all groups)

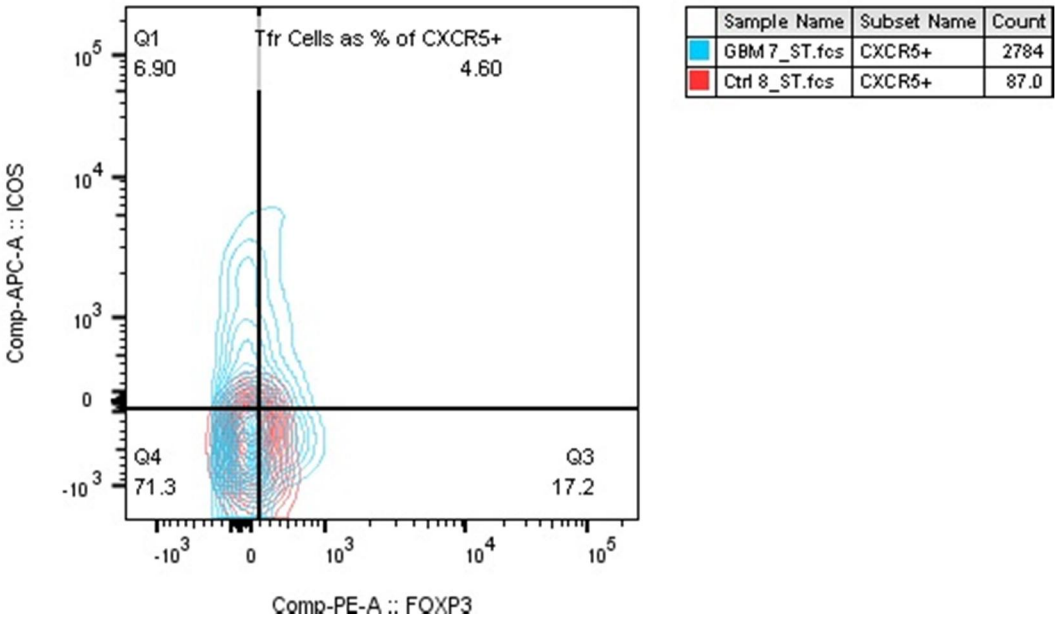

Figure 12. Representative histogram of the contribution of T_{fr} to the total pool of $CXCR5^+$ cells. Control CFA mouse is in red, GBM CFA mouse is in blue.

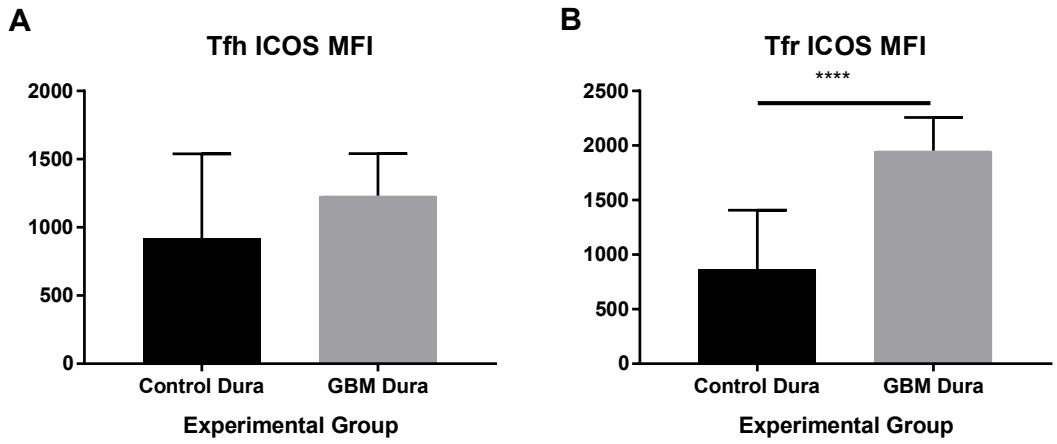

Figure 13. ICOS is significantly increased on T_{fr} cells in tumor-bearing mice. (A) MFI of ICOS on T_{fh} cells. (B) MFI of ICOS on T_{fr} cells. (n = 10 animals for all groups)

T_{fr} Upregulation is Not Observed in the Spleen

After we examined the dura, we investigated similar events in the spleen, as it is the largest secondary lymphoid organ and thus a major site from which immune cells are trafficked. Investigation of this site provides information on the status of the immune system on a global scale. We harvested and processed the spleens from the same mice from which we harvested the dura and stained them with the same antibodies. While we did see decreased amounts of T_{fh} and T_{fr} cells in the spleens of tumor-bearing mice, this ultimately translated into no significant difference between tumor-bearing and control mice in terms of T_{fr} as a percentage of $CXCR5^+$ cells (figure 14).

Splenic T_{fh} Cells in Tumor-Bearing Mice Express Less CD40L

After concluding that the T_{fr} ratios are upregulated in the dura, we sought to find other ways in which the humoral immune response is dysregulated. We hypothesized that there may be deficiencies not just in the relationship between the T_{fh} cells and T_{fr} cells, but also, possibly, in the T_{fh} cells themselves. To this end, we decided to examine the expression of CD40L on splenic T_{fh} cells, a ligand crucial to the survival of B cells. It is one of the important roles that T_{fh} cells play during the humoral response to antigen. We used this surface marker as a metric for the proficiency of the T_{fh} subset of cells. Control and experimental mice were treated with CFA as previously described. Spleens were then stained with antibodies for CD4, CD19, CXCR5, ICOS, FOXP3 and CD40L as well as Aqua for viability. Figure 15A shows that in GBM mice there are less $CD40L^+$ T_{fh} cells than there are in mice that received injections of DSCM. Additionally,

each T_{fh} cell expresses decreased amounts of CD40L (figure 15B) as indicated by the MFI.

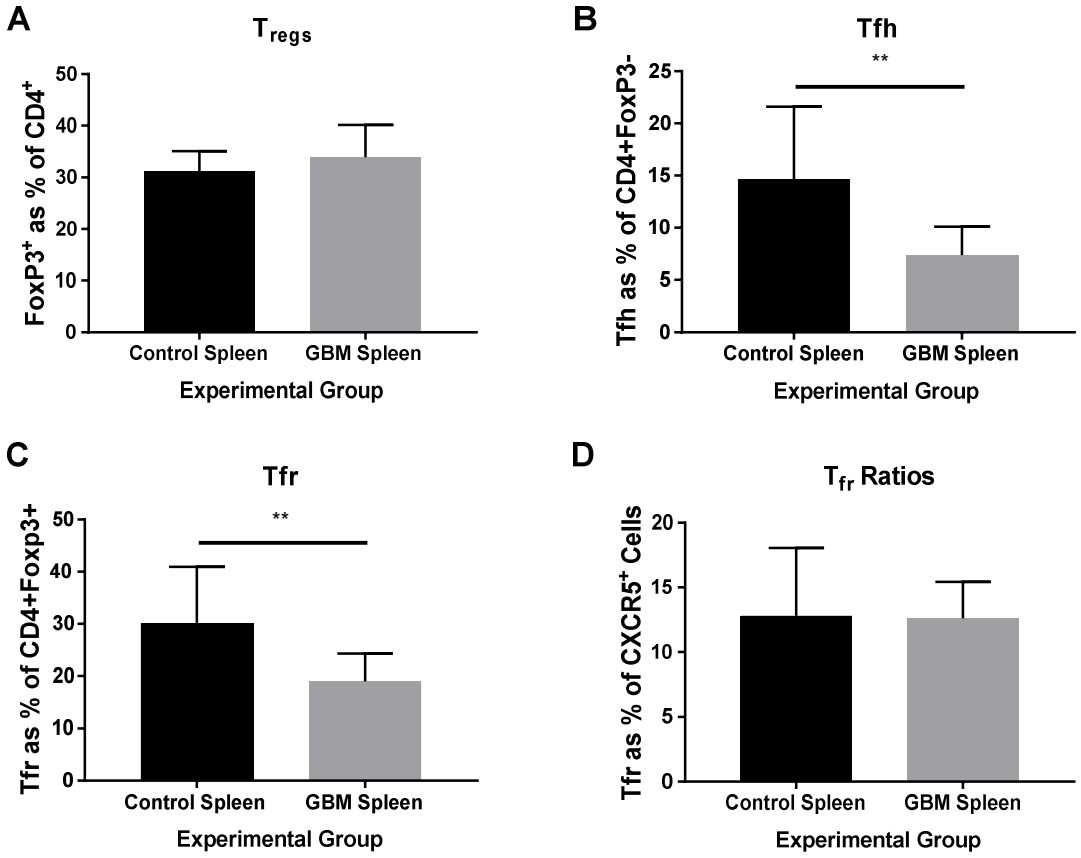

Figure 14. The ratio of Tfr cells is unaltered in the spleens of GBM-bearing mice. (A) Comparison of $CD4^{+}Foxp3^{+}$ levels between control and tumor-bearing mice. (B) Comparison of Tfh cells between control and tumor-bearing mice, 14 days after immunization. (C) Comparison of Tfr cells between control and tumor-bearing mice, 14 days after immunization. (D) Comparison of Tfr contribution to the pool of CXCR5+ cells (Tfr:Tfh ratio). (n = 10 animals for all groups)

