NEUTROPHIL ACTIVATION INDUCES PERIPHERAL IMMUNOSUPPRESSION IN GLIOBLASTOMA AND STROKE

by

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Neutrophil activation induces peripheral immunosuppression in glioblastoma and stroke
Thesis directed by Assistant Professor Allen Waziri.

ABSTRACT

Immunosuppression remains a challenge in the treatment of many cancers. Glioblastoma (GBM) is a highly malignant brain tumor that demonstrates significant tumor-associated immunosuppression. In recent years, cells of myeloid lineage have been increasingly associated with immunosuppression. The goals of this project were to characterize potential myeloid-derived suppressor cells in GBM, identify targets of their immunosuppressive effects, and explore pathology-specific factors associated with their development.

In the early phases of our work, we identified an expanded population of activated neutrophils within the peripheral circulation of patients with GBM. These cells were associated with elevated serum levels of Arginase I (ArgI) and suppressed T cell function in vitro. Critically, T cell suppression could be reversed in vitro through pharmacologic inhibition of ArgI or with L-arginine supplementation. As neutrophils are expected to transmigrate and persist within inflamed tissues, the activated circulating population in GBM patients was of unclear etiology. Through a series of phenotypic and functional analyses of the various neutrophil populations in GBM patients, we confirmed that 1) "resting" neutrophils in these patients appear normal in form and function, 2) intratumoral neutrophils express expected markers of activation, and 3) circulating activated neutrophils appear to be associated with failed transmigration.

This seminal observation drove us to explore potential tissue-related changes that could be associated with disruption of neutrophil migration. Given limited physiological parallels with available animal brain tumor models, we explored other
pathological conditions in which brain tissue damage is known to occur. Ischemic stroke shares pathological similarities to GBM, including necrosis and tissue edema, and transient suppression of cellular immunity is seen in affected patients. Therefore, through collaborative efforts we explored peripheral immunity in a mouse model of stroke. Critically, we confirmed the role of the activated-neutrophil/Arg1 in suppressing T cell function in affected animals, which once again could be reversed through L-arginine supplementation in vitro.

In summary, we have identified a novel source of immunosuppression in both GBM and stroke that offers a potential target for improving immune function in affected patients. This work has also opened new avenues of exploration into pathology-specific effects on circulating neutrophils.

The form and content of this abstract are approved. I recommend its publication.

Approved: Allen Waziri
I dedicate this work to my ever supporting and loving family.

I also dedicate this work to the generous patients who donated a part of themselves in order to help those who will follow them.
ACKNOWLEDGMENTS

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## LIST OF ABBREVIATIONS

**Abbreviation**

- ACT – Adoptive cell transfer.................................................................21
- AICD – Antibody induced cell death..................................................33
- AIM-2 – Absent in melanoma 2..........................................................28
- APC – Adenomatous polyposis coli.....................................................2
- APCs – Antigen presenting cells.........................................................23
- APG – Anaplastic glioma tumor patient..............................................59
- Arg I – Arginase I.............................................................................35
- BBB – Blood brain barrier.................................................................24
- BCECF – 2',7'-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein..........76
- bFGF – Basic Fibroblast Growth Factor............................................15
- BMI1 – B lymphoma Mo-MLV insertion region 1 homolog.................8
- BrdU – Bromodeoxyuridine...............................................................55
- BTSC – Brain Tumor Stem Cell...........................................................7
- CAR – Cimeric Antigen Receptor.......................................................21
- CDKN2A – Cyclin Dependent Kinase Inhibitor 2A...............................5
- CFSE – Carboxyfluorescein succinimidyl ester..................................52
- CHI34 – Chitinase-3-like protein 1.....................................................6
- CNS – Central Nervous System.........................................................3
- CTL – Cytotoxic T Lymphocyte..........................................................21
- CTLA-4 – Cytotoxic T lymphocyte-associated antigen 4.....................32
- DC – Dendritic cell.............................................................................22
- DT – *Diptheria*...............................................................................30
- EGFR – Epidermal Growth Factor Receptor...........................................5
EGFRvIII – Epidermal Growth Factor Receptor variant III

ENA-78 – Epithelial-derived neutrophil activating peptide 78

ESGL-1 – E-selectin liginand-1

FAK – Focal adhesion kinase

FDA – Food and Drug Administration

FLAIR – Fluid Attenuation Inversion Recovery

fMLP – Formyl-Methionyl-Leucyl-Phenylalanine

FoxP3 – Forkhead box P3

FSC – Forward Scatter

GABRA1 – Gamma-aminobutyric acid receptor subunit alpha-1

GBM – Glioblastoma

G-CSF – Granulocyte colony-stimulating factor

GCP-2 – Granulocyte chemotactic protein 2

GM-CSF – Granulocyte macrophage colony-stimulating factor

GRO-α – Growth-regulated protein α

HBSS – Hanks balanced Salt solution

H&E – Hemotoxylin and eosin

HER2 – Human epidermal growth factor receptor 2

HIF1α – Hypoxia Inducible Factor 1α

HLA – Human Leukocyte Antigen

HMEC – Human microvascular endothelial cells

HSP – Heat shock protein

ICAM-1 – Intracellular adhesion molecule 1

ICOS – Inducible T-cell Costimulator

IDH1 – Isocitrate Dehydrogenase 1

IDO – Indoleamine 2,3-dioxygenase
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<td>Interferon-α</td>
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<td>IL-1Ra</td>
<td>Interleukin 1 receptor agonist</td>
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<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>IL13Rα2</td>
<td>Interleukin 13 receptor α2</td>
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<tr>
<td>iNOS</td>
<td>Inducible nitric oxide synthase</td>
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<tr>
<td>KLH</td>
<td>Keyhole limpet hemocyanin</td>
<td>28</td>
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<tr>
<td>LAK</td>
<td>Lymphokine-activated killer cells</td>
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<td>LFA-1</td>
<td>Lymphocyte function-associated antigen 1</td>
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<td>LPS</td>
<td>Lipopolysaccharide</td>
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<td>MAC-1</td>
<td>Macrophage-1 antigen</td>
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<td>MCAO</td>
<td>Middle cerebral artery occlusion</td>
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<td>MAGE-1</td>
<td>Melanoma-associated antigen-1</td>
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<td>MDSC</td>
<td>Myeloid derived suppressor cell</td>
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<td>MEN</td>
<td>Meningioma tumor patient</td>
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<td>MERTK</td>
<td>c-Mer Proto-oncogene Tyrosine Kinase</td>
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<tr>
<td>MFI</td>
<td>Mean fluorescence intensity</td>
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<td>MGMT</td>
<td>O-6-methylguanine-DNA methyltransferase</td>
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<td>MHC</td>
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<td>MIF</td>
<td>Macrophage migration inhibitory factor</td>
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<td>MIP</td>
<td>Macrophage inflammatory protein</td>
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<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
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<td>MPO</td>
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<td>MLR</td>
<td>Mixed lymphoid reaction</td>
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<td>MRI</td>
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<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
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<td>NAP-2</td>
<td>Neutrophil activating peptide 2</td>
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RAR – Retinoic acid receptor ................................................................. 44
Rb – Retinoblastoma ........................................................................ 5
RNS – Reactive nitrogen species .........................................................128
SDF-1α – Stromal Cell-Derived Factor-1α ........................................ 15
SLC12A5 – Potassium-chloride transporter member 5 ...................... 6
SMC – Spleen mononuclear cells ..........................................................107
SNAP – Soluble NEM-sensitive factor attachment protein ..................40
SNARE – SNAP receptor .................................................................40
SOX2 – Sex determining region Y-box 2 ........................................... 8
SSC – Side scatter .......................................................... ........................ 62
STAT3 – Singal transducer and activator of transcription 3 ............... 44
SYT1 – synaptotagmin-1 ................................................................. 6
TAM – Tumor associated macrophage ..................................................34
TCGA – The Cancer Genome Atlas .................................................... 5
TCR – T cell Receptor .................................................................21
TGF-β – Transforming growth factor β ..............................................31
TIL – Tumor Infiltrating Lymphocyte ..................................................21
TLR – Toll-like Receptor .............................................................29
TNF-α – Tumor Necrosis Factor-α ....................................................34
Treg – Regulatory T cell .............................................................33
TRP-2 – Tyrosine-related protein 2 ..................................................28
TTC – 2,3,5-triphenyltetrazolium chloride .........................................106
VAMP – vesicle associated membrane protein ..................................40
VEGF – Vascular Endothelial Growth Factor ....................................9
WHO – World Health Organization .....................................................1
CHAPTER I

INTRODUCTION

Glioblastoma

Epidemiology and Prognosis

Glioblastoma (GBM) is the most common primary malignant brain tumor, making up approximately 50% of all gliomas (1). Though it represents only 2% of all diagnosed cancer, with approximately 10,000 new cases being diagnosed each year and an incidence rate of 3.2 new cases per 100,000 (2), it is one of the most aggressive and deadliest of all tumor types. The survival rate for newly diagnosed glioblastoma patients is only 12-15 months post diagnosis, with a 2 year survival rate of less than 10% and a 5 year survival rate of less than 5% (3). The median age of diagnosis is 64 years, making it a primarily adult disease, though it does occur at all ages. The incidence of GBM increases with increasing age, being most prevalent in the ages of 45-84 and the highest incidence occurring in the 75-84 age range at 15 per 100,000 (2). Survival rate also dramatically decreases with an increase in age. Patients diagnosed before the age of 40 have a median overall survival rate of 24 months, compared to only 7.5 months if the patient is over 70 (2).

