NOVEL MECHANISMS OF CYTOKINE SIGNALING ON T-CELL AND MDSC FUNCTION IN GLIOMA DEVELOPMENT

by

Gary Kohanbash

B.S, Neuroscience, University of Pittsburgh, 2004

M.S, Infectious Diseases and Microbiology, University of Pittsburgh, 2007

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This dissertation was presented

by

Gary Kohanbash

It was defended on

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and approved by

Dissertation Advisor:
Hideho Okada, MD PhD
Professor, Neurological Surgery, Surgery and Immunology
University of Pittsburgh School of Medicine
Co-Leader, Brain Tumor Program
University of Pittsburgh Cancer Institute

Committee Member:
Jeremy J. Martinson, DPhil
Assistant Professor, Infectious Diseases and Microbiology and Human Genetics
Associate Director, Bioscience of Infectious Diseases -MPH Program
Graduate School of Public Health, University of Pittsburgh

Committee Member:
Pawel Kalinski, MD PhD
Professor, Surgery and Immunology
University of Pittsburgh School of Medicine
Infectious Diseases and Microbiology
University of Pittsburgh Graduate School of Public Health
Melanoma Program Member
University of Pittsburgh Cancer Institute

Committee Member:
Paul D. Robbins, PhD
Professor, Microbiology and Molecular Genetics and Orthopedic Surgery
Member, Molecular Virology and Microbiology Graduate Program
Member, Biochemistry and Molecular Genetics Graduate Program
Member, Immunology Graduate Program
University of Pittsburgh School of Medicine

Committee Member:
Yue Chen, MD PhD
Assistant Professor, Department of Infectious Disease and Microbiology
Graduate School of Public Health, University of Pittsburgh

Hideho Okada MD PhD

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Malignant gliomas are the most common primary brain tumors with dismal prognosis. A growing line of evidence supports significant roles of immunosurveillance for prevention and regulation of cancer development. For example, tumor infiltrating T-cells are capable of killing tumor cells and are a positive prognostic factor for cancer patients. T-cell immune responses are classified into distinct effector cell types, type-1 or type-2, based on their cytokine-secreting profiles. We have demonstrated that tumor-specific type-1 T-cells, but not type-2 T-cells, can efficiently traffic into CNS tumor sites and mediate effective therapeutic efficacy via a type-1 chemokine CXCL10 and an integrin receptor VLA-4. Despite the importance of the type-1 T cell response, cancers, including GBMs, secrete numerous type-2 cytokines that promote tumor proliferation and immune escape. The hallmark cytokines of type-1 and type-2 immune responses are IFNs and IL-4, respectively. We therefore sought to better understand the role of IL-4 and IFN signaling in gliomas. We herein demonstrate that the miR-17-92 cluster is down-regulated in T-cells in both human and mouse tumors, dependent on IL-4R signaling. Further, ectopic expression of miR-17-92 cluster in T-cells resulted in enhanced IFN-γ and IL-2 production and resistance to activation induced cell death (AICD) **(Aim 1)**. We next examined IL-4Rα on immunosuppressive myeloid derived

suppressor cells (MDSCs). Interestingly we found that IL-4Rα was up-regulated on human and mouse glioma infiltrating, but not peripheral, MDSCs. Additionally, IL-4Rα expression promoted arginase activity, T-cell suppressing abilities and glioma growth **(Aim 2)**.

As type I IFNs are important for anti-glioma type-1 immunity, we further examined how type I IFNs impact glioma patient prognosis. As there are multiple type I IFNs, our collaborators assisted us to identify potentially important genes by single nucleotide polymorphism (SNP) analysis. We found that IFN-pathway genes IFN- alpha receptor-1 (IFNAR1) and the IFN-alpha-8 (IFNA8) promoter both had SNPs associated with glioma prognosis. By luciferase assay and electrophoretic mobility shift assay (EMSA) we demonstrated that the A-allele, which is associated with better glioma patient survival, but not the C-allele of rs12553612 in the promoter region of IFNA8 allows for OCT-1 binding and activity of the IFNA8 promoter **(Aim 3).**

Overall, our data suggests that type-2 promoting has a dual role in suppressing glioma immunity through decreased T-cell functioning and enhanced MDSC function. *Type-2 promoted suppression of glioma immunity can thus lead to better glioma patient prognosis, a significant public health achievement.*

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ABBREVIATIONS

AA	Anaplastic Astrocytoma
AICD	Activation Induced Cell Death
BIL	Brain Infiltrating Leukocytes
BM	Bone Marrow
CNS	Central Nervous system
COX	Cyclo-Oxygenase
CTL	Cytotoxic T Lymphocyte
EGFR	Epidermal Growth Factor Receptor
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
GAA	Glioma Associated Antigen
GBM	Glioblastoma
IFN	Interferon
IL-4Rα	Interleukin-4 Receptor Alpha Chain
MACs	Magnetic Activated Cell Sorting
mAb	Monoclonal Antibody
MDSC	Myeloid Derived Suppressor Cells
miRNA	MicroRNA
PTEN	Phosphatase and Tensin Homologue
RAS	Rat Sarcoma
SFS	Symptom Free Survival

sh	Short hairpin
siRNA	Short-interfering RNA
SNP	Single Nucleotide Polymorphism
SPC	Splenocytes
STAT	Signal Transducer and Activator of Transcription
TLR	Toll-like receptor
Th	T Helper Cell
Tc	T Cytotoxic Cell
TMZ	Temozolomide
TGF	Transforming Growth Factor
WHO	World Health Organization

1.0 INTRODUCTION

1.1 GLIOBLASTOMA MULTIFORME

1.1.1 Classification

Malignant gliomas are the most common type of primary brain tumor and a major unsolved public health problem, with more than 12,000 new cases diagnosed each year in the United States (Mulholland, Thirlwell et al. 2005).

Based on the World Health Organization (WHO) classification, the four main types of gliomas are astrocytomas, oligodendrogliomas, ependymomas, and mixed gliomas (usually oligoastrocytomas). Astrocytomas are typically classified as pilocytic (grade I), diffuse (grade II), anaplastic (grade III), or Glioblastoma (GBM) (grade IV) in order of increasing anaplasia. Categorization of astrocytomas as low (I and II) or high (III and IV) grade is generally dependent on nuclear atypia, mitotic activity, microvascular proliferation, and focal necrosis. GBM is by far the most common and most malignant glial tumor. Composed of poorly differentiated neoplastic astrocytes, GBM primarily affect adults, and they are located preferentially in the cerebral hemispheres.

Patients with GBM have a median survival of approximately 15 months, whereas those with anaplastic astrocytoma (AA) have a median survival of 24 to 36 months. For patients with recurrent malignant gliomas, the median time to further tumor progression, even with therapy, is only 8 weeks (Wen and Kesari 2004). In addition, low-grade gliomas often progress to more malignant gliomas when they recur (Ashby and Shapiro 2004). With over 12,000 new cases diagnosed in the United States each year, short survival time and the lack of curative treatment GBM tumors represent a signifi*cant public health problem.*

1.1.2 Epidemiology

As reviewed by Ohgaki (Ogaki 2009), GBM occurrence seems most prevalent in industrialized countries, and Caucasians have higher incidence than both African and Asian populations. Limited data is available on causes of GBM however occupational exposures have been shown to be associated with GBM such as plastics, formaldehyde and lead. Other factors such as smoking and electromagnetic field have shown no association with GBM in most studies. According to Ohgaki the only factor "unequivocally associate" with GBM is X-irradiation, a therapy used to treat acute lymphoblastic leukemia.

A single nucleotide polymorphism (SNP) is a single nucleotide variation that occurs within a gene of members of the same species. Several SNPs in immune regulatory genes correlate with glioma risks and/or prognosis (Rodero, Marie et al. 2008; Scheurer, Amirian et al. 2008; Fujita, Scheurer et al. 2010). Previous studies have

shown a significant impact of SNPs in innate immune pathways, such as ones in *Toll-Like Receptor (TLR)3* (Dhiman, Ovsyannikova et al. 2008; Yang, Stratton et al. 2008), *TLR4* (Apetoh, Ghiringhelli et al. 2007) as well as *interleukin (IL)-4 receptor (IL-4Rα),* which are associated with differential risk and prognosis of GBM (Schwartzbaum, Ahlbom et al. 2007; Scheurer, Amirian et al. 2008). SNPs in other genes that are associated with glioma prognosis include cyclo-oxygenase (COX)-2 (Fujita, Kohanbash et al. 2011) and CX3CR1 (Rodero, Marie et al. 2008).

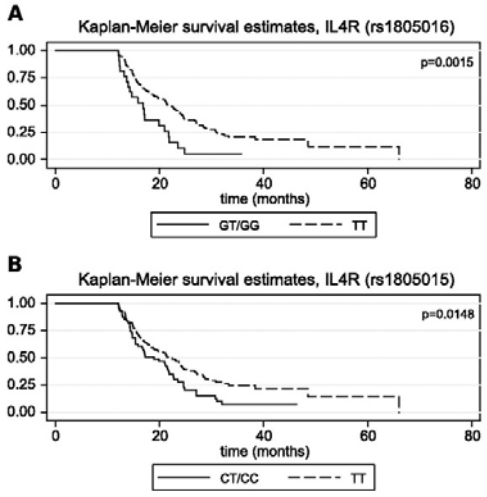


Figure 1: Kaplan-Meier survival curves beyond 12 months by genotype for IL4R SNPs among high-grade gliomas. (A), patients with the TT genotype for IL4R rs1805016 SNP experienced a median survival 4 mo longer than those with the GT/GG genotypes. (B), patients with the TT genotype for IL4R rs1805015 SNP experienced a median survival 5 mo longer than those with the CT/CC genotypes. The benefit of the TT genotypes seemed to increase as the patients lived longer. **(Figure and Caption taken from M Scheurer et al. Clinical Cancer Research, 2008.)**

1.1.3 Treatment

Despite extensive research, the treatment options for these tumors remain limited (Wen and Kesari 2004). No significant advancements in the treatment of GBM have occurred in the past 25 years except for chemotherapy with Temozolomide (TMZ) combined with radiotherapy, which demonstrates only a limited prolongation (approximately 3 months compared with radiotherapy only) of patients' survival (Stupp, Dietrich et al. 2002). The primary reason that no current treatment is curative is that the tumor is beyond the reach of local control when it is first detected clinically or radiologically. Clearly, there is an unmet clinical need for further improving treatment outcomes for patients with malignant gliomas.

Immunotherapy, based on the idea of taking advantage of the body's physiological mechanisms to defend itself, may develop as an effective and safe treatment modality for gliomas. However, the immunological microenvironment of the central nervous system (CNS) and tumors arising in the CNS are still believed to be sub-optimal for sufficient anti-tumor immune responses to mediate clinically-meaningful changes *in situ* [reviewed in (Prins and Liau 2004)]. Immunotherapies may be classified as either passive or active, either transferring the immune components or the stimulating of host immunity, respectively.

1.1.3.1 Passive Immunotherapy

Passive immunotherapy is defined as the use of products of a simulated immune response *ex vivo* to specifically target tumor cells. This consists of modalities that utilize

a variety of molecules, including monoclonal antibodies (mAb) and cytokines. Some potential therapeutics and targets are discussed below.

(a) Antibodies

An attractive candidate for Immunoglobulin (Ig)G-based inhibition has been epidermal growth factor receptor (EGFR). EGFR is overexpressed on 40-50% of tumors (Rivera, Vega-Villegas et al. 2008). EGFR overexpression is associated with increased tumor growth rate and shorter survival (Shinojima, Tada et al. 2003). EGFR, a transmembrane receptor tyrosine kinase, binds its ligands epidermal growth factor (EGF) and transforming growth factor (TGF)-α. EGFR activation generates gene transcription modulations resulting in stimulated proliferation, angiogenesis, and metastasis (Batra, Castelino-Prabhu et al. 1995). A mutation of EGFR, termed EGFR variant III (EGFRvIII), is frequently expressed in GBM and enhances tumorigenicity (Fukai, Nishio et al. 2008). There are several mAbs that specifically target EGFR including: Cetuximab and Nimotuxumab.

Cetuximab (Imclone, Bristol Meyers Squibb, New York, NY) has been shown to enhance the anti-tumor effects of chemotherapy and radiotherapy by inhibiting the EGFR pathway. Cetuximab specifically targets the extracellular domain of EGFR (Ramos, Figueredo et al. 2006). EGFRvIII can be bound by Cetuximab, and it has been suggested that Cetuximab has antitumor efficacy against $EGFRvIII^{+}$ glioma cells (Belda-Iniesta, Carpeno Jde et al. 2006).

Nimotuzumab (h-R3, YM Biosciences, Mississauga, ON, Canada) has been used in phase II trials for GBM (Rosenberg 2000). Ramos *et al*. observed a 17.5 month MST

for GBM patients. In high grade glioma patients, there was a 37.9% objective response rate and stable disease in another 41.4% of patients (Rosenberg 2000).

The efficient use of mAbs against brain tumors presents unique challenges. First, passage of therapeutic agents from circulation through the blood brain barrier (BBB) favors small, uncharged, lipid soluble molecules. The large size of antibodies as macromolecules requires novel delivery strategies to administer antibodies directly to the brain tumors. Indeed, an IgG antibody has a molecular weight of approximately 150 kDa whereas many chemotherapeutic agents have a molecular weight on the order of 1 kDa (DeVita, Hellman et al. 1995). While most small molecule drugs rely on diffusion as a mode of transport through tissue, antibodies must rely on bulk fluid flow (convection) in which antibodies flow down their pressure gradient. It has been shown that interstitial pressure in solid tumor mass is elevated above the interstitial pressure of the surrounding normal tissue (Butler, Grantham et al. 1975). This increased pressure may be a result of a less developed lymphatic system which is thereby less able to drain interstitial fluid, increased cell density of the tumor could be a contributing factor to this phenomenon (Jain and Baxter 1988; Williams, Duda et al. 1988).

The use of antibodies as inhibitors has the advantage of being precise and specific, but the challenges of delivery and penetrance remain. As cancer biology and the mechanisms of tumor cell proliferation and immune escape are increasingly understood, more candidates for therapeutic inhibition will be found.

(b) Coupled Targeted Toxins

A number of cytokine receptors have been observed to be up-regulated in glioma including IL-4R and IL-13Rα2. By harnessing these and other receptor-ligand interactions that are up-regulated in GBM, targeting of tumor cells can be achieved and by fusing cytokines with toxins, selective cytotoxicity can be achieved (Joshi, Leland et al. 2001; Liu, Yu et al. 2004).

IL-4R is up-regulated in glioma cells relative to normal tissue. In fact, 83% of GBM tumors and 86% of astrocytoma tumors were found to be moderately to highly positive for IL-4R *in situ* by Joshi *et al*. (Joshi, Leland et al. 2001) Puri et al. have developed a chimeric fusion protein including domains of IL-4 and *Pseudomonas exotoxin* (PE), which is produced by expressing chimeric genes in *E. coli* and purifying the protein using inclusion bodies (Puri, Hoon et al. 1996) . Phase I studies have been executed to determine safety and tolerability (Rand, Kreitman et al. 2000; Weber, Asher et al. 2003). Also, it was reported that in one preliminary study, 6 out of 9 patients with recurrent malignant glioma demonstrated tumor necrosis after receiving IL4-PE via convection-enhance delivery (CED) with multiple catheters as evidenced by gadolinium-enhanced MR images. One patient remained disease free for greater than 18 months (Rand, Kreitman et al. 2000).

IL-13R is also found to be overexpressed on a majority of glioma cell lines and resected GBM specimens (Debinski, Obiri et al. 1995). A mutated form of PE fused to human IL-13, named IL-13PE38QQR or cintredekin besudotox (CB), has been developed and has been shown to elicit specific cytotoxicity on glioma cell lines (Mut, Sherman et al. 2008). CB has been studied in a number of phase I clinical trials to

investigate dosimetry and catheter positioning and is reportedly more active against glioma cell lines than IL-4 targeted toxins *in vitro* (Mut, Sherman et al. 2008). A total of 51 GBM patients have been treated with CB with a median survival time of 42.7 weeks (Kunwar, Chang et al. 2006). Kioi et al. developed a strategy in which PE is conjugated to a smaller single chain variable fragment (scFv) anti-IL13R human antibody (Kioi, Seetharam et al. 2008) which may allow for better GMB penetration. Overall, IL-13-based toxins have potential to be utilized as an adjuvant therapy for malignant glioma pending further positive clinical studies.

(c) Adoptive T-cell Therapy

Adoptive transfer of tumor-reactive autologous cytotoxic $CD8^+$ T lymphocytes (CTLs) may hold promise as an attractive future immunotherapeutic intervention against malignant glioma. The earlier form of this therapeutic approach was mostly used to treat malignant melanoma, in which autologous lymphocytes infiltrating tumor nodules were isolated, expanded *in vitro* in the presence of IL-2 and subsequently returned to the patients (Rosenberg, Packard et al. 1988). In glioma, *ex vivo* activated lymphokine-activated killer (LAK) cells have been applied as an adjunct to surgery, often in combination with low-dose IL-2 (Barba, Saris et al. 1989; Hayes, Koslow et al. 1995). Although clinical responses have been observed in some cases, this approach relies upon innate immune effector cells (i.e., LAK cells), whose killing activity may not be tumor-specific. In contrast, antigen-specific $CD8^+$ CTLs survey the CNS parenchyma and selectively kill astrocytes that express a model antigen hemagglutinin (HA) without collaterally damaging neurons and oligodendrocytes or myelin. This was demonstrated

in an elegant study using HA-specific T-cells obtained from a TCR transgenic mouse line and recipient transgenic mice expressing HA in their astrocytes (Cabarrocas, Bauer et al. 2003). Importantly, i.v. injected tumor-specific CTLs have established their antitumor potency in syngeneic rodent models of glioma (Holladay, Heitz et al. 1992). Antigen-nonspecific LAK cells, in contrast, fail to eradicate tumor in most of these experimental models (Holladay, Heitz et al. 1992). Collectively, these studies demonstrate that CTLs have the capacity to migrate into brain parenchyma and have anti-tumor effects.

Recently, the approach has been vastly improved by the use of recent advances in several areas of human T-cell biology including *in vitro* human T-cell culture and *ex vivo* genetic manipulation. Although adoptive T-cell therapy remains the experimental therapy for a limited types of cancers (mainly malignant melanoma), there have been increasing attempts to widen the use of adoptive T-cell therapy to treat other types of tumors including malignant glioma (Ghazi, Ashoori et al. 2012).

1.1.4 Active Immunotherapy (Tumor Vaccines)

Although numerous preclinical studies in mouse models have shown the efficacy of peripheral vaccinations against intracranial gliomas, therapeutic vaccines face a substantial challenge in glioma patients since they must overcome a variety of immunoregulatory mechanisms that have already established the immune escape of tumors. Nevertheless, a number of clinical trials have been attempted to generate therapeutic immune responses against gliomas and shown some positive effects.

(a) T-Cell Epitopes Derived From Glioma-Associated Antigens

Many studies have demonstrated the safety and preliminary efficacy of whole glioma cell-based vaccine approaches (Liau, Prins et al. 2005; Okada, Lieberman et al. 2007; Wheeler, Black et al. 2008). However, the use of whole glioma cell-derived antigens, such as glioma lysate, may limit the feasibility and safety of the approach due to the cumbersome preparation procedures and theoretical concerns for autoimmune encephalitis (Bigner, Pitts et al. 1981). Therefore, the effectiveness and safety of T-cell-mediated immunotherapy of glioma depends on the proper selection of the targeted antigens; i.e. glioma-associated antigens (GAAs), and, more specifically, the CTL epitopes in GAAs. In addition, the use of "off the shelf" synthetic peptides coding GAA-derived CTL epitopes may be feasible, especially for multi-center clinical trials. Some, GAA epitopes known to elicit T-cell responses include: IL-13Rα2, Survivin, WT1 and EGFR-VIII (Okada, Kohanbash et al. 2009).

(i) Peptide-based vaccines targeting glioma-associated antigens

In vaccines using synthetic peptides for shared GAA-epitopes, advantages and disadvantages are distinct from those in whole glioma cell approaches. While synthetic GAA peptide-based vaccines may not adequately target antigens in each patient's tumor, these vaccines have less concern for autoimmunity and provide "off the shelf" feasibility. Indeed, a wide range of peptide-based vaccines have been evaluated. Yajima *et al*. reported a phase I study of peptide-based vaccinations in patients with recurrent malignant gliomas (Yajima, Yamanaka et al. 2005). In this study, prior to the first vaccine, each patient's PBMCs were evaluated *in vitro* for cellular and humoral

responses against a panel of antigens, and peptides that induced positive response were used for vaccinations. The regimen was well tolerated and resulted in an 89-week median survival of treated patients. However, there is little evidence that the antigens used in this study are expressed in gliomas at high levels. Izumoto *et al.* reported a Phase II clinical trial using a single peptide, WT1 (Izumoto, Tsuboi et al. 2008). In this study, they reported a median PFS of 20 weeks and a possible association between the WT1 expression levels and clinical responses. When single or oligo antigens are selected and targeted by vaccines, it also seems necessary to harness the concepts of epitope spreading to address the problems of tumor immune escape, while avoiding the augmentation of deleterious CNS autoimmune responses (Vanderlugt and Miller 2002).

(ii) Whole glioma cell vaccines

Initial vaccination strategies for gliomas consisted of subcutaneous inoculations of irradiated, autologous (Wikstrand and Bigner 1980) or allogeneic (Zhang, Eguchi et al. 2007) glioma cells. This type of vaccine has the advantage of providing a panel of multiple potential GAAs that are naturally expressed by glioma cells. Especially, autologous glioma cells should allow immunizations against the most relevant GAAs expressed in the patient's tumor (i.e. tailored medicine). Potential downsides of this approach, however, include: 1) cumbersome procedures and quality control (QC)/quality assurance (QA) issues associated with large scale cultures of autologous glioma cells and 2) theoretical risks of autoimmune encephalomyelitis (reviewed in (Wikstrand and Bigner 1980)). Nevertheless, this type of vaccine strategy has been carefully examined. Schneider *et al.* (Schneider, Gerhards et al. 2001) and Steiner *et*

al. (Steiner, Bonsanto et al. 2004) recently reported pilot clinical trials using autologous glioma cells modified with Newcastle-Disease-Virus (NDV), which is known to serve as an vaccine adjuvant and therefore to improve the efficacy of glioma vaccines. More recently, Ishikawa *et al.* reported a Phase I clinical trial using formalin-fixed glioma tissues as a source of antigens (Ishikawa, Tsuboi et al. 2007). The advantage of this strategy is that formalin fixation preserves the specific antigenicity of glioma cells. These studies reported no major adverse events.

(iii) Dendritic cell (DC) vaccines

DCs are the most potent antigen presenting cells, driving the activation of T-cells in response to invading microorganisms (Banchereau, Briere et al. 2000). The availability to culture DCs from human peripheral blood monocytes has generated significant interest in using DCs in novel cancer vaccination strategies (Banchereau and Palucka 2005).

Yu *et al.* reported a phase I trial of vaccinations using DCs pulsed with peptides eluted from autologous glioma cells (Yu, Wheeler et al. 2001). Later, Liau *et al.* also reported a phase I trial in patients with newly diagnosed GBM using DCs pulsed with acid-eluted glioma peptides (Liau, Prins et al. 2005). In this study, the authors reported a median overall survival of 23.4 months and that the benefit of the vaccine treatment was more evident in the subgroup of patients with slowly-progressing tumors and in those with tumors expressing low levels of TGF-β2.

However, pulsing DCs with eluted peptides requires a large culture of autologous glioma cells and time-consuming procedures, for which QC/QA is not always feasible.

To overcome this issue, glioma cell lysate has been used to pulse DCs in a number of trials (Yamanaka, Homma et al. 2005; De Vleeschouwer, Fieuws et al. 2008; Wheeler, Black et al. 2008). Yamanaka *et al.* reported a phase I/II study using DC pulsed with glioma lysate (Yamanaka, Homma et al. 2005). Patients received either DCs matured with OK-432 or DCs without OK-432-mediated maturation. GBM patients receiving matured DCs presented longer survival than those receiving DCs without OK-432-mediated maturation. Furthermore, patients receiving both intratumoral and intradermal DC administrations demonstrated longer overall survival periods than those with intradermal administrations alone (Yamanaka, Homma et al. 2005). Wheeler *et al.* has reported a phase II clinical trial with lysate-pulsed DCs (Wheeler, Black et al. 2008). IFN-γ production levels from post-vaccine PBMC correlated significantly with patients' survival and time to progression. We have recently reported the results of a phase I/II clinical trial using α-type-1 polarized DCs (αDC1) cells loaded with GAA-derived peptides in combination with the adjuvant poly-ICLC. The regime was safe, well tolerated and a type-1 skewed CD8 T-cell response was observed in patients following treatment (Okada, Kalinski et al. 2011).

Several pilot and phase I/II clinical studies of active vaccination have been undertaken in patients with glioma. Despite the fact that feasibility and safety have been sufficiently documented in most studies, clinical efficacy has not yet been convincingly proven. Although some studies demonstrated improved survival of patients and objective clinical responses, the ultimate judgment for clinical activity has to be made by rigorous evaluation in randomized studies.

1.1.5 Immunity

A large number of observations suggest that certain types of tumor microenvironment immune cells are not innocent bystanders at brain tumor sites, and that they actively promote or target tumor development and progression. Inflammatory cells, primarily macrophages/microglia, MDSCs and regulatory T-cells, may affect these processes via their ability to express a large variety of factors, including immunoregulatory cytokines. These cytokines may be secreted not only by inflammatory cells, but also by the tumor and stroma cells, together establishing a network of factors that significantly affects brain tumor.

1.1.5.1 Regulatory T-Cells (Treg)

The suppressive activity of Tregs has been implicated as an important factor limiting immune-mediated destruction of tumor cells. An increased $FoxP3^+$ Treg to $CD4^+$ T-cells ratio correlates with impairment of $CD4^+$ T-cell proliferation in peripheral blood specimens obtained from patients with GBM (Fecci, Mitchell et al. 2006). In this referenced study, *in vivo* depletion of Tregs led to glioma rejection in murine model systems. Other studies have shown that an immunosuppressive population of Tregs is present within the GBM microenvironment (Hussain, Yang et al. 2006; Andaloussi, Han et al. 2008). Moreover, it has been demonstrated that Tregs are not present in normal brain tissue and were very rarely found in low-grade gliomas and oligodendrogliomas (Heimberger, Abou-Ghazal et al. 2008). The same study also observed that Treg infiltration differed significantly in the tumors according to lineage, pathology, and grade.

Tregs seemed to have the highest predilection for gliomas of the astrocytic lineage (over oligodendroglioma) and specifically in the high-grade gliomas, such as GBM. In both univariate and multivariate analysis, the presence of Tregs in GBMs seemed to be prognostically neutral (Heimberger, Abou-Ghazal et al. 2008). However, in a study with a mouse GL261 glioma model (Mahaley, Bigner et al. 1983), treatment of glioma-bearing mice with anti-CD25 mAb delayed the tumor growth and prolonged the survival of mice, suggesting that CD4+CD25+ Treg cells play an important role in suppressing the immune response to CNS tumors (Andaloussi, Han et al. 2008).

Furthermore, Grauer *et al*. demonstrated a time-dependent accumulation of $CD4^+FoxP3^+$ Treg in brain tumors with syngeneic murine glioma GL261 model (Grauer, Nierkens et al. 2007). They observed that the expression of CD25, CTLA-4, GITR and CXCR4 on intratumoral $CD4^+FoxP3^+$ Treg during tumor growth is up-regulated in a time-dependent manner. They also demonstrate that treatment with anti-CD25 mAbs significantly provokes a CD4 and CD8 T-cell dependent destruction of the glioma cells. Moreover, combining Treg depletion with administration of blocking CTLA-4 mAbs further boosted glioma specific $CD4^+$ and $CD8^+$ effector T-cells as well as antiglioma IgG2a antibody titers resulting in complete tumor eradication. This study illustrated that intratumoral accumulation and activation of $CD4^+FoxP3^+$ Treg act as a dominant immune escape mechanism for gliomas and underline the importance of controlling tumor-infiltrating Treg in glioma immunotherapy (Grauer, Nierkens et al. 2007).

1.1.5.2 Macrophages/Microglia

In the CNS, macrophages/microglial cells constitute the first line of cellular defense against a variety of stressors, participating in the regulation of innate and adaptive immune responses in human and rat gliomas (Badie and Schartner 2001). Many human gliomas exhibit prominent macrophage/microglia infiltration. Glioma infiltrating macrophages (GIM) can account for as much as 30% of the tumor mass (Giometto, Bozza et al. 1996). GIM represent the largest subpopulation infiltrating human gliomas from postoperative tissue specimens of glioma patients (Hussain, Yang et al. 2006).