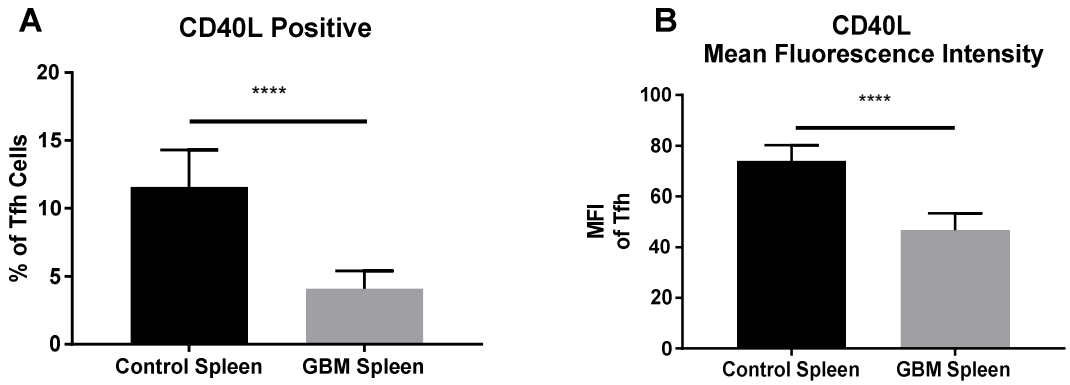

Figure 15. CD40L expression is impaired in tumor-bearing mouse splenic T_{fh} cells. (A) The percentage of $CD40L^+$ T_{fh} cells is decreased in GBM mice. (B) Average CD40L expression in tumor-bearing mouse splenic T_{fh} cells is decreased. (n = 10 animals for all groups)

Plasma Cell Generation Is Inhibited in Tumor-Bearing Mice

Following the examinations of T_{fr}:T_{fh} ratios and CD40L on T_{fh} cells, we determined that the downstream effector functions of T_{fh} cells may also be impaired. Because one of the end results of the humoral immune response to antigen is the production of antibodies to opsonize pathogen, we decided to examine the differences between tumor-bearing and control mice in terms of the cell types that generate antibodies, plasma cells and their precursors, plasmablasts. To this end, we processed the spleens of tumor-bearing and control mice and stained the resulting single cell suspension with antibodies for CD19, CD138 and Aqua for viability. CD138 is a positive marker for plasma cells. As GC B cells develop into plasmablasts, they acquire expression of CD138 and, as they develop into plasma B cells, expression of CD19 is lost. Live plasma cells were considered $CD19^-CD138^+$ and were examined in the live

gate as they are long-lived. Plasmablasts were considered $CD19^{+}CD138^{+}$ and were examined in the all-cells gate as they are very short-lived. The results can be seen in figure 16. There is a decreased number of both live plasma cells and total plasmablasts in the tumor-bearing group as compared to the group that received intracranial injections of DSCM.

T_{fr} Cells Are Extrathymic in Origin

We next examined the origin of the T_{fr} cells, in regards to whether they were peripheral or thymic, as this impacts the corresponding function of the T_{reg} as well as its relation to PD-L1. Chen et al. have described the PD-1:PD-L1 interaction as 'critical' for extrathymic pT_{reg} differentiation and 'dispensable' for natural thymic tT_{regs}. As their developmental origins affect a T_{reg}'s response to immunotherapy, this was deemed a necessary parameter to examine. To do this, we screened T_{reg} cells in the dura and the spleen for Nrp-1, a marker of the T_{reg} lineage. T_{regs} that were Nrp-1^{high} were considered to be natural tT_{regs}, whereas those that were Nrp-1^{low} were considered to be pT_{regs}. As can be seen in figure 17B, the bulk of T_{regs} in the dura of tumor-bearing mice are Nrp-1^{-}. This indicates that the bulk of these T_{regs} are peripheral, rather than thymic, in origin. This characteristic can also be seen in the spleens of tumor-bearing mice (figure 17A), but appears to be less pronounced.

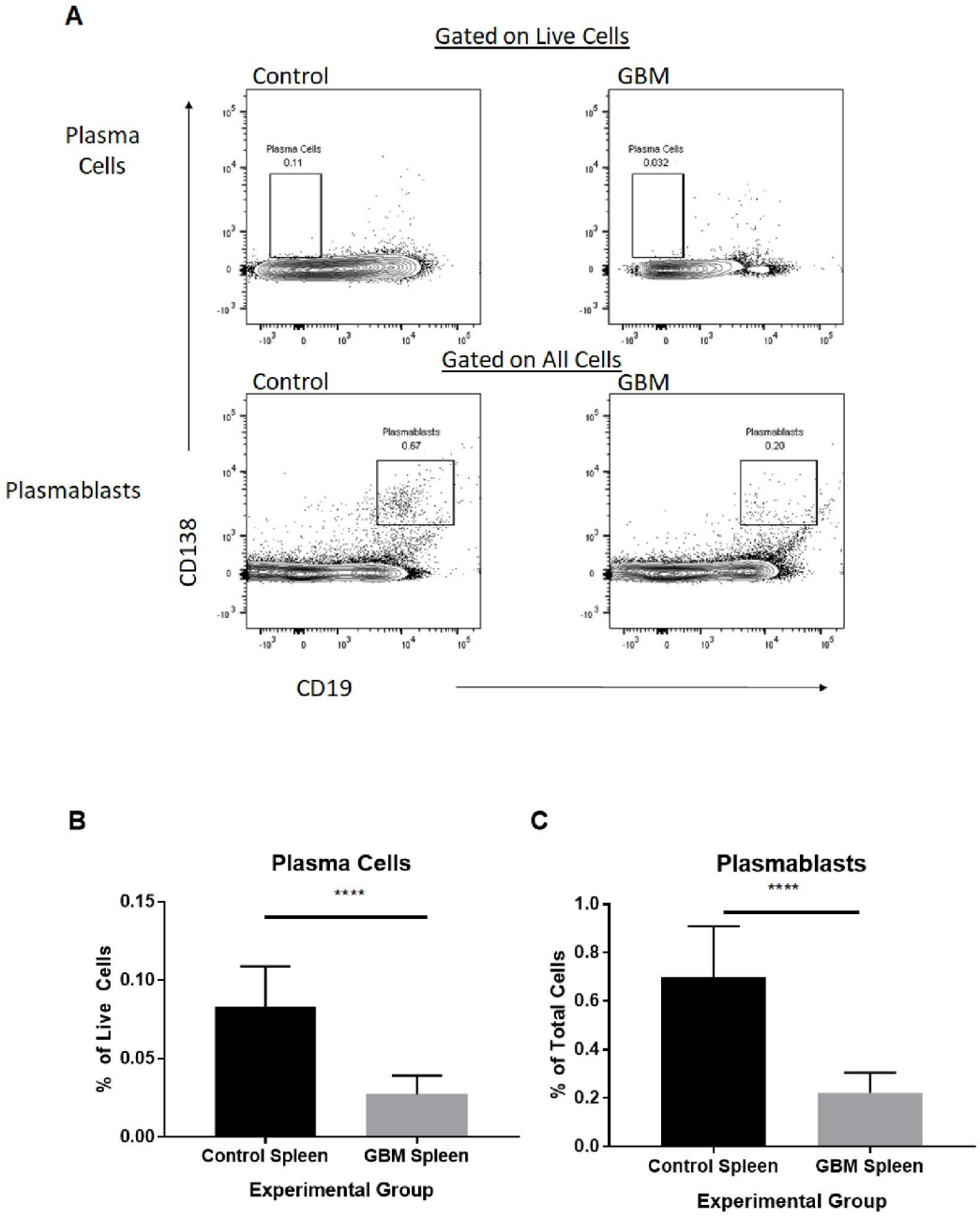

Figure 16. Generation of plasmablasts and long-lived plasma cells is inhibited in the spleens of tumor-bearing mice. (A) Representative contour plots of plasmablast and live cell gating. Increases in both live plasma cells and total plasmablasts can be seen in the control plots. (B) Live plasma cells presented as percentages of total live cells. (C) Plasmablasts presented as percentages of total cells. (n = 10 animals for all groups)