In addition to age, there are several prognostic factors associated with prolonged survival including patient mental status, postoperative Karnofsky score, World Health Organization (WHO) performance score, and the extent of surgical tumor resection (4). The incidence of GBM is increased in males, being 40% more common in men than women, and is two times more common in whites than in African Americans or Asians (2). Despite these associations, the underlying cause of glioblastoma has yet to be identified. Several risk factors have been proposed, including exposure to
electromagnetic fields, brain trauma, occupational risk factors, use of cellular telephones, and intake of N-nitroso compounds; however, no conclusive evidence for any of these factors have been identified (5). Only exposure to ionizing radiation has been established as a risk factor for GBM (5). Less than 5% of GBM are familial cases, with the underlying cause for these being unknown, though some are associated with rare genetic syndromes such as Li-Fraumeni (germ-line mutation in p53), neurofibromatosis 1 and 2 (neurofibromin and merlin mutations respectively), and Turcot’s syndrome (mutations in adenomatous polyposis coli (APC), MLHI, MSH2, MSH6 or PMS2) (6).

**Clinical Presentation and Diagnosis**

The presentation of glioblastoma is highly variable between patients, depending on the location of the tumor within the brain. The most common clinical symptoms are headaches, seizures, focal neurological signs (such as aphasia, paresthesia, hemiparesis, and visual disturbances), confusion, memory loss, and mood or personality changes (7). Increased intracranial pressure due to the tumor and tumor-associated edema commonly induces headaches, usually most severe in the morning. When severe, they can be associated with vomiting and nausea (7). The clinical course for most patients is very short, with symptoms emerging rapidly and usually requiring medical care within three months of development (8).

A patient presenting with neurological symptoms will commonly undergo magnetic resonance imaging (MRI) with gadolinium enhancement. This imaging modality will produce both T1 and T2 weighted images that can be used to identify several distinguishing features of GBM. T1-weighted MRI shows contrast between grey and white matter based on lipid to water ratio. Grey matter appears darker, due to higher water content from increased cellularity, while white matter appears brighter owing to
high lipid content. This depiction is the opposite for T2 images, where higher water content is brighter. Gadolinium, which is normally impermeable to the blood brain barrier, adds enhancement to T1 images by identifying areas where there is blood brain barrier breakdown (9). By T1-weighted gadolinium enhanced images, GBM appears heterogeneous, with areas of enhancement and necrosis. The classical GBM is comprised of an enhancing rim with central necrosis (10). Signs of mass effect can also be observed by compression of anatomical structures near the tumor. T2 images identify peritumoral edema by hyperintensive areas of fluid surrounding the tumor. As increased cellularity (with high water content) can also appear hyperintensive on T2 images, it is difficult to differentiate between edema and invasive tumor. To aide in this distinction, FLAIR (Fluid Attenuation Inversion Recovery) sequences can be used to filter out the water signal from edema and allow for only the hypercellular tumor to remain.

Once GBM is suspected, a biopsy is performed to confirm tumor presence by histology. This validation is important for determining treatment strategies, as other pathologies can have similar features as GBM on MRI. For instance, metastatic lesions, primary central nervous system (CNS) lymphoma, brain abscesses, multiple sclerosis plaques, and other lesions can have similar radiographic appearance as GBM but would require considerably different treatment paradigms (11). Biopsy can be done at the time of craniotomy for surgical resection if the tumor is in an anatomical location favorable for resection, or by using a minimally invasive stereotactic approach if the tumor is in an area not conducive to surgical resection.

**Pathology and Biological Hallmarks**

Gliomas can be comprised of astrocytic, oligodendroglial, or mixed oligoastrocytic components based on morphology. Glioblastoma is defined as grade IV astrocytoma, the highest and most aggressive grade of astrocytoma. Other members of
the astrocytoma lineage include pilocytic astrocytoma (grade I), diffuse astrocytoma (grade II), and anaplastic astrocytoma (grade III) (1). Most cases of glioblastoma develop de novo (primary GBM) and rapidly run their clinical course, with a clinical history of only a few weeks. Only a few cases (about 10% of GBM) present as secondary glioblastoma, progressing gradually from a lower grade glioma (12). Secondary glioblastoma is much more common in younger patients, with a median age of 45 years, and is associated with an increase survival rate (2). Though primary and secondary GBM have distinct molecular biomarkers, by histology and morphology they are indistinguishable (8).

By pathological evaluation, GBM is a heterogeneous tumor, containing both neoplastic and stromal tissues. GBM is classified using the same criteria as anaplastic astrocytoma, which includes pleomorphic cells, nuclear atypia, and mitosis, but additionally contains areas of necrosis and microvascular proliferation (13). Though macroscopically, by MRI, GBM appears well encapsulated, separating itself from the surrounding normal brain tissue, microscopically it appears highly invasive. Tumor samples collected from the tumor edge reveal individual tumor cells migrating away from the tumor mass and invading the surrounding parenchyma (14). In fact, autopsies performed on GBM patients reveal tumor cells that have traveled as far as the contralateral hemisphere (15). It is these invasive cells that are likely left behind during surgical resection and eventually lead to tumor recurrence. Though GBM is highly invasive, unlike other types of tumors, it does not metastasize to distant organs. Instead, it is the invasive disease within the brain itself that eventually leads to death (15).

Just as glioblastoma is described as heterogeneous histologically, the molecular alterations in GBM are complex and diverse. GBM contains many modifications in signaling pathways for cell proliferation, apoptosis, senescence, migration and cell-to-
cell communication that vary based on whether the GBM is primary or secondary. Primary GBM is associated with epidermal growth factor receptor (EGFR) amplification and mutation, loss of heterozygosity of chromosome 10q, deletion of phosphatase and tensin homolog (PTEN), and cyclin dependent kinase inhibitor 2A (CDKN2A, p16) deletion (7). Secondary GBM is associated with mutations in the p53 tumor suppressor gene, overexpression of platelet derived growth factor receptor (PDGFR), abnormalities in the p16 and retinoblastoma (Rb) pathways, and loss of heterozygosity of chromosome 10q (7). The most common of these pathways, affected in approximately 80% of all GBM, are the receptor tyrosine kinase/Ras/phosphatidylinositol 3-kinase (PI3K), p53, and Rb signaling pathways (16).

The most affected receptor tyrosine kinase is EGFR, which is amplified, overexpressed, or mutated in 40-60% of GBM (17). The mutated form of EGFR, known as EGFR variant III (EGFRvIII), contains a functional constitutively activated intracellular domain but lacks the extracellular ligand binding domain (18, 19). Both EGFR amplification and mutation are associated with poor survival (20, 21). For this reason, EGFR has become a focus of molecular targeted therapeutics for GBM. EGFR inhibitors including gefitinib and erlotinib have been used in clinical trials for GBM but have exhibited very low response rates in these studies (22, 23). This lack of response could partially be indicative of failure to account for PTEN status in patients receiving the EGFR targeted therapy. Loss of PTEN occurs in 70% of GBM patients and PTEN mutation occurs in 40% (17). When PTEN is absent or mutated, the PI3K pathway is constitutively active, regardless of upstream EGFR signaling, rendering EGFR inhibition ineffective in these patients.

Many of these aberrant signaling pathways were first identified or further verified by the work of The Cancer Genome Atlas (TCGA) (17). GBM was one of the first types of tumor to be fully sequenced for genomic abnormalities by TCGA. Stemming from this
work, Verhaak et al (24) used cluster analysis to divide GBM into four main subtypes based on gene expression: Classical, Mesenchymal, Proneural and Neural. Classical is enriched for EGFR amplification or mutation, and homozygous deletion of CDKN2A and respond well to chemotherapy. The mesenchymal subtype contains deletion or mutations in NF1 and high expression levels of mesenchymal genes including chitinase-3-like protein 1 (CHI3L1), MET, CD44, and c-mer proto-ongene tyrosine kinase (MERTK). They tend to have higher overall necrosis and are associated with inflammatory infiltrates. Proneural is defined by PDGFRA amplification, isocitrate dehydrogenase 1 (IDH1) mutation, and p53 mutation, and usually represents secondary GBM, occurring in younger patients. This type of GBM has increased survival compared to the other subtypes, but interestingly has no survival advantage when treated with aggressive chemotherapy. Lastly, the neural subtype is identified by expression of neuron related genes including neurofilament light polypeptide (NEFL), gamma-aminobutyric acid receptor subunit alpha-1 (GABRA1), synaptotagmin-1 (SYT1), and potassium-chloride transporter member 5 (SLC12A5).