With regard to distinction between resident microglia ($CD11b^{+}/CD45^{dim}$) versus macrophages ($CD11b^{+}/CD45^{high}$), most studies have demonstrated glioma infiltrating $CD11b^{+}$ cells are mostly $CD45^{high}$ macrophages (Ford, Goodsall et al. 1995). Indeed, it has been described that “microglia” in human gliomas appears mostly amoeboid and morphologically distinct from the resting microglia present in the intact brain (Graeber, Scheithauer et al. 2002). Intratumoural macrophage/microglia density is higher than in normal brain and abundance of microglia correlates with the grade of malignancy. In patients with gliomas, the number of macrophages in GBM (grade IV) is higher than that in grade II or III gliomas, and it is closely correlated with vascular density in the tumors (Nishie, Ono et al. 1999; Nishie, Masuda et al. 2001).

It is postulated that the defense functions of macrophage/microglia against glioma are compromised in the tumor microenvironment. These GIM expressed substantial levels of Toll-like receptors (TLRs), which are critical components for antigen presenting cells to mediate innate immune responses to any infectious or traumatic challenge and activating adaptive immune responses. However, GIM did not appear

stimulated to produce pro-inflammatory cytokines (TNF-α, IL-1, or IL-6), and *in vitro*, lipopolysaccharides could bind TLR-4 but could not induce GIM-mediated T-cell proliferation (Nishie, Ono et al. 1999). Moreover, it has been found that these GIM, in addition to decreased surface expression of MHC class II (Schartner, Hagar et al. 2005), lack expression of the costimulatory molecules CD86, CD80, and CD40 critical for T-cell activation, thereby unable to activate T-cells properly *ex vivo* (Hussain, Yang et al. 2006).

Macrophages/microglia can release many factors, including extracellular matrix proteases and cytokines, which may directly or indirectly influence tumor migration/invasiveness and proliferation (Platten, Kretz et al. 2003; Watters, Schartner et al. 2005). In Boyden chamber assays, glioma cell migration is stimulated by the presence of macrophage/microglia or macrophage/microglia-conditioned medium (Bettinger, Thanos et al. 2002). It has been recently demonstrated in an organotypic brain culture that the invasive potential of GBM was lower in macrophage/microglia-depleted slices and addition of microglial cells to microglia-depleted slices restored the invasiveness (Markovic, Glass et al. 2005). These findings suggest that macrophage/microglia in human gliomas may promote and support the invasive phenotype of these tumors.

1.2 MYELOID DERIVED SUPPRESSOR CELLS (MDSCS)

MDSCs represent a heterogenic population of immature myeloid cells (IMCs) that consists of myeloid progenitors and precursors of macrophages, granulocytes, and dendritic cells and has a strong ability to suppress a variety of T-cell and NK cell functions (Gabrilovich and Nagaraj 2009; Hoechst, Voigtlaender et al. 2009; Condamine and Gabrilovich 2011; Gabrilovich, Ostrand-Rosenberg et al. 2012)

In mice, MDSCs are identified as cells that simultaneously express the two markers CD11b and Gr-1 (Gabrilovich and Nagaraj 2009; Fujita, Scheurer et al. 2010; Fujita, Kohanbash et al. 2011). Recently, MDSCs were subdivided into two different subsets based on their expression of the two molecules Ly6C and Ly6G, both of which react with anti-Gr1 mAb RB6-8C5 (Hestdal, Ruscetti et al. 1991; Youn, Nagaraj et al. 2008). $CD11b^{+}Ly\text{-}6G^{-}Ly6C^{high}$ cells have monocytic-like morphology and are termed monocytic-MDSCs (M-MDSCs). $CD11b^{+}Ly6G^{+}Ly6C^{low}$ cells have granulocyte-like morphology and are termed granulocytic-MDSCs (G-MDSCs).

In cancer patients, MDSCs are defined as cells that express the common myeloid marker CD33 but lack markers of mature myeloid cells, such as the MHC class II molecule HLA-DR (Almand, Clark et al. 2001; Schmielau and Finn 2001; Zea, Rodriguez et al. 2005; Filipazzi, Valenti et al. 2007; Hoechst, Ormandy et al. 2008). Expression of the granulocytic marker CD15 divides patient MDSCs into at least two subsets that likely parallel those in the mouse model, such that the $CD15^{+}$ (human) and the $Gr1^{hi}$ (mouse) MDSC are G-MDSCs, and the $CD15^{-}$ (human) and the $Gr1^{-/lo}$ (mouse) MDSC are M-MDSCs(Movahedi, Guilliams et al. 2008). M-MDSCs with the

phenotype $CD14^{+}CD11b^{+}HLA\text{-}DR^{low/neg}$ have been detected in melanoma patients (Filipazzi, Valenti et al. 2007; Poschke, Mougiakakos et al. 2010). In patients with melanoma or colon carcinoma, the two main subpopulations, $CD14^{+}$ monocytes and $CD15^{+}$ neutrophils, both of which express IL-4 receptor-α (CD124), functionally suppress immune responses (Mandruzzato, Solito et al. 2009). MDSCs are also defined as $CD11b^{+}CD14^{-}CD15^{+}CD33^{+}$ cells in patients with advanced non-small cell lung cancer (Srivastava, Bosch et al. 2008; Liu, Wang et al. 2010).

MDSCs utilize a number of mechanisms to suppress T-cell function, including a high level of arginase activity as well as the production of nitric oxide (NO) and reactive oxygen species (ROS) (Gabrilovich and Nagaraj 2009; Condamine and Gabrilovich 2011). These main pathways are linked with different subsets of MDSCs: ROS with G-MDSCs, and arginase and NO with M-MDSCs (Movahedi, Guilliams et al. 2008; Youn, Nagaraj et al. 2008; Youn and Gabrilovich 2010). Several other suppressive mechanisms have recently been suggested: secretion of TGF-β (Yang, Huang et al. 2008; Li, Han et al. 2009), induction of regulatory T-cells (Huang and et al. 2006; Serafini, Mgebroff et al. 2008; Pan, Ma et al. 2010), depletion of cysteine (Srivastava, Sinha et al. 2010), and up-regulation of COX-2 and prostaglandin E2 (PGE2) (Rodriguez, Hernandez et al. 2005).

1.2.1 MDSCs in Glioma

We have reported substantial numbers of glioma-infiltrating $CD11b^{+}Gr1^{+}$ cells in both *de novo* (Fujita, Scheurer et al. 2010; Fujita, Kohanbash et al. 2011) and transplantable syngeneic GL261 cell line mouse models (Zhu, Fujita et al. 2011), depletion of which can inhibit the development of gliomas. In humans, healthy donor-derived human $CD14^{+}$ monocytes exposed to glioma cells acquire MDSC-like properties, including increased production of immunosuppressive IL-10, TGF-β, and B7-H1 and the increased ability to induce apoptosis in activated lymphocytes (Rodrigues, Gonzalez et al. 2010). Patients with GBM have increased circulating $CD33^{+}HLA\text{-}DR^{-}$ MDSC in peripheral blood compared with healthy donors (Rodrigues, Gonzalez et al. 2010; Raychaudhuri, Rayman et al. 2011). Furthermore, significant increases in arginase 1 activity and G-CSF levels were observed in plasma specimens obtained from patients with GBM (Raychaudhuri, Rayman et al. 2011; Sippel, White et al. 2011). Interestingly, T-cell suppression in GBM was completely reversed through the pharmacologic inhibition of arginase 1 or with arginine supplementation (Sippel, White et al. 2011). Several studies have demonstrated that human glioma-infiltrating macrophages express CD45 and MHC Class I and II but lack CD14, suggesting that the cells are immature inflammatory antigen-presenting cells from circulation rather than microglia, a phenotype similar to what is observed in rodent models (Ford, Goodsall et al. 1995; Hussain, Yang et al. 2006; Parney, Waldron et al. 2009; Kees, Lohr et al. 2012).

Although GBM are highly infiltrated by monocytes/macrophages, there are no published studies on human glioma-infiltrating MDSC to date, thus warranting further studies into human GBM-associated MDSCs in the tumor microenvironment.

1.2.2 Translational Approaches Targeting MDSCs in Gliomas

As we understand more about the biological mechanisms underlying the MDSC-mediated immunosuppression, the list of pharmacologic agents that can antagonize the MDSC effects expands. Those can be directed against a variety of MDSC properties: blockade of MDSC generation, differentiation of these cells into mature myeloid cells which lack immunosuppressive properties, and targeting individual suppressive features. Especially in combination with glioma immunotherapies such as vaccination, targeting either MDSCs or their suppressive molecules may allow for enhanced anti-tumor immunity and better clinical outcomes.

1.2.2.1 Direct MDSC Depletion

In murine glioma models, antibody-mediated depletion of MDSCs is often achieved with the anti-Gr-1 mAb RB6-8C5 (Fujita, Scheurer et al. 2010; Fujita, Kohanbash et al. 2011), which can deplete both G-MDSCs and M-MDSCs in the brain. While the ability of the antibodies to cross the blood brain barrier may be limited, as MDSCs are known to be recruited from bone marrow and systemic circulation with a relatively rapid turnover rate (Tadmor, Attias et al. 2011), antibody-based approaches may have a potential to block further MDSC infiltration of the brain. However, a human antibody-mediated

depletion of MDSCs is challenging as human MDSCs demonstrate significant heterogeneity and not a single unique MDSC marker has been identified. CD33 for example is found on other myeloid cell but is also present on human activated T and NK cells (Hernandez-Caselles, Martinez-Esparza et al. 2006), thus its use would be self-defeating as it would also target anti-tumor immune cells. For example, Gemtuzabam, a toxin conjugated anti-CD33 mAb previously used for treating AML, has been withdrawn from the market in the United States due to adverse events such as myelo-suppression and possible hypersensitivity reactions, (Sievers, Larson et al. 2001; Hanbali, Wollner et al. 2007).

A tumor-associated receptor tyrosine kinase, vascular endothelial growth factor receptor (VEGFR) signaling has been implicated in MDSC generation (Ko, Zea et al. 2009). A receptor tyrosine kinase inhibitor (RTKI) Sunitinib reverses M- and G-MDSC-mediated immunosuppression (Ko, Zea et al. 2009) and their accumulation in tumor-bearing mice (Ko, Rayman et al. 2010) and G-MDSC accumulation renal cell carcinoma patients (Ko, Zea et al. 2009). While many chemotherapeutics cause lymphopenia, Sunitinib promotes type-1 immunity without interfering with the induction of antigen specific T-cells and also reduces the immune suppressive T-regulatory cells (Tregs) (Finke, Rini et al. 2008; Hipp, Hilf et al. 2008). Although Sunitinib may have no direct anti-tumor effect on recurrent malignant gliomas (Reardon, Vredenburgh et al. 2011), Sunitinib may be used solely for its ability to promote anti-tumor immunity. Another RTKI Sorafenib has also been shown to reduce MDSCs (Cao, Xu et al. 2011). However, Sorafenib can also reduce the induction of antigen specific anti-tumor T-cells (Hipp, Hilf et al. 2008). Thus, more research is warranted to identify the most suitable

chemotherapeutic agents and/or RTKIs, which can reduce MDSCs and promote tumor antigen specific T-cell induction as well as direct anti-tumor affects. Separately, it is important to note that signal transducer and activator of transcription (STAT)3, an important signaling molecule for MDSC development (Wu, Du et al. 2011), might be targeted by inhibitors such as miRNAs to block MDSC generation and/or function (Kohanbash and Okada 2012).

One of the surface markers commonly found on MDSCs is the IL-4R-α chain (IL-4Rα). IL-4Rα mediates the signaling of both IL-4 and IL-13 and is important for the suppressive activity of MDSCs mediated by arginase and TGF-β (Terabe, Matsui et al. 2003; Highfill, Rodriguez et al. 2010). A recent study demonstrated that RNA aptamer-mediated blocking of IL-4Rα on MDSCs resulted in MDSC apoptosis through suppression of STAT6 signaling (Roth, De La Fuente et al. 2012). Importantly, while the IL-4Rα aptamer could bind to both M- and G-MDSCs, the aptamer displayed preferential binding to M-MDSCs compared to G-MDSCs (Roth, De La Fuente et al. 2012). Furthermore, aptamer-mediated blocking of IL-4Rα was associated with an increased number of tumor-infiltrating T-cells and suppressed growth of 4T1 mammary carcinoma (Roth, De La Fuente et al. 2012). Thus, blockade of IL-4Rα may provide a mechanism by which MDSCs can be depleted in glioma patients.

1.2.2.2 Promotion of MDSC Maturation

Since MDSCs are immature cells, multiple studies have attempted to promote MDSC maturation in which they lose their suppressive phenotype. This strategy may be effective to promote the transition of MDSCs from suppressive cells into cells that

contribute to anti-tumor immunity. Pak and colleagues demonstrated that treatment of mice with low dose IFN-γ and TNF-α could promote maturation of granulocyte macrophage suppressive progenitor cells into mature macrophages which lost the ability to suppress T-cells (Pak, Ip et al. 1995). Following maturation of these progenitor cells, intratumoral T-cells had an increased cytolytic capacity toward autologous tumor cells and an increased capacity to proliferate and secrete IL-2, leading to reduced growth and metastasis of murine metastatic Lewis lung carcinoma cell line (Pak, Ip et al. 1995).

Type I interferons (IFNs) have been extensively evaluated for their anti-tumor effects. Recent studies demonstrate that hematopoietic cells in the host (rather than tumor cells) are the crucial mediators of the antitumor activity elicited by endogenous type I IFNs (Dunn, Koebel et al. 2006; Hervas-Stubbs, Perez-Gracia et al. 2011). The TLR9 agonist CpG can promote a type I IFN, IFN-α to force the maturation of $Ly6G^{hi}$ MDSCs into plasmacytoid dendritic cells and promote anti-tumor immunity (Zoglmeier, Bauer et al. 2011). This has particular relevance to gliomas as we have demonstrated increased MDSCs in IFN-alpha deficient (*Ifna*$^{-/-}$) mice using a *de novo* glioma model, and that glioma patients with the SNP rs12553612 in *IFNA8* promoter is associated with altered *IFNA8* promoter activity and survival of glioma patients (Fujita, Scheurer et al. 2010; Kohanbash, Ishikawa et al. 2012). These studies demonstrate the relevance of IFN-α-mediated MDSC differentiation for gliomas. Similarly, the adjuvant polyinosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose (poly-ICLC) which has been shown to be safe and effective in glioma patients (Okada, Kalinski et al. 2011) is

also a potent inducer of type I IFNs (Zhu, Nishimura et al. 2007) and may reduce MDSCs.

Curcumin, the major component found in turmeric, can directly reduce survival of human glioma cell lines (U87MG, T98G and T67) and sensitize them to both chemotherapeutic reagents and radiation (Dhandapani, Mahesh et al. 2007). Further, athymic mice bearing intracerebral U87 glioma xenografts receiving curcumin experience a decrease in angiogenesis and better survival (Perry, Demeule et al. 2010). Using an *in vitro* BBB model curcumin can efficiently cross the BBB (Perry, Demeule et al. 2010). More recently curcumin has been shown to inhibit STAT3 and NF-κB activation in MDSCs (Tu, Jin et al. 2012). Curcumin treatment also decreases MDSC expansion and promotes the MDSC differentiation into M-1 macrophage phenotype (Tu, Jin et al. 2012), which are known to kill tumor cells and produce copious amounts of pro-inflammatory cytokines (Mantovani, Sozzani et al. 2002). Thus, glioma patients may be administered curcumin to reduce MDSCs and promote anti-tumor immunity.

Other promising approaches for the therapeutic targeting of MDSC development are anti-inflammatory therapies, since pro-inflammatory cytokines such as IL-1β and IL-6 are frequently present in the tumor microenvironment and promote MDSC accumulation (Ostrand-Rosenberg and Sinha 2009; Ostrand-Rosenberg 2010). The reduction of inflammation through the use of the naturally occurring IL-1 receptor antagonist, IL-1 receptor blockade, or PGE2 blockade (Gabrilovich, Ostrand-Rosenberg et al. 2012) can reverse MDSC development and accumulation. Additionally, as G-CSF can promote MDSC development, particularly G-MDSCs, a blockade of G-CSF remains a possible therapeutic target (Waight, Hu et al. 2011; Luyckx, Schouppe et al. 2012).

1.2.3 Translational Approaches Blocking MDSC-Associated Suppressor Molecules

Although completely ridding the body of MDSCs may seem most effective, strategies to reduce individual components may be more feasible and more controlled. Further, MDSC in different tumors have a variety of phenotypes, thus direct targeting of specific pathways may be more appropriate than less targeted approaches. In the next section, we discuss suppression of individual MDSC-mediated immune suppressive molecules.

1.2.3.1 Arginase inhibitors or arginine supplementation and gliomas

Arginase-1 can be secreted by MDSCs directly into the tumor microenvironment or the serum of tumor-bearing mice and deplete l-arginine (Gabrilovich, Ostrand-Rosenberg et al. 2012). Further, MDSCs express the cationic amino acid transporter CAT-2B, which allows them to sequester and deplete their microenvironment of arginine (Ochoa, Zea et al. 2007; Gabrilovich, Ostrand-Rosenberg et al. 2012). Depletion of arginine in the tumor microenvironment leads to suppression of T-cells through inhibition of the CD3-zeta chain and IL-2 production, resulting in T-cell apoptosis (Rodriguez, Zea et al. 2003; Sica and Bronte 2007; Klink, Kielbik et al. 2012). There are multiple compounds which can suppress arginase, including N^{w}-hydroxy-nor-arginine (nor-Noha), 2(S)-amino-6-boronohexanoic acid (ABH), (S)-(2-Boronoethyl)-L-cysteine(BEC), and DL-alfa-Difluoromethylornithine (DFMO) (Baggio, Emig et al. 1999; Berkowitz, White et al. 2003; Santhanam, Christianson et al. 2008). While there are some reports of arginase inhibitors used *in vivo* in animal studies (Reviewed in (Morris 2009)), future studies are

warranted to evaluate the feasibility of these compounds in patients. Alternatively, l-arginine supplementation may be used to overcome the arginase-mediated arginine depletion. L-arginine supplements are relatively cost effective and can be purchases as an over-the-counter supplement. When arginine was supplemented to T-cells which were attenuated by glioma patient-derived $CD11b^+$ cells (Sippel, White et al. 2011), it completely reversed the suppression and restored the T-cells' ability to produce IFN-γ (Sippel, White et al. 2011). Lastly, CAT-2B may be targeted by inhibitors, such as lysine (Yang, Ma et al. 2002) to prevent MDSC uptake of arginine, blocking the ability of MDSC-intracellular arginase. Despite the fact that arginase may have some direct anti-tumor properties (Lam, Wong et al. 2011), the use of arginase inhibitors in glioma patients may provide enhanced anti-tumor immunity as a monotherapy or in combination with immunotherapies. Future studies in patients are warranted to establish such benefits.

1.2.3.2 COX-2 inhibitors

Recent epidemiological studies have suggested associations between the regular use of nonsteroidal anti-inflammatory drugs (NSAIDs) and reduced glioma risks in humans (Sivak-Sears, Schwartzbaum et al. 2004; Scheurer, El-Zein et al. 2008). Significantly, host MDSCs have receptors for PGE2, which induces the differentiation of $Gr\text{-}1^+CD11b^+$ MDSC from bone marrow stem cells (Sinha, Clements et al. 2007). Treatment of tumor-bearing mice with the COX-2 inhibitor, SC58236, delayed primary tumor growth and reduced MDSC accumulation (Sinha, Clements et al. 2007). Furthermore, PGE2 produced by the tumor induced arginase I and CAT-2B (the arginase transporter) in

MDSC, both of which lead to depletion of arginine from the tumor microenvironment and impaired T-cell signal transduction and function (Rodriguez, Quiceno et al. 2007). MDSC derived from mice bearing GL261 murine glioma suppress the proliferation of activated splenic $CD8^+$ T-cells (Umemura, Saio et al. 2008). With regard to the role of glucocorticoids (GC), which are often used in clinical management of patients with glioma, GC-treated murine monocytes not only demonstrated immunosuppressive effects, but also they were $CD11b^+Gr\text{-}1^+$ (Varga, Ehrchen et al. 2008). We recently demonstrated that deletion of *Cox2* or treatment with acetylsalicylic acid or celecoxib decreased tumor-infiltration of G-MDSC in the *SB*-induced *de novo* glioma model (Fujita, Kohanbash et al. 2011). Hence, COX-2 blockade may promote the tumor-immune surveillance by reducing the induction of MDSC.

These data provide strong rationale for the use of COX-2 inhibitors for blockade of MDSC activity. In high grade glioma patients, the use of COX-2 inhibitors have been investigated in combination with retinoids (Giglio and Levin 2004), low-dose chemotherapy (capecitabine or temozolomide) (Hau, Kunz-Schughart et al. 2007), or irinotecan (Reardon, Quinn et al. 2005) , and these regimens were well-tolerated. Celecoxib has been approved by the US food and drug administration (FDA) to prevent development of colon cancers in patients with familial adenomatous polyposis as a premalignant condition, while celecoxib increases cardiovascular risk (Solomon, Wittes et al. 2008) and gastrointestinal hemorrhage (Fidler, Argiris et al. 2008). Although aforementioned celecoxib-containing regime only demonstrated modest therapeutic activity in patients with high grade glioma (Giglio and Levin 2004; Reardon, Quinn et al. 2005; Hau, Kunz-Schughart et al. 2007), investigation of COX-2-inhibitors, such as

celecoxib, may be justified in low-grade glioma patients as they are at extremely high risks for recurrence with high grade glioma. Indeed, our recent study with *de novo* mouse gliomas demonstrated that these gliomas have low-grade glioma characteristics on day 21 and treatment of those with celecoxib lead to rejection or inhibition of glioma growth (Fujita, Kohanbash et al. 2011).

1.2.3.3 Antihistamines

Histamine is a biogenic amine that has well-defined roles in allergic responses and gastric acid secretion and has also been linked to the modulation of immune responses. For example, histamine has been shown to enhance T helper type 1 (T_H1) responses through the H_1 receptor and down-regulate both T_H1 and T_H2 responses through the H_2 receptor (Jutel, Watanabe et al. 2001). Furthermore, histamine is believed to have an immune regulatory function in myeloid cells (Elenkov, Webster et al. 1998; van der Pouw Kraan, Snijders et al. 1998). Histamine can be taken up in the diet, but endogenous histamine is generated through the conversion of L-histidine to histamine by the action of a unique enzyme, histidine decarboxylase (HDC). Although mast cells are known to store histamine, recent studies have suggested that other types of myeloid cells may be key sources of histamine production (Zwadlo-Klarwasser, Vogts et al. 1998; Higuchi, Tanimoto et al. 2001; Sasaguri, Wang et al. 2005). In mouse models of atherosclerosis, HDC-expressing myeloid cells are primarily bone marrow-derived and appear more like monocytic precursors rather than mature macrophages (Sasaguri, Wang et al. 2005). Knockout of the *Hdc* gene has been reported (Ohtsu, Tanaka et al. 2001; Ohtsu and Watanabe 2003; Ercan-Sencicek, Stillman et al. 2010), but little is

known regarding the role of histamine in carcinogenesis. Individuals with atopic allergy and excessive histamine release are reported to have a reduced incidence of cancer, but patients who chronically use anti-histamines rather have increased glioma risks (Scheurer, Amirian et al. 2010). However, the mechanism underlying these observations remains unclear (Prizment, Folsom et al. 2007; Vajdic, Falster et al. 2009).

Wang's group recently showed that *Hdc* is primarily expressed in $CD11b^{+}Ly6G^{+}$ immature myeloid cells (IMCs) within the bone marrow, where histamine promotes myeloid cell differentiation, thus suppressing carcinogenesis (Yang, Ai et al. 2011). In this study, *Hdc*-knockout mice had a high rate of colon and skin cancer. Using *Hdc-EGFP* bacterial artificial chromosome transgenic mice in which EGFP expression is controlled by the *Hdc* promoter, they showed that *Hdc* is expressed primarily in $CD11b^{+}Ly6G^{+}$ IMCs that are recruited early on in chemical carcinogenesis. Transplant of *Hdc*-deficient bone marrow to wild-type recipients results in increased $CD11b^{+}Ly6G^{+}$ cell mobilization and reproduced the cancer susceptibility phenotype seen in *Hdc*-knockout mice. In addition, *Hdc*-deficient IMCs promoted the growth of tumor allografts, whereas mouse CT26 colon cancer cells down-regulated *Hdc* expression through promoter hypermethylation and inhibited myeloid cell maturation. Exogenous histamine induced the differentiation of IMCs and suppressed their ability to support the growth of tumor allografts. These data indicate key roles for *Hdc* and histamine in myeloid cell differentiation and $CD11b^{+}Ly6G^{+}$ IMCs in early cancer development. Thus the use of anti-histamines may be used to enhance anti-tumor immunity though the reduction of IMCs and/or MDSCs.

1.2.3.4 TGF-β regulation

TGF-β is produced by GBM cells, Tregs and MDSCs and can both promote tumor growth and suppress anti-tumor immunity (Uhm, Kettering et al. 1993; Bierie and Moses 2010). The anti-mouse TGFβ1, 2 and 3 antibody 1D11 can cross-react with bovine, chicken, mouse, and human TGF-β. Further Cat-192 a human anti-TGF-β1 mAb has been developed by Genzyme corporation however in a phase I/II clinical trial in patients with systemic sclerosis treatment with Cat-192 resulted in more serious adverse events than patients in the placebo control group (Denton, Merkel et al. 2007). We have demonstrated that TGF-β neutralization by 1D11 enhances the induction, persistence and IFN-γ production of antigen-specific CTLs in glioma-bearing mice. Neutralization of TGF-β also up-regulates plasma levels of IL-12, macrophage inflammatory protein-1α and CXCL-10, suggesting a promotion of type-1 immune response.

Suramin is a polysulfonated naphthyl urea originally developed as a treatment for African trypanosomiasis and later found useful in the treatment of onchocerciasis (Grossman, Phuphanich et al. 2001). Suramin has been shown to block TGF-β-binding to its receptor at clinically achievable concentrations (50-400 micrograms/ml) (Kloen, Jennings et al. 1994). One study examining human glioma xenografts in nude mice treated with suramin revealed a decrease in hypoxia, more abundant but decreased blood vessel size, and a decrease in tumor growth (Bernsen, Rijken et al. 1999). Despite the lack of immunity in the nude mouse model, the suramin mediated decrease in hypoxia and change in the tumor vasculature may allow for a more potent and viable anti-tumor immune response (Sun, Zhang et al. 2010). A clinical trial evaluating suramin in high grade recurrent glioma patients demonstrated achievable levels of suramin in

patients between 100-700 micrograms/ml with levels remaining at between 100 and 400 micrograms/ml 5 days after treatment (Grossman, Phuphanich et al. 2001). In this study, toxicity was modest and reversible, and positive response was observed in 3 patients (of 12 who were enrolled on the trial) with 2 patients achieving disease stabilization and survival of 16 and 27 months (Grossman, Phuphanich et al. 2001). Further, 1 patient had a marked reduction in tumor size and maintained a partial response for over 2 years without other therapy (Grossman, Phuphanich et al. 2001). In a phase II clinical trial administering suramin therapy in an intermittent fixed-dosing regimen during cranial RT was generally well tolerated. However no benefit in overall survival was observed compared with the New Approaches to Brain Tumor Therapy GBM database or other comparable patient population (Laterra, Grossman et al. 2004). Why only a subpopulation of patients responded warrants further studies, however such treatment may be more suitable and potent in patients with low-grade gliomas or in combination with immunotherapies.

Another class of molecules that can interfere with TGF-β signaling are the TGF-β receptor (TGFβR)-I kinase inhibitors, particularly SD-208 and SB-431542 (Hjelmeland, Hjelmeland et al. 2004; Uhl, Aulwurm et al. 2004; Halder, Beauchamp et al. 2005). SB-431542 is a small molecule inhibitor with the ability to block the tumor-promoting effects of TGF-β, including cell motility, migration, invasion, and vascular endothelial growth factor (VEGF) secretion in human colon cancer cells (HT29) (Halder, Beauchamp et al. 2005). Further, treatment of human glioma cell lines, including U87MG cells, with SB-431542 blocks TGF-β-induced activity of SMAD as a transcription factor, VEGF expression and morphological changes in U87MG cells (Hjelmeland, Hjelmeland et al.

2004). Interestingly, SD-208, another TGFβR-1 kinase inhibitor, reduces SMAD2 phosphorylation in both the brain and spleen 3 days following oral administration. Further, systemic SD-208 treatment prolongs survival of mice challenged with SMA-560 glioma cells into the brains. Interestingly, SD-208 did not alter tumor cell growth or vascularization; rather, an increased tumor infiltration by natural killer cells, CD8 T-cells and macrophages was observed (Uhl, Aulwurm et al. 2004). Thus the confirmed bioavailability in the brain together with the ability to improve anti-tumor immunity supports the use for SD-208 for treatment of glioma patients.