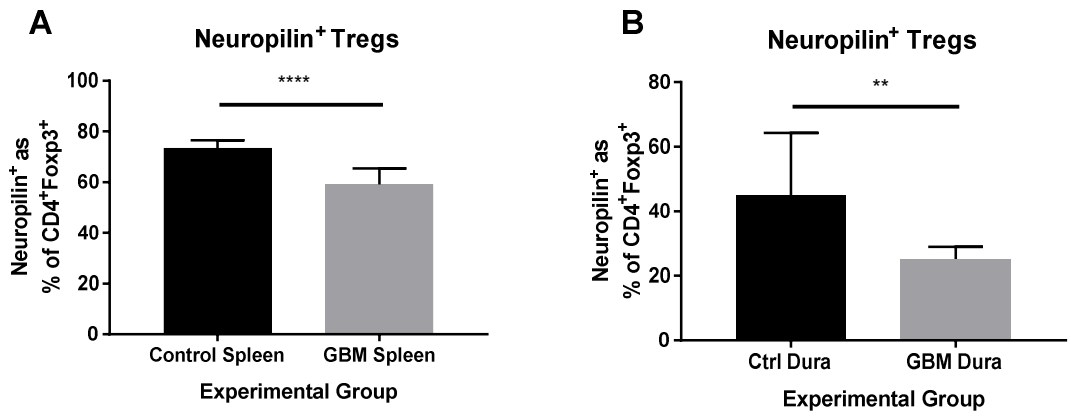

Figure 17. T_{regs} in tumor-bearing mice under inflammatory conditions are predominantly extrathymic in nature. (A) Neuropilin-1^+ T_{regs} are downregulated in the spleens of tumor-bearing mice. (B) Neuropilin-1^+ T_{regs} are downregulated in the dura of tumor-bearing mice. (n = 10 animals for all groups)

PD-L1 is Increased in Neurons Near the Tumor Bulk

As we have suggested several ways in which the humoral immune response is impaired, we attempted to elucidate a pathway through which this impairment is mediated. We chose the PD-L1:PD-1 axis as our research had shown that the T_{fr} cells that are elevated are of extrathymic origin.(Chen et al., 2014) Blockade of PD-L1 has been shown to cause regression of melanoma if MHCII is expressed by the tumor.(Johnson et al., 2016) However, prior research found that PD-L1 expression on neurons close to the tumor bulk is beneficial to the survival of patients with GBM, though the mechanism through which this effect is mediated is not immune related.(Liu et al., 2013) In light of these facts, we examined the expression of PD-L1 in neurons close to the tumor using immunohistochemistry.

As the tumor was visible without any microscopy, obtaining the tumor and brain tissue in the same slice did not require any extra staining. Slices of 30 microns or less were used for immunohistochemistry. Tissue was stained for GFAP in glial cells using FITC-conjugated antibody and PD-L1 using APC-conjugated antibody. We observed (figure 18) that there was an increased number of neurons expressing PD-L1 in tumor-bearing mice compared to control mice. Expression of PD-L1 diminished as distance from the tumor increased. When anti-PD-L1 mAb was injected intraperitoneally into tumor-bearing mice, PD-L1 fluorescence near glial cells diminished. The precise mechanism as to how PD-L1 fluorescence in the brain is diminished is unclear. One possible explanation is that the injected mAb is able to attach to PD-L1 and block the fluorescent antibody from binding. Another possible mechanism is that the injection of the mAb induces an immune reaction that leads to a global decrease in the expression of PD-L1. Nonetheless, PD-L1 expression in the brain was inhibited. This serves as proof that the intraperitoneal route of administration for the antibody is appropriate to block PD-L1 in the microenvironment.

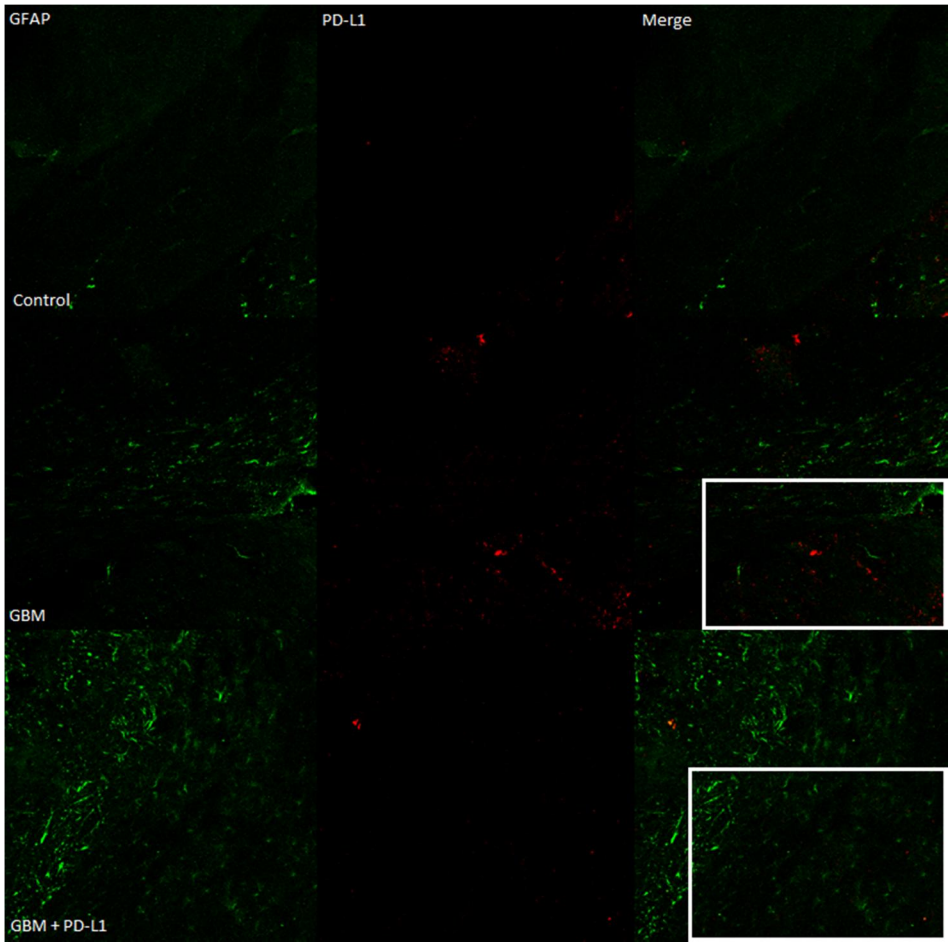

Figure 18. There is increased PD-L1 expression in tumor-bearing mice. The first column is GFAP fluorescence. The middle column is PD-L1 fluorescence. The third column contains the two images merged. The first row is control cells. The second role is GBM mice that have not received i.p. injections of PD-L1 mAb. The final row is GBM mice that have received both GBM injections and i.p. injections of PD-L1 mAb.

PD-L1 Blockade Leads to a Decrease in T_{regs} and T_{fr}:T_{fh} Ratios

To demonstrate the role of the PD-L1:PD-1 axis in the impairment of the humoral immune response, to further prove the extrathymic lineage of Tfr cells and to demonstrate inhibition of PD-L1 as a potential pathway for therapeutic benefit, we decided to compare the humoral immune response of tumor-bearing mice to CFA with the humoral immune response of control mice to CFA. To accomplish this, we administered intraperitoneal injections of PD-L1 mAb to tumor-bearing mice 14, 17, 19, and 21 days post intracranial injections. CFA was administered to both sets of mice 14 days post intracranial injections. The initial dose was a 500 μg loading dose, followed by three 200 μg maintenance doses of the antibody. We chose this late injection schedule to allow the tumor to establish itself and to allow the immune system to react accordingly. We decided that this timeline is necessary for this mouse model to accurately recapitulate the disease pathology.