The goal of this classification system was to eventually personalize treatment modalities by identifying key genetic hits in each patient’s tumor that could be targeted with molecular therapies. While this original study appears promising for such a regimen, a recent study has shown that in addition to intertumor heterogeneity, intratumor heterogeneity also exists. Sottoriva et al (25) demonstrated that biopsying several areas of an individual tumor lead to classification of different subtypes within the same tumor. Based on this study, it is unlikely that a single molecular targeted agent will succeed in treating an individual’s tumor. Instead, a molecular signature created from multiple biopsies may be needed to identify an appropriate multimodal therapy to eliminate as many tumor cells as possible.
Though there have been many oncogenic and tumor suppressor mutations associated with GBM, the initiating events leading to its formation have yet to be identified. It is uncertain in which cell the initial transformation takes place. Several studies have suggested that inducing mutations commonly found in human glioblastoma in neural stem or progenitor cells will give rise to a tumor, whereas more differentiated cells are less susceptible to transformation (26). However, Bachoo et al (27) demonstrated that more terminally differentiated cells are capable of dedifferentiating into progenitor cells that produced glioblastoma by stimulating mature INK4a/Arf-astrocytes with EGF or expressing the mutant EGFRvIII. Dai et al (28) also demonstrated this concept by inducing dedifferentiation of INK4a/Arf-astrocytes with PDGF.

Regardless of the initiating cell that is transformed, it is thought that a population of brain tumor stem cells (BTSC), also referred to as tumor-initiating cells, is responsible for inducing and maintaining tumor growth. As in other cancers, these stems cells are defined by their ability to self-renew, proliferative extensively, and differentiate into multiple lineages. According to the glioma stem cell hypothesis (29, 30), BTSC are thought to make up only a minority of cells within the tumor mass, but could be considered a critical target for treatment of glioblastoma because they are the principal drivers of gliomagenesis. In addition, they contribute to the eventual recurrence of the tumor due to their resistance to conventional therapies (31). BTSC acquire resistance to radiotherapy by activation of DNA-damage-response pathways and chemotherapy by overexpression of O-6-methylguanine-DNA methyltransferase (MGMT), up-regulation of multidrug resistance genes, and inhibition of apoptosis.

Based on this evidence, it suggests that targeting BTSC could improve outcomes when combined with conventional therapies; however, there have been discrepancies in defining a phenotype for these cells. BTSC were first defined by surface marker
expression of CD133, but recent evidence has shown that CD133 negative cells are also capable of tumor formation (32). More recent markers including CD15 and A2B5 have been used to define glioma stem cells (33); however, further investigation is needed to clearly define the phenotype of these cells. Instead, a more logical goal might be to target pathways essential for stem cell self-renewal and maintenance of their immature phenotype such as notch, hedgehog, PI3K, and Wnt or to target transcription factors involved in neural stem cell growth such as sex determining region Y-box 2 (Sox2), octamer-binding transcription factor 4 (Oct4), Nanog, oligodendrocyte transcription factor (Olig2), B lymphoma Mo-MLV insertion region 1 homolog (BMI1) and c-Myc (34).

In addition to proliferating tumor cells and brain tumor stem cells, GBM contains a milieu of additional, nontransformed cells within its microenvironment that play an important role in tumor growth [reviewed in (35)]. The most prominent of these cells is the macrophage/microglia population (36, 37). Microglia are considered resident macrophages of the brain. Despite discrepancies over the origin of microglia within GBM, whether they are resident microglia recruited from the population within the brain or differentiate from monocytes entering the tumor from the peripheral blood, microglia/macrophages have been associated with suppressed immune function and promoting glioma migration and tumor growth (35).

Despite original thoughts that the brain is an immunoprivileged site and the blood brain barrier blocks trafficking of immune cells into the brain, recent studies have demonstrated several types of peripheral immune cells infiltrate the tumor and are associated with significant effects on tumor growth and overall survival of the patient. These cells will be discussed in greater detail below.

Other prominent cell types within GBM are endothelial cells, vascular smooth muscle cells, pericytes, and astrocytes that make up the tumor vasculature (35). These cells help to maintain the blood brain barrier. Failure to recruit these cells to areas of
angiogenesis can lead to increased tumor vascular permeability (38). Directly associated with the vasculature is an area recently identified as the perivascular niche, which houses neuronal stem cells in both normal and malignant tissues (39, 40). It’s thought that brain tumor stem cells reside within this area and are maintained through signaling events between BTSCs and the surrounding stromal cells (41).

These cells are also directly involved with maintaining the tumor vasculature and creating new networks as the tumor outgrows its blood supply. Microvascular proliferation is a hallmark of GBM (7), with active angiogenesis contributing to tumor progression. Angiogenesis occurs when tumor tissue becomes deprived of oxygen and other nutrients needed for tumor growth. This induces hypoxia that allows for the accumulation of hypoxia inducible factor 1- α (HIF-1α), which acts as a transcription factor in combination with HIF-1β to induce vascular endothelial growth factor (VEGF) expression, one of the main drivers of angiogenesis (42). The newly formed blood vessels tend to be highly aberrant and incomplete, creating new areas of hypoxia and contributing to peritumoral edema (43). Due to its central role in angiogenesis, VEGF has become a target of antiangiogenic therapies for GBM.

**Treatment and Management of Symptoms**

The current standard of care for treatment of GBM is to first perform surgical resection when feasible, followed by radiation and chemotherapy. Because of its highly infiltrative nature and presence within a highly sensitive tissue, unlike other types of tumors where a margin around the tumor can be taken, GBM cannot be completely removed by surgical resection. Even when it appears by MRI that gross total resection has been achieved, individual tumor cells invading the surrounding parenchyma still exist and can contribute to recurrent disease. Advances in MRI-guided navigation, intraoperative MRI (44), functional MRI (44), intraoperative mapping (45), and
fluorescence-guided surgery (46) have improved the amount of tumor that can be removed, especially in high risk areas of the brain, and help to reduce the risk of permanent disabilities. Recent studies have shown that patients who receive gross total surgical resection have a significant survival advantage over those who receive a subtotal resection, implying that maximal safe resection improves survival (47).

In addition to surgical resection, GBM patients will undergo a regimen of radiation and chemotherapy. In 2005, Stupp et al (3) demonstrated that radiotherapy in combination with temozolomide followed by adjuvant temozolomide improved survival over radiotherapy alone. The combination treatment improved survival from 12 to 14.6 months, making it the new standard of care for GBM. More importantly, it improved the 2 year survival rate from 10.4% to 26.5% (3). It has more recently been shown that this survival rate can be improved even more when patients are stratified according to their MGMT status. MGMT is epigenetically regulated in GBM patients, with promoter methylation preventing its expression. When MGMT is expressed it is capable of reversing DNA damage induced by alkylating chemotherapies such as temozolamide making them less effective (48). GBM patients with MGMT promoter methylation who are treated with temozolomide have a median survival rate of 21.7 months and a 2 year survival rate of 46% (49). Presently GBM patients are treated with temozolomide regardless of their MGMT promoter methylation status; however treatments to target MGMT function, such as the MGMT inhibitor O6-benzylguanine (50, 51) or a metronomic schedule of temozolomide to deplete MGMT (52), are under investigation.

There are many therapeutic strategies under investigation for the treatment of GBM in hopes of improving this survival rate even further. Most of these therapies have focused on the recent insights gained into the molecular events necessary for tumor growth. In addition to targeting the receptor tyrosine kinase EGFR, as mentioned above, several other inhibitors of tyrosine kinases have been investigated including imatinib
(PDGF/BCR-ABL/c-KIT) (53-55), AMG102 (c-MET) (56), and enzastaurin (protein kinase Cβ – PKCβ) (57). Similar to the EGFR inhibitors, the response to these agents has been mainly disappointing. One reason for these failures, in addition to intratumor heterogeneity as mentioned above, could be compensation of the inhibition of one receptor tyrosine kinase by the upregulation/activation of another. This compensation is evident in EGFR targeted tumors where an increase in PDGFR and c-MET is observed (58). For this reason, agents that target common downstream signaling pathways of these RTKs, such as PI3K, Akt, Ras, or mammalian target of rapamycin (mTOR) are also under investigation [reviewed in (59)]. It is also evident that successful elimination of all tumor cells within GBM will require the targeting of multiple pathways, not just a single oncogenic protein.