1.2.3.5 MDSC attracting chemokines

Multiple chemokines can attract MDSCs toward the tumor. These include inflammatory chemokines S100A8, S100A9 and CCL-2. CCL-2, also known as macrophage chemoattracting protein (MCP)-1, was originally isolated from glioma cells (Yoshimura, Robinson et al. 1989). We have demonstrated that intraperitoneal injection of CCL-2 neutralizing antibodies into C57BL/6 murine GL261 glioma bearing mice or severe combined immunodeficiency (SCID) mice bearing intracranial human U87 glioma xenografts results in significantly prolonged symptom free survival compared to control treated animals(Zhu, Fujita et al. 2011). Further, brain infiltrating leukocytes from treated animals reveal a significant decrease of $CD11b^{+}Gr\text{-}1^{+}$ infiltrating cells.

The S100 family members S100A8 and S100A9 chemokines are expressed both by tumor cells and immune infiltrating cells (Gebhardt, Nemeth et al. 2006). In addition to forming homodimers, S100A8 and S100A9 often form heterodimers and heterotetramers, which may be important for their biological activity (Sparvero, Asafu-

Adjei et al. 2009). MDSCs are known to express S100A8 and S100A9 at high levels and have receptors for both molecules (Gabrilovich and Nagaraj 2009), suggesting that they can be recruited to a tumor and support additional MDSC recruitment to tumors. In a recent study of human $CD14^{+}HLA\text{-}DR^{-}$ MDSCs from colon cancer patients, Zhao and colleagues found these MDSC express S100A8, S100A9, and S100A12 (Zhao, Hoechst et al. 2012). Analysis of whole blood from colon cancer patients also demonstrated increased MDSCs compared to healthy donors. A couple of studies have assessed the role of S100 proteins and glioma MDSCs (Deininger, Pater et al. 2001; Murat, Migliavacca et al. 2009). In a gene expression set from 80 GBM patients, S100A8 and S100A9 expression levels were inversely associated with survival (Murat, Migliavacca et al. 2009). Further, in recurrent GBM tissues following radiation therapy, there are significantly more macrophages/microglial cells expressing S100A9 compared with recurrent GBM tissues without prior radiation therapy (Deininger, Pater et al. 2001). Thus, treatment courses may have a critical impact on MDSCs and their effector functions. Based on the limited data on S100 proteins and gliomas, more studies are warranted to determine if blockade of S100A8 and A9 can improve T-cell functioning and reduce MDSCs in glioma patients.

1.2.4 Summary

With a growing amount of supporting literature, strategies to overcome the detrimental impact of MDSC on anti-tumor immunity in glioma patients may serve as potential therapeutics **(Summarized in Table 1 and Figure 2)**. With direct mechanisms to

reduce MDSCs by depletion or forced maturation, as well as indirect interventions by targeting immune suppressive features of MDSCs available as potential therapies, further studies are warranted to elucidate the benefits and pitfalls of each strategy. The ideal therapy would not only reduce immune cell suppression but also promote type-1 immunity, reduce hypoxia, and have some direct tumor suppressing effects. In addition, multiple MDSC-related strategies may be combined for maximal effect, and these strategies may be used in combination with other immunotherapies to further enhance their response.

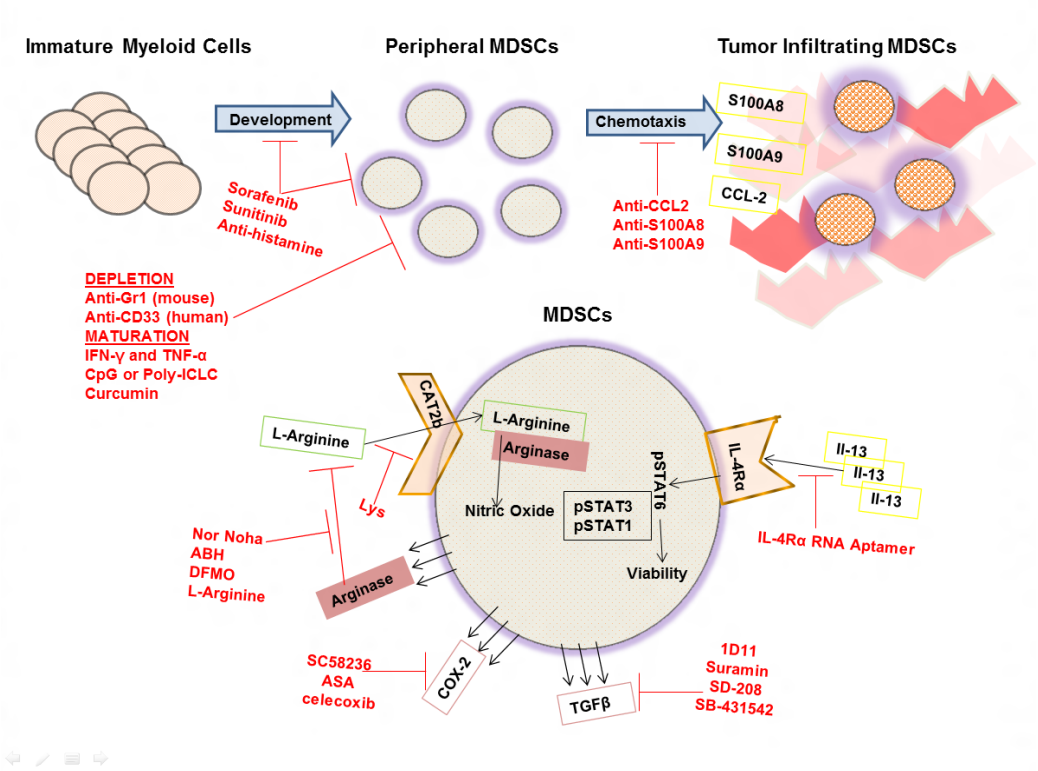


Figure 2: Strategies to Block MDSC Development and Function in Gliomas

Table 1: Summary of Mechanisms to Block MDSC-Mediated Suppression

Category	Function	Blocking agent/mechanism	References
MDSC Inhibition	Block MDSCs generation	Sunitinib, Sorafenib, IL-4Ra aptamer,	*Ko, et al. 2009, Cao, et al. 2011, Roth, et al. 2012*
MDSC Maturation	Mature MDSCs to cells with anti-tumor properties	IFN-γ and TNF-α, CpG or Poly-ICLC adjuvant, Curcumin	*Pak, et al. 1995, Zoglmeier, et al. 2011 ,Zhu, et al. 2007, Tu, et al. 2012*
Arginase	Depletes arginine necessary for T-cell growth and function	Arginase inhibitors (nor-Noha, ABH, DFMO), L-arginine supplementation, Lys (CAT-2B inhibitor)	*Baggio, et al. 1999, Sippel, et al. 2011, Yang, et al. 2002,*
COX-2	Prostaglandin E2 synthesis and MDSC recruitment from bone marrow	SC58236, Acetylsalicylic Acid (ASA), celecoxib	*Condamine, et al. 2010, Fujita, et al. 2011*
Histamine	Recruitment of immune suppressor cells such as MDSCs	Anti-histamines	*Scheurer et al 2010, Yang et al. 2011*
TGF-β	CTL and NK cell inhibition and Treg recruitment	1D11, Suramin, SD-208, SB-431542	*Ueda, et al. 2009, Grossman, et al. 2001, Halder, et al. 2005, Uhl, et al. 2004*
Chemokines	Chemotaxis of immunesuppressor cells to the tumor including MDSCs	Antibody mediated blockade of S100A8, S100A9 and CCL-2	*Zhu, et al. 2011, Murat, et al. 2009*

1.3 MICRORNA

1.3.1 MicroRNA Biology

MicroRNAs (miRNAs) are endogenous small single-stranded RNA molecules which are 18-24 nucleotides long. MiRNAs are highly conserved between species and have been identified in plants, animals and viruses (Carrington and Ambros 2003). These small RNA are located in various parts of the genome, usually in segments not associated with known genes. Mature miRNA molecules have the ability to repress translation and therefore serve an important role in regulating post transcriptional activities(Elmen, Lindow et al. 2008). There are predicted to be over 800 microRNAs in the human genome which are predicted to regulate 2/3 of all genes (Bentwich, Avniel et al. 2005; Hammond 2006; Ueda, Kohanbash et al. 2009).

1.3.2 MiRNA Processing and Function

Genes encoding miRNAs are transcribed by RNA polymerase II into long primary miRNA sequences (pri-miRNAs) with a 5’ cap, 3’ untranslated region (UTR), and a hairpin sequence that encodes the mature miRNA. The hairpin of the pri-miR is then cleaved by the enzyme Drosha to form precursor microRNAs (pre-miRNAs). Pre-miRNAs are then transported via Exportin V to the cytoplasm. Once in the cytoplasm dicer, an Rnase III superfamily member cleaves one of the strands and attaches the mature miRNA to an RNA-induced silencing complex (RISC). The full RISC complex

(miRNA and RISC) are then able to bind to 3' UTR regions of mRNAs, and inhibit translation. Translational inhibition may occur either through mRNA degradation or translational suppression. When there is complete complementarity of the miRNA to the mRNA 3' UTR, the mRNA is degraded, however, partial complementarity of the miRNA to the 3' UTR sequence results in inhibition of the circularization of the mRNA needed for ribosomal attachment(Ying and Lin 2009).

1.3.3 Previous Findings from my Master's Degree

Data generated from my master's thesis concluded that the miRNA cluster, miR-17-92 is expressed at higher levels in type-1 helper T-cells (Th1) cells compared with type-2 helper T-cells (Th2) cells. IL-4 suppresses miR-17-92 through the STAT6 signaling pathway. MiR-17-92 cluster expression levels in T-cells correlate with cell proliferation, and overexpression of miR-17-92 downregulates E2F1 and E2F2. We proposed a model in which IL-4 from Th2 cells or from the tumor environment (Roussel, Gingras et al. 1996) is able to decrease the proliferative ability of T-cells. However, more work is necessary to determine other biological effects of the miR-17-92 cluster, the effects of ectopic overexpression of miR-17-92 cluster in T-cells and the relevance to gliomas.

2.0 THESIS AIMS

Enhancing the host immunological response to tumors remains a challenge for glioma researchers. We have previously demonstrated the importance of type-1 T-cells for anti-glioma immunity. However cancers, including gliomas, secrete numerous type-2 cytokines that promote tumor proliferation and immune escape. The hallmark cytokines of type-1 and type-2 skewing are IFNs and IL-4, respectively. The overall goal of this study was to better understand the role of IFN and IL-4 signaling in glioma prognosis. Specifically we examine in the context of gliomas: (1) the role of IL-4R regulated miR-17-92 expression on T-cells **(Aim 1)** (2) the role of IL-4Rα on the suppressive activity of MDSCs **(Aim 2)** and (3) the beneficial effects of SNPs in type I IFN genes that are associated with glioma patient survival **(Aim 3)**.

2.1 SPECIFIC AIMS

Specific Aim #1 (miR-17-92 cluster in T-cells): To determine whether IL-4R signaling in the glioma microenvironment inhibits miR-17-92 expression in human and mouse glioma-infiltrating T-cells and suppresses T-cell function.

Hypothesis: We hypothesize that miR-17-92 expression is down-regulated in T-cells from human glioma patients and glioma bearing wild type (WT) but not Stat6 deficient mice and that ectopic expression of miR-17-92 in T-cells enhances their function.

Results: miR-17-92 cluster members are down-regulated in T-cells of glioma patients and in glioma-bearing WT but not Stat6 deficient mice. Ectopic expression of miR-17-92 in T-cells promotes IFN-γ, IL-2 and VLA4 expression and reduces activation induced cell death (AICD).

Specific Aim #2 (IL-4Rα on MDSCs): IL-4Rα enhances MDSC function and promotes glioma development.

Hypothesis: *Il4ra* deficient (*Il4ra*$^{-/-}$) mice exhibit prolonged survival following *de novo* glioma challenge and decreased MDSC expression of suppressive molecules.

Results: Human and mouse glioma up-regulates IL-4Rα expression on tumor infiltrating myeloid cells via GM-CSF. *Il4ra*$^{-/-}$ MDSCs have reduced arginase activity and suppressive activity on T-cells. *Il4ra*$^{-/-}$ mice challenged with de novo gliomas exhibit prolonged survival compared to WT mice.

Specific Aim #3 (SNP in IFNA8): To determine whether SNPs in the type I IFN genes associated with glioma prognosis impact downstream signaling and IFNA expression.

Hypothesis: A SNP in the promoter of a type I IFN gene that is associated with glioma prognosis impacts binding of transcription factors to the IFN promoter and reduces promoter activity.

Results: The A- genotype of the rs12553612 SNP in the *IFNA8* promoter which is associated with better survival of glioma patients enhances *IFNA8* activity and Oct-1 binding compared with the C-genotype of the rs1255612 SNP in the *IFNA8* promoter region.

3.0 AIM 1 BACKGROUND (MIR-17-92 CLUSTER IN T-CELLS)

MiRs in miR-17-92 cluster have been reported to be amplified in various tumor types, such as B-cell lymphoma and lung cancer, and are found to promote proliferation and confer anti-apoptotic function in tumors, thereby promoting tumor-progression (Hayashita, Osada et al. 2005; He, Thomson et al. 2005; Lawrie 2007; Matsubara, Takeuchi et al. 2007; Rinaldi, Poretti et al. 2007). Knockout and transgenic studies of the miR-17-92 cluster in mice have demonstrated the importance of this cluster in mammalian biology (Xiao and Rajewsky 2009). Transgenic mice with miR-17-92 overexpressed in lymphocytes develop lymphoproliferative disorder and autoimmunity but not cancer (Xiao, Srinivasan et al. 2008). These findings demonstrate a critical role for miR-17-92 cluster in T-cell biology.

During the course of my M.S degree I demonstrated that miR-17-92 cluster is up-regulated in Th1 cells compared with Th2 cells as determined by microRNA microarray **(not shown)** and by RT-PCR analysis **(Figure 3).** The down-regulation of miR-17-92 cluster was mediated by IL-4 signaling as blockade of IL-4 **(Figure 4A)** and T-cells from mice deficient of STAT6 **(Figure 4B)**, the major IL-4 receptor signaling molecule, blocked the down-regulation of miR-17-92 in Th2 cells. Furthermore, miR-17-92 expression correlated with the proliferation of T-cells **(Figure 5)**.

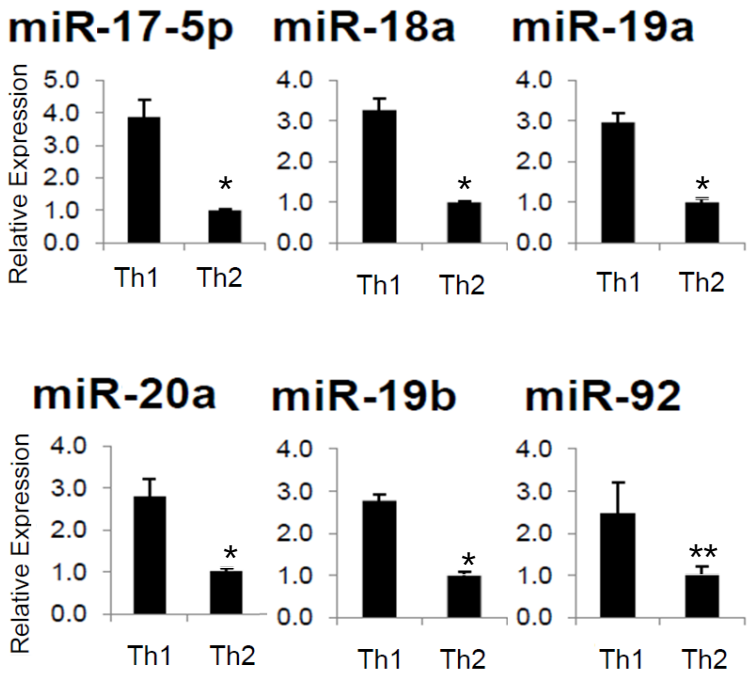


Figure 3: RT-PCR analysis of all miRs in the miR-17-92 cluster. Data represent relative expression of mature miRs in Th1 compared with Th2 cells. SNO202 was used as the internal control and 2ΔΔCT method was used to examine expression relative to the Th2 cell value. Relative expression is shown for miR-17-92 cluster members. Error Bars indicate standard deviation of the triplicate samples. Each experiment was repeated at least 3 times. Up-regulation in Th1 vs. Th2 is significant in with $p<.01$ for miR-92 and $p<.0001$ for all other miRs.

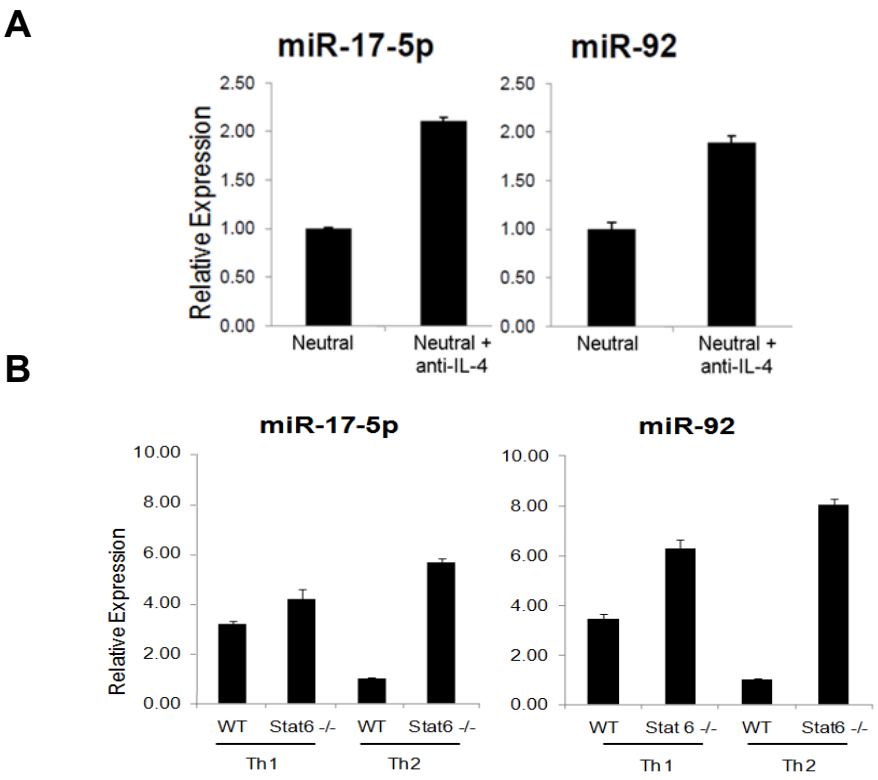


Figure 4: Down-regulation of miR-17-5p and miR-92 by IL-4 and STAT6. (A) Immuno-magnetically isolated mouse splenic CD4+ T-cells were cultured with 5 µg/ml plated anti-CD3, feeder cells and 100U/mL hIL-2 ("Neutral" condition). Anti-IL-4 (2.5 µg/ml) or isotype control mAb was added to the appropriate wells and cultured for 5 days prior to extraction of total RNA. Statistical analysis was carried out using the student t test. Blockade of IL-4 up-regulates MiR-17-5p and miR-92 significantly with $p<.001$ and $p<.005$, respectively. (B), Th1 and Th2 cells were induced from splenic CD4+ T-cells isolated from either wild-type or STAT6-/- mice. Total RNA was extracted and RT-PCR was performed using specific primers against miR-17-5p and miR-92. Data is representative from one of 2 two independent experiments with similar results, and error bars represent standard deviations. STAT6-/- cells demonstrated significantly higher levels of miR-17-5p and miR-92 compared with WT cells in both Th1 and Th2 conditions ($p<.001$) using the student t test.

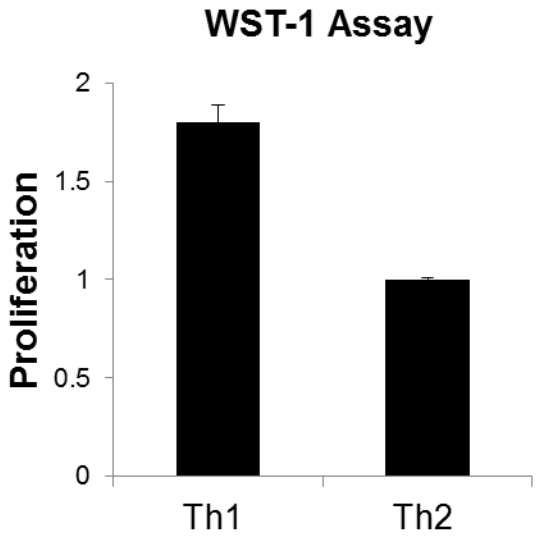


Figure 5: WST-1 Assay of Th1 and Th2 cultured cells. 1x 10^4 cells were cultured in a 96 well plate for 24-48 hours in 100ul of complete media. After this time 10ul of WST-1 reagent was added to each well. Cells were incubated at 370C, 5% CO2 for 4 hours, and placed on a shaker for 1 min. The plates were then read on a micro plate reader with a wavelength of 420 nm. Columns represent the mean of 2 separate Th1 and Th2 cultures, each run in quadruplicate; error bars represent standard deviation of all 8 samples. Statistical analysis was carried out on Graphpad prism using the student t test. Values are significant with a $p<.01$

Based on the findings from work completed during my M.S degree, we suggested the following mechanism **(Figure 6).** As SNPs in the *Il4ra* gene are associated with glioma prognosis further exploration of IL-4-regulated miR-17-92 cluster in T-cells in cancer conditions seemed relevant and could explain a possible mechanism for better survival of some patients. It remained unknown if miR-17-92 is down-regulated in cancer conditions and the effect of ectopic expression of miR-17-92 cluster in T-cells.

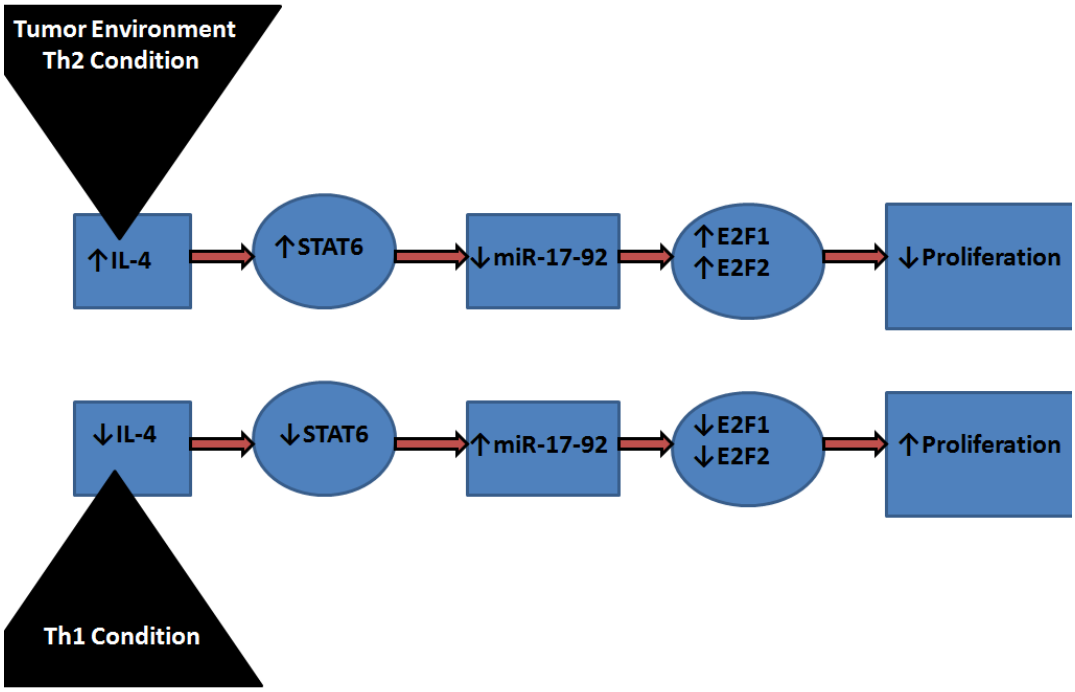


Figure 6: Model of miR-17-92 signaling pathway in T-cells. Based on our current data we propose that IL-4 from Th2 skewing conditions such as the tumor environment down-regulates miR-17-92 through the STAT6 pathway in T-cells. This down-regulation of mIR-17-92 results in up-regulation of anti-proliferative E2F1 and E2F2 molecules resulting in decreased proliferation relative to Th1. Conversely Th1 conditions lack activation of STAT6 and therefore have up-regulation of miR-17-92, decreased E2F1 and E2F2, and increase proliferation relative to Th2.

4.0 AIM 1 MATERIALS AND METHODS (MIR-17-92 CLUSTER IN T-CELLS)

4.1 REAGENTS

RPMI 1640, fetal bovine serum, sodium pyruvate, 2-mercaptoethanol, nonessential amino acids, and penicillin/streptomycin were obtained from Invitrogen Life Technologies. Recombinant murine (rm) IL-12 was purchased from Cell Sciences Technologies. RmIL-4, recombinant human (rh) IL-4 and rhIL-2 were purchased from PeproTech. Purified mAbs against IL-12 (C15.6), IFN-*γ* (R4–6A2), IL-4 (11B11), CD3 (145-2C11), CD4 (RM4-5), CD8 (53-6.7) and CD49d (R1-2) were all purchased from BD Pharmingen. Purified mAbs against CD3 (UCHT1) and CD28 (CD28.2) and IL-4 (MP4-25D2) were purchased from Biolegend. RT-PCR reagents and primers were purchased from Applied Biosystems and analyzed on a BioRad IQ5. WST-1 reagent was purchased from Roche. For isolation of T-cells, immunomagnetic isolation kits from Miltenyi Biotec were used. All reagents and vectors for lentiviral production were purchased from System Biosciences with the exception of Lipofectamine 2000, which was from Invitrogen.

4.2 MICE

C57BL/6 mice and C57BL/6 background STAT6 deficient mice (B6.129S2[C]-*Stat6*^{tm1Gru}/J; The Jackson lab) (both 5–9 wk of age) were purchased from The Jackson Laboratory. C57BL/6-background miR-17-92 transgenic (TG) mice (C57BL/6-*Gt[ROSA]26Sor*$^{tm3(CAG-MIRN17-92,-EGFP)Rsky}$/J; The Jackson Lab) were maintained in the Hillman Cancer Center Animal Facility at University of Pittsburgh as breeding colonies and bred to C57BL/6-background mice transgenic for *Cre* recombinase gene under the control of the *Lck* promoter (B6.Cg-Tg[Lck-cre]548Jxm/J, the Jackson Lab) to obtain mice, in which T-cells expressed miR-17-92 at high levels (miR-17-92 TG/TG). For mouse tumor experiments, C57BL/6 mice and C57BL/6 background *STAT6*$^{-/-}$ mice received subcutaneous injection of 1x 10^6 B16 tumor cells resuspended in PBS into the right flank. On day 15 following tumor inoculation, mice were sacrificed and splenic T-cells were isolated. Animals were handled in the Hillman Cancer Center Animal Facility at University of Pittsburgh per an Institutional Animal Care and Use Committee-approved protocol.

4.3 SUBCUTANEOUS TUMOR MODEL

B16 melanoma cells were harvested in the exponential growth phase by trypsinization, washed twice with ice-cold PBS and then resuspended in ice-cold PBS. C57BL/6

background WT or *Stat6*$^{-/-}$ mice were subcutaneous injected with B16 cells (2 x10^5 cells/mouse) on the right back flank. Mice were sacrificed on day 15 post-tumor cell injection.

4.4 T-CELLS FROM HEALTHY DONORS AND PATIENTS WITH GBM

This study was approved by the local ethical review board of University of Pittsburgh. All healthy donors and patients with GBM signed informed consent before blood samples were obtained. To determine the impact of IL-4, healthy donor-derived $CD4^+$ T-cells were isolated with immunomagentic-separation and stimulated with 100 IU/ml rhIL-2, anti-CD3 and anti-CD28 mAbs (1 µg/ml for each) in the presence or absence of rhIL-4 (10ng/ml). RT-PCR analyses were performed with both healthy donor- and patient-derived T-cells to determine the expression of miR-17-92 as described in the relevant section.

4.5 QUANTITATIVE RT-PCR

Total RNA was extracted using the Qiagen RNeasy kit and quality was confirmed with a A260/A280 ratio greater than 1.85. RNA was subjected to RT-PCR analysis using the TaqMan microRNA Reverse Transcription Kit, microRNA Assays (Applied Biosystems), and the Real-Time thermocycler iQ5 (Bio-Rad). The small nucleolar SNO202 was used as the housekeeping small RNA reference gene for all murine samples and RNU43 for human samples. All reactions were done in triplicate and relative expression of RNAs was calculated using the $\Delta\Delta C_T$ method (Livak and Schmittgen 2001).