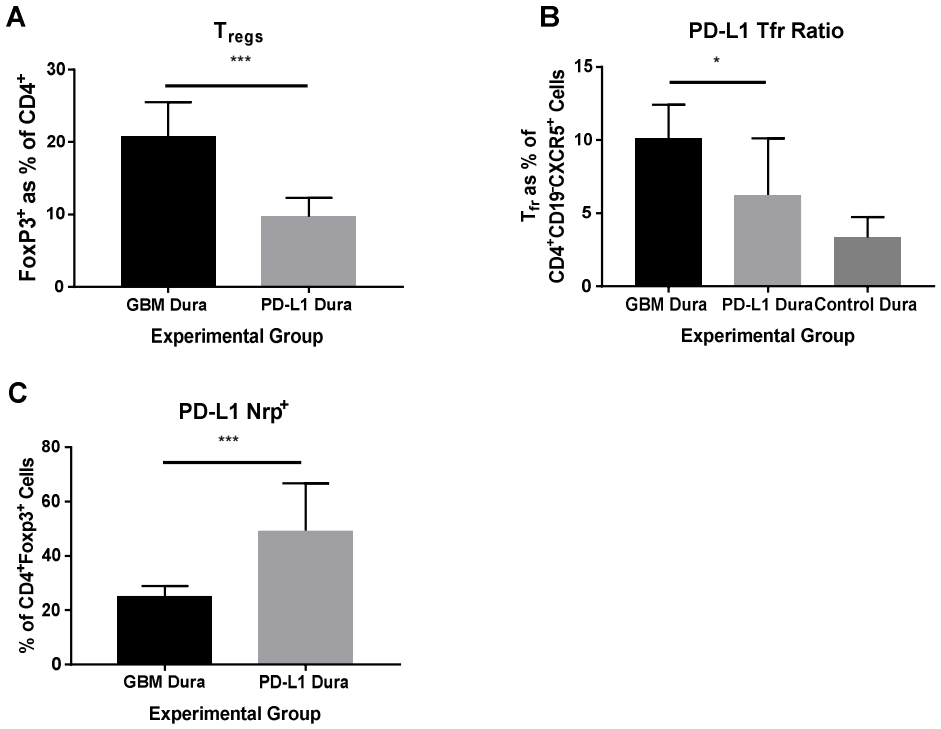

Figure 19. PD-L1 blockade prevents the expansion of Foxp3$^+$ T_{regs} and T_{fr} cells in the dura. (A) PD-L1 treatment leads to a decreased amount of Foxp3$^+$ T_{regs} when compared with control tumor-bearing mice. (B) PD-L1 treatment decreases the amount of T_{fr} cells that contribute to the total pool of CXCR5$^+$ cells. (C) PD-L1 treatment causes a switch to thymic neurons in the dural meninges under inflammatory conditions. (n =10 animals for control GBM, n = 5 animals for PD-L1 treatment)

We found that when we compared the treatment (PD-L1, CFA and tumor) to the control group (saline, CFA and tumor), the amount of FoxP3$^+$ cells decreased in treated mice (figure 19A). When we blocked the ligand, the transcription factor responsible for tT_{reg} differentiation additionally decreased. Furthermore, as we noted in figure 17, the majority of T_{regs} in GBM are thymic in nature. Due to the nature of their pT_{reg} origin, the T_{fr} cells were also prevented from expanding when treated with PD-L1 mAb (figure 19A, 19C). Accordingly, the proportion of T_{fr} cells that comprise CXCR5$^+$ cells was

significantly decreased when animals were treated with PD-L1 mAb (figure 19B). This leads us to believe that expression of PD-L1 by the tumor, as seen in figure 18, is at least in part responsible for the expansion of T_{regs} and T_{fr} cells in the glioblastoma disease state. In addition, we further believe that application of a PD-L1 inhibitor is responsible for a decrease in T_{fr} cells and, consequently, a decrease in the proportion of these cells that comprise all $CXCR5^{+}$ cells.

PD-L1 Blockade Rescues Plasmablast Generation and Live Plasma Cell Populations

As we had shown that anti-PD-L1 immunotherapy prevents expansion of T_{fr} cells in tumor-bearing during the humoral immune response to CFA, we sought to prove the downstream effects of this expansion are also prevented by anti-PD-L1 mAb treatment. To complete our study, we examined the generation of plasmablasts and live plasma cell populations in the spleens of tumor-bearing mice that had received PD-L1 mAb immunotherapy and were immunized with CFA. These mice were compared with mice that had received both intracranial injections of tumor and subcutaneous injections of CFA but not the injections of PD-L1 mAb. We examined live plasma cells and total plasmablast counts (figure 20). Figure 20A shows that there are more live plasma cells in the spleens of the PD-L1 antibody-treatment group than there are in the spleens of the control group. Figure 20B shows that there are plasmablasts in the PD-L1 treatment group make up a higher percentage of the total splenic population than do the plasmablasts in the control group.

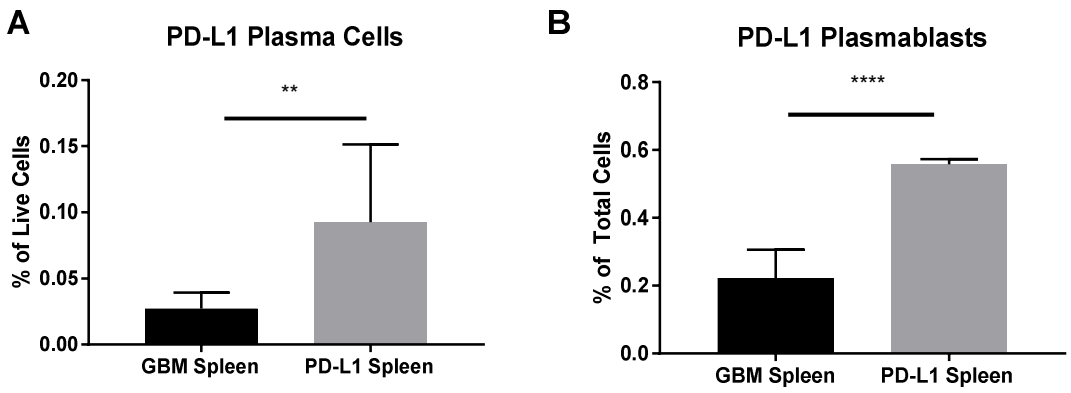

Figure 20. Live plasma cells and plasmablast generation are rescued by administration of PD-L1 mAb. (A) Live plasma cells comprise a larger portion of total live cells in the spleens of PD-L1 antibody-treated mice than they do in the spleens of control GBM mice. (B) Plasmablasts comprise a larger portion of total cells in the spleens of PD-L1 antibody-treated mice than they do in the spleens of control GBM mice. (n=5 animals)

PD-L1 Blockade Confers Survival Advantage When Combined with CFA

Although there were no initial plans to examine the role that anti-PD-L1 treatment played in survival times for tumor-bearing mice, the reduction of T_{fr} ratios and expansion of plasmablasts resulted in an increased survival time for PD-L1 antibody-treated mice (figure 21). As can be seen in the survival curve, PD-L1 antibody-treated mice survived a median of 54 days, as compared to non-treated GBM mice, which survived a median of 45 days. In one of the mice from the PD-L1 treatment group, remission was seen and the mouse was sacrificed at 100 days, when the protocol for the study ended. Unfortunately, in this particular study neurospheres were improperly prepared. The DSCM prepared had insufficient L-glutamine to cause transformation.

The mice used in this experiment did not display symptoms of glioma until roughly 30-40 days after intracranial injection. Symptoms of glioma using properly prepared neurospheres are exhibited in mice 15-21 days after injection. However, as both mice received the same group of prepared cells, comparisons can be made between the two groups. There is no significant difference between the two groups, though there does seem to be a slight advantage when treating mice with PD-L1.

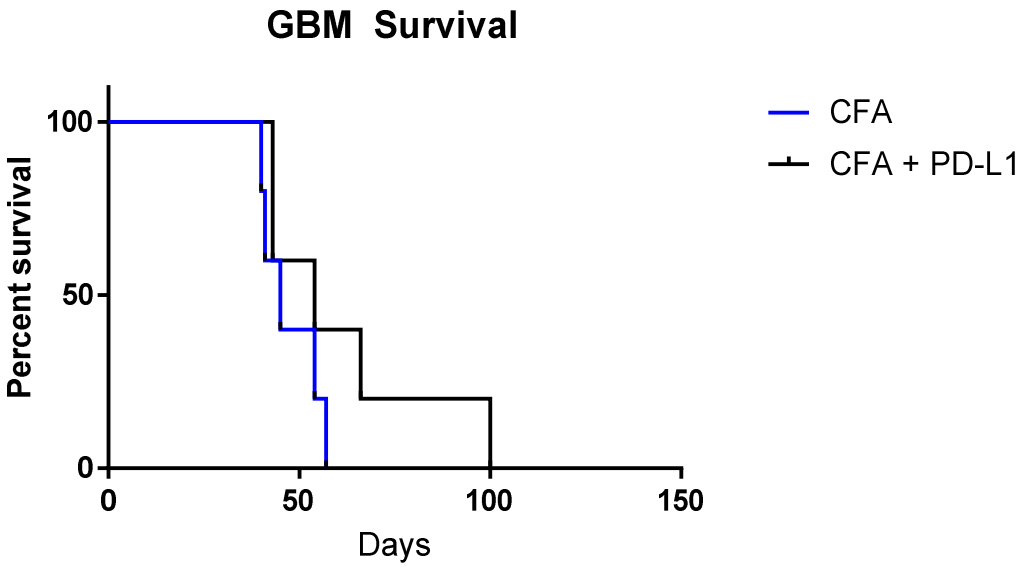

Figure 21. Survival curve for mice treated with intracranially-injected, incompletely transformed 261 neurospheres. (n = 5 animals for each group. p = 0.29)