A major difficulty of using chemotherapy or other targeted therapeutics for treatment of GBM is attaining sufficient concentration of the drug within the tumor to have maximal effect. Though it is usually broken down in the main center of the tumor, the blood brain barrier remains intact at the infiltrative edge (60). Here it is still effective at keeping these molecules from entering areas where single tumor cells are invading the healthy brain. To overcome this phenomenon, several methods of enhanced delivery have been developed to try and improve drug concentration within the area surrounding the tumor. One of these methods is the Gliadel Wafer (61), a biodegradable polymer containing carmustine that is placed directly in the tumor cavity during surgical resection. As the polymer breaks down it releases chemotherapy directly to the tumor bed over the course of several weeks. Another method is to use convection-enhanced delivery where catheters are placed into the surgical cavity immediately following resection and therapy can be delivered by convection, which enhances the distribution in surrounding tissue (62).
One of the main clinical problems for GBM patients results from this break down of the blood brain barrier. Edematous swelling dramatically increases intracranial pressure that can also affect healthy areas of the brain and lead to headaches, vertigo, nausea/vomiting, and sometimes life threatening conditions such as brainstem compression and herniation (8). To reduce swelling, GBM patients are commonly put on corticosteroids such as dexamethasone. While this treatment is extremely effective, long-term exposure to corticosteroids have many side effects, including Cushing’s syndrome, myopathy, increased risk for pneumonia, osteoporosis, and compression fractures (8). For this reason, novel therapeutics for decreasing peritumoral edema are under investigation.

**Recurrence**

Despite these aggressive treatments, all patients with glioblastoma will ultimately recur. In fact, the average progression free survival is as little as 7 months post diagnosis (3), even after receiving chemotherapy and radiation. Post resection or biopsy, patients are carefully followed over the course of their treatment and in the months following. Routine MRI scans are performed to determine tumor progression. However, recurrent disease can be difficult to distinguish from treatment effects present on MRI due to radiation, chemotherapy, or antiangiogenic therapy. Patients undergoing fractionated radiotherapy who have changes in enhancement within the first 3 months following treatment are considered to have pseudoprogession (63). The enhancement represents treatment effect rather than recurrent disease. The irradiated region usually contains regions of blood brain barrier breakdown, resulting in increased permeability and leakage of gadolidium (64). It usually improves over time and is not associated with worsening symptoms. The percentage of patients that experience psuedoproggression
has increased from 10% to 30% with the addition of concomitant chemotherapy to radiotherapy (65).

Enhancement occurring after 3 months following radiation can also be considered treatment effect and is referred to as radiation necrosis (66, 67). It also occurs secondary to blood brain barrier breakdown and gadolinium leakage, but produces a ring-enhancing lesion that can easily be confused with recurrence (68). Some patients also have symptoms associated with radiation necrosis that can easily suggest recurrence such as headaches, seizures, behavioral changes, and focal neurological defects (69). It is imperative to distinguish progression from radiation necrosis to determine treatment modalities for these patients. Incorrect treatment can lead to unnecessary morbidity. The best way to distinguish radiation necrosis from recurrence is histologically, however this requires additional surgery that can also lead to unnecessary morbidity if the enhancement is from radiation necrosis. For this reason, several radiographic modalities are being developed to distinguish treatment effect from tumor progression. If the majority of the new enhancement occurs outside the radiation field it is most likely progressive disease (70).

Progression can ultimately be determined using a set of guidelines called the Macdonald Criteria (71), which takes into account radiographic, treatment, and clinical variables. According to this system, progression is defined as: a greater than 25% increase in the enhancing lesions compared to baseline (image taken immediately following resection or prior to treatment), stable or increasing doses of steroid treatment, a significant increase in T2/FLAIR nonenhancing lesion, appearance of new lesions, or clinical deterioration not due to other known causes. If there is any uncertainty in whether the patient has recurrent disease, the patient will usually continue on their current treatment and return at 4 week intervals to reassess radiographic progression (70).
Once recurrence is confirmed, the patient may undergo re-resection, re-irradiation, salvage chemotherapy, or antiangiogenic therapy. The treatment received depends mostly on recurrence location, performance status, and the expected survival of the patient with intervention. Most tumors recur within 2 cm of the primary tumor site (72, 73) and resection of the recurrence can improve symptoms from mass effect while also improving efficacy of adjuvant therapy by decreasing tumor burden. Patients that have a good performance score can choose to undergo salvage chemotherapy, usually with temozolamide. Re-irradiation can also be performed, however the usefulness of this treatment remains debatable (74).

Most recurrent GBM patients will also be considered for antiangiogenic therapy with Avastin (bevacizumab), a humanized monoclonal anti-VEGF antibody. It was first approved by the food and drug administration (FDA) for treatment of recurrent GBM in 2009 and has since shown promising results alone or in combination with the chemotherapy irinotecan (a topoisomerase inhibitor), improving 6 month progression free survival to 35-50% compared to 21% for patients receiving only temozolamide (75). In addition to targeting angiogenesis, VEGF treatment allows for normalization of the vasculature, which reduces peritumoral edema and decreases the dependency of these patients on steroid treatment. This can be observed radiographically by decreased leakage of gadolinium within tumor capillaries and therefore decreased enhancement on T1-weighted images. This phenomenon is often referred to as “pseudoresponse” because the tumor is no longer visible by T1-weighted images as a result of vascular normalization rather than anti-tumor effect (70). Therefore progression free survival (PFS) acts as a better judgment of anti-tumor effect rather than radiologic response; although a radiologic response persisting for at least 4 weeks can be considered a true response (70).
Just as with other molecular targeted therapies, patients receiving bevacizumab ultimately acquire resistance to its antiangiogenic effects. This resistance most likely occurs from increased expression of several other growth factors involved in vascularization including basic fibroblast growth factor (bFGF), stromal cell-derived factor-1α (SDF-1α) (76) and PDGF (77). In addition, several studies have suggested that anti-VEGF therapy can lead to increased invasion into surrounding brain parenchyma (78-81). Here the tumor cells can take advantage of normal vasculature and eventually propagate into recurrent tumor. For ideal use in treatment of glioblastoma, anti-VEGF therapy will likely need to be combined with a treatment targeted against tumor cell migration and invasion.

Though there have been many developments in the treatment of glioblastoma, the improvement in survival rate has been negligible and all patients will eventually succumb to their disease. While this mostly relates to the complex nature of GBM, it also is attributable to the fact that there are no models of GBM that accurately recapitulate the intricacy of this tumor. There have been many advancements made in the understanding of the underlying biology and molecular mechanisms of GBM due to \textit{in vitro} and \textit{in vivo} model systems; however translating treatment modalities developed in animal models to human patients has had little success. For this reason, there is a desperate need for GBM model systems that more accurately reiterate the human disease.

\textbf{Models to Study Glioblastoma}

There are three main types of \textit{in vivo} rodent models used to study glioblastoma: chemically induced, genetically engineered, and xenografts. Each has their advantages and disadvantages but none fully recapitulate the human disease [reviewed in (82)]. As reviewed by Huszthey et al, there are several reasons why the successful treatment of
GBM in rodent models fails to translate to the clinical setting. First of all, most rodent models do a poor job of reiterating the biological properties of human tumors in terms of histological features, microenvironmental factors, and cellular heterogeneity. Second, the animals used do not have the same pharmacokinetics as humans. Regardless of these disadvantages, the progress made thus far in the treatment of GBM would not have been accomplished without these in vivo models.

Chemical models are induced by exposure to N-nitroso compounds locally, orally, intravenously, or tryploblastically. Though this exposure induces glioma formation in rats, it is less efficient at doing so in mice (83) and has not been correlated to glioblastoma in humans, making it a poor model for the etiology of human tumors (84, 85). Commonly used chemically induced syngeneic models include the 9L, C6, F98, and CNS-1 (86). Most are now maintained as cells lines and used as syngeneic models by intracranial injection. These models tend to be less invasive than the human disease, failing to produce contralateral infiltration (87). In addition, very little is known about the molecular profile of these tumors, making targeted therapies impossible to test in these systems. One advantage of the syngeneic models is that they have an intact immune system; however immune reactions in these systems tend to vary from those observed in human patients.