4.6 ASSAYS USING JURKAT LYMPHOMA CELLS TRANSDUCED WITH MIR-17-92

Jurkat human T-cell lymphoma cells (American Type Culture Collection) were transduced by either one of the following pseudotype lentiviral vectors: 1) control vector encoding GFP; 2) the 17-92-1 expression vector encoding miR-17 18 and 19a, or 3) the 17-92-2 expression vector encoding miR 20, 19b-1, and 92a-1. All vectors were purchased from Systems Biosciences (SBI). Lentiviral particles were produced by co-transfecting confluent 293TN cells (SBI) with pPACK-H1 Lentivirus Packaging Kit (SBI) and the miR containing expression vectors (SBI) noted above using Lipofectamine 2000 reagent (Invitrogen). Supernatant was collected after 48 hour incubation at 37°C with 5% CO_2 and placed at 4°C with PEG-it Virus Concentration Solution (SBI) for 24 hrs.

Supernatants/PEG solutions were then centrifuged and the pellet was resuspended in a reduced volume of media as viral stock. Jurkat cells were further resuspended in the viral stock together with polybrene (8µg/ml) for 24 hrs. Fresh media was then added to the cells and transduction efficiency was evaluated by GFP expressing cells. For IL-2 production, transduced Jurkat cells were stimulated with Phorbol 12-myristate 13-acetate (PMA) (10ng/ml) and ionomycin (500nM) for overnight and supernatant was assayed for IL-2 by a human IL-2 ELISA kit. For activation induced cell death (AICD), cells were treated with 10 µg/ml purified anti-CD3 mAb (UCHT1) from Biolegend for 24 hours and then cell viability was measured using WST-1 reagent.

4.7 STATISTICAL METHODS

All statistical analyses were carried out on Graphpad Prism software. The statistical significance of differences between groups was determined using student t- test. We considered differences significant when $p < 0.05$.

5.0 AIM 1 RESULTS (MIR-17-92 CLUSTER IN T-CELLS)

5.1 SUPPRESSION OF MIR-17-92 MAY OCCUR IN CANCER-BEARING HOSTS

Data from our previous experiments **(Aim 1 background)** led us to hypothesize that suppression of miR-17-92 would occur in cancer-bearing hosts where tumor-derived factors likely promote Th2-skewed immune responses and secretion of IL-4(Roussel, Gingras et al. 1996). Indeed, $CD4^+$ and $CD8^+$ splenocytes (SPCs) derived from WT C57BL/6 mice bearing B16 subcutaneous tumors expressed lower levels of miR-17-5p when compared with those derived from non-tumor bearing mice (**Figure 7A**). Interestingly, the tumor bearing condition did not suppress miR-17-5p expression in $CD4^+$ T-cells from *Stat6*$^{-/-}$ mice. Furthermore, $CD8^+$ T-cells in *Stat6*$^{-/-}$ mice demonstrated enhanced levels of miR-17-5p expression when these mice bore B16 tumors when compared with non-tumor bearing mice. When WT $CD4^+$ T-cells were stimulated with anti-CD3 mAb *in vitro* for 24 hours, the $CD4^+$ T-cells from tumor-bearing mice produced lower levels of IFN-γ when compared with ones from non-tumor bearing WT mice (**Figure 7B**). Tumor-associated immunosuppression may involve the down-regulation of miR-17-92 through a STAT6-dependent pathway.

We next evaluated whether the observed IL-4-mediated and tumor-induced suppression of miR-17-92 are relevant in human T-cells. Healthy donor-derived $CD4^+$ T-

cells were stimulated with rhIL-2, anti-CD3 and anti-CD28 mAbs, consistent with the mouse data, addition of rhIL-4 in the cultures suppressed expression of miR-17-5p (**Figure 7C**). Moreover, $CD4^+$ T-cells obtained from patients with GBM exhibited significantly decreased levels of miR-17-5p when compared with ones from healthy donors (**Figure 7D**). Thus both IL-4 and GBM-bearing conditions suppress miR-17-5p expression in $CD4^+$ T-cells. Although not statistically significant, we observed a similar trend with $CD8^+$ T-cells demonstrating decreased levels of miR-17-5p expression in GBM patient-derived $CD8^+$ T-cells when compared with healthy donor-derived $CD8^+$ T-cells (not shown).

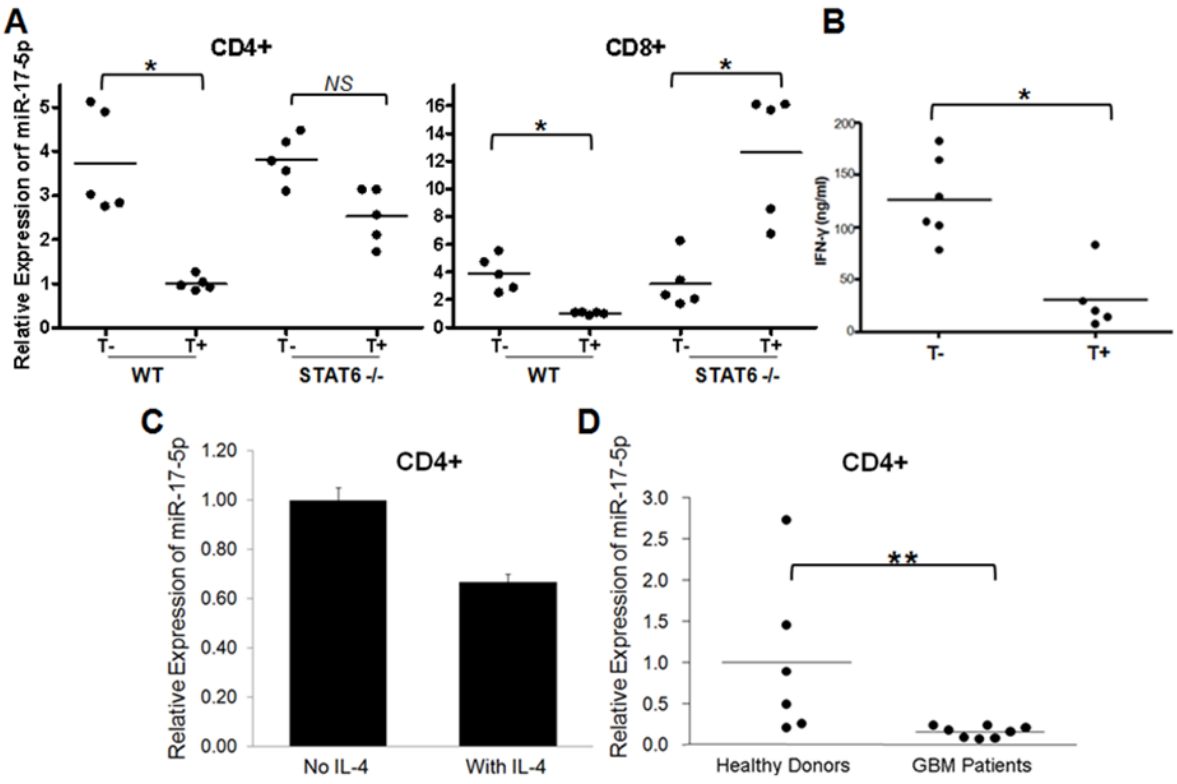


Figure 7: Tumor bearing conditions down-regulate miR-17-5p expression in T-cells. SPCs were harvested from C57BL/6 or *Stat6*$^{-/-}$ mice bearing day 15 subcutaneous B16 melanoma (T+) or control non-tumor bearing mice (T-). (A), CD4^{+} and CD8^{+} T-cells were isolated by immuno-magnetic bead separation, and evaluated for miR17-5p expression. (B), 1 x 10^{6} CD4^{+} cells from WT mice were briefly stimulated with anti-CD3 mAb for 6 hours. Concentration of IFN-γ secreted in culture media was evaluated by specific ELISA. (C), CD4^{+} T-cells were isolated from healthy donor-derived peripheral blood mononuclear cells (PBMC) and stimulated with 5 µg/ml plated anti-CD3, feeder cells (irradiated PBMC) and 100IU/ml hIL2 in the presence or absence of hIL-4 (10 ng/ml) for 5 days prior to extraction of total RNA. (D), Non-stimulated CD4^{+} T-cells were isolated by immuno-magnetic beads from PBMC derived from healthy donors (n=6) or patients with GBM (n=8) and miR-17-5p expression was analyzed by RT-PCR. Data in (A), (B) and (C), are representative of 2 identical experiments with similar results. Columns represent the mean of triplicates from a single experiment and error bars represent standard deviation. * indicates $p<0.01$ and ** indicates $p<0.05$ between the two groups using the student t test.

5.2 T-CELLS DERIVED FROM MIR-17-92 TRANSGENIC ANIMALS DISPLAY AN ENHANCED TYPE-1 PHENOTYPE

The data discussed above strongly suggest GBM-associated factors, and a type-2 promoting cytokine (IL-4) down-regulate miR-17-92 in T-cells. MiR-17-92 is expected to play pivotal roles in T cell functions. We therefore sought to determine whether ectopic expression of miR-17-92 would promote the type-1 phenotype of T-cells. As detailed in Materials and Methods, we produced mice that overexpress miR-17-92 specifically in T-cells (miR-17-92 TG/TG). We isolated $CD4^+$ SPCs from these mice and evaluated the expression of miR-17-5p (**Figure 8A**). $CD4^+$ cells from TG/TG mice displayed a >15 fold increase in miR-17-p5 expression as compared with controls. These cells also expressed elevated levels of CD49d, which is a subunit composing a type-1 T-cell marker VLA-4 (**Figure 8B**). Although CD49d (α4) can form heterodimers with both β1 (CD29) and β7 integrins, α4β7 complexes are not expressed by either Th1 cells or Th2 cells, suggesting that CD49d is a suitable surrogate for VLA-4 expression levels (Zhu, Nishimura et al. 2007; Sasaki, Zhao et al. 2008). MiR-17-92-TG/TG $CD4^+$ cells also demonstrated enhanced ability to produce IFN-γ upon stimulation (**Figure 8C**). Similar data were obtained with $CD8^+$ T-cells isolated from these TG/TG mice (data not shown). These findings suggest that miR-17-92 promotes the type-1 phenotype in differentiating T-cells.

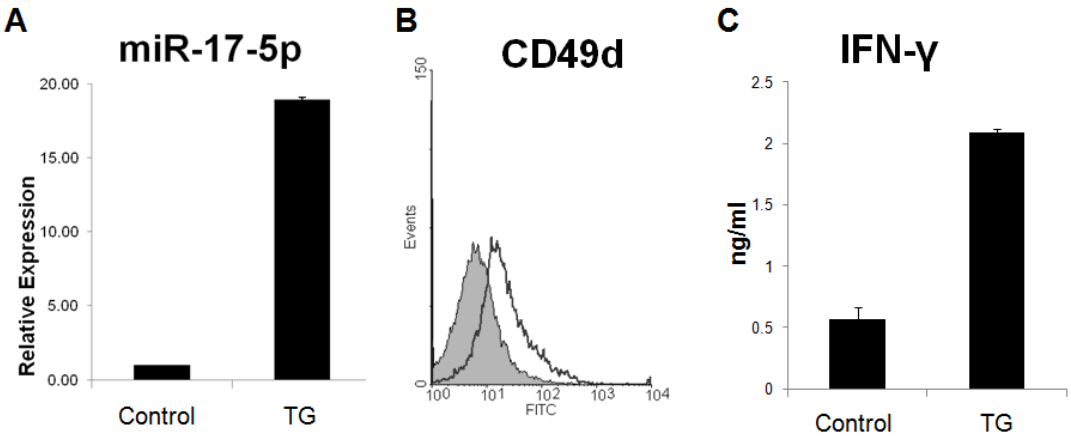


Figure 8: T-cells from miR-17-92 transgenic mice demonstrate enhanced Th1 phenotype. Splenic CD4+ T-cells were Immuno-magnetically isolated from miR-17-92 TG/TG or control animals. (A), miR-17-5p expression was analyzed in total RNA extracted from these freshly isolated cells. (B), Flow analysis was carried out on these freshly isolated cells for surface expression of CD49d, a subunit composing VLA-4. The grey shaded region represents CD4+ T-cells isolated from control animals and the un-shaded region represents CD4+ T-cells from miR-17-92 TG/TG mice (C), Isolated cells were stimulated in Th1 skewing condition for 9 days and 5 x 10^6 cells were then plated in fresh media for 24 hours, at which point supernatant was collected and analyzed for IFN-γ by ELISA. Both in (A), and (C), values in the two groups were statistically different with $p<.01$ using the student t test.

5.3 ECTOPIC EXPRESSION OF MIR-17-92 PROMOTES IL-2 PRODUCTION AND RESISTANCE AGAINST ACTIVATION-INDUCED CELL DEATH (AICD) IN JURKAT CELLS

Based on our discussion in the background section, miR-17-92 is expected to play pivotal roles in T cell survival as well as functions. To evaluate these aspects, we transduced Jurkat human T lymphoma cells with lentiviral vectors encoding green fluorescence protein (GFP) and either the miR-17-92-1 expression vector encoding miR-17 18 and 19a, or the 17-92-2 expression vector encoding miR 20, 19b, and 92. The control vector encodes GFP, but not miRs. Transduced Jurkat cells were stimulated with PMA and ionomycin overnight before the supernatants were assayed for IL-2 production by ELISA (**Figure 9A**). Transduction of either miR-vector promoted IL-2 production in Jurkat cells. It was somewhat expected the both vectors would have similar effects to one another as miRs from both miR-17-92-1 and miR-17-92-2 share similar seed sequences.

AICD and chemotherapy-induced suppression of T-cells represent major obstacles for efficient T cell-based cancer immunotherapy (Brenner, Krammer et al. 2008; Kennedy and Celis 2008). We next examined whether transfection of Jurkat cells with miR-17-92 makes T-cells resistant to AICD. AICD was induced by cultivation of Jurkat cells in the presence of 10 µg/ml anti-CD3 mAb, which is hyper-stimulatory and used as a standard method to induce AICD (Jiang, Han et al. 2009). As demonstrated (**Figure 9B)**, the growth of control Jurkat cells was significantly suppressed by nearly 25% in the AICD inducing condition compared with the same cells with the regular (growth-

promoting) dose of anti-CD3 mAb (1 μg/ml). In contrast, the growth of Jurkat cells transduced with either miR-17-92-1 or miR-17-92-2 was not significantly altered by the high dose (10 μg/ml) of anti-CD3 mAb, suggesting that the miR-17-92 transfection confers T-cells with substantial resistance against AICD. These findings point to a potential utility for engineered T-cells in cancer immunotherapy.

*

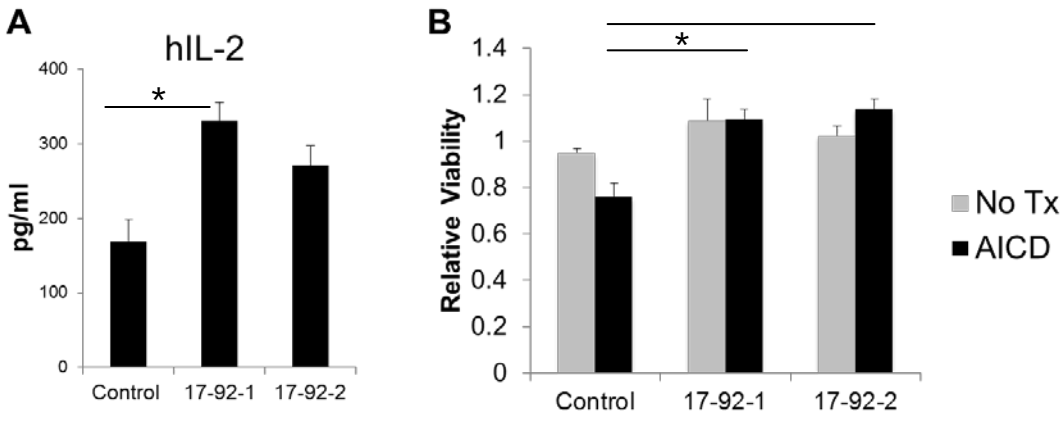


Figure 9: Ectopic expression of miR-17-92 cluster members in the human Jurkat T-cell line confers increased IL-2 production and resistance to AICD. Jurkat cells were transduced by either one of the following pseudo typed lentivirus vectors: 1) control vector encoding GFP; 2) the 17-92-1 expression vector encoding miR-17 18 and 19a, or 3) the 17-92-2 expression vector encoding miR 20, 19b-1, and 92a-1. (A), Transduced Jurkat cells (5x104) in the triplicate wells were stimulated with PMA (10 ng/ml) and ionomycin (500 nM) for overnight and supernatant was harvested and tested for the presence of IL-2 by specific ELISA. The figure shows mean values and standard deviations of the amount of IL-2 released from each group. Statistical analysis was carried out using the student t test, and significant ($p<.005$) increase of IL-2 production was confirmed in both 17-92-1 and the 17-92-2 transduced groups compared with the control group. (B), Transduced Jurkat cells were treated with the AICD inducing condition (10 µg/ml anti-CD3 mAb) or in complete media (No Tx) for 24 hrs. Then, the relative numbers of viable cells were evaluated using the WST-1 assay (Roche). The figure shows mean values and standard deviations of 8 wells/group each containing 5×10^5 cells. For each group, the relative OD readings at 450 nm of AICD-treated cells compared with control Jurkat cells without AICD-treatment is indicated. * indicates $p<0.05$ between the two groups using student t test.

6.0 AIM 1 CONCLUSION (MIR-17-92 CLUSTER IN T-CELLS)

Attaining effective anti-tumor immunity is a major goal of modern biologic therapy, limited by the tumor microenvironment and profound regulatory mechanisms that limit T-cell and NK cell effectors. In Aim 1 we show that the type-2-skewing tumor microenvironment induces down-regulation of miR-17-92 expression in T-cells, thereby hampering anti-tumor T-cell responses. It also suggests that development of immunotherapy using miR-17-92-transduced T-cells is warranted based on our findings demonstrating that ectopic expression of miR-17-92 in T-cells leads to improved type-1 functions, including increased VLA-4 expression and IFN-γ production.

It remains obscure as to how IL-4 and the STAT6 signaling pathway negatively influence miR-17-92 expression at molecular levels. Our findings indicate that the tumor-bearing host down-regulates miR-17-92 in T-cells, systemically **(Figure 7)**. Interestingly, not only are *Stat6*$^{-/-}$ T-cells resistant to tumor-induced inhibition of miR-17-5p, but CD8^{+} T-cells in tumor bearing *Stat6*$^{-/-}$ mice exhibited higher levels of miR-17-5p when compared with CD8^{+} T-cells obtained from non-tumor bearing *Stat6*$^{-/-}$ mice. In addition to IL-4, other tumor-derived factors are likely to be involved in these events. Additionally, IL-13 expression in tumor conditions may also signal through the IL-4R/STAT6 pathway and suppress miR-17-92 expression.

Ectopic expression of miR-17-92 cluster members in Jurkat cells lead to increased IL-2 production levels and viability following treatment with AICD condition **(Figure 9)**. The Jurkat cell line was isolated from the peripheral blood of a T-cell leukemia patient in the 1970s, they are often used to recapitulate what would happen in humans T-cells as they retain many T-cell properties such as CD4, a T cell receptor, and ability to produce IL-2. Additionally, being a cell line, Jurkat cells survive after many passages making them ideal in experiments. For these reasons we chose to use Jurkat cells in our study. We recognize that this cell line may have pitfalls since they are an immortalized cell line and have better survival than non-immortalized human T-cells.

Messenger RNA that are targeted by miR-17-92 cluster miRs include: E2F1, E2F2, E2F3 (O'Donnell, Wentzel et al. 2005; Sylvestre, De Guire et al. 2007), P21 (Inomata, Tagawa et al. 2009), anti-angiogenic thrombospondin-1 and connective tissue growth factor (Dews, Homayouni et al. 2006), proapoptotic Bim, and phosphatase and tensin homolog (PTEN) (Xiao, Srinivasan et al. 2008). These proteins are all involved in cell cycle regulation or apoptotic cell death, further supporting the importance of miR-17-92 cluster in T cell biology. In fact, Bim and PTEN are down-regulated in T-cells overexpressing miR-17-92 (Xiao, Srinivasan et al. 2008). Furthermore, TGF-β receptor II (TGFBRII) is one of the established targets of miR-17-92 (Volinia, Calin et al. 2006). We predict based on this that miR-17-92 transgenic T-cells should show down-regulated TGFBRII and decreased sensitivity to TGF-β.

In agreement with others (Xiao, Srinivasan et al. 2008), our findings demonstrating increased IFN-γ production from miR-17-92 TG/TG T-cells compared

with control cells suggests that miR-17-92 may promote the type-1 skewing of T-cells **(Figure 8)**. As miR-17-92 targets hypoxia-inducible factor (HIF)-1α in lung cancer cells (Taguchi, Yanagisawa et al. 2008), enhanced miR-17-92 expression in activated T-cells may promote the type-1 function of T-cells at least partially through down-regulation of HIF-1α. HIF-1 expression provides an important adaptation mechanism of cells to low oxygen tension (Semenza 1998; Sitkovsky and Lukashev 2005).However, it does not appear to be critical for survival of T-cells, unlike its apparent role in macrophages (Cramer, Yamanishi et al. 2003). T-cells do not depend on HIF-1α for survival to the same degree as macrophages since activated T-cells produce ATP by both glycolysis and oxidative phosphorylation (Brand and Hermfisse 1997). Rather, HIF-1α in T-cells appears to play an anti-inflammatory and tissue-protecting role by negatively regulating T-cell functions (Eltzschig, Thompson et al. 2004; Neumann, Yang et al. 2005; Sitkovsky and Lukashev 2005). T-cell-targeted disruption of HIF-1α leads to increased IFN-γ secretion and/or improved effector functions (Kojima, Gu et al. 2002; Lukashev, Klebanov et al. 2006; Thiel, Caldwell et al. 2007; Guo, Lu et al. 2009). These data collectively suggest that miR-17-92 expression in activated T-cells may promote the type-1 function of T-cells at least partially through down-regulation of HIF-1α.

MiRs in the miR-17-92 clusters are amplified in various tumor types including B-cell lymphoma and lung cancer, and promote proliferation and confer anti-apoptotic function in tumors, thereby promoting tumor-progression and functioning as oncogenes (Hayashita, Osada et al. 2005; He, Thomson et al. 2005; Lawrie 2007; Matsubara, Takeuchi et al. 2007; Rinaldi, Poretti et al. 2007). However, miR-17-92 by itself may not

be responsible for oncogenesis as transgenic mice with miR-17-92 overexpressed in lymphocytes develop lymphoproliferative disorder and autoimmunity but not cancer (Xiao, Srinivasan et al. 2008). MiR-17-92 may cooperate with other oncogenes to promote the oncogenic process. Transgenic mice overexpressing both miR-17-92 and c-Myc in lymphocytes develop early-onset lymphomagenesis disorders (He, Thomson et al. 2005). On the other hand, knockout studies of the miR-17-92 cluster in mice have demonstrated the importance of this cluster in mammalian biology. While knockout of the miR-17-92 cluster results in immediate post-natal death of all progeny with lung and heart defects, knockout of either or both the miR-106a or miR-106b (miR-17-92 homologs) clusters are viable without an apparent phenotype(Ventura, Young et al. 2008). However, disruption of the miR-17-92 cluster together with miR-106a or 106b cluster results in embryonic lethality (Xiao and Rajewsky 2009). During lymphocyte development, miR-17-92 miRs are highly expressed in progenitor cells, with the expression level decreasing 2- to 3-fold following maturation (Xiao, Srinivasan et al. 2008).

These studies reviewed above provide us with critical insights as to what has to be expected if we develop therapeutic strategies by modulating miR-17-92 expression. One major barrier for successful T-cell–based cancer immunotherapy is the low persistence of tumor antigen (TA)-specific T-cells in tumor-bearing hosts (Morgan, Dudley et al. 2006; Pule, Savoldo et al. 2008). It seems promising to generate genetically modified TA-specific T-cells *ex vivo* that are resistant to tumor-mediated immune suppression and mediate robust and long-lived anti-tumor responses. MiR-17-92 miRs have the potential to confer resistance to tumor-derived immunosuppressive

factors and to improve type-1 reactivity. Further characterization of the role of miR-17-92 cluster in tumor antigen (TA)-specific CTLs is clearly warranted and may provide us with ability to develop novel immunotherapy strategies with genetically engineered T-cells. Additionally, identification of diminished miR-17-92 expression in the peripheral blood may emerge as an important biomarker in patients with malignancy.

7.0 AIM 2 BACKGROUND (IL-4RA ON MDSCS)

As mentioned previously, while the risk factors for gliomas still remain unclear, genome wide analyses have demonstrated an association of SNPs with glioma risk and prognosis (Scheurer, Amirian et al. 2008; Shete, Hosking et al. 2009). Of particular interest are the glioma-related SNPs in the *Il4rα* gene, rs1805015 and rs1805016, which are associated with altered glioma prognosis. To follow our findings from Aim 1 demonstrating the negative impact of IL-4R signaling in T-cells through the regulation of mIR-17-92, we next sought to understand whether IL-4rα could also impact other cells which would promote tumor growth. As IL-4rα is expressed on murine MDSCs and is important for their suppressive activity, we examined whether IL-4Rα expression on MDSCs plays a role in glioma development. Of note, IL-4Rα chain is a necessary component of both the IL-4 and IL-13 receptors.

MDSCs in the tumor contribute to the harsh tumor environment promoting angiogenesis, recruitment of other inhibitory cells and blocking anti-tumor T-cell activation and function (Serafini, Meckel et al. 2006; Ostrand-Rosenberg and Sinha 2009) through molecules including CCL2, arginase, TGFβ, S100A8/A9 and IL-10 (Ostrand-Rosenberg and Sinha 2009; Boelte, Gordy et al. 2011). We therefore sought to examine the role of IL-4Rα on the suppressive function of MDSCs in gliomas.

Animal models that mimic the complexity of human gliomas would be useful in understanding glioma biology and in predicting therapeutic responses. In this regard, a novel Sleeping Beauty (SB) transposon–mediated *de novo* murine glioma model has been recently developed in which tumor initiation and progression can be monitored by bioluminescent imaging (WIESNER, DECKER ET AL. 2009). These murine tumors share many features with the human disease including glial marker expression, pseudopalisading necrosis, and invasive growth into the surrounding brain. In contrast to traditional models with transplantation of cultured glioma cells, these tumors evolve with the host immune system; herein, we show that they are profoundly infiltrated by regulatory immune cells that suppress antitumor immunity, which is similar to human gliomas (Parney, Waldron et al. 2009). Therefore, this *de novo* glioma model allows us to address biological mechanisms of gliomagenesis in a clinically relevant manner.

8.0 AIM 2 MATERIALS AND METHODS (IL-4RA ON MDSCS)

8.1 ANIMALS

WT Balb/c and Balb/c-background *Il4ra*$^{-/-}$ mice were obtained from The Jackson Laboratory. Animals were bred and handled in the Animal Facility at the University of Pittsburgh per an Institutional Animal Care and Use Committee–approved protocol.

8.2 FLOW CYTOMETRY

The procedure used in the current study has been described previously (Fujita, Scheurer et al. 2010). Briefly, single cell suspensions were surface-stained with fluorescent dye-conjugated antibodies. Due to the small number of BILs obtained per mouse, BILs obtained from all mice in a given group (5 mice/group) were pooled and then evaluated for the relative number and phenotype of monocyte-gated brain infiltrating leukocytes (BILs) between groups by Accuri C6 Flow cytometer (Beckman Coulter, Fullerton, CA).

8.3 BONE MARROW (BM)-MDSC GENERATION

A similar procedure has been previously described (Highfill, Rodriguez et al. 2010) . BM was harvested from Balb/c-background femur and tibia bones, depleted of red blood cells and plated at $2x10^5$ cells/ml in DMEM, 10% FBS, 50mM 2-ME, 10mM HEPES buffer, 1mM sodium pyruvate, 100U/ml penicillin, 100mg/ml streptomycin, and amino acid supplements (MEM NEAA). G-CSF (Peprotech) was added at 100ng/ml and mouse GM-CSF (Peprotech) was added at 250U/ml. Cultures were incubated at 37ºC 10% CO2. On day 4 and 9, recombinant murine IL-13 (Peprotech) was added at 80ng/ml and on day 10 cells were positively selected with CD11b by MACS immunomagnetic separation and used for experiments.

8.4 ARGINASE ACTIVITY ASSAY

The QuantiChrome™ arginase assay detection kit was used (DARG-200) according to the manufacturer's recommendations. Briefly, 40µl of cell culture supernatant was incubated with 10 µl of 5x substrate buffer and incubated at 37ºC for 2 hours. Urea reagent was then added to the wells to stop the reaction and following a 60 minute incubation at room temperature optical density was determined at 430nm using a multiscan RC plate reader (Thermo).