CHAPTER IV

CONCLUSIONS

GBM Mice Have an Increased Number of T_{fr} Cells Before Stimulation with CFA

While it has been shown that prior to immunization with CFA, the immune system of experimental GBM mice does in fact harbor an increased number of T_{regs} compared to normal mice, it has yet to be shown that a similar increase in T_{fr} cells can be seen. In figure 10**,** we demonstrate that, before CFA challenge, the number of T_{fr} cells in the spleens and inguinal lymph nodes of tumor-bearing mice is elevated when compared with control mice. More importantly, the percentage of T_{fr} cells that contribute to the total amount of $CXCR5^{+}$ cells is elevated (figure 10C, 10F). This percentage is important as it dictates the magnitude of the immune reaction. We believe this percentage to partially be a byproduct of an increased number of total $Foxp3^{+}$ T_{regs}. Another possibility may be that the tumor is able to disseminate cancer antigens that cause minor immune reactions in parts of the body distant from the tumor site. To tease out these possible confounding variables, we challenged both the control and tumor-bearing groups with CFA, a TLR9 agonist, to stimulate an immune response.

The Dural Meninges Harbors an Elevated Percentage of T_{fh} and T_{fr} Cells

The current literature supports the notion that elderly mice have higher concentrations of T_{regs} than do healthy young mice.(Jagger et al., 2014) Recent work has shown that without treatment with CFA, GBM-bearing mice also have higher levels of T_{regs} than do healthy mice.(Wainwright et al., 2013) In the referenced study, the investigators examined the draining cervical lymph nodes of mice for evidence of

increased T_{regs}, which they found. Other researchers have found that preventing the development of T_{regs} leads to a prolongation in survival time for tumor-bearing mice.(Andaloussi et al., 2006) To date, the dura remains a place that has yet to be examined for the $Foxp3^+$ T_{regs} and T_{fr} cells. As the dura is a location for immune activity with increased proximity to the brain as compared to the cervical lymph nodes, we deemed it necessary to examine this tissue for evidence of immunosuppression.

We started by examining the levels of both T_{fr} and T_{fh} cells in control mice and GBM tumor-bearing mice, both of which had been subjected to injections of CFA. We considered T_{fr} cells as $CD4^+CD19^-CXCR5^+ICOS^+FOXP3^+$ and T_{fh} cells as $CD4^+CD19^-CXCR5^+ICOS^+FOXP3^-$. As can be seen in figure 11B and 11C, the number of T_{fr} and T_{fh} cells is upregulated in the dural meninges of tumor-bearing mice. There is a roughly five-fold increase in the populations of T_{fr} and a roughly tenfold increase in the population of T_{fh} cells in tumor-bearing mice. This effect appears to be driven by an upregulation in ICOS expression on T_{fr} cells in the tumor-bearing groups when compared with the control groups (figure 12). There are more T_{fr} cells that express ICOS in the dura of tumor-bearing mice, but the amount of ICOS on each individual T_{fh} cell does not differ (figure 13A). Regarding T_{fr} cells however, there is a marked increase in the amount of ICOS expressed on each T_{fr} cell (figure 13B). In immunosenescent mice, the opposite was found, i.e., T_{fr} cells generally expressed decreased amounts of ICOS. Upregulation of ICOSL, the cognate ligand for ICOS, has been found in both human glioma and in vitro in glioma cell lines. (Schreiner et al., 2003) Schreiner et al. have additionally demonstrated that this effect is mediated by TNF-α. In the context of

immunosenescence, decreased ICOS expression is tied to decreased antigen specificity.(Sage et al., 2015) The ICOS: ICOSL interaction results in the expression of IL-10. IL-10 is a cytokine that inhibits proinflammatory responses and drives the expansion of T_{regs} in melanomas.(Martin-Orozco et al., 2010) Additionally, ICOS helps downregulate klf2, a factor that causes T_{fh} cells to revert to pre-T_{fh} cells. A possible reason that T_{fr} cells may upregulate ICOS expression may be to promote their own proliferation and inhibit GCs as ICOS:ICOSL engagement results in both of these effects.

We also report that the number of T_{regs} is elevated in the dural meninges of tumor-bearing mice that have been challenged with CFA when compared with control mice that have also been challenged with CFA (figure 11A). This is to be expected as $Foxp3^+$ T_{regs} are widely known to be upregulated in GBM disease states.(Liu et al., 2016; Wainwright et al., 2013) It is interesting that these are upregulated under the context of inflammation, close to the tumor bulk when compared with control mice, as we saw no similar effect in the spleen (figure 14A). From this information, we assumed that the immunosuppression in the tumor is a localized effect, rather than a global one. It is possible that cytokines or soluble cancer antigens produced by or in response to the tumor cause the upregulation of $Foxp3^+$ cells.

We additionally report that the number of T_{fr} cells that contribute to the total number of $CXCR5^+$ cells is elevated in the dura of tumor-bearing mice (figure 11D). This is more important than the individual T_{fr} and T_{fh} percentages because it is the balance between the two that determines the magnitude of the humoral immune response

in regards to antibodies.(Sage et al., 2014) Because this percentage is increased in tumor-bearing mice, we believe that the antibody response in GBM, especially near the tumor site is impaired. One possible explanation for this is that there is an increase in the number of $Foxp3^+$ cells in the $CD4^+$ population from GBM mice, which may partially account for the expansion of T_{fr} cells and, therefore, the increased T_{fr}:T_{fh} ratios. There is a twofold increase in the population of $Foxp3^+$ cells as a percentage of $CD4^+$ cells and a threefold increase in the population of T_{fr} cells that contribute to the total population of $CXCR5^+$ cells. Whether the increase in $Foxp3^+$ cells can fully account for the increase in the T_{fr}:T_{fh} ratio remains unclear.

An intriguing finding about this elevation in the T_{fr}:T_{fh} ratio is that it seems to be localized to the dura (figures 11 and 14). There does not seem to be a corresponding increase in T_{fr} cells in the spleen. When examined, the spleen and blood did not show increased ratios of Tfr:Tfh cells. Although there is a decreased number of both T_{fr} and T_{fh} cells in the spleen of tumor-bearing mice, this did not translate to a significant difference in either direction in percentage of T_{fr} cells that contributes to the $CXCR5^+$ subset of cells, and hence, the strength of the antibody response between the tumor-bearing mice and control mice should be similar. Because the dural meninges would contain the lymphatic vessel closest to the tumor site, this location may be the location most likely to be associated with increased signs of immunosuppression and immunosenescence. The difference between the meningeal lymphatics and the spleen suggests that the differences may be due to the closer anatomical location to the tumor and, therefore, the altered systemic levels of cytokines or protein expression, rather than

global immunosuppression. Furthermore, the dural lymphatic system is a major source of drainage of macromolecules and interstitial fluid from the brain.(Aspelund et al., 2015) One of the proteins upregulated by GBM is IDO.(Wainwright et al., 2013) A possible reason that the immunosuppression is more localized may be that kynurenine, a product of tryptophan metabolism by IDO, causes recruitment of T_{regs} through activation of the AhR. If kynurenine is only able to migrate into the dural lymphatic vessel before it is processed for AhR:kynurenine signaling, the argument can be made that availability of kynurenine is an important factor in the difference in T_{fr}:T_{fh} ratios in the spleen and the dura of tumor-bearing mice. Identification of the molecules responsible for this effect may be a viable avenue for development of future immunotherapy. The possibility of these therapeutics not requiring the ability to penetrate the BBB makes treating this aspect of immunosuppression interesting.

Furthermore, there seems to be no similar effect in the spleen (figures 11D and 14D), suggesting that this suppression of the humoral immune system is localized to sites near the tumor. Although there is a trend toward increased T_{fr} cells in the spleen, this may be due to the fact that the spleen acts as a reservoir for lymphocytes, some of which may be intended for trafficking to the meninges. In parallel, this increase in T_{fr} cells is matched by an increase in T_{fh} cells, causing there to be essentially no difference in the magnitude of the antibody response. Because there is no difference in the ratios in the blood, either, we surmise that immunosuppression is localized to the tumor draining lymph node. One possible reason for no significant increase in T_{regs} in the spleen in

GBM mice is that the injection of CFA causes an increased production of IL-6, leading to inhibition of T_{reg} development.