With the recent developments in the understanding of the genetic oncogenic drivers of GBM, genetically engineered models have emerged. These models are instigated in a number of ways. Genes can be gained or lost in specific cells at specific time points in development using a tet-regulation or cre-inducible systems. Retro- or adenoviral vectors delivering cre recombinase can be used for somatic cell gene transfer. These methods are used to alter key signaling pathways known to be involved in GBM including EGFR, Rb, Ras, Akt, and PDGF (88-91). Another method involves the direct injection of retrovirus engineered to express an oncogene such as PDGF or EGFR
into areas of the brain rich in neuronal stem cells (92, 93). These cells take up the virus, incorporate it into their genome, and subsequently overexpress the oncogene, leading to tumor formation. These models have the advantages of being able to study the minimum number of oncogenic events required for tumor formation and allowing for the study of immune interactions with the tumor. Many of them also acquire the defining histological features of GBM, such as necrosis and angiogenesis (82). On the other hand, the tumor is composed of cells with homogeneous genetic alterations and do not reflect the genomic and phenotypic heterogeneity found in human tumors.

The invention of the immunodeficient rodents, which lack an adaptive immune system, allowed for the development of xenograft models using human derived GBM implanted orthotopically in rodent brains. Xenograft models can be split into two categories: those made from human cell lines and those made from biopsies of human tumors. In general, xenografts from human cell lines have a reproducible engraftment rate and show expansive growth with characteristics similar to human GBM such as angiogenesis and some invasion from the main tumor, but still lack the true single cell invasion (82). Necrosis and microvascular abnormalities often found in human GBM are frequently absent from these models (94). Improvements in these characteristics can be made when cell lines are grown as neurospheres in serum-free medium (such as the recently developed neurobasal medium) rather than as monolayers in serum-containing medium. Neurospheres represent populations of brain tumor stem cells that are highly tumorigenic and tend to create a more heterogeneous tumor with marked angiogenesis and extensive invasion. The ability to grow neurospheres, specifically from primary tumor samples, can be rather difficult, with highly variable success rates. However, neurosphere cultures tend to maintain their genomic stability compared to monolayer cultures that tend to lose their chromosomal profile over time (95-99).
Xenografts developed from fresh tumor biopsies can be done in two ways. The tumor sample can be minced into serum-supplemented medium and transferred to flasks with agar-coated surfaces. Here they will form spheroids that can be transferred intracranially. The other option is to first directly transplant the human tissue into a mouse flank and then serially passage the tumor intracranially. Both methods tend to take at reproducible rates, though the growth rate tends to be much longer than that of cell lines, with engraftment taking anywhere from 2 to 11 months (100). These xenografts tend to acquire GBM histological features such as necrosis and angiogenesis with serial passages, but vary depending on the features of the original tumor (82). Despite the acquisition of histological features, they tend to be genomically stable, making them promising models for the study of targeted therapeutics (82).

The obvious disadvantage of these xenograft models is that they are lacking an adaptive immune system. While innate cells may still interact with the tumor and have their effects, these rodents lack T cells that play a vital role in antitumor immunity. Immunodeficient animals lack the immunesurveillance mechanisms of immunocompetent animals, which the tumor would normally have to overcome in order to successfully grow.

It is clear that the maximum understanding of GBM would come from studying the human disease itself. Though there are limited mechanistic studies that can be done ex vivo with human tissue, there are systems being developed to overcome these limitations. One such system, developed by Parker et al (101), involves culturing thin slices of tumor in neurobasal medium. This maintains the tumor architecture while also allowing for continued proliferation of the tumor cells. Using this system, cell movements can be tracked and observed to gain important insights in the mechanisms of GBM tumor cell invasion. Actively migrating microglia were also observed in this system. It is
possible the interactions of microglia and tumor cells could also be studied using this model.

For the current studies, we have chosen to use human GBM tissue and blood to study immune reactions occurring in these patients. For the study of the immune system in GBM this provides the most accurate depiction of the disease. While the ability to derive mechanism using only tissue and blood samples is limited, the significance of observations made from studying cells within these compartments is instrumental to fully understanding this disease. While there are rodent models of GBM that have intact immune systems and allow for the study of the immune interaction with the tumor, we and others have found the observations made in these systems do not translate to those observed in humans. This discrepancy is most likely due to the difference in prevalence of immune cell populations in the blood between rodents and humans (102). Until an animal model is developed that accurately depicts the immune reactions observed in humans, we feel it is best to study humans themselves. That being said, to accurately complete preclinical testing of immunotherapies, the development of a precise rodent model will be necessary.

**Immunotherapy as a Treatment Option for Glioblastoma**

Though there has been a modest increase in the survival rate of GBM patients with the development of new treatment options, in comparison to advancements made in other types of tumors this success is abysmal. In addition to the lack of accurate model system, this is most likely a result of the complex biology of GBM, its unfavorable location, and its highly invasive nature. Many strides have been made in understanding the molecular events influencing tumor formation; however therapies targeted at these specific pathways have so far failed to significantly improve survival. As discussed
above, the reasons for this are multivariable. For instance, other molecules can become overexpressed or mutated to compensate for the loss of the target and maintain downstream signaling events. The therapy can also have a hard time reaching single cells that have invaded the surrounding parenchyma and will eventually grow into a recurrent tumor.

These resistance mechanisms achieved by GBM actually make it an ideal candidate for immunotherapy. Cells of the immune system are capable of distinguishing between tumor and normal cells based on the antigens presented by both. If activated in the right way by immunotherapy, theoretically these immune cells would actively search for tumor cells within the surrounding normal brain parenchyma and eliminate these cells, unlike chemo or molecular therapies which are unable to access them. In addition, mutations or changes in gene expression compensating for targeted therapies create new antigens for immune cells to target. The dynamic ability of the immune system allows it adapt to changes within the tumor. Once an immune response is activated against the tumor, lasting immunity will be created against the tumor antigens, even after the tumor has been eliminated, allowing for continued surveillance and therefore preventing recurrence.

**Successful Immunotherapies in Other Types of Cancer**

The influence of the immune system on tumor progression has become increasing clear, whether it is promoting tumor growth or preventing tumor development. In fact, Hanahan and Weinberg have recently updated their defining hallmarks of cancer to include “avoidance of immune destruction” and have added “tumor-promoting inflammation” as one of their enabling characteristics (103). For this reason it is no surprise that the development of immunotherapies to take advantage of the immune
system’s ability to selectively destroy cancer cells has become increasingly prevalent and successful.

Most immunotherapies are aimed at the activation of CD8+ cytotoxic T cells (CTLs) against tumor antigens. CTLs selectively induce cell death in their targets, which is a desirable effect for cancer therapy. The first immunotherapeutic treatments targeted the non-specific activation of T cell proliferation with cytokines such as interleukin (IL)-2 and interferon (IFN)-α. While these cytokines improved survival in patients with melanoma and renal cell carcinoma (104, 105), these therapies can be difficult for the patient to tolerate, as they induce a global non-specific immune reaction. Patients often experience flu-like symptoms and can even develop serious systemic toxicities. An improvement on this strategy is the use of adoptive T cell transfer (ACT). This involves the isolation of tumor infiltrating lymphocytes (TIL) collected either from tumor tissue or tumor draining lymph nodes. After ex vivo expansion, TIL are infused back into the patient. ACT has been done in melanoma patients with high success rates (106, 107). Both unfractionated TIL or TIL with increasing specificity to tumor antigens have been evaluated, although the more ambiguous fractions tend to have a greater response, possibly due to the ability to target multiple tumor antigens (108).

A variation of ACT that has recently been developed is the chimeric antigen receptor (CAR) T cell. CARs are composed of an Ig variable domain of an antibody fused to the T cell receptor (TCR) signalling domain. This fusion allows for antibody recognition of a specific antigen to give a T cell activation signal (109). CARs have been designed to overcome the natural limitation of major histocompatibility complex (MHC) restriction to certain antigens and allow for T cell activation without signaling through human leukocyte antigen (HLA) molecules. Depending on the generation of CAR, the TCR domain is often fused to intracellular T cell signaling molecules such as CD3ζ, CD28, 41BB, inducible T-cell costimulator (ICOS), or OX40 to allow for increased
activation of signaling cascades (110). CAR T cells are similar to adoptive cell transfer in that T cells are isolated from the patient, CAR are introduced into the T cells and the CAR T cells are infused back into the patient. This method has shown promising results in several types of cancer, with the most promising results observed in early clinical trials of anti-CD19 CARs for patients with lymphoma (111, 112). While these results are encouraging, there is also the risk of autoimmunity when the tumor antigen targeted is also found on normal tissues.

Another approach to use the body’s natural ability to respond to tumor antigens is through vaccination. Cancer vaccines are designed to deliver tumor antigens systemically where they are processed by dendritic cells that will travel to the lymph node and activate T cells specific to that antigen. The identification of tumor specific antigens has brought significant improvement to the design of cancer vaccines. An ideal tumor specific antigen would be one that is present on tumor cells but not normal cells, found in the majority of patients with the cancer, and vital to the cancer’s growth to avoid recurrence from resistant antigen negative tumor cells. Once these antigens are identified, they are used in peptide vaccines to target the specific antigen. Peptide vaccines are often paired with some sort of vaccine adjuvant to aide in the activation of dendritic cells to engulf and process the antigen.