8.5 MDSC T-CELL INHIBITION ASSAY

T-cells were isolated from WT Balb/c mouse spleen using MACs bead negative separation, labeled with .1µM CFDA SE (Invitrogen) and incubated with varying amounts of day 10 cultured BM-MDSCs for 5 days in the presence of anti-CD3/anti-CD28 Dynabeads (Invitrogen) and 30U/ml of hIL-2 (Peprotech). Cells were then analyzed by flow cytometry on an AccuriC6 (BD) for proliferation (reduced CFSE levels).

8.6 ANTIBODY-MEDIATED IMMUNE CELL DEPLETION ASSAY

The procedure has been described previously (Fujita, Scheurer et al. 2010). Anti–Gr-1 (RB6-8C5) mAb was obtained from Taconic; control IgG was obtained from Sigma-Aldrich. Mice with developing gliomas received intraperitoneal (i.p.) injections of mAb (0.25 mg/dose) 3x/week starting on day 21 after SB plasmid DNA transfection. A similar procedure was used for depletion of CD4 (GK1.5) and CD8 (TIB105) with 0.5 mg/dose give 2x/week. Depletion was confirmed to be effective with greater than 95% depletion of cells by flow cytometry.

8.7 REAL-TIME PCR

The procedure has been described previously (Muthuswamy, Urban et al. 2008). Primers and probes were obtained from Applied Biosystems: The following primers and probes were used for murine samples: *Arg1, Il4ra, Ccl2, Inos, Il13, Gmcsf, Cd49d* and the following primers for human samples *TGF-β*, *CD49d* and *Arg1*. Human or mouse *GAPDH* was used as the internal control.

8.8 INTRACEREBROVENTRICULAR DNA INJECTION FOR SLEEPING BEAUTY-SPONTANEOUS GLIOMA INDUCTION

The procedure has been described previously (Wiesner, Decker et al. 2009). Briefly, DNA transfection reagent (*In vivo*-JetPEI) was obtained from Polyplus Transfection. The following DNA plasmids were used for glioma induction: *pT2/C-Luc//PGK-SB13* (0.125 µg), *pT/CAGGS-NRASV12* (0.125 µg), *pT2/shP53* (0.125 µg) and PT3.5/CMV-EGFRvIII (0.125 µg). For survival studies mice were sacrificed at the first sign of symptoms (symptom free survival) which included weight loss, inability to self-feed and drink water, seizures, hemiparesis, weight-loss as well as hunch-back. For immunological evaluation of WT and *Il4ra*$^{-/-}$ tumors we conducted bioluminescence imagining (BLI) using an IVIS200 (Caliper Life Sciences) and evaluated tumors of comparable size (BLI of $2x10^8$).

8.9 BONE MARROW CHIMERA

Donor WT or *Il4ra*$^{-/-}$ mice were sacrificed and placed in a 70% ethanol solution to sterilize skin, and femur and tibia bones were removed. Bone marrow cells were isolated by flushing the femur and tibia with PBS. Cells were then centrifuged for 10 min at 2000 X G followed by RBC lysis with ACK buffer and filtering through a 45 micron filer. Host Balb/c background WT mice received 10 Gy of total body irradiation. At 24 hours after irradiation mice received an i.v (tail vein) injection of 100 µl (1-2 X 10^6) viable BM cells. Mice were >90% chimeric by day 15 and were used for subsequent experiments.

9.0 AIM 2 RESULTS (IL-4RA ON MDSCS)

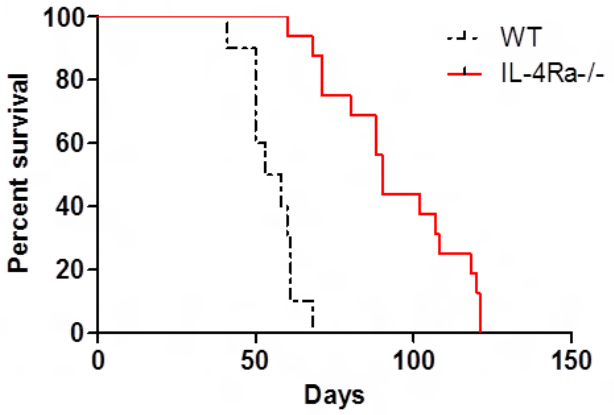
9.1 IL4RA$^{-/-}$ MICE EXHIBIT DELAYED GROWTH OF SB *DE NOVO* GLIOMAS COMPARED WITH WT MICE.

To evaluate the role of IL-4Rα on glioma growth we induced *de novo* gliomas by *SB* transposon-mediated intracerebroventricular transfection of *EGFRvIII*, *NRAS*, and short hairpin (*sh)P53* in neonatal Balb/c-background WT and *Il4ra*$^{-/-}$ mice **(Figure 10A)**. We have previously shown that pathological characteristics of *SB* tumors closely resemble those of human gliomas (Wiesner, Decker et al. 2009; Fujita, Scheurer et al. 2010). While the median symptom-free survival (SFS) for WT mice was 55.5 days, *Il4ra*$^{-/-}$ mice exhibited prolonged survival with a median SFS of 90 days. As IL-4Rα is expressed on some myeloid derived suppressor cells (MDSC) (Terabe, Matsui et al. 2003; Mandruzzato, Solito et al. 2009; Ostrand-Rosenberg and Sinha 2009), we next evaluated whether the genetic deletion of *Il4ra* impacts the glioma infiltration of myeloid cells, such as CD11b^{+}Gr-1^{+} cells, which are likely MDSC **(Figure 10B)**. In WT mice, *SB* glioma-bearing brains demonstrated higher numbers of CD11b^{+}Gr-1^{+} cells compared with non-tumor bearing brains. Further, in the presence of the *SB* gliomas, WT CD11b^{+}Gr-1^{+} expressed higher levels of IL-4Rα than those in non-tumor bearing animals. Compared with WT animals, *Il4ra*$^{-/-}$ hosts had a significantly fewer number of

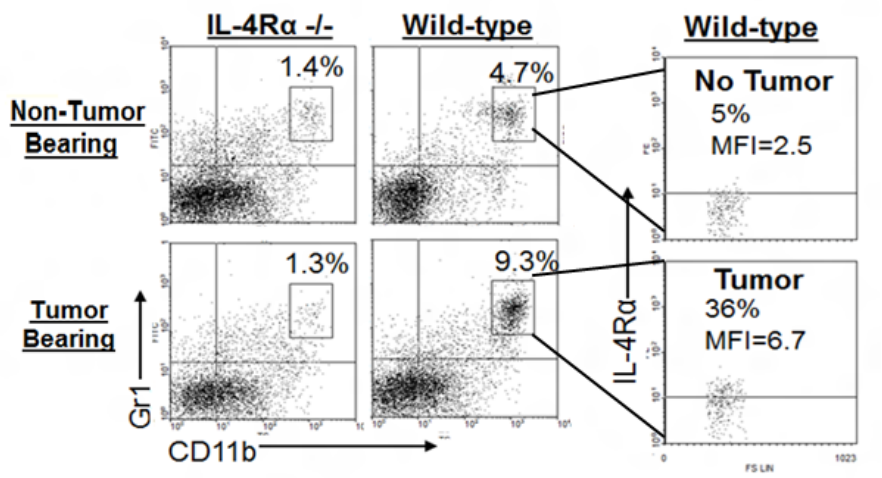
$CD11b^{+}Gr\text{-}1^{+}$ cells in the non-tumor bearing brain which did not increase significantly in the presence of the *SB* tumor (**Figure 10B**).

To determine whether the different expression level of IL-4Rα and numbers of $CD11b^{+}Gr\text{-}1^{+}$ cells in tumor-bearing hosts also in periphery, we analyzed splenic cells from *SB* glioma bearing mice (**Figure 10C**). Similar to our observation in the brain, WT but not *Il4ra*$^{-/-}$ hosts demonstrated an increase of $CD11b^{+}Gr\text{-}1^{+}$ cells in the spleen following induction of *SB* glioma. However, unlike the brain, peripheral $CD11b^{+}Gr\text{-}1^{+}$ cells maintained low IL-4Rα expression even in tumor-bearing animals. Thus IL-4Rα expression on MDSCs in the SB glioma model appears to be a tumor-brain specific phenomenon.

A


100
80
60
40
20
0
Percent survival
0
50
100
150
Days
WT
IL-4Ra-/-

B


IL-4Rα -/-
Wild-type
Wild-type
Non-Tumor Bearing
Tumor Bearing
1.4%
4.7%
1.3%
9.3%
No Tumor
5%
MFI=2.5
Tumor
36%
MFI=6.7
IL-4Rα
Gr1
CD11b

C

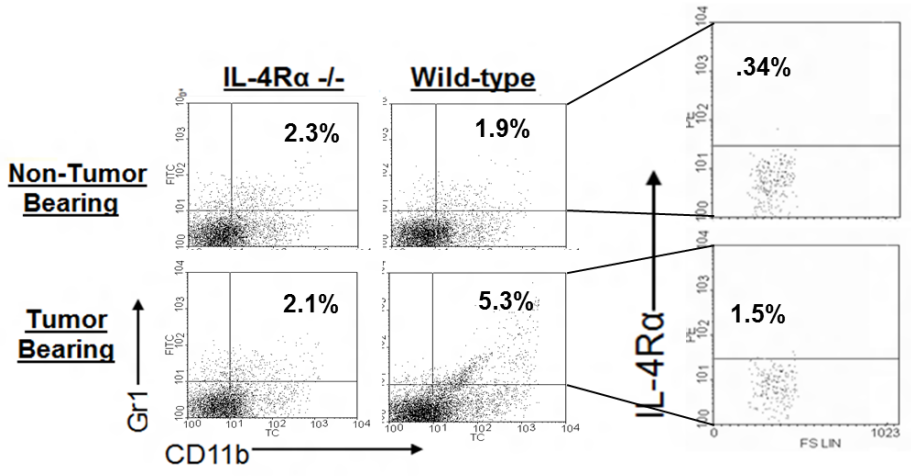
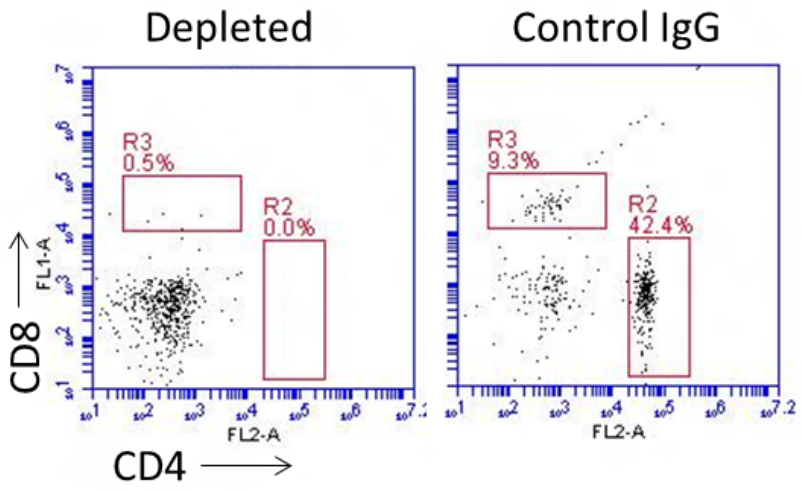


Figure 10: IL4Rα in glioma development. Gliomas were induced in neonatal mice by intracerebroventricular transfection of the following plasmids: pT2/C-Luc//PGK-SB13 (0.125 μg), pT/CAGGS-NRASV12 (0.125 μg), PT35/CMV-EGFR(0.125 μg) and pT2/shp53 (0.125 μg). (A), SFS following SB challenge plotted on a Kaplan Meier plot. The survival of *Il4ra*$^{-/-}$ mice with SB tumors was significantly longer than WT mice ($p < 0.0001$, by log-rank test). (B), Brains from tumor bearing and non-tumor bearing mice were removed from BALB/C (WT) and *Il4ra*$^{-/-}$ mice with tumors of similar size (BLI ~3x10^{8}). Brain infiltrating leukocytes were isolated by percol separation, pooled and were analyzed for CD11b^{+}Gr-1^{+} cells and IL-4Rα expression. (C) Peripheral cells were isolated from the spleen and were analyzed for CD11b^{+}Gr-1^{+} cells and IL-4Rα expression.

9.2 IL4RA$^{-/-}$ MICE EXHIBIT DELAYED GROWTH OF SB *DE NOVO* GLIOMAS COMPARED WITH WT MICE IN THE ABSENCE OF CD4^{+} AND CD8^{+} T-CELLS.

T-cells deficient of IL-4R or its major signaling molecule, signal transducer and activator of transcription (STAT)-6 are typically skewed towards type-1 which is known to promote better anti-tumor immunity (Fujita, Zhu et al. 2009; Okada 2009; Okada, Kohanbash et al. 2009; Sasaki, Pardee et al. 2009). To exclude a possibility that the prolonged survival of *Il4ra*$^{-/-}$ mice is due solely to enhanced anti-tumor type-1 T-cell skewing, we induced *SB* gliomas in WT and *Il4ra*$^{-/-}$ hosts in which CD4^{+} and CD8^{+} T-cells were depleted **(Figure 11A).** Although depletion of T-cells significantly accelerated the growth of gliomas in WT and *Il4ra*$^{-/-}$ mice, *Il4ra*$^{-/-}$ mice still demonstrated improved SFS over WT mice when both were depleted of T-cells **(Figure 11B)**. These data demonstrate that the improved survival of *Il4ra*$^{-/-}$ mice is partially independent of type-1 T-cell skewing of *Il4ra*$^{-/-}$ T-cells and suggest the involvement of other immune cells (possibly MDSCs).

A



B

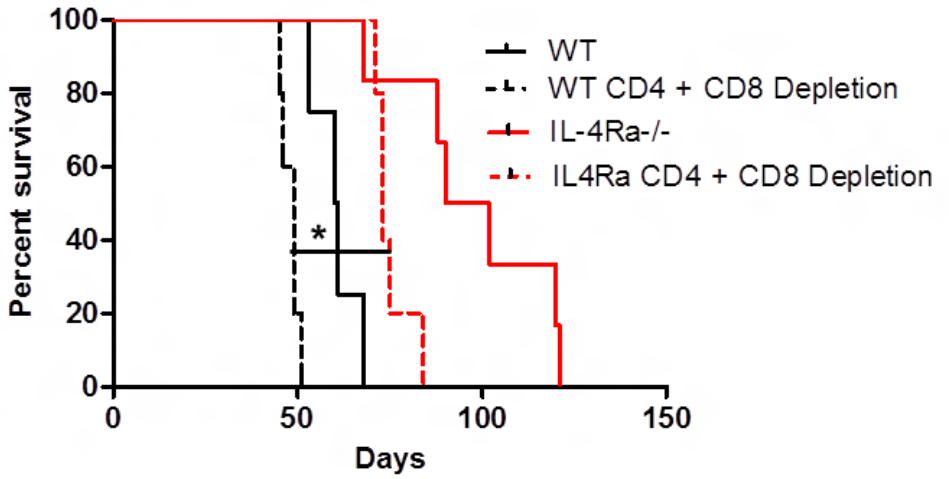


Figure 11: Effects of IL-4Rα on glioma development in the absence of T-cells. (A), Efficiency of T-cell depletion was evaluated in SPCs of WT animals by flow cytometry from mice receiving 3x, 50µg doses of anti-CD4 (GK1.5) and anti-CD8 (TIB105) (depleted) or control rat igG (Control IgG). (B), SFS was monitored in SB-bearing Balb/c and *Il4ra*$^{-/-}$ mice receiving i.p. injections of 50µg of neutralizing mAbs for CD4 (GK1.5) and CD8 (TIB105) 2x/week beginning when mice reached 23 days old.

9.3 IL4RA$^{-/-}$ TUMOR TISSUE AND TUMOR-DERIVED MDSCS HAVE REDUCED EXPRESSION OF INHIBITORY MOLECULES COMPARED TO WT TUMOR TISSUE.

To examine the impact of IL-4Rα on the effector function of CD11b^{+}Gr-1^{+} cells, total RNA was isolated from WT or *Il4ra*$^{-/-}$ SB glioma tumors of similar size and using RT-PCR expression of MDSC-associated genes was evaluated **(Figure 12A)**. SB tumor tissue from WT mice overall demonstrated significantly higher levels of *Tgfb* and *Arg1* than *Il4ra*$^{-/-}$, which directly suppresses T-cell induction and anti-tumor immune surveillance (Terabe, Matsui et al. 2003; Kropf, Baud et al. 2007). Our data also demonstrated a trend of increased CCL-2, a primary MDSC chemo-attractant in WT compared with *Il4ra*$^{-/-}$ tumor tissue. Notably, while similar levels of IL-13 were detected in WT and *Il4ra*$^{-/-}$ tumors, IL-4 expression was undetectable. These data suggest an important role of IL-13 on MDSC function in SB tumor bearing mice.

To better understand the significance of MDSCs in SB tumors we isolated 2 subsets of CD11b^{+}Gr-1^{+} cells by flourescence-activated cell sorting (FACs), CD11b^{+}Ly6C^{high} monocytic cells and CD11b^{+}Ly6G^{high} granulocytic cells, and analyzed MDSC-associated genes by RT-PCR **(Figure 12B)**. While CD11b^{+}Ly6C^{high} cells expressed higher levels of both *Tgfb* and *Arg1* than CD11b^{+}Ly6G^{high} cells, *Arg1* expression was significantly lower in *Il4ra*$^{-/-}$ CD11b^{+}Ly6C^{high} cells than WT cells. Conversely, *Inos* which can suppress tumor cells (Chang, Liao et al. 2001) is expressed in both subsets of *Il4ra*$^{-/-}$ CD11b^{+}Gr-1^{+} cells at elevated levels compared with WT CD11b^{+}Gr-1^{+} cells. Surprisingly, our data did not support a difference in *Tgfb* expression in sorted WT and *Il4ra*$^{-/-}$ MDSCs, thus the observed difference in the bulk

tumor expression of *Tgfb* appears to be due to other cells, possibly ones induced by MDSCs, like T-regs. Thus, IL-4Rα appears important for MDSC inhibitory function mediated through Arg1 and possibly Inos and Tgfb.

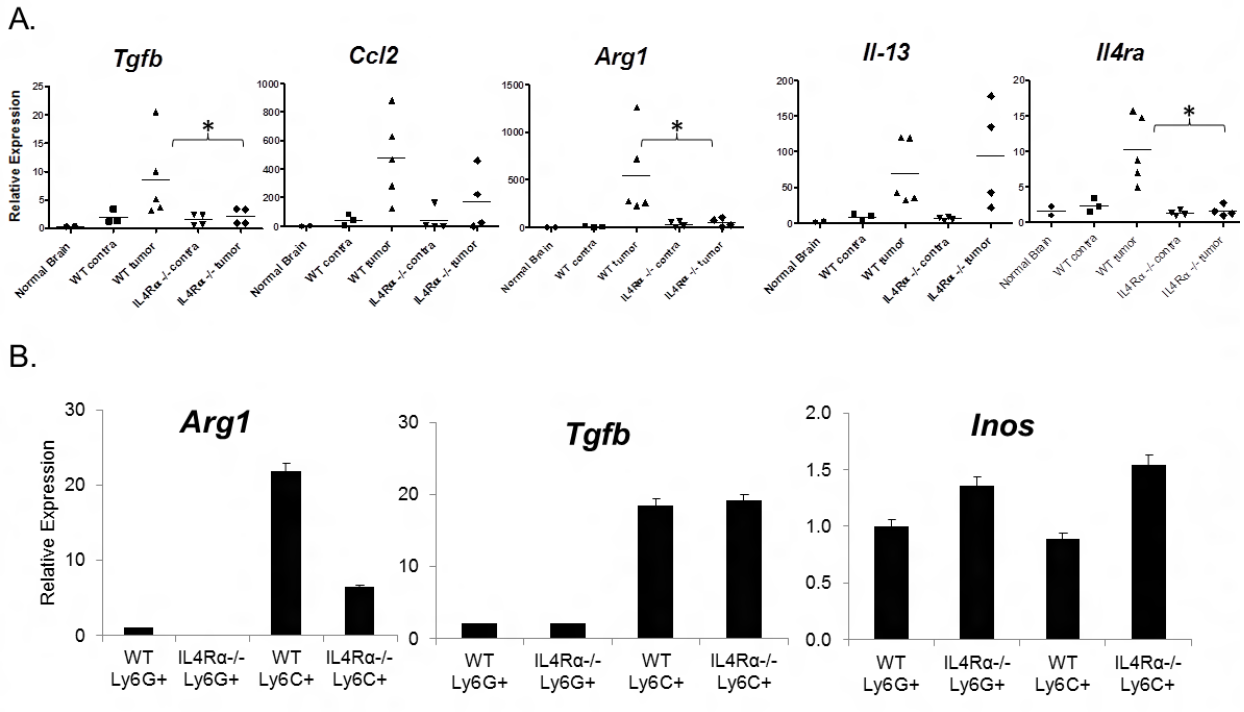


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Figure 12: Effect of IL-4Rα on tumor infiltrating MDSCs and the tumor microenvironment. (A), Total RNA was isolated from either brain of non-tumor bearing mice (normal brain), or the contralateral (contra) or tumor-bearing (tumor) hemisphere of WT and Il4r*a*$^{-/-}$ mice bearing SB glioma. RT-PCR for mRNA expression levels of *Tgfb, Il-13, Arg1, Ccl2* and *Il4ra* were analyzed. (B), BILs from WT or *Il4ra*$^{-/-}$ mice were sorted for double positive cells with either CD11b and Ly6C or CD11b and Ly6G. Total RNA was immediately isolated and RT-PCR was used to examine *Arg1, Tgfb, Inos* mRNA levels from these cells.

9.4 MDSC DEPLETION PROLONGS SURVIVAL OF MICE CHALLENGED WITH SB GLIOMAS.

We next examined whether depletion of $CD11b^{+}Gr\text{-}1^{+}$ cells prolongs survival in mice challenged with SB gliomas. While there are multiple methods to deplete $CD11b^{+}Gr\text{-}1^{+}$ cells, many of them also have direct anti-tumor activities. Thus, to deplete $CD11b^{+}Gr\text{-}1^{+}$ cells, we used anti-Gr-1 (RB6-8C5) mAb which should have no direct anti-tumor activity. As SB challenged mice start developing detectable tumors at around 30 days (data not shown), we started anti-Gr-1 treatments at 23 days. To maintain complete depletion we administered 50mg/dose anti-Gr-1 3x/week **(Figure 13A)** (Fujita, Scheurer et al. 2010; Fujita, Kohanbash et al. 2011) . Mice depleted of $CD11b^{+}Gr\text{-}1^{+}$ cells experienced significantly prolonged SFS as shown by Kaplan Meier curve **(Figure 13B)** with some animals surviving past 120 days. BLI of mice revealed that some anti-Gr-1 treated mice even experienced tumor regression below the level of detection **(Figure 13C).** These data demonstrate the importance of $CD11b^{+}Gr\text{-}1^{+}$ cells in promoting tumor growth.

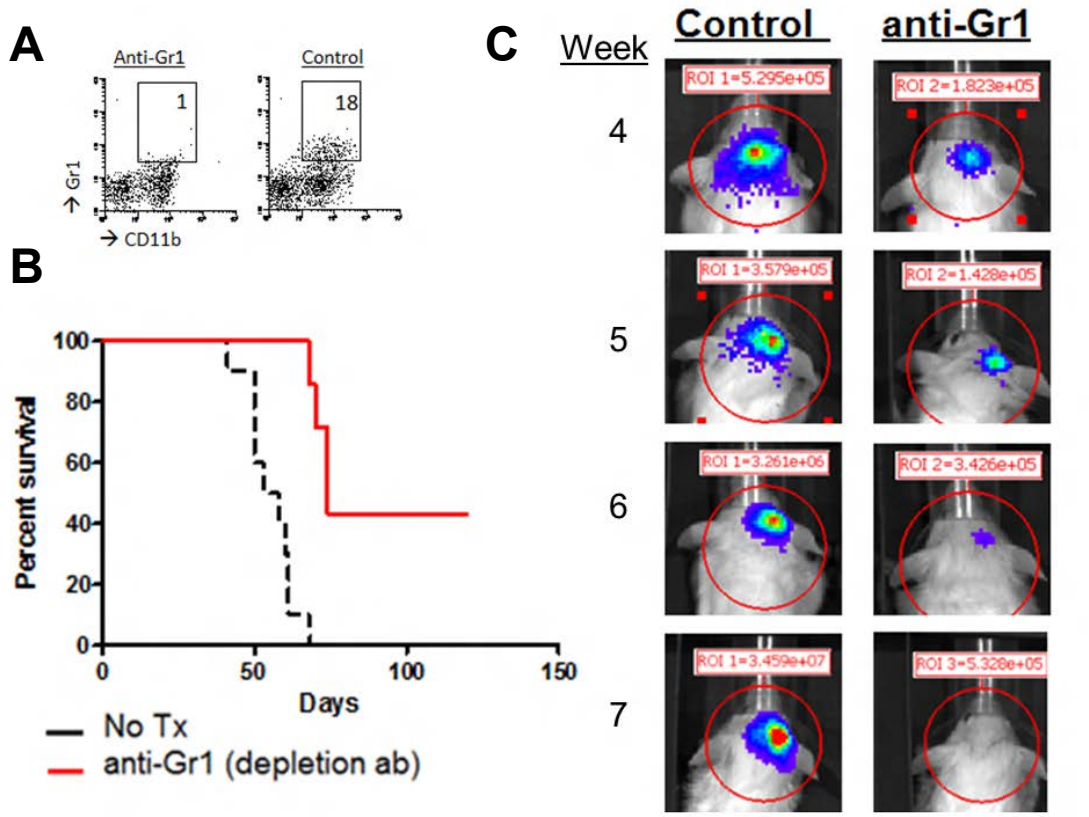


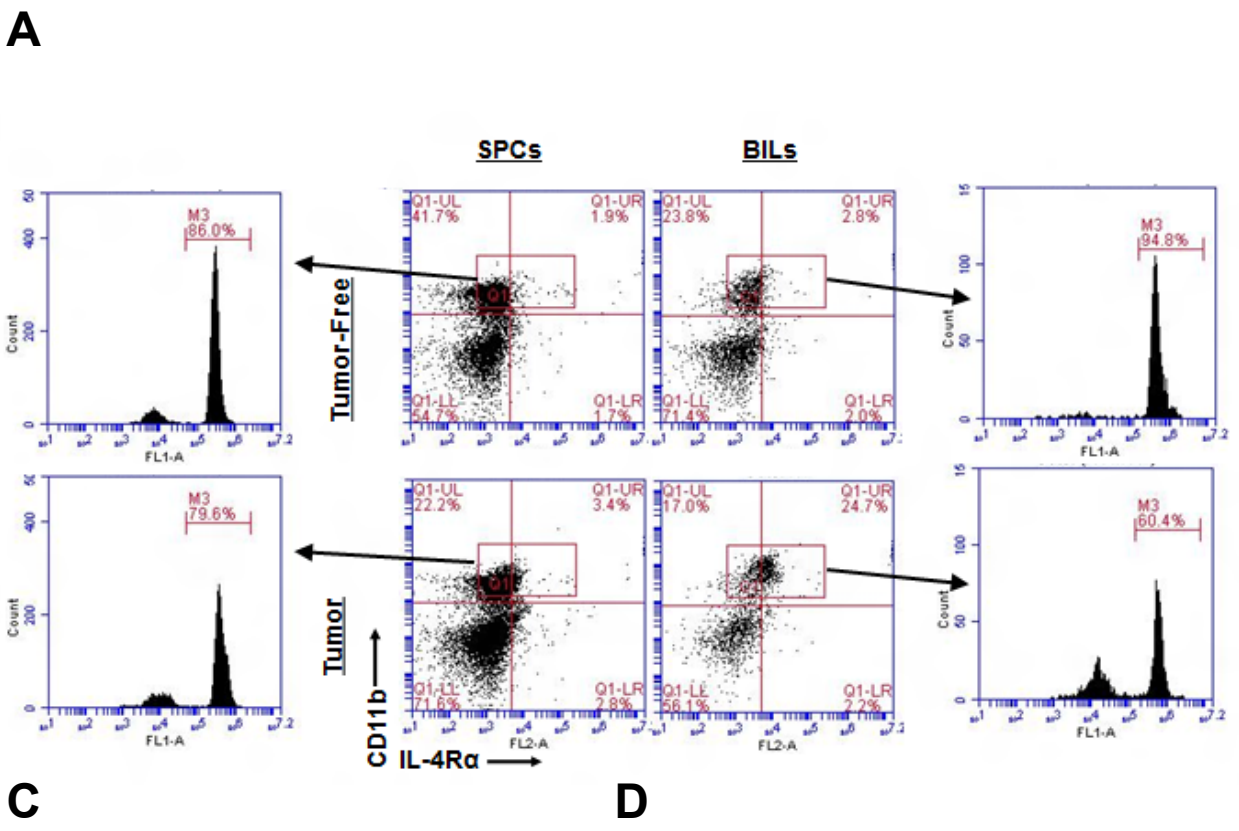
Figure 13: Depletion of MDSCs in SB tumor bearing animals. Sleeping beauty tumor bearing animals received anti–Gr-1 mAb (RB6-8C5; 0.25 mg/dose) 3 times per week beginning at 23 days old. (A), CD11b+Gr-1+ cells in BILs from mice receiving control rat IgG or anti-Gr-1 antibody. (B), SFS was monitored in mice bearing SB gliomas until day 120. (C), Representative of BLI imaging showing tumor growth in control treated animals and tumor regression below the limit of detection in anti-Gr-1 treated animal.