Tfh Function in the Spleen is Impaired

In addition to dysregulated numbers of T_{fr} cells, we hypothesized that T_{fh} cells would be dysfunctional in tumor-bearing mice. As other researchers found that the function of T_{fh} cells was impaired in immunosenescent mice, we surmised that the same could be true for GBM tumor-bearing mice.(Sage et al., 2015) Whereas those researchers focused on the specific-antigen stimulatory capability of T_{fh} and antibody production in regards to class switching, we focused on the ability of T_{fh} cells to express CD40L. To this end, we examined the amount of CD40L expressed on T_{fh} cells. We chose CD40L as a marker of T_{fh} function because the expression of CD40L and its interaction with CD40 on B cells is crucial to the development of GCs. This interaction allows B cells to become resistant to CD95-induced apoptosis.(Koopman et al., 1997) Thus, in the context of the development of the humoral immune response, displaying CD40L to B cells is the primary function of T_{fh} cells. Furthermore, studies have shown that CD40L and CD40 expression is associated with a better prognosis in human GBM.(Chonan et al., 2015) Chonan et al. further proved that enhancing CD40 expression via addition of an agonistic antibody for CD40 to a tumor-lysate vaccination prolonged survival time in mice when compared to mice that had received a similar tumor-lysate vaccination without antibody. We hypothesized that there would be fewer CD40L-positive T_{fh} cells derived from tumor-bearing mice when compared to control

mice and that there would be decreased total expression of CD40L on T_{fh} cells. Figure 15 shows that our hypothesis was correct.

There is a roughly threefold increase in the number of T_{fh} cells that are $CD40L^+$ in healthy control mice when compared to tumor-bearing mice. In addition, the amount of CD40L expressed by each T_{fh} cell in healthy mice is also increased (figure 15B). From this information, we concluded that T_{fh} cells in tumor-bearing mice are indeed impaired when compared with T_{fh} cells from healthy mice. We hypothesize that this effect is responsible for the decrease in the number of plasmablasts and plasma cells that comprise total live cells in tumor-bearing mice. Furthermore, as plasmablasts and plasma cell differentiation is shown to be restored in tumor-bearing mice upon anti-PD-L1 treatment, we believe that there is a positive correlation between increased expression of PD-L1 and inhibition of CD40L expression. Whether one influences the other or whether the two share a similar cause remains to be seen. We further hypothesize that increased CD40L expression may also be beneficial in survival time in human patients.

Germinal Cell Development in Tumor-Bearing Mice Is Impaired

The most apparent downstream effect of the upregulation of T_{fr} cell subsets and the impairment in T_{fh} cells we observed would be the inhibition of the development of plasma cells. Plasma cells are crucial to the development of the antibody response to pathogens. The role of plasma cell development in GBM remains poorly understood as not many studies have investigated the role of these cells in GBM. However, a few studies have tangentially approached the topic. Daga et al. have shown that treatment with IL-21 in tandem with immunotherapy causes rejection of tumor xenografts. They

show that this IL-21 administration causes the development of host antibodies to tumor antigens. IL-21 is crucial in the development of an antibody response. It does this by acting on B cells to upregulate Bcl-6, a transcription factor necessary for the formation of GCs.(Linterman et al., 2010; Ozaki, 2002) These GC B cells are the precursors to plasmablasts, and ultimately, to antibody-producing plasma cells. In addition, research has also shown B cells to play a critical role in combined immune-stimulatory/conditionally cytotoxic therapy.(Candolfi et al., 2011)

To this end, we examined the spleens of tumor-bearing and control mice, both of which had been treated with CFA, for evidence of live plasma cells ($CD19^{-}CD138^{+}$). As figure 16 shows, there is a marked decrease in the amount of live plasma cells in the spleens of tumor-bearing mice. We additionally examined cells in the spleen for evidence of plasmablasts, plasma cell precursors. As plasmablasts are very short-lived, we decided to look at all splenic cells, including dead cells. We also found that plasmablast generation was decreased in the spleens of tumor-bearing mice as compared to GC precursor cells that are able to differentiate into plasmablasts and then antibody-producing plasma cells in the spleens of control mice. This data may seem incongruous with the data obtained in figure 14, that there should be no difference in the magnitude of the humoral immune response between spleens, due to the fact there is no difference in the percentage of T_{fr} cells that comprise the $CXCR5^{+}$ subset of cells. A possible explanation is that plasmablasts and plasma cells in the spleens of the control mice may be intended for trafficking to the dura where the humoral immune response is still robust in comparison to the dura of the tumor-bearing mice. As the spleen contains a large

reserve of B lymphocytes, it is possible that some of the lymphocytes in the spleen are intended for migration to the dura.

Another possible explanation, however, is that the development of plasmablasts and plasma cells in itself is not crucial to the therapeutic effect of PD-L1, but rather, a downstream consequence of healthy B cell development. Candolfi et al. demonstrated that therapy using the immunostimulatory cytokine fms-like tyrosine kinase 3 ligand was effective in causing T-cell dependent tumor regression in wild-type mice with intracranially injected tumor-cells. This tumor regression was not seen in tumor-bearing mice that were deficient in B cells, demonstrating the role of B cells in the immune system's anti-tumor response. However, when the researchers used a model of mice that had B cells that were deficient in prdm-1, which is critical in the differentiation of B cells into plasmablasts and then plasma cells, tumor-regression was still seen. This suggests that it is the antigen-presenting capability of B cells that is necessary for tumor-regression, rather than the ability to differentiate into plasma cells. This information does not discount the use of plasma cells in the clinical setting, however. It may still be possible to measure plasma cells and production of antibodies in the blood as a metric of B cell activity when planning to treat GBM patients with newly developed immunotherapies.

T_{fr} Cells in Tumor-Bearing Mice Are Primarily of Inducible Origin

Because the origin of a T_{reg}, in regards to it being natural versus it being inducible, affects the way it responds to treatment with PD-L1 mAb, we decided to examine the origin of T_{regs} and T_{fr} cells in tumor-bearing mice. Specifically, PD-L1

blockade spurs the development of thymic tT_{regs} while inhibiting the development of extrathymic pT_{regs}.(Chen et al., 2014) Furthermore, as PD-L1 expression on neurons close to the tumor results in a beneficial prognosis, we additionally believed that there might be a connection between the neuronal expression of PD-L1 and the upregulation of tT_{regs}.(Liu et al., 2013) There is disagreement as to whether T_{fr} cells are of thymic or extrathymic origin. While the Sharpe group has shown that in immunosenescent mice, T_{fr} cells are differentiated from tT_{regs}, the Lintermann group has shown that, in healthy mice, T_{fr} cells can also arise from naïve $CD4^+Foxp3^-$ T cells which include extrathymic T cells.(Aloulou et al., 2016; Sage et al., 2012) The Lintermann group justifies this discrepancy by noting that they had immunized their mice with incomplete Freund's adjuvant, which supports T cell plasticity, allowing for the development of pT_{regs}, whereas the Sharpe group had immunized their mice with CFA, which does not allow for the same level of plasticity.

Extrathymic inducible pT_{regs} were deemed to be $CD4^+Foxp3^+Nrp1^-$, whereas thymic natural tT_{regs} were deemed to be $CD4^+Foxp3^+Nrp1^+$. As can be seen in figure 17B, there are more extrathymic pT_{regs} in the dura of tumor-bearing mice. This finding is contrary to what we predicted. Previous studies showed that tTregs dominate in mouse and human tumors if there is no treatment with an immune stimulating agent.(Wainwright et al., 2011) Our data show that under conditions of inflammation, it is extrathymic T_{regs} that predominate. The most likely explanation for this discrepancy in regards to the previous researchers who found that the bulk of T_{regs} in GBM are of

thymic origin is that we immunized mice with CFA, whereas Wainwright et al. did not immunize their mice.

PD-L1 Expression and T Follicular Regulatory Cells

Our first aim was to prove that T_{fr}:T_{fh} ratio expansion is the result of decreased PD-L1 expression near the tumor bulk. We based this hypothesis on three ideas. First, it has been recently found that PD-L1 expression on neurons near the tumor bulk is beneficial to prognosis in GBM, though the detailed mechanism is not immune based.(Liu et al., 2013) An increased T_{fr}:T_{fh} ratio is indicative of an impaired immune response and as most recruited T_{regs} in unimmunized tumor-bearing mice are tT_{regs}, we previously assumed T_{fr} cells were of tT_{reg} origin.(Wainwright et al., 2011) Finally, previous studies have shown that tT_{reg} differentiation is enhanced in the absence of PD-1, a ligand for PD-L1.(Chen et al., 2014) When these concepts were taken together, it led us to believe that PD-L1 expression would be decreased on the tumor bulk, that T_{regs} in tumor-bearing mice would be of thymic origin, and that inhibition of this process would cause an increase in these T_{regs} and their T_{fr} subsets.