The most recent success in cancer vaccines came with the development of the dendritic cell (DC) vaccine. This vaccine eliminates the antigen processing step in vivo by presenting the antigen to peripheral blood mononuclear cells (containing dendritic cells) ex vivo. The prostate cancer DC vaccine Sipuleucel-T (Provenge) uses the prostate cancer antigen prostatic acid phosphatase (PAP) linked to the cytokine granulocyte macrophage colony-stimulating factor (GM-CSF) to promote dendritic cell growth and differentiation (113). This vaccine provided a 4 month overall survival benefit
compared to control, leading to its approval by the FDA in 2010. This was the first therapeutic cancer vaccine to be approved for use by the FDA.

Perhaps the most successful use of the immune system for cancer treatment is monoclonal antibodies. In fact, three of the top selling cancer drugs rituximab, trastuzumab, and bevacizumab are monoclonal antibodies specific to CD20, human epidermal growth factor receptor 2 (HER2), and VEGF, respectively. Most of these were not designed to necessarily have immunologic effects, but rather act as an antagonist to its target or downregulate the target from the cell surface. However, many of these monoclonal antibodies have the added effect of inducing cell lysis through a process called antibody-dependent cellular toxicity (114, 115). Once the antibody has bound its target, the Fc portion binds to the Fc receptor on natural killer (NK) cells that then become activated and induce cell death. Cell lysis can also be achieved through another mechanism involving complement binding to the Fc portion of the antibody and activating the complement cascade.

Immunotherapies have the potential to have a huge impact on the treatment of cancer. The promising studies that have emerged over the past few years are prime examples of that potential. As we begin to further understand the spontaneous interactions of the immune system and the mechanisms used by the tumor to interfere with this immune reaction, we will be able to design immunotherapies that overcome these barriers and ultimately eliminate the tumor.

**GBM Immunology**

There are several steps in the immunological process that must be fulfilled in order for immunotherapies to be effective. First of all, antigens must be consumed and processed by antigen presenting cells (APCs). These APCs must travel to the lymph node and interact with antigen specific T cells in a way that activates the T cells. Then
the T cells must leave the lymph node and home to the source of antigen to target cells expressing the antigen. While GBM was originally thought to lack many of the immunologic features required for these steps to occur, due to its anatomical location, recent advances in GBM immunology have shown that this is not the case.

For many years it was thought that the brain was an immunoprivileged site, with very little interaction with the peripheral immune system. This was assumed because of early experiments using dyes or toxins injected intravenously that induced staining and symptoms systemically, but had no cerebral effect (116). It was these experiments that lead to the identification of the blood brain barrier (BBB), a distinctive property of the central nervous system that restricts the passage of substances into and out of the brain. The BBB is a result of unique vascular structures found at the tight junctions of endothelial cells within intracerebral capillaries. In combination with astrocyte foot processes surrounding the endothelium, these tight junctions create a firm barricade to limit the paracellular transport of molecules between endothelial cells. Thought to be included in this restriction is the passage of immune cells (117). However, with the recent understanding of inflammatory conditions within the CNS such as multiple sclerosis (MS) and experimental autoimmune encephalitis have demonstrated that immune cells are capable of entering the brain and inducing immunity (118).

While the process of immune cell trafficking to the brain is well understood in these autoimmune diseases (118), the process is not as fully described in the case of glioblastoma. It is well known that the blood brain barrier is disrupted in GBM (43, 119); however, the role of this increased permeability in the trafficking of immune cells to the tumor is not understood. While it is possible the increased permeability may allow for passive transport of cells into the tumor, it is also thought that the severe dysregulation of the vascular microenvironment may also hinder the expression of target molecules needed to cross the endothelium. That being said, GBM endothelium has been
observed to express several of these adhesion molecules necessary for T cell migration including intracellular adhesion molecule-1 (ICAM-1), CD44, sialylated Lewis\(^x\), and integrin \(\alpha4\) (120, 121), although the influence of these adhesion molecules on lymphocyte migration in GBM has not been assessed. In addition to the necessary adhesion molecule expression, T cells respond to proinflammatory cytokines which aid in their trafficking across the endothelium and toward the site of inflammation. Once again, GBM has been shown to express several proinflammatory cytokines that could play a role in this process [reviewed in (122, 123)], but they have not been studied in the context of lymphocyte migration. Despite the lack of knowledge as to how lymphocytes enter GBM, there have been many observations of the presence of lymphocytes within GBM, though found at varying densities [reviewed in (124)].

For T cells to traffic to the brain in the first place, they must be activated by antigen presenting cells. This activation occurs in the draining lymph node of the tissues where the antigen presenting cells has identified the dangerous antigen. For a long time it was unknown whether this process could occur in the brain due to the lack of normal lymphatic channels within the brain parenchyma and the unknown identity of APCs in the CNS. Recently it has been demonstrated that antigens from within the brain drain to the cervical lymph nodes (125, 126), despite the lack of lymphatic channels. For example, APCs containing myelin-specific proteins have been identified in the cervical lymph nodes of MS patients (127). Though they were thought to have limited activity in the brain, it has recently been shown that dendritic cells can act as antigen presenting cells within the brain (128, 129). Resident microglia and recruited monocytes/macrophages also present antigens and are found within GBM (124, 130).

As with any targeted immune response, adaptive immune cells need an antigen to respond to. As mentioned earlier, in the case of cancer, this antigen would ideally be expressed by tumor cells but not be present on normal cells. Several GBM specific
antigens have been identified, making them favorable targets for an immune response [reviewed in (124)]. One such antigen is formed from the EGFRvIII protein. The mutation of EGFR gives rise to a tumor-specific mutant peptide that is not found in normal tissue (131). Another antigen that shares this property is melanoma-associated antigen-1 (MAGE-1), a cancer/testis antigen that is expressed in GBM cell lines but is not found in normal brain tissue (132). There have also been studies evaluating the immunogenicity of antigens detectable in GBM that are also found in normal tissues such as interleukin 13 receptor-α2 (IL13Ra2) (133, 134).

As a final step in this process, T cells need to respond to these antigens and become activated with cytotoxic anti-tumor effects. This anti-tumor response requires not only a response to the specific tumor antigen, but also migration into the tumor to have access to the tumor cells. As mentioned above, T cells have been observed to be present in GBM, but potential effects in the tumor remain unclear. Studies with MAGE-1 and EGFRvIII in vitro have demonstrated the ability of antigen specific CTLs to become activated by primary glioblastoma cells (as measured by IFN-γ production in the case of MAGE-1) (135) or lysed cells from a glioblastoma cell line engineered to express the EGFRvIII mutant protein (136). This provides evidence that such tumor specific antigens could be used for an anti-tumor response.

There is also evidence of naturally occurring immune responses to GBM. Several studies have attempted to correlate the presence of T cells within the tumor and survival. While they are not all in agreement, the majority of these studies found a positive correlation between the presence of T cells and patients survival (137-142). GBM patients have also demonstrated spontaneous immunogenicity to their tumors. Tang et al (143) found that PBMCs isolated from tumor patients have the ability to lyse tumor cells in vitro. This finding suggests that peripheral immune cells exist within GBM patients that fight off the tumor but are prevented in doing so by the tumor environment.
This inhibition is due to the potent immunosuppressive ability of GBM as discussed further below.

**Immunotherapies Developed for GBM**

Given the advantageous immunological properties of GBM, there have been several attempts to exploit these assets and design an immunotherapy for GBM. In fact, many of the types of successful immunotherapies for other types of cancer are also being developed for GBM. Similar to the initial immunotherapies explored, non-specific activation of the immune system by proinflammatory cytokines has been used in the treatment of GBM. These studies used IFN-α, IFN-γ, and/or IL-2 injected systemically or intrathecally in combination with radiotherapy (144-149). There was no significant improvement in survival with any of these treatments; however, patients experienced considerable toxicities associated with non-specific immune activation. Investigations are underway to define a method of local delivery for these cytokines to avoid systemic toxicities and acquire a more potent effect.

Adoptive cell transfer has been attempted with lymphocytes stimulated non-specifically *ex vivo* with IL-2 and administered into the surgical resection cavity of recurrent GBM patients (150). These cells contain both T and NK cells and are termed lymphokine-activated killer cells (LAK). Original administration of LAKs to recurrent GBM patients did not show a significant survival advantage, but demonstrated its safety with minimal side effects (151-153). A more recent study showed similar promising results when LAKs were placed in the resection cavity of recurrent GBM patients where median survival was increased to 17.5 months compared to 13.6 months in controls (154). Tumor specific CTLs have also been used for ACT by co-culturing patient PBMC with autologous tumor cells. These again were injected back into the tumor site at the resection cavity. There was a slight radiographic response observed in these patients,
however there was no significant improvement in survival despite marker T cell
infiltration at the time of autopsy (155).