9.5 BONE MARROW CHIMERIC MICE REVEAL THAT IL4RA ON HEMATEPOETIC CELLS IS CRITICAL FOR MDSC ACCUMALATION IN THE BRAIN

We observed that gliomas in *Il4ra*$^{-/-}$ mice had fewer infiltrating MDSCs that in WT controls. To assess whether the difference was due to intrinsic factors in MDSCs or other factors such as the IL-4Rα void nature of the CNS and tumor microenvironment in *Il4ra*$^{-/-}$ mice we evaluated MDSC infiltration using a bone marrow chimera system. To confirm the efficiency of the system, mice received 10 Gy of whole body irradiation followed by (within 24 hours) tail vein injection 1 x 10^6 RBC-depleted BM cells from GFP transgenic mice. On day 15 post irradiation mice received SB-derived glioma cells. Fifteen days after tumor cell inoculation mice were sacrificed and BILs were analyzed by flow cytometry for CD11b$^+$IL-4Rα$^+$ cells **(Figure 14A)**. Consistent with our previous results, peripheral CD11b$^+$ cells in both tumor-free and tumor-bearing mice lacked IL-4Rα expression. However, BILs in tumor-bearing mice but not tumor-free mice had high expression of IL-4Rα, suggesting that the tumor microenvironment, but not the brain specifically induces IL-4Rα. Further CD11b$^+$ cells were mostly GFP$^+$ confirming the efficiency of our BM chimera system.

After validating the BM chimera system we next asked whether tumor depletion of IL-4Rα in BM cells impacts the infiltration in MDSCs and T-cells in the tumor. We observed greater numbers of CD11b$^+$Gr-1$^+$ cells in mice with WT BM compared with ones with *Il4ra*$^{-/-}$ BM both in the spleen (26.5% WT vs. 11.7% *Il4rα*$^{-/-}$) and brain (20.9% WT vs. 8.4% *Il4ra*$^{-/-}$). Consistently, we observed increased numbers of CD4$^+$ and CD8$^+$

T-cells in the spleen **(Figure 14C)** and BILs **(Figure 14D)** of mice receiving *Il4ra*$^{-/-}$ BM, compared to mice receiving WT BM. This data further supports the more suppressive nature of WT MDSCs compared to *Il4ra*$^{-/-}$ MDSCs.



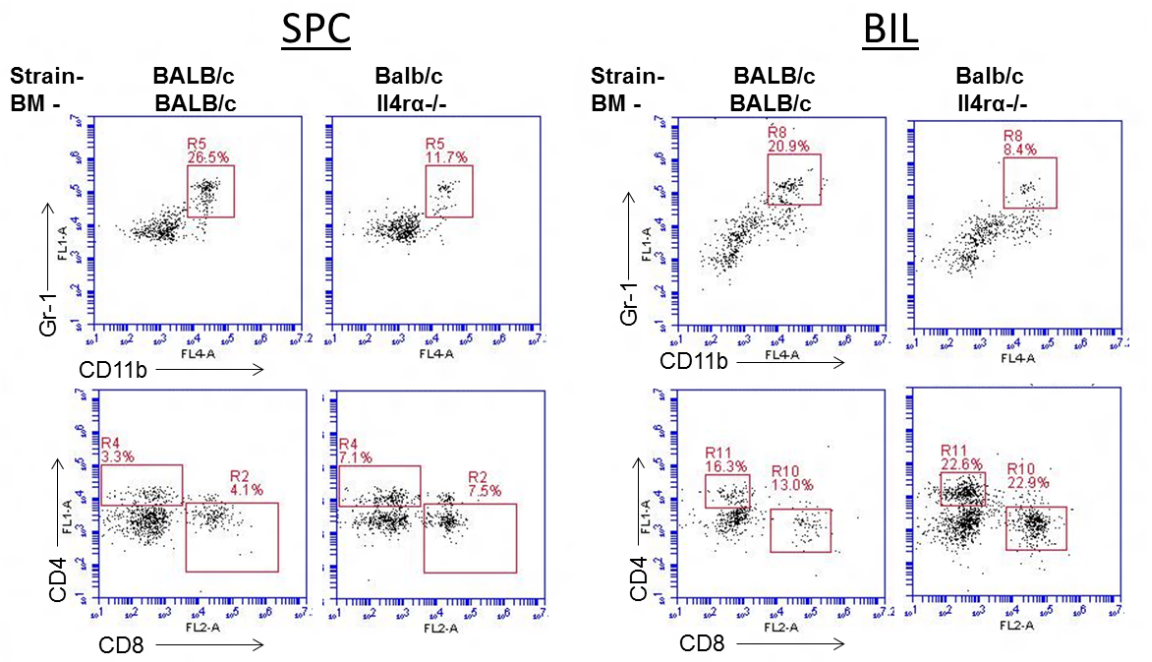


Figure 14: A critical role of IL-4Rα on BM cells in the immunological environment of glioma. Host Balb/c background WT mice received 10 Gy of total body irradiation. At 24 hours after irradiation mice received an i.v (tail vein) injection of 100 µl (1 X 10^6) viable bone marrow cells from either WT, *Il4rα*$^{-/-}$ or GFP transgenic mice. (A), Mice received GFP-transgenic mouse derived from BM and tumor cells derived from SB glioma bearing mice on day 15. BILs were isolated on day 15 following tumor cell injection and analyzed for CD11b$^+$IL-4Rα$^+$ cells and the % of these cells that were GFP$^+$ to determine the extent of chimerism by flow cytometry. (C-D), 15 days after whole body irradiation mice received either WT or *Il4ra*$^{-/-}$ donor BM followed by i.c injections of 5x10^5 glioma cells derived from SB beauty animals. Three weeks after glioma cell injection splenocytes (SPC) and BILs were harvested and analyzed by flow cytometry for Gr-1, CD11b, CD4 and CD8 expression.

9.6 IL-13 BUT NOT IL-4 PROMOTES BONE MARROW (BM)-CD11B^{+}GR-1^{+} CELL GROWTH AND FUNCTION

As IL-4Rα is a key component of both the IL-4 and IL-13 cytokine receptors, we evaluated the ability of each cytokine to promote MDSC function. To establish suppressive CD11b^{+}Gr-1^{+} cells for *in vitro* evaluation, BM cells from WT or *Il4ra*$^{-/-}$ mice were isolated and treated with G-CSF (.1µg/ml) and GM-CSF (250U/ml) on days 0,3 and 9, with 80 ng/ml IL-4 or IL-13 on days 3 and 9, and BM cells were analyzed on day 10. On day 10 greater than 75% of the cells expressed CD11b and Gr1 (Not shown). Culture supernatant from BM-CD11b^{+}Gr-1^{+} cells treated with IL-13 displayed enhanced arginase activity **(Figure 15A)** and arginase expression **(Figure 15B)** compared with BM cells not treated with IL-13. Additionally, WT-CD11b^{+}Gr-1^{+} cells displayed elevated *Tgfb* expression levels compared with *Il4ra*$^{-/-}$ BM-CD11b^{+}Gr-1^{+} cells. However this was not dependent on IL-13, as IL-13 treatment did not increase *Tgfb* expression in WT-BM-CD11b^{+}Gr-1^{+} cells **(Figure 15B)**.

We next determined whether the suppressive function of BM-CD11b^{+}Gr-1^{+} cells was specifically dependent on IL-13, but not IL-4. Unlike with IL-13, treatment of BM-CD11b^{+}Gr-1^{+} cells IL-4 did not promote the suppressive function of CD11b+Gr-1+ cells **(Figure 15C)** as determined by arginase expression. Our data that IL-13 but not IL-4 is important for MDSC function are consistent with our data that IL-13 but not IL-4 is detectable in SB tumors.

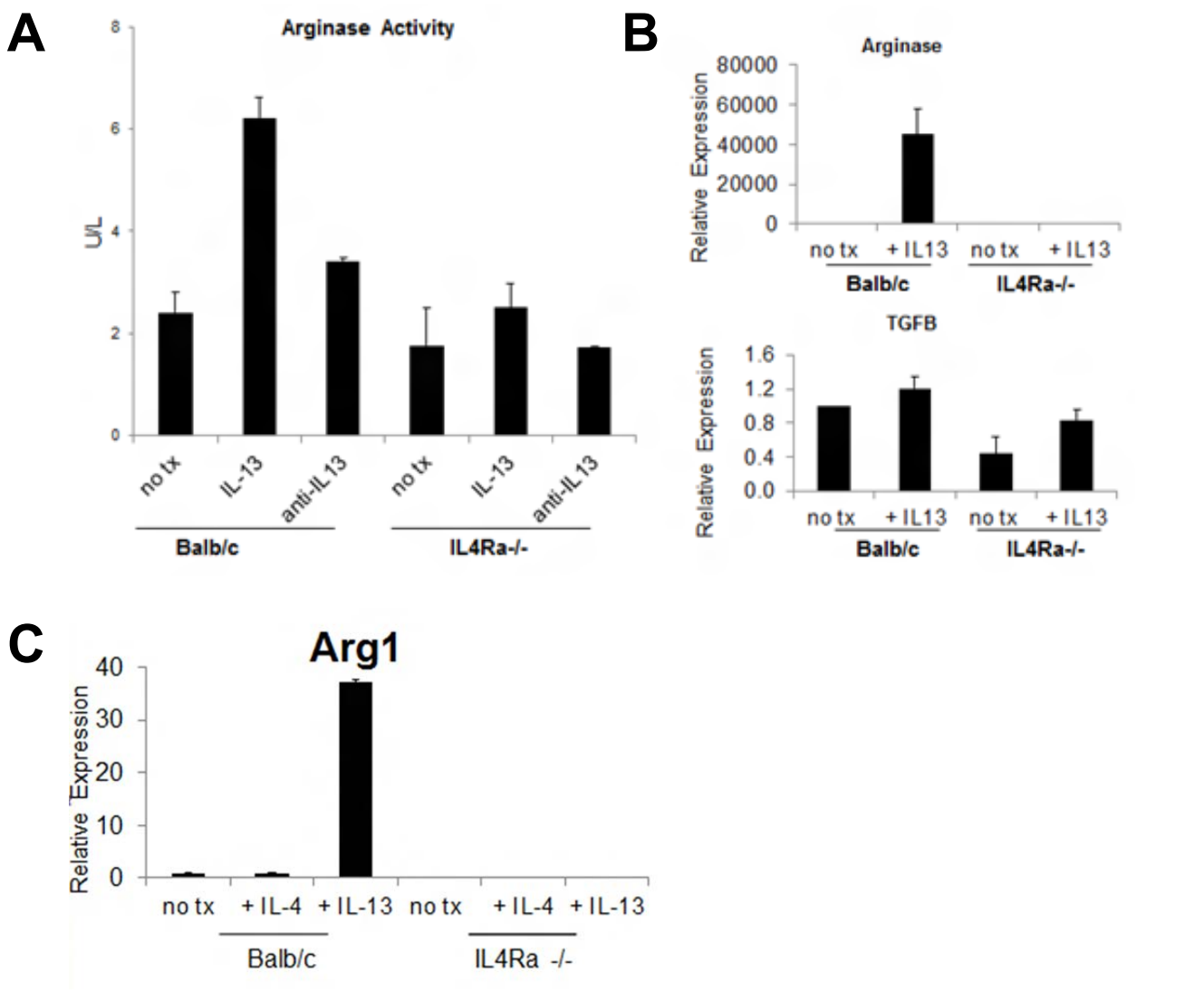
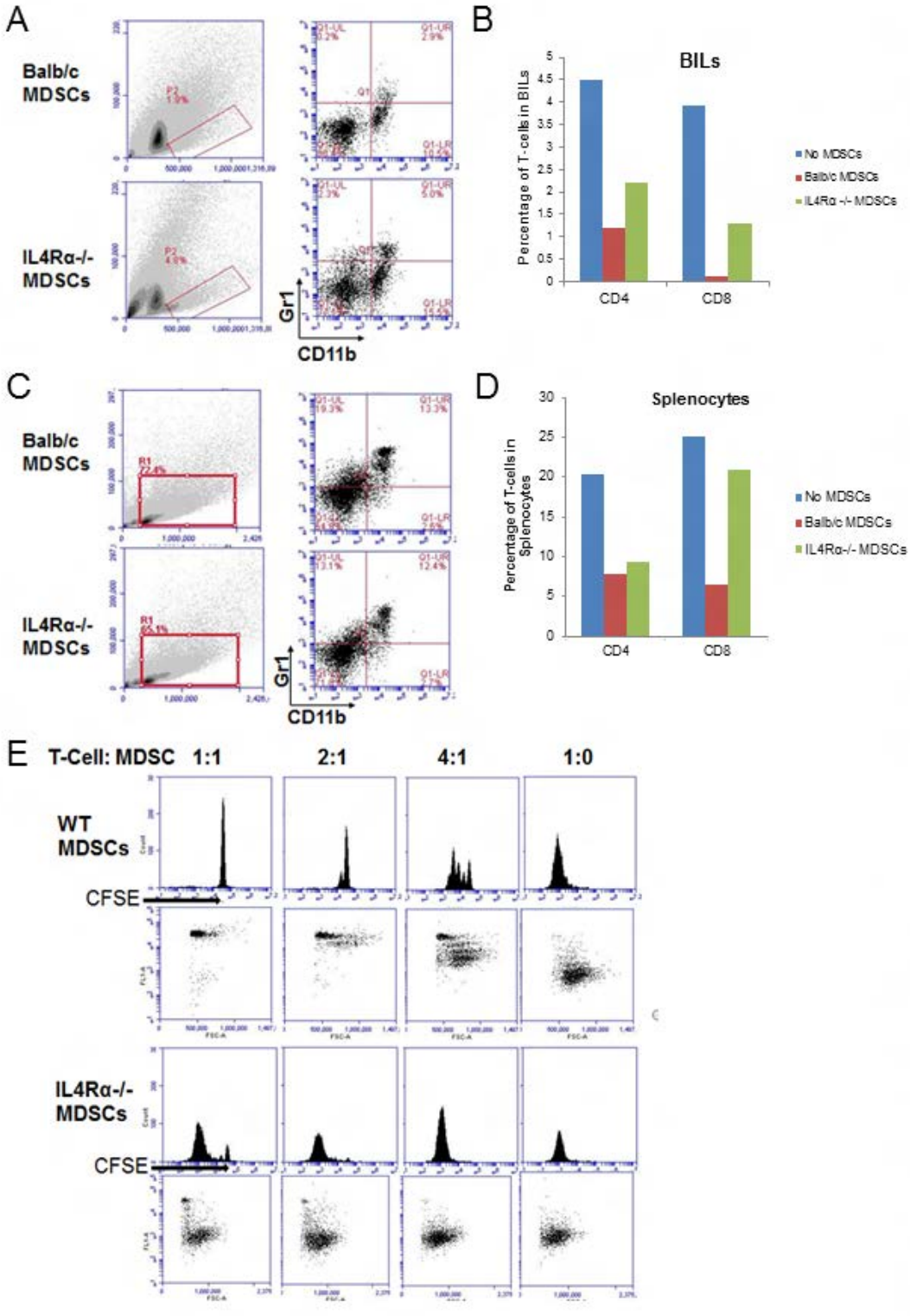


Figure 15: Effects of IL-13 on MDSC generation and phenotype. BM cells derived from WT-Balb/c or *Il4ra*$^{-/-}$ mice were cultured in complete DMEM media with G-CSF and GM-CSF on days 0, 3 and 9 and rIL-13 or rIL-4 on days 3 and 9. (A), On day 10 of MDSC culture the QuantiChrom™ arginase activity assay was carried out on 40 µl of supernatant (B), Total RNA was extracted from cultured cells and expressions of *Arg1* and *Tgfb1* were analyzed. (C), Balb/c BM cells cultured with G-CSF and GM-CSF (no tx) or with the addition of mrIL-4 (+IL-4) or mrIL-13 (+IL-13) *Arg1* expression by RT-PCR.

9.7 IL4RA$^{-/-}$ BM-DERIVED CD11B^{+}GR-1^{+} CELLS HAVE REDUCED ABILITY TO SUPPRESS T-CELLS BOTH *IN VIVO* AND *IN VITRO.*

One of the hallmark characteristics of MDSCs is their ability to inhibit T-cells. To determine if *Il4ra*$^{-/-}$ BM-derived CD11b^{+}Gr-1^{+} cells have reduced capacity to inhibit T-cells compared with WT cells, we challenged WT mice with SB gliomas and administered 1x10^{6} BM derived MDSCs via tail vein injection. Mice with small SB tumors were used, when endogenous MDSCs levels were undetectable. Seventy two hours after the MDSC injection, brains were harvested and MDSCs were evaluated **(Figure 16A)**. While both WT and *Il4ra*$^{-/-}$ BM-MDSCs migrated to the brain, more *Il4ra*$^{-/-}$ BM-MDSCs migrated to the brain than WT BM-MDSCs. This may be attributed to the elevated levels of VLA-4 expression on *Il4ra*$^{-/-}$ cells, which is important for immune cell-trafficking into the brain (Sasaki, Pardee et al. 2009). We then evaluated both CD4^{+} and CD8^{+} T-cells in BIL samples **(Figure 16B)**. We observed that WT BM-MDSCs displayed a greater capacity to inhibit CD4^{+} and CD8^{+} T-cells than the *Il4ra*$^{-/-}$ BM-MDSC in the brain. We next examined the effect of the adoptive BM-MDSCs transfer on splenic T-cells **(Figure 16C)**. Unlike the brain, at 72 hours after injection, spleens display comparable numbers of WT and *Il4ra*$^{-/-}$ CD11b^{+}Gr-1^{+} cells. Consistent with what we observed in the brain, mice receiving WT BM-CD11b^{+}Gr-1^{+} cells had fewer T-cells in the spleen than mice treated with *Il4ra*$^{-/-}$ MDSCs **(Figure 16D)**. These data further support the importance of IL-4Rα for the function of MDSCs, and their ability to suppress T-cell proliferation.

To directly assess the capacity of WT or *Il4ra*$^{-/-}$ BM derived MDSCs to inhibit T-cell proliferation we co-cultured varying ratios of MDSCs with 5x10^5 CFSE labeled naïve WT CD8$^+$ T-cells in the presence of anti-CD3/anti-CD28 dynabeads^{TM} and 30U/ml of hIL-2. After a 5 day co-culture T-cell proliferation was determined by examining the reduction of CFSE intensity levels by flow cytometry **(Figure 16E)**. Consistent with our *in vivo* data WT MDSCs suppressed T-cells at a lower MDSC: T-cell ratio than *Il4ra*$^{-/-}$ MDSCs. Furthermore, an arginase inhibitors nor-noha and L-arginine supplementation inhibited the T-cell suppressor activity of WT MDSCs **(Figure 16F)**. Thus, IL-4Rα is important for the direct T-cell suppressive activity of MDSCs.


A
Balb/c MDSCs
IL4Rα-/- MDSCs
Gr1
CD11b
B
BILs
Percentage of T-cells in BILs
CD4
CD8
No MDSCs
Balb/c MDSCs
IL4Rα -/- MDSCs
C
Balb/c MDSCs
IL4Rα-/- MDSCs
Gr1
CD11b
D
Splenocytes
Percentage of T-cells in Splenocytes
CD4
CD8
No MDSCs
Balb/c MDSCs
IL4Rα-/- MDSCs
E
T-Cell: MDSC 1:1
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1:0
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IL4Rα-/- MDSCs
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F

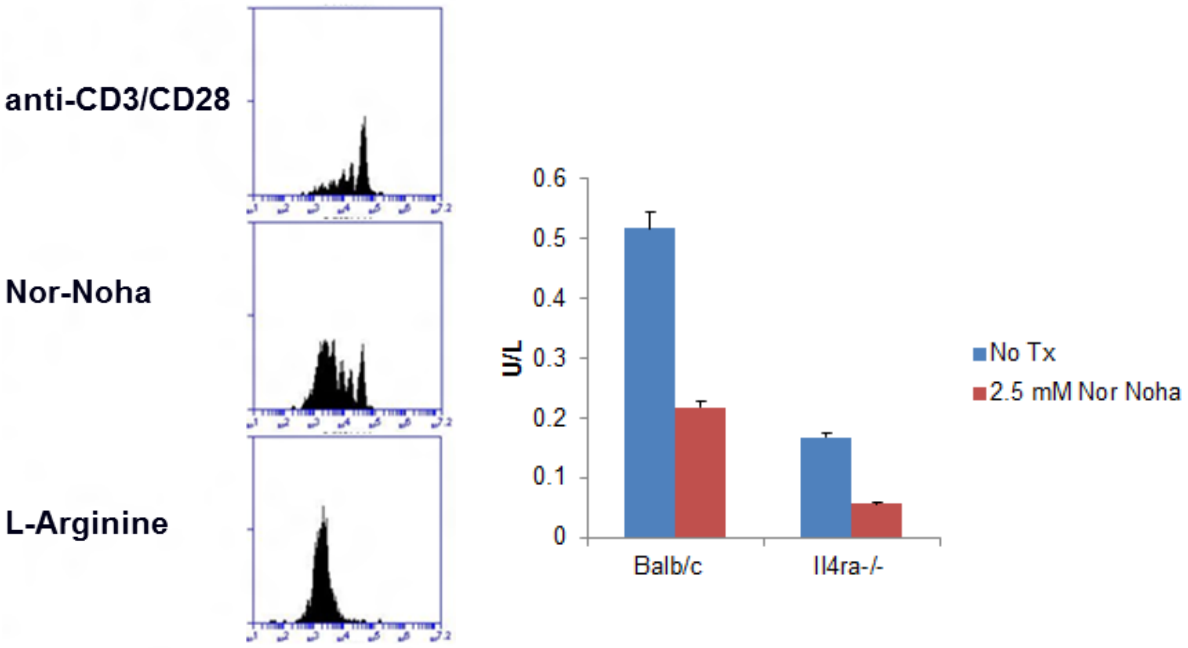

Figure 16: Function of *Il4ra*$^{-/-}$ MDSCs. MDSCs were induced from BM cells derived from WT-Balb/c or *Il4ra*$^{-/-}$ mice. BM cells were administered via tail vein injection to Balb/c mice with small SB gliomas (day 35, BLI of 5 x 10^7). (A), At 72 hours after MDSC treatment, brains were harvested and (A), MDSCs percentages and (B), T-cells were analyzed by flow cytometry. Spleens from these animals were also analyzed for (C), MDSCs and (D), T-cells by flow cytometry. E, 1 x10^5 CFSE (1µM) labeled CD8+ T-cells were stimulated with anti-CD3/CD28 microbeads and 30 U/ml and hIL-2 in a 96 well plates with varying ratios of MDSCs for 5 days. After the 5 day culture CFSE levels were analyzed on gated T-cells. All results are representative of at least 3 independent experiments. (F), BM-MDSCs were co-cultured with CFSE labeled $CD8^+$ T-cells at a 1:4 ratio in the presence of 2.5 mM Nor Noha or 5mM L-argininase activity and CFSE was levels were assessed after a 5 day co-culture.

9.8 GM-CSF UP-REGULATES IL-4RA ON BM CELLS AND IS OVEREXPRESSED IN GLIOMAS.

As IL-4Rα expression on $CD11b^{+}Gr\text{-}1^{+}$ cells is increased by SB gliomas in the brain **(Figures 10B and 14A)** we next sought to determine the factors in the brain that lead to the up-regulation of IL-4Rα. BM cells were cultured with either G-CSF, GM-CSF, IL-13 or tumor condition media (TCM) from SB tumor cell culture for 4 days and IL-4Rα expression was then measured by flow cytometry **(Figure 17A)**. While, G-CSF, GM-CSF and TCM treatment all up-regulated IL-4Rα expression, GM-CSF treatment had the most pronounced effect. We thus assessed GM-CSF expression in mouse SB-induced (**Figure 17B)** and human **(Figure 17C)** glioma tissues. Compared with normal brain and PBMCs, SB and human glioma tissues displayed increased GM-CSF expression.

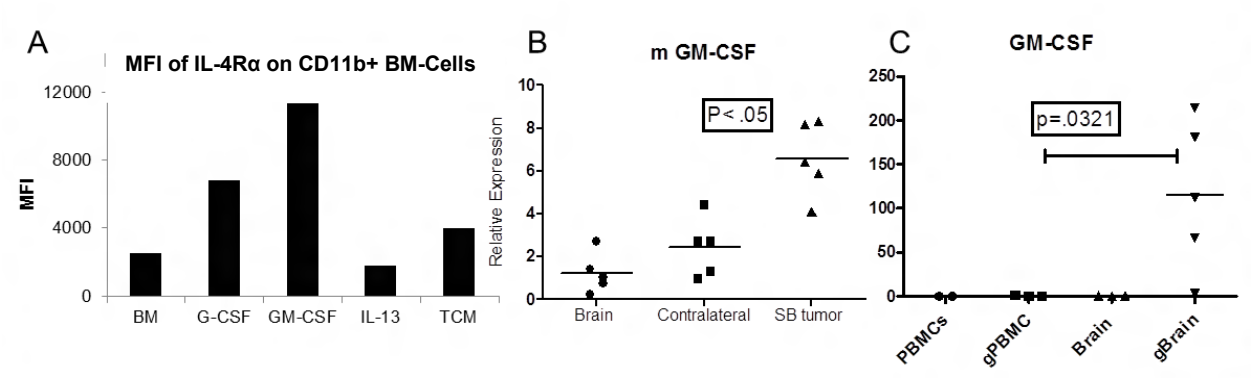

Figure 17: GM-CSF promotes IL4Rα expression on MDSCs and is up-regulated in tumor settings. BM cells depleted of RBCs and cultured in either 80ng/ml of G-CSF, GM-CSF, rmIL-13, or tumor culture media for 4 days. (A), IL-4Rα expression was determined by flow cytometry. Data is representative of 2 independent experiments with similar results. (B), Total RNA was isolated from either brain of non-tumor bearing mice (Normal brain), or the contralateral (contra) or tumor-bearing (tumor) hemisphere of WT and Il4r*a*-/- mice bearing SB glioma, mGM-CSF was then evaluated by RT-PCR. C, hGM-CSF expression was evaluated from total RNA isolated from normal human brain or glioma samples.

9.9 HUMAN GLIOMA INFILTRATING CD14^{+}HLA-DR^{-} MONOCYTES EXPRESS IL-4RA ASSOCIATED WITH SUPPRESSOR FUNCTION.

As murine monocytic CD11b^{+}Ly6C^{high} cells have enhanced expression of immune suppressor molecules **(Figures 12B)** and as it has been reported that human monocytic CD14^{+}HLA-DR^{-} cells have suppressive function (Poschke, Mougiakakos et al. 2010; Vuk-Pavlovic, Bulur et al. 2010; Lin, Gustafson et al. 2011), we next evaluated IL-4Rα expression on human CD14^{+}HLA-DR^{-} cells from fresh glioma samples (n=7) and glioma patient derived PBMCs (n=5) by flow cytometry **(Figure 18A)**. Using identical forward and side scatter gating on CD14^{+}HLA-DR^{-} monocytes in both the tumor infiltrating

lymphocyte (TIL) samples and PBMCs we observed that IL-4Rα is detected on 20-30% of $CD14^{+}HLA\text{-}DR^{-}$ in the brain while in the periphery IL-4Rα is barely detectable on corresponding populations. We further examined IL-4Rα expression on frozen tumor-infiltrating cells (n=13) and glioma patient derived PBMCs (n=10) **(Figure 18b)**. Consistently, all glioma infiltrating $CD14^{+}HLA\text{-}DR^{-}$ cells, but not peripheral $CD14^{+}HLA\text{-}DR^{-}$ cells had detectable IL-4Rα expression.