Sage et al. claimed that T_{fr} cells are of natural thymic origin, i.e., Nrp^{hi}.(Sage et al., 2012) However, other studies by other researchers claim findings that argue against this. Aloulou et al. claim that after inhibition of PD-L1, the number of Nrp^{lo} T_{fr} cells decreases. They believe that this decrease supports the idea that T_{fr} cells do not always arise from $Foxp3^{+}$ precursors, i.e., that T_{fr} cells do not always have T_{reg} origins.(Aloulou et al., 2016) The main difference between these two studies is that in Sage's study CFA was used to immunize mice, whereas in Aloulou's study, incomplete Freund's adjuvant,

that is, CFA without inactivated mycobacterium tuberculosis, was used. The difference between them is the ability of CFA to induce the production of IL-6 , which inhibits the conversion of naïve $CD4^+$ T cells into potential pT_{regs}. In this study we used CFA and we found that PD-L1 mAb did indeed prevent the expansion of T_{fr} cells relative to the total number of $CXCR5^+$ cells. Additionally, there is a wealth of evidence that demonstrates that T_{regs} in GBM are of thymic rather than induced origin when there is no treatment with an immune stimulatory agent.(Delgoffe et al., 2013; Wainwright et al., 2011) Furthermore, targeting the thymic nature of these T_{regs} allows the immunosuppressive aspects of GBM to be manipulated while preventing deleterious inflammation. Our working hypothesis was that the T_{regs} and T_{fr} cells in tumor-bearing mice were of thymic rather than extrathymic origin. To this end we examined the dura of tumor-bearing mice for Nrp levels in T_{regs}.

Figure 17 shows that T_{regs} in the dura are primarily $Nrp1^{lo}$. This phenotype is more pronounced in the dura than it is in the spleen. From this we can conclude that the bulk of these T_{regs} most likely are not of thymic origin, but rather of inducible extrathymic origin. Thus, when PD-L1 is blocked by mAb, the number of $Nrp1^{hi}$ T_{regs} should increase, as it has been shown that inhibiting PD-L1 causes tT_{regs} to proliferate. It has also been shown that inhibiting PD-L1 prevents the proliferation of pT_{regs}.(Chen et al., 2014) While tT_{regs} may be predominant in GBM prior to immunization, it is possible that under inflammatory conditions, pT_{regs} may be recruited to curtail inflammation related to CFA treatment and subsequent TLR9 activation.

A more striking discrepancy, however, is that in immunosenescent mice in the study by Sage et al., T_{fr} cells are derived from tT_{regs}, whereas the T_{fr} cells in GBM mice from our study are derived from pT_{regs}, even though both groups of mice have been immunized with CFA.(Sage et al., 2012) The tumor may cause the release of cytokines or other macromolecules that prevent the CFA from inhibiting $CD4^+$ T $Foxp3^-$ T cell plasticity, allowing for the polarization of naïve $CD4^+$ to $Foxp3^+$ pT_{regs}, which are then able to further differentiate into T_{fr} cells.

Fornara et al. have attempted to draw correlations between immunosenescence and glioblastoma by using immunosenescence, which itself was determined by levels of $CD3^+$ T cells and certain subsets of $CD4^+$ T cells, as a measure of poor prognosis in GBM patients.(Fornara et al., 2015) Beyond this study, however, immunosenescence remains poorly understood within the context of GBM patients. While glioblastoma and old age, and therefore, immunosenescence, are linked, the manner in which the humoral immune system is impaired in each case appears to be different.

PD-L1 Expression Is Increased in Both the Tumor and In the Brain near the Tumor Bulk

Given our initial hypothesis was that T_{regs} in GBM mice under inflammatory conditions would be largely of thymic lineage, given that the PD-1:PD-L1 interaction causes an expansion in T_{regs} that are of thymic lineage, given that expression of PD-L1 on neurons near the tumor bulk is beneficial for prognosis in human GBM and given that increased T_{regs} in the GBM mouse models is detrimental to survival, we reasoned that there would be a decreased amount of PD-L1 in the brain near the tumor bulk.(Chen et

al., 2014; Liu et al., 2013; Wainwright et al., 2011, 2013) To this end we stained tissue from the brain of control mice, tumor-bearing mice and PD-L1-treated, tumor-bearing mice, all of which had received injections of CFA.

Figure 18 shows that PD-L1 expression is increased in the brain tissue near the tumor of GBM tumor-bearing mice. Again, this is counter to our initial hypothesis, that PD-L1 expression would be decreased near the tumor. It is important to make the distinction that we did not hypothesize that the tumor itself would have a decreased amount of PD-L1. Though PD-L1 expression on neurons near the tumor bulk being better for prognosis is not immune related,(Liu et al., 2013) we made the assumption that it would be possible for there to be pleiotropic effects in which the increased amount of PD-L1 would translate into an increased T_{fr}:T_{fh} ratio. Additionally, figure 18 shows that the PD-L1 mAb is able to penetrate the BBB and inhibit the expression of PD-L1 in the treated groups. As can be seen, there is less total fluorescence in regards to PD-L1. From this, we can assume that the availability of the PD-L1 mAb in the dura of mice is not negligible. Liu et al. claim that expression of PD-L1 is beneficial due to the neurons being able to recognize and eliminate glioma cells.(Liu et al., 2013) In this explanation there is no role of PD-L1 interacting with the immune system to either help or hamper the expansion of T_{regs}.

PD-L1 Blockade Helps Rescue T_{fr}:T_{fh} Ratios

Once again, previous studies have found that PD-L1 is capable of preventing the differentiation of T_{fr} cells and preventing their downstream effector functions in immunosenescent mice.(Sage et al., 2015) Although we initially believed that this

would not be true in the case of GBM-bearing mice, the results are similar. As previously mentioned, it is the number of T_{fr} cells that contributes to the total pool of $CXCR5^{+}$ cells that dictates the strength of the humoral immune response.(Sage et al., 2012) As previous research had shown that i.p. injection of PD-L1 was able to cause regression of GBM when used in tandem with other immune therapies, we decided to use this route of administration and injection regimen when planning this set of experiments.(Wainwright et al., 2014)

Figure 19B shows that instead of the predicted increased T_{fr}:T_{fh} ratio, PD-L1 mAb decreases the number of T_{fr} cells that contributes to the pool of $CXCR5^{+}$ cells. A rescue function can be seen in this figure. Once again, figure 19C shows T_{regs} as primarily being of extrathymic origin. It would make sense that inhibition of the PD-1:PD-L1 interaction, which is necessary for the development of extrathymic $T_{regs,}$ would then lead to an overall decrease in the number of both T_{fr} cells and their progenitors, $T_{regs.}$ Furthermore, as the total percentage of Nrp^{lo} T_{regs} decreases, the number of Nrp^{hi} T_{regs} must also increase. PD-L1 mAb does not seem to bring about a complete rescue, however, and it can be seen that there still is a difference between the control and the PD-L1 mAb-treated mice, as seen in figure 19B. Perhaps a higher dose of mAb would correct this.