Several tumor vaccines have also been developed for GBM, taking advantage of
both the peptide-based and dendritic cell vaccine strategies. The most commonly used
antigen in both of these cases is EGFRvIII due to its expression by tumor cells but lack
of expression by normal brain. The peptide vaccine using EGFRvIII involves a 14 amino
acid peptide linked to a foreign molecule, keyhole limpet hemocyanin (KLH), to aide in
APC activation. Administration of this vaccine to patients with known expression of
EGFRvIII improved 6 month progression free survival compared to historical controls
(156). Interestingly, similar to treatment with EGFR inhibitors, the recurrent tumors
lacked EGFRvIII expression (156). This demonstrated the specificity of the vaccine but
also reiterated the heterogeneity of GBM and the importance of targeting more than just
a single tumor antigen.

EGFRvIII has also been targeted using DC vaccines. This initial study (157) used
the same 14 amino acid peptide conjugated to KLH to preload DCs and expanded them
ex vivo. These DCs were then used to vaccinate patients with newly diagnosed GBM.
This study demonstrated that the vaccine was safe, as patients had very few adverse
events. They also demonstrated immunogenicity, as cellular and humoral immune
responses to the peptide were detected post-vaccination. GBM patients receiving the
vaccine also demonstrated a survival benefit compared to historical controls with a
median survival rate of 22.8 months (157). However, this study only involved 13 patients
and a larger cohort will need to be analyzed to accurately determine its efficiency.

Several other DC vaccines are also being developed for the treatment of GBM
[reviewed in (158)]. The ICT-107 vaccine uses a number of tumor associated antigens
loaded onto DCs including tyrosine-related protein 2 (TRP-2), GP100, HER2, MAGE1,
IL13Ra2, and absent in melanoma 2 (AIM-2), supporting the idea that multiple targets
will help to eliminate more tumor cells (159). This vaccine has completed phase I study with promising results in median progression free and overall survival, but again needs further testing in larger studies. Another approach, termed DC-Vax Brain, uses DCs pulsed with autologous tumor lysate in combination with toll-like receptor (TLR) agonists to help in the activation of DCs (160). Once again both of these vaccines have been used in patients with minimal toxicities.

Another type of vaccine being developed for GBM is the heat shock protein (HSP) vaccine. Heat shock proteins are known for their role in aiding in protein folding, but are also involved in regulating apoptosis and modulating immune responses (161, 162). They have been found to be overexpressed in the cytoplasm and on the surface of GBM tumor cells (163). HSP vaccines are designed to isolate HSP from tumor cells along with their naturally associated tumor antigens such as EGFR, PDGFR, focal adhesion kinase (FAK), AKT, p53, and PI3K (164, 165). When used as a vaccine, HSP will interact with CD91, TLR4, or CD14 on the surface of APCs, allowing for their internalization along with their associated tumor antigens (166-169). One HSP vaccine has undergone phase I clinical trial for recurrent GBM patients with promising results.

As mentioned above the most successful use of immunotherapy to date is monoclonal antibodies. This is most certainly also the case for GBM. Bevacizumab (Avastin), the monoclonal antibody specific to VEGF is currently approved for treatment of recurrent tumors. Interestingly, although it is an antibody and therefore considered an immunotherapy, the mechanism of action of Avastin does not involve the immune system (170). It works by binding VEGF and preventing ligand binding to the receptor. Though it retains the Fc component capable of binding complement and NK cells, this is not necessary for its function.

A variation on the use of monoclonal antibodies to target tumor specific antigens that are required for tumor growth and progression is the immunotoxin. Immunotoxins
are composed of tumor-specific antibodies conjugated to a toxin derived from pathogens such as *Pseudomonas aeruginosa* (PE) or *Diptheria* (DT). The antibody will bind specifically to tumor cells, inducing internalization and allowing for the delivery of the toxin to induce apoptosis. Once such immunotoxin has been evaluated in clinical trials for GBM. This immunotoxin is targeted at IL-13R and conjugated to PE. While phase I and II trials had promising clinical results (62, 171), a phase III clinical trial failed to show a significant survival advantage (172). This failure may be the result of the lack of verification of IL-13R expression prior to enrollment, as IL-13R expression is highly variable between GBM patients.

Unlike the success seen in other types of cancer, immunotherapy has been relatively unsuccessful for the treatment of GBM thus far. Though there have been promising outcomes from some phase I and II clinical trials with a small number of patients, these results need to be confirmed in larger cohorts. The strong advantage of the immunotherapies developed so far is that they have relatively few side effects, especially when targeted at a specific antigen. Despite these initial hopeful results, patients on these trials still have recurrent disease and the ideal lasting immunity of immunotherapy has not been achieved.

**Challenges of Immunotherapies**

The reason for the failure of many immunotherapies in GBM (and other tumor types, despite the mentioned success stories) is the ability of the tumor to control and override the immune reaction. In fact, this manipulation is how the tumor escapes immune surveillance in the first place. Tumors take advantage of the immune system’s own checkpoints that are crucial for maintaining self-tolerance and controlling the duration and extent of immune responses. These pathways are critical for preventing collateral damage during physiologic immune reactions. For immunotherapies to be
successful, they must overcome these immune checkpoints implicated by tumors to prevent their own destruction by the immune system. A further understanding of the strategies used by tumors to suppress the immune system will allow us to shut down these mechanisms and design immunotherapies that are more effective.

**Tumor Induced Immunosuppression**

**Molecular Mechanisms**

Tumor cells in many different types of cancer have derived mechanisms to avoid immune surveillance. The most simplistic of these is to downregulate MHC expression on their surface (173). All cells express MHC I, while MHC II is restricted to only antigen presenting cells of the immune system. Immune cells use MHC molecules and the antigens presented within them to survey the health of the cell. Therefore a tumor cell presenting immunogenic peptides in its MHC I would be detected by T cells and targeted for an immune response (vs. self peptides found in a normal cell that would not elicit an immune response). By downregulating MHC expression, through mutation or transcriptional regulation, tumor cells avoid this interaction. However, there is a fine line between decreasing MHC I expression and completely getting rid of it, as NK cells detect cells lacking MHC expression and attack them. Therefore, tumor cells may also decrease antigen presentation without decreasing MHC I expression by acquiring mutations in proteins involved in the antigen presentation pathway.

Tumors may also produce immunosuppressive molecules to decrease infiltrating immune cell function. The tumor cells themselves may make these molecules, or immune cells entering the tumor microenvironment may produce them as discussed below. Tumor cells have been shown to produce transforming growth factor-β (TGF-β), IL-10, and prostaglandin E\textsubscript{2} (PGE\textsubscript{2}). TGF-β works to suppress the CTL response by
preventing the formation of several cytolytic gene products necessary for tumor toxicity (174, 175). IL-10 suppresses DC function and inhibits antigen presentation by tumor cells (176, 177). PGE$_2$ reduces T cell function by inhibiting IL-2 activation of T cells (178).

There are numerous co-stimulatory or inhibitory molecules present on the surface of APCs and T cells that interact when an APC’s MHC interacts with the TCR. Some tumors have taken advantage of the inhibitory signals and express them on their surface. The two most studied of these in tumor immunotherapy are cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed cell death protein 1 (PD-1). CTLA-4 is expressed exclusively by T cells and helps to regulate the early stages of T cell activation by binding its ligands CD80 (B7.1) and CD86 (B7.2) on the APC (179-182). These ligands also bind CD28 on the T cell, which amplifies signaling from the TCR interaction with the MHC (183, 184). By competing for their interaction, CTLA-4 dampens the T cell activation signal and prevents over activation. CTLA-4 has become a target for immunotherapy due to this ability to prevent immune stimulation. By blocking CTLA-4 activity, the endogenous T cell response can be enhanced and naturally occurring T cells specific to tumor antigen will have greater effects [reviewed in (185)].

While CTLA-4 is involved in early activation, PD-1 acts at the late stages of activation, during the execution of an immune response. PD-1 is also found on T cells and interacts with its ligands PD-1 ligand (PD-1L, B7-H1) and PDL2 (B7-DC) on APCs (186, 187). It is upregulated after a T cell becomes activated (188) and also inhibits TCR signaling when bound to either of its ligands, thereby limiting the duration of T cell contact with APCs or their target cells (189). Tumor cells have taken advantage of this interaction and express PD1 ligands. By doing so they are able to dampen the naturally occurring antitumor immune response [reviewed in (185)].
Another cell surface molecule involved in the regulation of T cell activation is Fas (CD95), which is constitutively expressed on activated T cells. It interacts with its ligand, FasL, to induce activation induced cell death (AICD), a process essential for the regulation of activated T cell population at the end of an immune response (190). This helps to maintain T cell homeostasis and prevent autoimmunity. Many different tumor types have been shown to express FasL (191-194) resulting in apoptosis of activated T cells expressing Fas that enter the tumor [reviewed in (195)].