We next sought to address whether IL-4Rα expression on $CD14^{+}HLA\text{-}DR^{-}$ monocytes was associated with immune suppressor function. Using FACS we isolated $CD14^{+}HLA\text{-}DR^{-}IL\text{-}4R\alpha^{+}$ and $CD14^{+}HLA\text{-}DR^{-}IL\text{-}4R\alpha^{-}$ negative cell populations, extracted total RNA and analyzed *ARG1*, *TGFB, COX-2*, *indoleamine 2,3-dioxygenase (IDO*), *IL10* and *VLA4* (*CD49d*) **(Figure 18C)**. We found that the $CD14^{+}HLA\text{-}DR^{-}IL\text{-}4R\alpha^{+}$ cells had higher expression of *TGFB* than their $IL\text{-}4R\alpha^{-}$ counterparts. Further, consistent with our murine data VLA-4 expression was significantly higher in the $IL\text{-}4R\alpha^{-}$ cells. Possibly due to limited amounts of human tissue, expression of other suppressor molecules was below our limit of detection in both IL-4Rα positive and negative $CD14^{+}HLA\text{-}DR^{-}$ monocytes. We therefore examined the IL-4Rα expressing cells in a leukopheresis sample obtained from a glioma patient **(Figure 19A)**. Although there was a much smaller percentage of $IL\text{-}4R\alpha^{+}$ cells compared with in TILs, $CD14^{+}HLA\text{-}DR^{-}IL4r\alpha^{+}$ cells had higher levels of *ARG1* and *COX2* expression than their $IL\text{-}4R^{-}$ counterpart. However, IL-10 and IDO was not detectable in any of the samples. Thus, IL-4Rα on $CD14^{+}HLA\text{-}DR^{-}$ cells in the tumor microenvironment seems important for the immunosuppressive activity of human patient MDSCs.

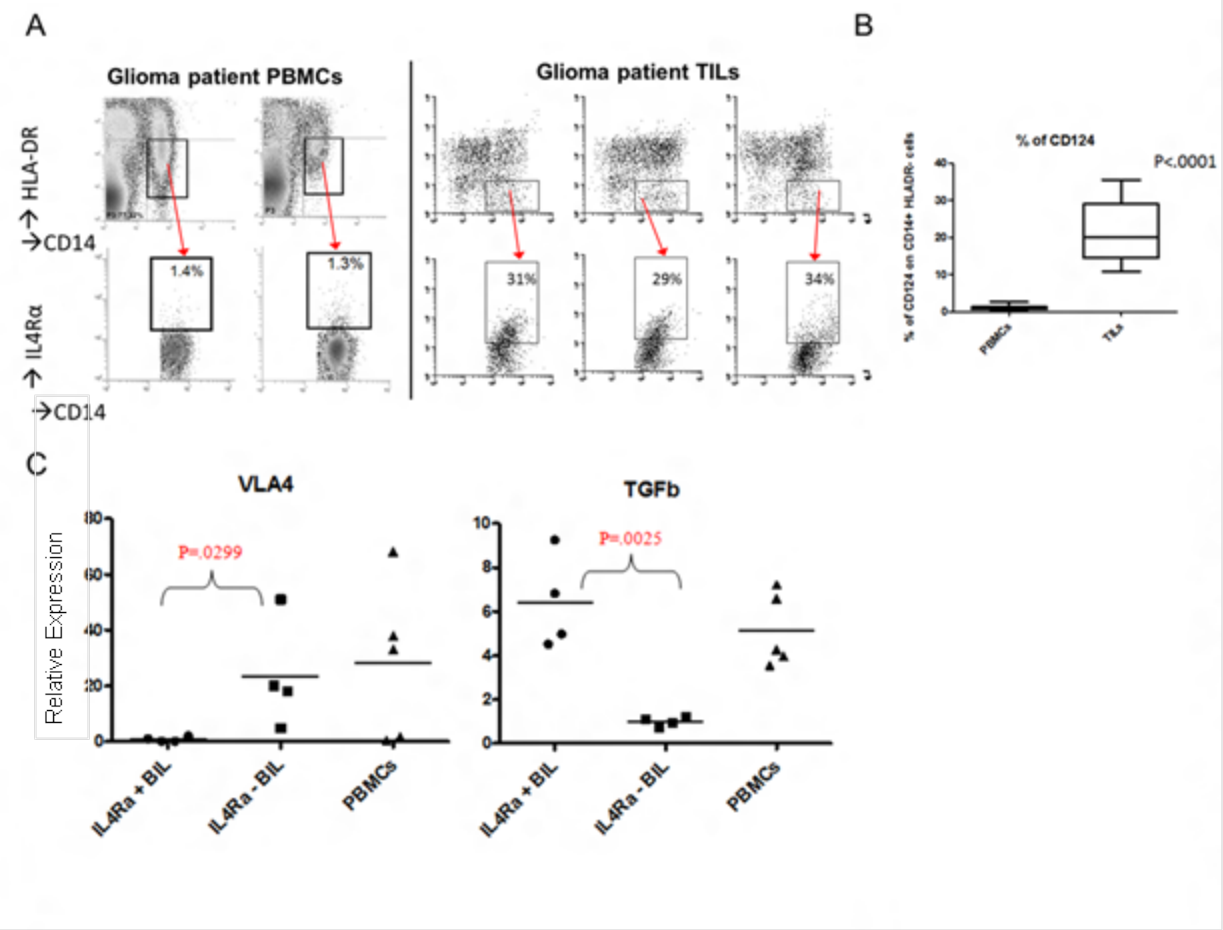

Figure 18: IL-4Rα expression on human glioma infiltrating monocyte. BILs were sorted from fresh glioma tissue by percol density separation or glioma patient derived PBMCs were collected by ficol method. (A), IL-4Rα was analyzed on CD14+HLA-DR- cells. (B), Percentage of $CD14^{+}HLA\text{-}DR^{-}$ cells expressing IL-4Rα in the brain and PBMCs from 12 patient samples. C, Total RNA was extracted from sorted $CD14^{+}HLA\text{-}DR^{-}$ cells that were either IL-4Rα+ or IL-4Rα- and analyzed with RT-PCR for *VLA-4* and *TGFB*.

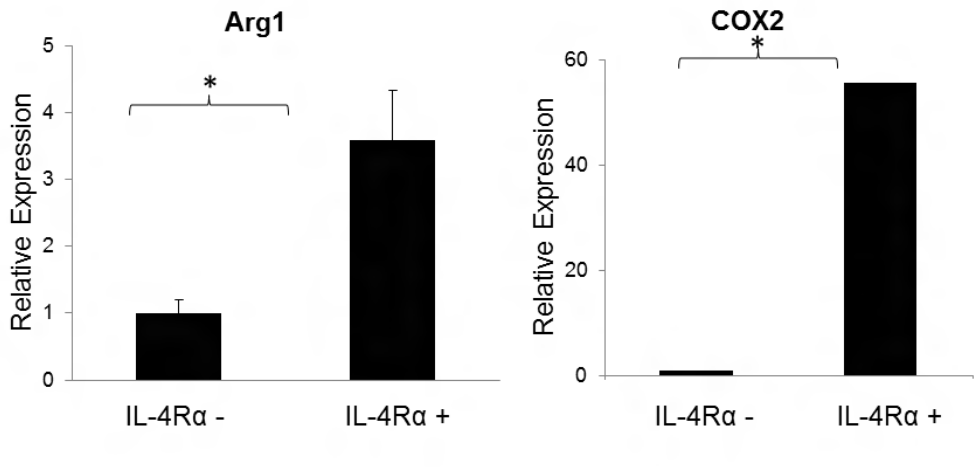

Figure 19: IL-4Rα is associated with increased *ARG1* and *COX2* expression on human glioma patient MDSCs

RNA was isolated from FACs sorted $CD14^{+}HLA\text{-}DR^{-}$ cells separated into either IL-4Rα^{+} or IL-4Rα^{-} fractions from a glioma patient leukopheresis sample and ARG1 and COX2 were evaluated. * indicated $p < .05$ by student's t test.

10.0 AIM 2 CONCLUSION (IL-4RA ON MDSCS)

An ideal immunotherapy for gliomas would improve both anti-tumor immune cell-functions and inhibition of the immune suppressor cells. Our data demonstrate for the first time in glioma patients and the *de novo* murine glioma model, that IL-4Rα is up-regulated on MDSCs specifically in the tumor but not in the periphery. *In vitro* data indicate that GM-CSF and to a lesser degree G-CSF are the primary inducers of IL-4Rα. Consistently, both murine and patient glioma tissues had high levels of gmcsf expression. Further, our BM chimera experiments demonstrate the up-regulation of IL-4Ra on MDSCs in the tumor conditions is specific to the brain tumor and not the brain alone. Our finding that the tumor-free brain expresses low GM-CSF compared to the tumor further supports the idea that GM-CSF in the tumor microenvironment up-regulates IL-4Ra expression on MDSCs.

Il4ra$^{-/-}$ gliomas are infiltrated by significantly fewer MDSCs than gliomas in WT mice. We still do not know whether this is due to differential recruitment to the tumor or growth. Based on a study examining anti-IL-4Rα aptamer treatment(Roth, De La Fuente et al. 2012), blockade of IL-4Rα resulted in increased MDSC apoptosis, suggesting that *Il4ra*$^{-/-}$ mouse MDSCs may be apoptotic. However, as we were able to culture similar amounts of BM-MDSCs from WT and *Il4ra*$^{-/-}$ mice, it seems plausible that (albeit with

different effector capabilities) the IL-4Rα status does not impact the generation of MDSCs. Our BM chimera experiments **(Figure 20)** further revealed that the increased amount of MDSCs in WT mice is intrinsic to hematopoietic cells as total body irradiated mice receiving *Il4ra*$^{-/-}$ BM had fewer brain infiltrating MDSCs than mice receiving WT BM. More work is thus necessary to determine the cause of reduced MDSCs in *Il4ra*$^{-/-}$ mice compared with WT mice.

IL-4Rα is a component of both the IL-4 and IL-13 receptors. However, based on our BM-MDSC data indicating that IL-13 but not IL-4 can regulate arginase expression and activity, IL-13 appears to have a primary role in the suppressive activity of MDSCs. Further, while SB gliomas express detectable levels of IL-13, we could not detect IL-4 by RT-PCR. The finding that IL-13 but not IL-4 promotes MDSC activity (Arg1 expression) is especially relevant for clinical applications of MDSC as blocking IL-4 may not be suitable to target MDSCs in glioma patients.

Our data indicates an important role for arginase as the primary mediator of WT-MDSC suppressive activity compared with *Il4ra*$^{-/-}$ MDSCs. This is supported by the following findings: 1) WT tumor-infiltrating MDSCs express significantly higher levels of Arg1 compared *Il4ra*$^{-/-}$ MDSCs; 2) WT BM-MDSCs suppress T-cell robustly compared with *Il4ra*$^{-/-}$ MDSCs and 3) The suppressive activity of WT MDSC is reversed by the arginase inhibitor, nor noha or L-arginine supplementation. Our findings are consistent with previous reports on IL-4Rα signaling for Arg1 expression (Terabe, Matsui et al. 2003; Highfill, Rodriguez et al. 2010). Interestingly *Arg1* expression is higher in Ly6C^{+} monocytic MDSCs but much less in Ly6G^{+} granulocytic MDSCs. While we also found higher levels of *Tgfb* in WT over *Il4ra*$^{-/-}$ glioma tissues, MDSCs do not appear to be the

source of *Tgfb* as we could not detect any differential expression between MDSCs sorted from WT or *Il4ra*$^{-/-}$ mice. Nevertheless elevated *Tgfb* expression in WT tumors may be an indirect mechanism of MDSC such as MDSC promoted $CD4^{+}CD25^{+}$ Treg cells that may also infiltrate in gliomas (Nakamura, Kitani et al. 2001; Ostrand-Rosenberg 2010).

Herein, we have demonstrated the impact and importance of MDSCs and IL-4Rα expression on glioma immunity and tumor progression. Our finding that *Il4ra*$^{-/-}$ mice have prolonged SFS compared with WT mice in the absence of T-cells suggests that cells other than T-cells are also important for the better survival of *Il4ra*$^{-/-}$ mice challenged with SB gliomas (possibly MDSCs). Thus it is important to note that in the absence of T-cells MDSCs may exert suppressive function on other cells such as NK cells or possibly direct promotion of tumor cell growth. Further studies are being conducted to determine the *in vivo* ability of Il4ra$^{-/-}$ MDSCs compared with WT MDSCs to suppress WT T-cells. Interesting depletion of CD4 and CD8 T-cells influenced survival of *Il4ra*$^{-/-}$ mice to a greater extent than WT mice. This may be attributed to the specific nature of SB gliomas: when tumors start to develop and their specific growth rates. These findings suggest that inhibited MDSC function in *Il4ra*$^{-/-}$ mice is a major contributor to tumor suppression, strongly supported by the better overall survival of mice depleted by MDSCs using anti-Gr1 mAb.

We propose based on our findings in Aim 2, a mechanism in which GM-CSF in the glioma microenvironment promotes IL-4Rα expression on MDSCs, leading to IL-13 mediated production of arginase. Arginase activity can then suppress anti-tumor immune cells, including T-cells (and possibly other cells) and promote the development of glioma growth **(Figure 20)**.

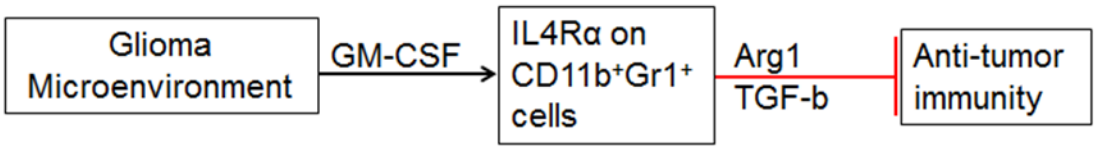

Figure 20: Proposed mechanism of IL-4Rα mediated inhibition of anti-tumor immunity.

11.0 AIM 3 BACKGROUND (SNP IN IFNA8)

Among a variety of cytokines and their signaling pathways, the type I IFNs, IFN-α and IFN-β, appear to play a key role in immunosurveillance against tumors. Although they have been long known to induce tumor cell apoptosis and angiogenesis inhibition (Hervas-Stubbs, Perez-Gracia et al. 2011), hematopoietic cells in the host (rather than tumor cells) are the crucial targets of the antitumor activity of endogenous type I IFNs (Dunn, Koebel et al. 2006). More recent studies with melanoma have demonstrated that host type I IFNs are critical for the innate immune recognition of a growing melanoma through signaling on $CD8\alpha^+$ DCs. (Diamond, Kinder et al. 2011; Fuertes, Kacha et al. 2011)

Previous studies have shown a significant impact of SNPs in innate immune pathways, such as ones in *Toll-Like Receptor (TLR) 3,* (Dhiman, Ovsyannikova et al. 2008; Yang, Stratton et al. 2008) *TLR4* (Apetoh, Ghiringhelli et al. 2007) as well as *IL-4Rα,* which is associated with differential risk and prognosis of GBM. (Schwartzbaum, Ahlbom et al. 2007; Scheurer, Amirian et al. 2008) Recently, we reported a previously undefined protective role of the type I IFN pathway in the surveillance against *de novo* mouse gliomas and that SNPs in *IFNAR1* and *IFNA8* are associated with significantly altered overall survival of patients with WHO grade 2 to 3 gliomas **(Figure 21)**. (Fujita,

Scheurer et al. 2010) Specifically, the SNP rs12553612 is located at 335 base pairs (bp) upstream of the *IFNA8* initiation codon, which is in the *IFNA8* promoter region. As a SNP in a promoter region may affect the promoter activity and therefore the gene expression levels, (Shastry 2009) we hypothesized that the SNP in the *IFNA8* promoter (rs12553612) affects the interaction of transcription factors with the DNA region involving the SNP, thereby affecting the activity of the *IFNA8* promoter.

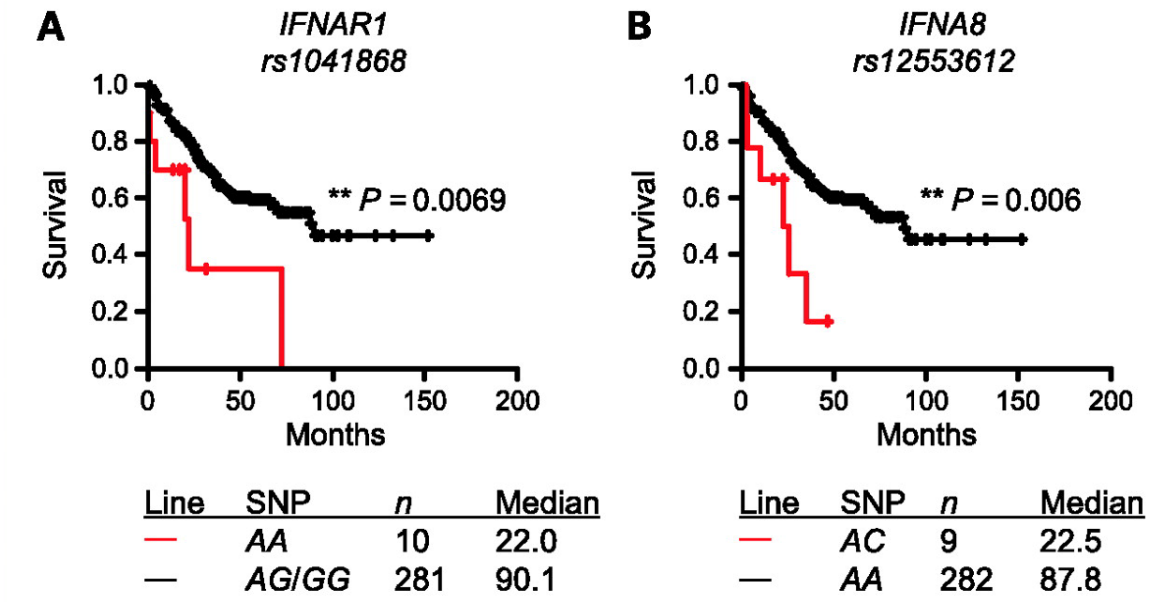

Figure 21: Association of SNPs in IFN-related genes and the survival of patients with WHO grade 2 to 3 gliomas. Overall survival was evaluated among 304 glioma patients with grade 2 to 3 gliomas by genotype for SNPs in IFN-related genes. (A), patients with AA genotype (red line) for IFNAR1 rs1041868 exhibited a significantly shorter survival than those with the AG/GG genotypes (black line). (B), patients with AC genotype (red line) for IFNA8 rs12553612 exhibited a significantly shorter survival than those with the AA genotype (black line).

12.0 AIM 3 MATERIALS AND METHODS (SNP IN IFNA8)

12.1 REAGENTS

RPMI 1640, FBS, L-glutamine, sodium pyruvate, 2-mercaptoethanol, nonessential amino acids, and penicillin/streptomycin and all reagents for DNA transfection were purchased from Invitrogen, including Lipofectamine 2000 (11668-02), with the exception of luciferase reporter genes and dual-luciferase reporter assay system, which were from Promega (E1910). Plasmids containing human cDNA clones were purchased from Origene. Oligonucleotides for the *IFNA8* electrophoretic mobility shift assays (EMSA) were obtained from Integrated DNA technologies; EMSA were done using the Thermo LightShift Chemiluminescent EMSA Kit (20148). Oct-1 (clone C-21, sc-232) and Elk-1 (cloneI-20, sc-355) antibodies were purchased from Santa Cruz Biotechnology.

12.2 CELL CULTURE

The THP-1 human monocyte cell line was maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, 50 units/mL penicillin, 50µg/mL streptomycin, 1mM Sodium Pyruvate, 55µM 2-Mercaptoethanol, and MEM non-essential amino acids (Life Technologies Invitrogen) in a humidified incubator in 5% CO2 at 37°C.

12.3 DNA TRANSFECTION

Mixture of plasmid-DNA (pDNA) was prepared as 0.08-0.2 µg (total pDNA value)/25 µl/well. Usually, 0.02 µg/well pGL4.73 (hRluc/SV40) vector (Rluc) as internal control and 0.06-0.18 µg/well pGL4.20 (luc2/Puro) vector (Fluc) containing the A or C genotype of *IFNA8* promoter gene were combined with diluted Lipofectamine 2000 (0.5 µl/well), and incubated for 20 min at room temperature. After the incubation, the complexes were added to each well containing 1 x 10^5 THP-1 cells and incubated at 37 °C in a CO2 incubator for 24 hours. During the incubation, 100-150 µl/well complete medium was added 18-20 hours after the transfection. When using stimulant such as poly-ICLC (Oncovir, Inc) and LPS, the stimulant was added 2 hours before stopping the incubation.

12.4 DUAL-LUCIFERASE ASSAY

Cultured cells with complexes for DNA transfection were centrifuged at three times at 1,500 rpm for 2-3 min. 80 µl/well passive lysis buffer (PLB) was added into the well containing the pellet and incubated for 15 min at room temperature. Aliquots of PLB lysate (20 µl) were transferred into luminometer tube containing 70 µl of LARII and inserted into luminometer machine to measure Fluc activity. After addition of 100 µl of Stop&Glo Reagent, Rluc activity was measured immediately. Relative Luciferase activity (A- [or C-] Fluc / A- [or C-] Rluc) / (untreated C-Fluc / untreated C-Rluc) was measured using OD data from triplicate cell-lysates.

12.5 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

The non-radioactive LightShift Chemiluminescent EMSA Kit (Thermo, 20148) was used to detect DNA-protein interactions. The 5′biotin end-labeled DNA oligonucleotides each containing one SNP in the IFNA8 promoter, were used as probes for the EMSA. The probe DNAs were 40-bp double stranded DNA made by annealing (95 °C for 5 minutes) single stranded oligonucleotides for the A-SNP (5’-Biotin-TAGGAATGTAGTACATTCAAATATGTGCATAATATATCTG and 5’-Biotin CAGATATA TTATGCACATATTTGAATGTACTACATTCCTA) and the C-SNP (5’-Biotin-TAG GAATGTAGTACATTCACATATGTGCATAATATATCTG and 5’-Biotin-CAGATATATTATGCACATATGTGAATGTACTACATTCCTA). Specificity was

determined by a competition assay with the addition of 200 molar excess of unlabeled double stranded IFNA8 promoter oligonucleotide.

Nuclear extracts (10 µg) were isolated from the THP-1 human monocyte cell line using the Thermo Subcellular Protein Fractionation Kit (Thermo, 7884) and protein concentration was determined by the Bradford assay. Aliquots of nuclear extracts (10ug) were incubated with 20 femtomole (fM) AT or GC IFNA8 probe in 1x binding buffer, 500 mM KCl, 0.1% NP40, 2.5% glycerol, 50 ng/µl poly dI-dC and 5mM MgCl2 for 20 minutes and were then electrophoresed through a 6% DNA retardation gel at 70V for ~45 minutes. For the supershift assay, nuclear extracts were incubated with antibodies for Elk-1 or Oct-1 for 2 hours on ice prior to incubation with probes. The gels were electrophoretically transferred at 380mA for 1 hour on ice to a positively charged nylon membrane and immediately cross-linked for 15 minutes with a UV transilluminator equipped with a 312 nm bulb. Streptavidin-horseradish peroxidase conjugate and the LightShift Chemiluminescent Substrate (Thermo, 89880) were used to detect the biotin end-labeled DNA. The nylon membranes were exposed to x-ray film for 0.5–2 minutes for detection of the signal.

12.6 STATISTICAL ANALYSES

The statistical significance (P value) of differences between groups was calculated by unpaired two-tailed Student's *t* test. Differences were considered as significant when $p < 0.05$. All statistical analyses were carried out on Graphpad Prism software.

13.0 AIM 3 RESULTS (SNP IN IFNA8)

13.1 THE A-GENOTYPE LEADS TO SUPERIOR PROMOTER ACTIVITY COMPARED WITH THE C-GENOTYPE

Glioma patients with the AA-genotype in the rs12553612 SNP in the *IFNA8* promoter exhibit prolonged overall survival compared with patients with the AC-genotype **(Figure 21)**. (Fujita, Scheurer et al. 2010) Additionally, as type I IFNs promote immune cell functions, we examined whether *IFNA8* promoter activities in the A-genotype were superior to those in the C-genotype. To understand the underlying molecular basis, we created *IFNA8* promoter luciferase constructs by cloning the promoter region of *IFNA8* (-1528~-27 upstream the *IFNA8* precursor) with either A or C nucleotide at position -335 into the pGL4.20 luciferase vector at the XhoI and HindIII sites in the multiple cloning site . Human monocyte derived THP-1 cells were co-transfected with these firefly luciferase reporter plasmids with the A- or C- genotype (Fluc) and Renilla luciferase plasmid for internal control (Rluc). Relative luciferase activities (Fluc/Rluc) were obtained at 24 hours after the co-transfection **(Figure 22A)**. We found that the *IFNA8* A-genotype reporter plasmid demonstrated significantly higher activity than the C-genotype. The immunoadjuvant poly-ICLC has been shown to enhance the efficacy of glioma vaccines, as we previously demonstrated in glioma-bearing mice (Zhu, Fallert-

Junecko et al. 2010; Maes and Van Gool 2011) and humans. (Okada, Kalinski et al. 2011) As poly-ICLC and lipopolysaccharide (LPS) are potent inducers of type I IFNs, we further examined whether they could enhance *IFNA8* promoter activity of the A and C genotype. Following 2 hour treatment with 10 µg/ml poly-ICLC or LPS, THP-1 cells still exhibited increased activity of the A-genotype *IFNA8* promoter over the C-genotype in the presence of LPS **(Figure 22B)** or poly-ICLC **(Figure 22C)**.

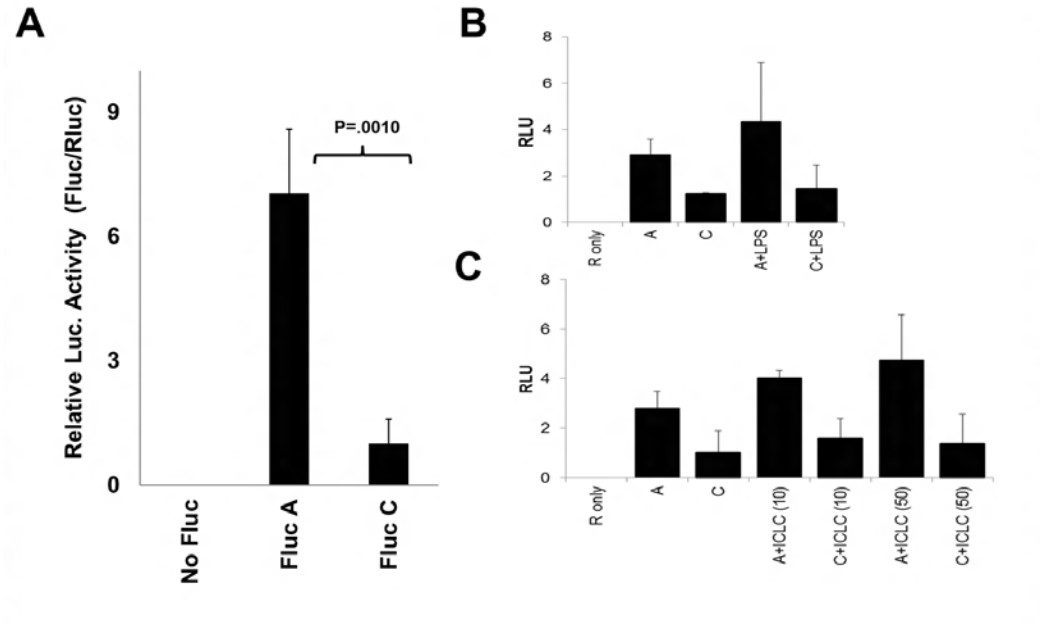

Figure 22: *IFNA8* promoter activity with the A-genotype at -335 is superior to that with the C-genotype. (A), 1 x 105 THP-1 cells were co-transfected with 0.02 µg of pGL4 vector encoding Rluc as internal control and 0.18 µg of pGL4 vector encoding Fluc downstream of IFNA8 promoter with A- or C-genotype (A-Fluc and C-Fluc). Twenty-four hours after the co-transfection, luc activity was measured from triplicate cell-lysates and relative luciferase was calculated (Fluc/Rluc). (Band C), Twenty-four hours after the co-transfection, the cells were stimulated with either (B) 10µg/ml of LPS or (C) 10 or 50 µg/ml of poly-ICLC. Two hours after the stimulation, relative luciferase activity was measured. Results are from one of three experiments with similar results. The P value was calculated by an unpaired two-tailed Student's t test. Error bars indicate standard deviation among triplicate samples.

13.2 THE A-GENOTYPE IFN-A8 PROMOTER SPECIFICALLY BINDS MORE NUCLEAR PROTEINS THAN THE C-GENOTYPE.

We hypothesized that the observed differential activities of the promoter constructs reflecting the two SNPs were mediated by altered binding of transcription factors at the site of SNPs. We therefore extracted nuclear proteins from THP-1 cells and incubated them with biotin-labeled 40-mers derived from the *IFNA8* promoter (nucleotide -354 to -314) with either A- or C- genotype. By using EMSA to detect protein bound DNA, we found that the probe with A-genotype SNP binds to either more protein and/or with a higher affinity than the C-genotype SNP **(Figure 23A)** as seen in both the shifted blot and densitometry plot. To demonstrate that the observed binding is sequence-specific, a competition assay was conducted using 200 fold more non-biotin labeled (but otherwise identical) 40-mers added to the sample. Indeed, the protein-DNA interaction was specific as the non-biotin labeled competitive inhibitors blocked the binding of protein both with the A- and C- genotype **(Figure 23B)**.