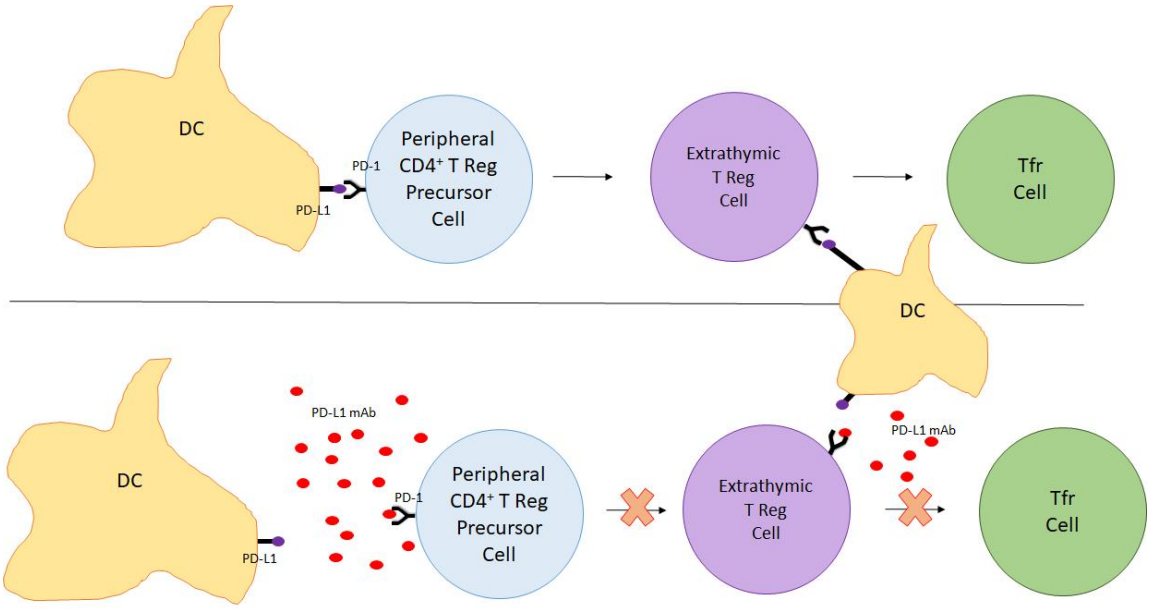

Figure **22.** PD-L1 inhibits the development of both T_{fr} and T_{reg} cells. Antigen presenting cells are unable to signal to $CD4^+$ T_{reg} precursors to stimulate differentiation into T_{regs}. The upper figure shows development of T_{regs} and T_{fr} cells under normal conditions. The bottom panel shows that blockade of PD-L1 using antibody therapy (the red circles) prevents the development of T_{regs} and, additionally, the development of T_{fr} cells.

Plasma Cell Development Is Inhibited in GBM Mice but Rescued by PD-L1 Blockade

After examining the role of PD-L1 in the development of T_{fr} cells, we looked at its downstream effects in CFA-treated GBM mice. We found that PD-L1 mAb-treated GBM mice had an elevated level of plasmablasts and total live plasma cells when compared with GBM mice that had not been treated (figure 20). This data is in agreement with our finding that PD-L1 mAb-treated mice have a decreased number of T_{fr} cells that contribute to the total pool of $CXCR5^+$ cells. The fact that not only is the

percentage of live plasma cells increased in PD-L1 mAb-treated mice, but also that plasmablast formation is increased in PD-L1 mAb-treated mice, leads us to believe that PD-L1 mAb treatment rescues the formation of GCs, since a plasmablast arises from GC B cells. We assume that PD-L1 was able to rescue CD40L, but unfortunately did not have an opportunity to confirm this.

CFA and PD-L1 Combinatorial Treatment Results in Improved Survival Times

In the course of this study, one set of neurospheres that failed to transform completely was injected into the brains of 10 mice. We believe the reason that this set of glioma cells failed to completely transform is due to an insufficient amount of L-glutamine. L-glutamine is often added to cell culture media as a metabolic energy source. In its absence, cells in culture are unable to survive and proliferate. Both sets of mice received CFA treatment. An experimental group received intraperitoneal injections of PD-L1 mAb. When completely transformed neurospheres are intracranially injected into the caudate nucleus of mice, GBM symptoms, e.g, seizure, motor problems, etc., are typically visible within 15-20 days. In this set of mice, tumor symptoms took 30-40 days to appear. The mice in this study were then used to examine survival times in regards to PD-L1 mAb treatment. Although there are studies demonstrating the efficacy of PD-L1 mAb therapy, these studies used 261 adherent cells as opposed to neurospheres, which more accurately recapitulate human glioblastoma symptoms. Furthermore, in these studies, PD-L1 mAb was administered relatively early, preventing the tumor from establishing itself.(Wainwright et al., 2014) This does not recreate the typical clinical case in which the tumor has already established itself in the patient

before treatment begins. Additionally, early clinical trials of PD-L1 mAb in human patients has not yet shown any promising results. Here, we report that treatment with CFA and PD-L1 mAb leads to increased survival when compared with CFA alone.

Figure 21 shows a better prognosis for mice that received CFA treatment alongside PD-L1 blockade as opposed to mice that received only CFA treatment. The average survival time for mice without PD-L1 mAb treatment was 45 days whereas PD-L1 administration extended this survival time to 54 days. Furthermore, in the case of one of the PD-L1-treated mice, complete remission was seen. This mouse was later sacrificed at 100 days as the study had ended. The results of this study are in agreement with a recent study showing that administration of TLR9 agonist, CpG, and OX40, another immune checkpoint inhibitor, led to eradication of various tumors implanted at various locations in immunocompetent mice(Sagiv-Barfi et al., 2018). Although this group used CpG, to stimulate TLR9, CFA should theoretically have the same effect, as it also stimulates the TLR9 pathway. A possible explanation for this remission is that immune checkpoint inhibitors by themselves are not enough to eradicate tumors, and remission requires additional activation of the immune system. It may be necessary for the immune system to be primed by activation of TLR agonists in addition to inhibition of immune checkpoints.

This evidence suggested that our initial beliefs about the thymic nature of these cells was incorrect. Perhaps when the immune system is activated by external signals such as CFA, extrathymic T_{regs} are recruited to dampen immune activity following

inflammation. Figure 22 illustrates the ways in which PD-L1 mAb treatment could prevent the expansion of T_{fr} and of T_{regs}.

Unfortunately, human studies of PD-L1 mAb treatment have not proven successful. Although use of PD-L1 mAb has been successful in treating melanoma in human patients, similar results have not been found in GBM patients.(Bertrand et al., 2017; Filley et al., 2017) Wainwright et al. have shown that treatment with only PD-L1 mAb is capable of prolonging survival in tumor-bearing mice.(Wainwright et al., 2014) One possible reason for this is that though Wainwright et al. use the 261 cell line, they use the adherent version of the cell line and begin their anti PD-L1 treatment regimen 7 days after injection. This may not be enough time for the tumor to establish itself and would, therefore, not accurately recapitulate glioblastoma in the clinical setting, as glioblastoma is usually diagnosed only after it becomes symptomatic in humans.

Future Studies

Although this study proved that the humoral immune response in tumor-bearing mice is inhibited, there are certain aspects we did not have the opportunity to explore. In particular, we would have liked to take a closer look at the role of IL-21 in glioblastoma in regards to T_{fh} cells. We think IL-21 would play an important role in enhancing the ability of various lymphocytes in regards to presentation of cancer antigen. As IL-21 enhances immunotherapy in GBM model mice, we believe that it is most likely downregulated in GBM mice compared to control mice. We hypothesize that there would be less total IL-21 in the spleens of tumor-bearing mice. To test this, we would have two groups of mice, one of which would receive intracranial injections of 261

neurospheres and the other of which would receive sham injections of DSCM. We would then process the spleens of both groups and perform an ELISA for IL-21.

We additionally would have liked to perform an in-depth examination of the role of class-switching in tumor-bearing mice. We believe that this is inhibited in tumor-bearing mice. In particular, we believe that there would be lower concentrations of IgE and higher concentrations of IgM in tumor-bearing mice. We believe this because IgE is elevated in patients with rhinitis with which GBM is negatively correlated. (Schoemaker et al., 2006) Linking a decrease in IgE with the presence of GBM would create a stronger argument for the therapeutic role of IL-21 in GBM, as a decrease in IgE is correlated with an increase in IL-21.(Jen et al., 2015) Conversely, in the absence of IL-21, an increase in IgE was noted after immunization with sheep red blood cells. (Ozaki, 2002) These two experiments imply that an increase in IgE, which could be potentially induced by allergic rhinitis, causes an increase in IL-21. This would additionally provide a mechanism for allergic rhinitis leading to decreased likelihood for GBM later in life.

One other non-related experiment we would have liked to perform in regards to GBM, but not the humoral immune system, is the role of GFAP and chaperone-mediated autophagy in "tumor stemness." As GFAP is a terminal marker on glial cells that prevents anaplasia and as GFAP is a positive regulator of chaperone-mediated autophagy, we surmise that chaperone-mediated autophagy is inversely correlated with tumor stemness and is correlated with a better prognosis in GBM.

Finally, if it were possible, it would be interesting to examine all of the mentioned ideas not just in mice, but also in humans. The end goal of translational medicine is for the results found in laboratory studies to benefit clinical patients. As has been mentioned previously, recent immunotherapy for GBM has been shown to be disappointing. We would like to determine if the results we found in this study would be able to explain why the recent clinical trials failed. It may also be that our studies would be able to provide biomarkers by which to divide patients into groups that would receive a higher level of benefit from the failed PD-L1mAb trial. Furthermore, PD-L1 mAb in combination with IL-21 or a TLR9 agonist may have more potent benefits than the PD-L1 mAb alone. As the field of cancer immunotherapy, and in particular monoclonal antibodies for immune checkpoint inhibitors, grows, it will be necessary to examine different combinations involving not just antibodies that prevent inhibition of the immune system, but also of therapies that stimulate it.

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