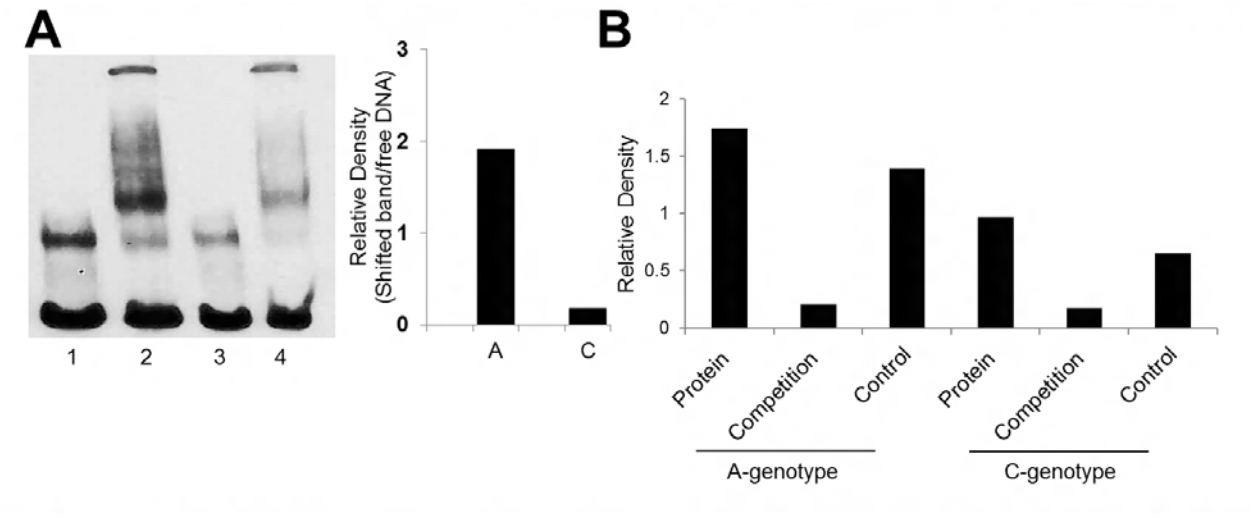

Figure 23: The DNA probe with the A-genotype in the *IFNA8* promoter demonstrates higher binding to THP-1 nuclear lysate than one with the C-genotype by EMSA. (A), An EMSA was performed with biotin labeled DNA 40mers (20 fmol) with either the A-genotype or C-genotype SNP using THP-1-derived nuclear lysate (10 µg) (protein lysate). Lane 1, A-genotype DNA alone; Lane 2, A-genotype DNA incubated with protein lysate; Lane 3, C-genotype DNA alone; Lane 4, C-genotype DNA incubated with protein lysate. Quantification of the bands in Lanes 2 and 4 was done using ImageJ (National Institutes of Health) software. **(B)**, A competition assay with or without 200 fold excess non-labeled A- or C-genotype DNA over the biotin labeled SNP DNA (control) to compete specifically with DNA binding site or EBNA DNA (control). Quantification of the bands was done using ImageJ (National Institutes of Health) software. Results are from one of three experiments with similar results.

13.3 TRANSCRIPTION FACTOR OCT-1 BINDS AND ENHANCES THE PROMOTER ACTIVITY OF THE IFNA8 A-GENOTYPE

On the basis of predicted binding sites to the promoter region, we next performed a supershift assay to determine which proteins bind in greater amounts to the A-genotype.

We selected Oct-1 and Elk-1 as our *in silico* analysis with TFsearch (Heinemeyer, Wingender et al. 1998) predicted that Oct-1 and Elk-1 may bind to this region. Further, TFsearch predicted that Oct-1 would bind to the A- but not C- genotype. Surprisingly, although Elk-1 is expressed in THP-1 cells (not shown) we could not detect any supershift in either the A- or C-genotype when THP-1 nuclear protein extracts were pre-incubated with Elk-1 specific antibody **(Figure 24A)**. However, when the nuclear extracts were incubated with Oct-1 antibody we observed a supershift in the A- but not C- genotype, suggesting the binding of Oct-1 to the *IFNA8* promoter, as predicted *in silico*. These results suggest that Oct-1 may be the transcription factor involved in the activation of *IFNA8* promoter, and failure of Oct-1 to bind to the C-genotype results in lower *IFNA8* activity.

We further assessed whether overexpression of Elk-1 or Oct-1 could lead to enhanced activity of the *IFNA8* promoter. We therefore performed a promoter luciferase assay using the *IFNA8* promoter A- genotype. Consistently, overexpression of Oct-1 but not Elk-1 lead to a statistically significant increase in luciferase activity of the *IFNA8* promoter **(Figure 24b)**, further supporting the role of Oct-1 in the differential IFNA8 promoter activities between the A- and C- genotype.

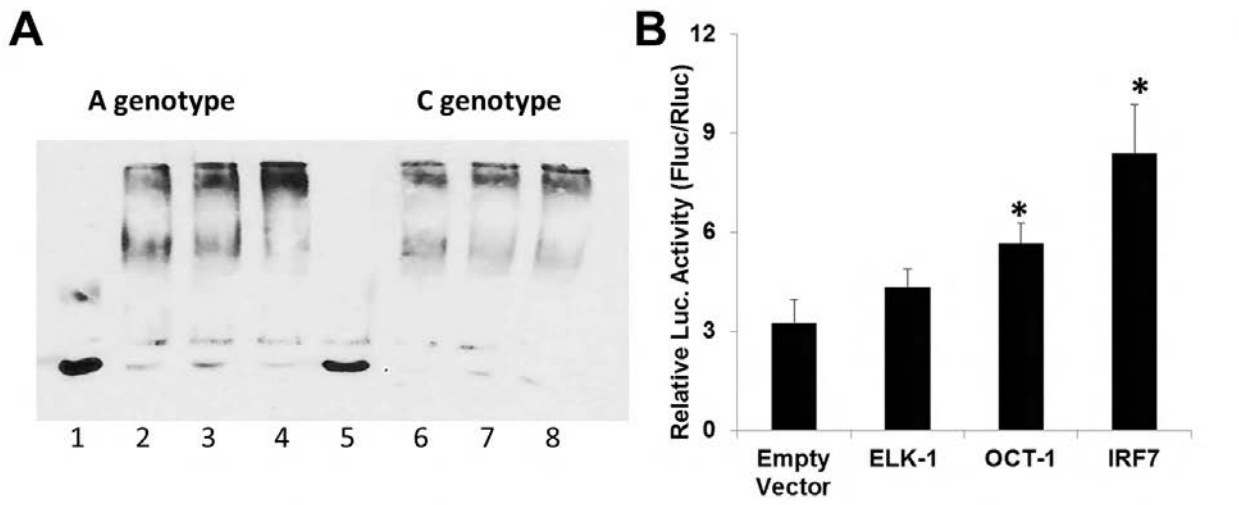

Figure 24: The A-genotype demonstrates superior binding to Oct-1 compared with the C-genotype. (A), Supershift assay was performed by pre-incubation of THP-1 cell nuclear lysate with either anti-Elk-1 or Oct-1 mAb prior to DNA binding assay. Lanes 1-4, A-genotype DNA probe; lanes 5-8,C-genotype DNA probe, lanes 1 and 5, DNA probes alone; lanes 2 and 6, DNA probes incubated with THP-1 lysate alone; lanes 3 and 7, DNA probes with THP-1 lysate preincubated with anti-Elk-1 mAb; lanes 4 and 8,DNA probes with THP-1 lysate preincubated with anti-Oct-1 mAb. The supershifted bands are marked with arrows. (B), THP-1 cells were transfected with the A-genotype Fluc-reporter plasmid and the internal control Rluc plasmid as well as an expression plasmid encoding either Elk-1, Oct-1 or IRF-7 as a positive control. Relative luciferase was calculated as Fluc/Rluc. Results are from one of two experiments with similar results. * Indicates that the values were statistically different ($P < .05$) from the control samples with the empty vector by unpaired two-tailed Student's t Test. Error bars indicate standard deviation among triplicate sample.

14.0 AIM 3 CONCLUSION (SNP IN IFNA8)

We herein described in Aim 3 that the A-genotype in the *IFNA8* promoter SNP rs125553612 confers a better promoter activity compared with the C-genotype. As we previously reported that WHO grade II-III glioma patients with the AA-genotype have better overall survival than patients with the AC-alleles, higher IFN-α8 expression levels may indeed contribute to the better survival of patients. Importantly this demonstrates the dominant effect of the C-allele (as patients have both the A and C allele) .Through a series of experiments, we provide the following molecular mechanism to explain this observation. As depicted by **Figure 25**, the rs12553612 SNP results in a change of Oct-1 binding site of *IFNA8* promoter at position -335, a change at this site from A to C allele causes substantial loss of transcription factor Oct-1 binding affinity to the promoter resulting in down-regulation of *IFNA8* transcription. As we have shown before, type I IFN signaling plays a major role in promoting anti-glioma immune surveillance. (Fujita, Scheurer et al. 2010) Thus a decreased *IFNA8* transcription activity may potentially affect the immune surveillance resulting in lower survival. Using overexpression experiments, we demonstrated that Oct-1 but not Elk-1 regulates the *IFNA8* promoter activity in the A- but not C- genotype.

Little is known about the roles and regulation of the individual *IFNA* genes of which there are 14 in humans. To date the primary activators of *IFNA* promoters that have been described are IFN regulatory factor (IRF) family members. (Colonna 2007) For example, upon Newcastle disease virus (NDV) infection, infected cells overexpress IRF-5 that induces *IFNA8*. (Barnes, Field et al. 2003) Therefore, IRF-5 may be a key transcription factor for *IFNA8*. However, IRF-5 can act as both an activator and a repressor of *IFN* gene induction dependent on the IRF-interacting partner including IRF-3 and IRF-7 (Barnes, Field et al. 2003). Further, *IFNA8* has been suggested to have the most potent anti-tumor activity in chronic myelogenous leukemia (CML)-derived cell lines (Yanai, Sanou et al. 2002), suggesting the importance of IFNA*8*.

Our data demonstrate that Oct-1 can bind and promote *IFNA8* promoter activity. Oct-1, also known as POU domain class 2 transcription factor 1 (POU2F1) is known to be post transcriptionally regulated at least in part by p34cdc2-related protein kinase which is active during mitosis as well as multiple other kinases and phosphatases. (Roberts, Segil et al. 1991) Thus activated, proliferating immune cells may have enhanced Oct-1 activity which can lead directly to type I IFN production. Other studies have demonstrated additional mechanisms by which Oct-1 function is regulated, such as hydrogen peroxide which can stimulate the nuclear import of Oct-1 (Wang and Jin 2010) and the glucocorticoid receptor which can synergize with Oct-1 and promote recruitment of the complex (Oct-1 and the glucocorticoid receptor) to glucocorticoid response elements on DNA. (Prefontaine, Lemieux et al. 1998) Oct-1 can also be activated in response to DNA damage. (Zhao, Jin et al. 2000) These may still be partial

mechanisms with which Oct-1 is regulated in glioma tissues and IFNA8 is induced in patients with the A- genotype. Interestingly, Oct-1 can inhibit IRF-7- and IRF-3-mediated IFNA11 expression in a virus infection model. (Mesplede, Island et al. 2005) Further investigations are warranted to evaluate the role of Oct-1 in the entire type-I IFN families and anti-tumor immunity.

Further investigations using samples obtained from human donors with AA-, AC- or CC genotype would have strengthened our study. However, unfortunately, analysis of patient samples was not feasible as we have previously reported that of about 300 patients analyzed few patients (n=9) have the AC- genotype and we identified no individuals with the CC-genotype. (Fujita, Scheurer et al. 2010) Accordingly, the National Center for Biotechnology Information (NCBI) data base for the current SNP (rs12553612) indicates that among a total 947 individuals analyzed AA, AC and CC genotypes were found in 719, 122 and 14 individuals, respectively, with a dominant prevalence of AC and CC genotypes in Asian populations (http://www.ncbi.nlm.nih.gov/projects/SNP/snp_ref.cgi?rs=12553612#Diversity).

Taken together, our data suggest that Oct-1 can regulate IFNA8 promoter in the A-genotype but not the C-genotype allele. We predict based on our data that patients with the AA- genotype should have higher expression of IFNA8 than patients with the AC- or CC- genotype.

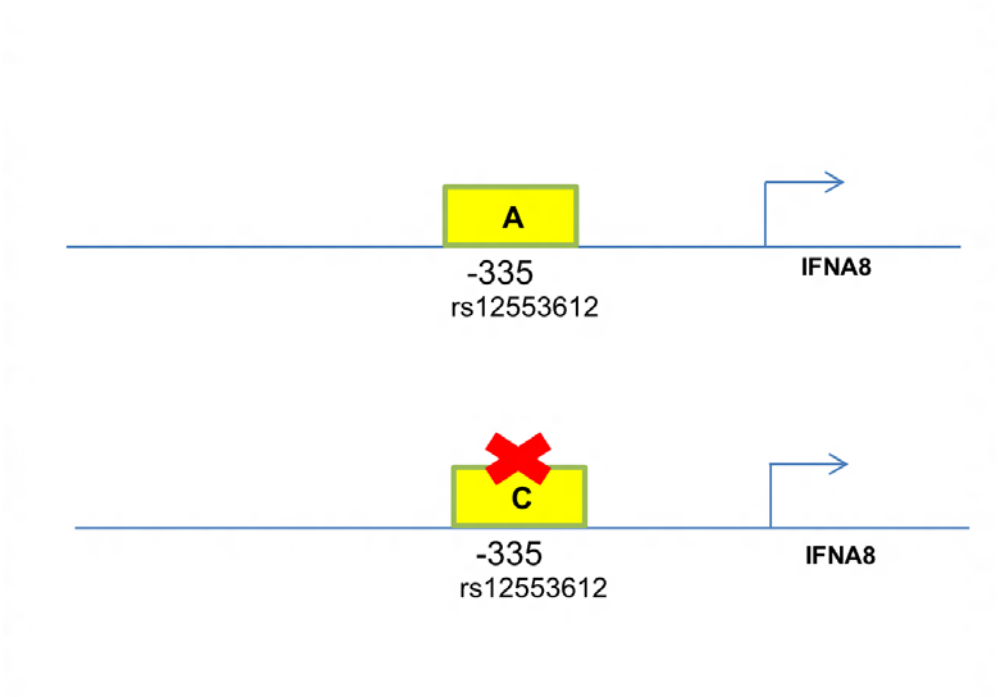

Figure 25: Schematic, demonstrating the Oct-1 binding ability to the IFNA8 promoter region containing the rs12553612 SNP.

15.0 OVERALL DISCUSSION

Overall herein we have demonstrated relevant roles of the type-1/IFN pathway and the type-2/IL-4 and 13 pathways in glioma development. Our finding on the IL-4R signaling regulation of miR-17-92 cluster is highly relevant to cancer immunotherapy and addresses some of the major barriers to effective immunotherapy: T-cell persistence and effector function. Further, miR-17-92 may be used as a biomarker in cancer patients, as patients with less effective IL-4R signaling presumably will have increased miR-17-92 and improved anti-tumor immunity. We have a patent pending on the use of miR-17-92 in T-cell adoptive transfer (application # 20100322909) and believe this will have potential as use in cancer immunotherapy.

The up-regulation of IL-4Rα on human glioma-infiltrating MDSCs and its association with suppressive molecules demonstrate the feasibility to target these cells in glioma patients. Further, in mice we demonstrated the importance of IL-4Rα on MDSCs using *Il4ra*$^{-/-}$ mice, which had few glioma infiltrating MDSCs and reduced ability to inhibit T-cells, through a mechanism at least partially mediated by arginase. As discussed in the introduction, there are multiple mechanisms to deplete MDSCs and each should be evaluated for both efficacy and off-target effects.

We believe blocking IL-4R (by antibody, aptamer or other mechanism) signaling may be useful to both improve T-cells and suppress MDSCs. However, based on our data solely blocking the suppression of miR-17-92 is not likely to be as effective as overexpressing miR-17-92 which allows for ectopic expression. On MDSCs blockade of IL-4Rα signaling may be promising to reduce MDSC production of arginase however this may be harder to accomplish as antibodies have difficulties crossing the BBB while a small molecule inhibitor of arginase may be more effective. Our studies demonstrate the importance of understanding the biological mechanisms of action mediated by specific genes for the development of effective therapeutics.

While we detected IL-13 in SB glioma tissue this was not the case for IL-4, which was undetectable by RT-PCR. In Aim 1 we proposed tumor IL-4-mediated down regulation of miR-17-92 cluster as the primary mechanism. While in Aim 2 we did not detect IL-4, the alternate model used in Aim 1 may have expressed IL-4. Aim 1 utilized C57Bl/6-background mice and B16 tumors while Aim 2 utilized BALB/c-background mice with SB gliomas. Alternatively, as IL-13 signaling also is mediated by STAT6, IL-13 expression may have contributed to the miR-17-92 down-regulation observed in WT but not Stat6$^{-/-}$ tumor bearing mice.

One benefit of SNP analysis is that it provides specific genes which may be evaluated. Based on our findings on the importance of type I IFNs in glioma development (Fujita, Scheurer et al. 2010), our collaborators examined SNPs in a variety of IFN-related genes. This led to the finding that SNPs in *IFNAR1* and *IFNA8* are associated with glioma prognosis. Thus we were able to focus on genes with clinical significance of the many type I IFN genes. We thus analyzed the specific impact of

IFNA8 and found that the C-genotype of rs12553612 in the promoter region of *IFNA8* leads to decreased *IFNA8* promoter activity through inhibited binding of Oct-1 compared to the A-genotype. As patients with the C-genotype also have a worse outcome, this SNP may be used as a biomarker and patients with the C-genotype may be specifically suitable for type I IFN therapy.

The data described herein support the role for further SNP guided studies to understand the importance of specific genes in anti-glioma immunity. Future studies using this method will likely lead to identification of risk factors, novel preventative and therapeutic strategies based on firm biological mechanism.

16.0 FUTURE DIRECTIONS

16.1 EVALUATE THE MOLECULAR MECHANISM FOR IL-4RA SNPS.

In aims 1 and 2, we have demonstrated the tumor promoting effects of IL-4R signaling on T-cells and MDSCs in both humans and mice. Our interest in IL-4R signaling stems from type-2 immune biology and the epidemiologic studies demonstrating that SNPs rs1805015 and rs1805016 are associated with glioma prognosis. We have demonstrated that IL-4Rα signaling may both suppress miR-17-92 cluster in T-cells and promote MDSC immune suppressor function. It remains important to evaluate the impact of each SNP in IL-4Rα signaling.

SNP rs1805015 affects the cytoplasmic domain of IL-4Rα with the reference/alternative allele T/C resulting in S503/P503. This region has been postulated to be required for IRS1 (*Insulin receptor substrate 1)* activation(Kruse, Japha et al. 1999). SNP rs1805016 affects position 752, with the reference/alternative allele T/G resulting in S752/P752. Aside from conferring susceptibility to asthma, atopy and prognosis of GBM patients no functional consequence is currently known for this variant(Ober, Leavitt et al. 2000). In IL-4Rα, both 752 and 753 are serine (S) and have very high potential to be phosphorylated (NetPhos 2.0). Additionally, the nearby T756 has been reported to be phosphorylated in high-throughput proteomic analysis(Wu,

Wang et al. 2010). Further, the serine or proline amino acides may account for structural variations which impacts signaling. To evaluate the effect of each SNP on intracellular signaling we will use 2 different models: 1) transfection and 2) patient sample analysis.

16.1.1 Transfection Approach to Evaluate IL-4Rα SNPs.

We will use two cell lines, THP-1 and HEK293, to study IL-4Rα signaling. Unlike THP-1 cells which express IL-4Rα, HEK293 cells do not express STAT6 or IL-4Rα and are not sensitive to IL-4/IL-13 stimulation. However, exogenous expression of STAT6 has been successfully used in these cells for IL-4 signaling studies (Ohmori and Hamilton 2000). As discussed below, we will take advantage of the IL-4Rα-null feature of HEK293 cells to investigate STAT6 binding and phosphorylation status in presence of different *IL4Rα* variants.

We will establish THP-1 cells in which endogenous IL-4Rα expression is silenced using commercially available shRNA. This will allow us to express recombinant IL-4Rα devoid of 3'UTR in the same cells with shRNA. We will express different variants of IL-4Rα which we have already created and demonstrated to express in HEK293 cells **(Figure 26)**, and assay for STAT6 phosphorylation in the presence of IL-4 and IL-13 by Western blot. We will also measure signaling output in presence of these constructs by assaying transcription of GATA3 and miR-17-92 cluster expression, target genes of STAT6, by RT-PCR. Besides GATA3 and miR-17-92 cluster, in response to IL-4 or IL-13, STAT6 is also involved in the transcriptional induction of a number of genes, such

as *Arg1* and MHC II, in myeloid cells (Elo, Jarvenpaa et al. 2010; Goenka and Kaplan 2011). These findings will indicate the critical role for SNPs in the IL-4Rα-pathway for myeloid cell production of arginase and mir-17-92 cluster expression in T-cells.

One major benefit of this system over the use of human primary samples with known SNPs is the lack of confounding variables, as we are using cell lines. However as cell lines are immortalized they may have disrupted signaling molecules which may influence observations.

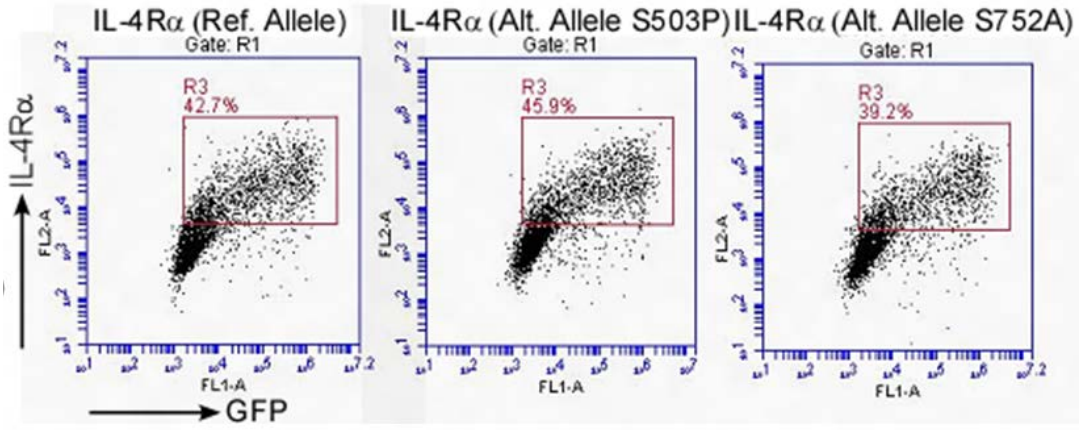

Figure 26: Expression of recombinant *IL4RA* SNP variants in HEK293 cells. HEK293 cells were transfected with plasmids containing a GFP-tagged *IL4RA* gene with either the reference allele or alternate alleles S503P (rs1805015) or S752A (rs1805106), using the Lipofectamine 2000 protocol. 48 hours after transfection cells were surface stained for IL-4Rα and GFP and analyze by flow cytometry.

16.1.2 Describe the Function of *IL4RA* Polymorphisms in Healthy Donor and Glioma Patient PBMCs

We will identify and compare the intracellular signaling capabilities of healthy donor PBMCs and/or glioma patient PBMCs either the dominant or alternative alleles in rs1805015 and rs1805016 following stimulation with either IL-4 or IL-13. Using patient samples we examined each donors genotype for the 2 SNPs by RT-PCR. We then plotted the results **(Figure 27)** and identified which samples were homozygous for the dominant allele (Blue box), homozygous for the alternative allele (yellow box) or heterozygous (green box). Our system allowed for tight grouping of samples. Based on these data we plan to obtain additional PBMC from patients in each group, stimulate the cells with IL-4 or IL-13 and then evaluate STAT6 phosphorylation, GATA3 and other molecules in a similar manner discussed previously with the transfection method **(16.1.1)**

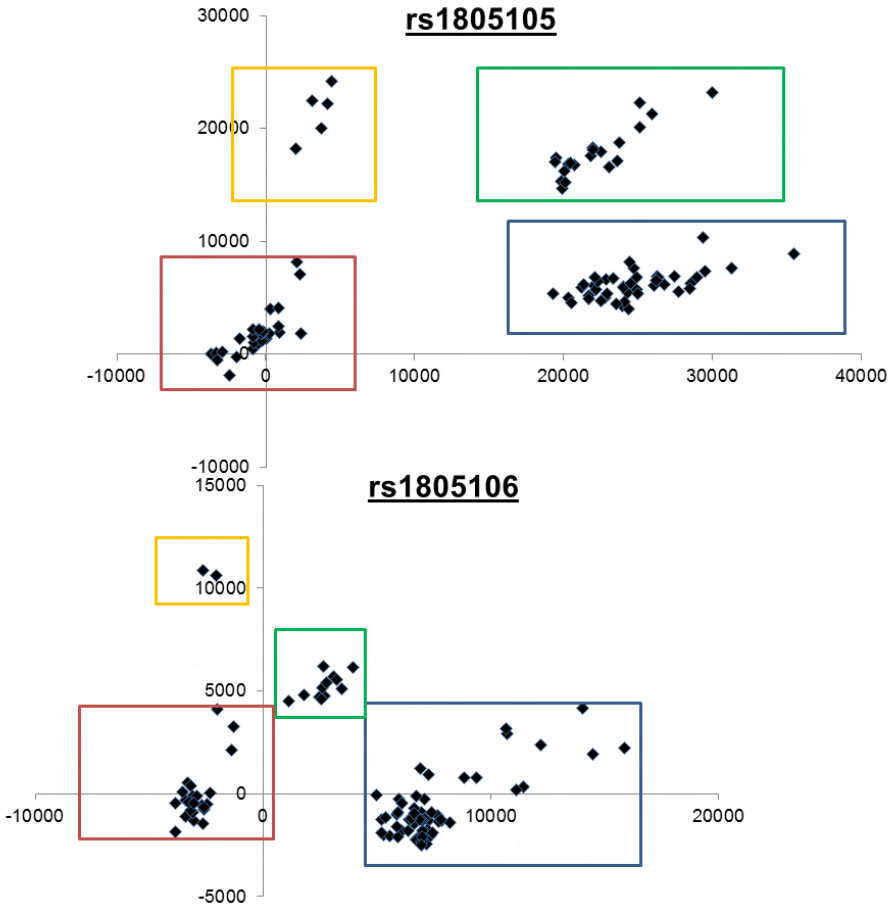

Figure 27: Single Nucleotide Polymorphism identification in human samples. Genomic DNA was isolated from donor PBMCs using the Qiagen DNeasy kit. Primer/probe kits for each SNP were obtained from Applied Biosciences and were used according to the manufacturer's protocol. FAM and VIC endpoint readings were plotted and each genotype group received a box. Blue-Homozygous for dominant allele, yellow-Homozygous for alternate allele and green, Heterozygous. Samples in red did not have detectable levels of either allele due to do to failed PCR.

16.2 EVALUATE TUMOR GROWTH IN MIR-17-92 TG/TG MICE

Our data from AIM 1 demonstrates the ability of miR-17-92 overexpression in T-cells to resist AICD, with increased IFN-γ and lL-2 production. Further, we have demonstrated that tumor conditions suppress miR-17-92 expression. Therefor we would like to examine glioma growth in both miR-17-92 TG/TG mice and in WT mice receiving adoptive transfer of miR-17-92 TG//TG T-cells. We may also generate mIR-17-92 TG/TG mice that express the pmel gene making all $CD8^+$ T-cells specific for GP100 and use these cells for adoptive transfer (for these experiments we would use murine gliomas that express GP100). We will monitor mice for SFS and immunological response, such as intratumoral T-cell numbers and viability IFN-γ production. We expect that following glioma challenge miR-17-92 expression will remain high in T-cells, however based on our data we will evaluate if IL-4 down regulates miR-17-92 in the transgenic mice. We will also carefully monitor these mice for any signs of autoimmunity, however we believe this is unlikely based on our previous pathological analysis of miR-17-92 TG/TG mice. These studies will further support the rational for a clinical trial evaluating adoptive transfer of miR-17-92 overexpressing T-cells for glioma patients.

16.3 CORRELATION OF PATIENT DATA WITH FINDINGS

Based on our findings that miR-17-92, arginase and IL-4Rα play critical roles in glioma immunity it would be important to understand which factors play the most significant role in glioma patients. To accomplish this we would like to evaluate expression of these genes/molecules by RT-PCR and where available flow cytometry. Then using patient records try to correlate each with glioma prognosis. We have an IRB protocol that allows us to obtain de-identified patient samples and then get patient information through an intermediate broker. We expect to find that expression of some of these genes may correlate with patient outcome.

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