**Cellular Sources**

In addition to factors found on or released by the tumor cells themselves, tumors are also capable of inducing immunosuppression by regulating or recruiting cells of the immune system. Regulatory T cells (Tregs) are CD4+ T cells that actively inhibit the function of other immune cells including CD4+ T helper cells, CD8+ cytotoxic T cells, DCs, NK cells, natural killer T cells and B cells [reviewed in (196)]. Although there are numerous subests of Tregs, most are defined by their expression of high levels of CD25 and the transcription factor forkhead box P3 (FOXP3) in addition to CD4 (197, 198). They also express high levels of CTLA-4 and PD-1, which play a role in their suppressive ability (199, 200). Their physiologic immune function is to suppress the activation of self-reactive cells and thereby prevent autoimmunity (201). Tumors can take advantage of these cells to suppress antitumor immunity by recruiting them to the tumor microenvironment or activating them to patrol the peripheral blood.

Tregs suppress T cell function through the release of IL-10 and TGF-β (202, 203). They also interact with effector T cells either directly through binding of their CTLA-4 to effector cell CD80 or CD86 which induces suppression, or indirectly through the same molecular interaction with antigen presenting cells (204). The interaction with APCs induces the activation of indoleamine 2,3-dioxygenase (IDO) which reduces the
amount of free tryptophan, an essential amino acid for T cell activation (205). Several human tumors have been described to have increased frequencies of Tregs within the tumor and within the peripheral blood of tumor patients [reviewd in (196)]. In patients with gastrointestinal malignancies Tregs were observed at high frequencies but were significantly reduced after tumor resection and increased again with tumor progression. The frequency of Tregs also correlated with poor prognosis and decreased survival (206, 207).

Another type of immune cell frequently found in solid tumors that has been shown to have a role in immunosuppression is the macrophage. Macrophages can be categorized into two general groups, depending on the microenvironment they are formed in [reviewed in (208)]. M1 macrophages are described as classically activated, usually by IFN-γ or bacterial lipopolysaccharide (LPS), and tend to express inducible nitric oxide synthase (iNOS), IL-12, IL-6, and tumor necrosis factor-α (TNF-α). Their main function is to kill microorganisms and tumor cells and produce proinflammatory cytokines. M2 alternatively activated macrophages form in response to IL-4, IL-13, and IL-10 and produce Arginase I, IL-10, and IL-1 receptor agonist (IL-1Ra). These cells have roles in wound healing and tissue repair as well as controlling immune responses. Macrophages in the tumor microenvironment are often referred to as tumor associated macrophages (TAMs) and thought to be of the M2 phenotype. They are capable of promoting angiogenesis, invasion, and metastasis as well as inducing immunosuppression (208). They do so through expression of immunosuppressive cytokines, such as IL-10, recruiting Tregs through CCL22 expression, or expressing PD-1 ligands on their surface.

In addition to macrophages, cells of myeloid lineage have been increasingly associated with immunosuppression in various forms of cancer. Myeloid-derived suppressor cells (MDSCs) have been identified as potent inactivators of both CD4+ and
CD8+ T cells. As recently reviewed by Montero et al (209), there are discrepancies over the phenotype of these cells between cancer types. In early descriptions, MDSC were defined as a heterogenous population made up immature myeloid cells, consisting of early stage myeloid precursors, immature monocytes, and immature DCs (210-213). More recent identification of MDSCs has described them as a more differentiated population of cells, most commonly expressing CD11b and CD33. These mature MDSC can be broken down into two groups based on the markers CD14 and CD15. Granulocytic MDSC tend to be CD15hiCD14- (214-218), while monocytic are CD15-CD14+ (219-223) depending on the type of cancer.

Regardless of phenotype, the primary mechanism by which MDSCs confer immunosuppression is through the metabolism of L-arginine. T cells require uptake of L-arginine to become activated and proliferate in response to a stimulus through their TCR. L-arginine is essential for expression of the T cell co-receptor CD3ζ, which acts as an intracellular signal following TCR stimulation. Reduced expression of CD3ζ due to insufficient L-arginine inhibits T cell activation (224). The reduction of L-arginine occurs through the activity of the enzymes Arginase I (ArgI) and nitric oxide synthase 2 (NOS2).

Arg I converts L-arginine to ornithine and urea. It is expressed by both monocytic and granulocytic MDSCs, though in different cellular compartments. Granulocytic MDSCs express ArgI in preformed granules, which upon activation are released to the extracellular environment, where Arg I metabolizes L-arginine (218). Monocytic MDSCs express Arg I within their cytosol and metabolize L-arginine after transport from the extracellular environment by the transporter CAT2B (225). NOS2 is also expressed by both populations of MDSCs (226). It converts L-arginine to nitric oxide (NO) and citrulline. In addition to metabolizing L-arginine, it has been shown to have roles in immunosuppression through NO production, which directly impairs signaling pathways downstream of the IL-2 receptor (227).
MDSCs can be difficult to study in preclinical models due to the disconnect between markers used to define MDSCs in humans and mice. In mouse models, MDSCs are described as CD11b+Gr-1+ and are made up of two subsets, defined as granulocytic CD11b+LY6+LY6Clo and monocytic CD11b+LY6GnegLY6Chi (209, 228-230). The Ly6G and Ly6C molecules, both recognized by the Gr-1 antibody, are mouse specific markers and their counterpart has not been identified in humans. Both monocytic and granulocytic populations have been shown to be expanded in various mouse tumor models, with some tumors inducing the formation of both populations (228). Both subsets suppress T cell function, though similar to human MDSC, through different mechanisms. The differences in surface marker expression used to define MDSCs in humans and mice make therapies targeted at MDSC depletion difficult to contrive without directly studying the human disease. Despite these differences, there is agreement that MDSC are a valuable therapeutic target, especially if combined with cancer specific immunotherapies.

Neutrophils are cells of the myeloid lineage that have also been implicated in immunosuppression (231). The suppression of T cell function by neutrophils has been described in several cancer types including renal cell carcinoma (215), and breast, lung, and prostate cancer (216). The markers used to identify these cells are identical to those used to describe granulocytic MDSCs, making the distinction between the two populations unclear. While some groups describe granulocytic MDSCs as a subset of neutrophils that are capable of inducing immunosuppression (232), others insist they are a unique population of cells formed by interaction with tumor associated factors (233), and still others agree with the notion that granulocytic MDSCs are simply activated neutrophils (215, 234). Further studies of the suppressive populations in each type of cancer would be needed to determine if a distinction should be made.
Neutrophils involved with immunosuppression are described as activated, causing them to appear in the low-density PBMC fraction of blood where as unactivated/resting neutrophils will separate with the red blood cell fraction (215, 234). Activation of neutrophils is required for their immunosuppressive abilities, as their immunosuppressive molecules are housed within their granules (231, 235). Resting neutrophils that have not released their granules have no effect on T cell function. Regardless of the nomenclature, granulocytic MDSCs and neutrophils have been described to suppress immune function using the same mechanism of L-arginine depletion from the extracellular environment by release of the enzyme Arg I (231).

Normal Neutrophil Function

In addition to having immunosuppressive effects when activated in certain types of cancer, neutrophils have numerous roles in the function of the innate immune system and activation of the adaptive immune system. As part of the innate immune system, they are among the first responders to tissue damage and infection. Their primary role is to nonspecifically destroy and ingest pathogens within the inflammatory site. Neutrophils are attracted to sites of inflammation through adhesion molecules expressed on the endothelium surrounding the inflammatory site as well as chemotactic cytokines released from the inflamed tissue. In response to these signals, neutrophils will undergo a well defined process of rolling, adhesion, and transmigration through the endothelium and into the tissue [reviewed in (236, 237)].

The molecular interactions involved in the transmigration process have been well described for each step. At the site of inflammation, neutrophils will first bind to the activated endothelium through interactions between CD62L (L-selectin), P-selectin ligand 1 (PSGL-1) and E-selectin ligand 1 (ESGL-1) and their corresponding ligands P-selectin and E-selectin, expressed on the endothelium. These initial contacts initiate
rolling of the neutrophil along the endothelium. Interaction with inflammatory cytokines present on the activated endothelium will induce shedding of CD62L from the neutrophil surface and allow for the binding of lymphocyte function-associated antigen 1 (LFA-1 – a complex formed between CD11a and CD18) with its ligand intracellular adhesion molecule 1 (ICAM-1) on the endothelium to start the process of adhesion. Next, macrophage-1 antigen (MAC-1 – a complex formed between CD11b and CD18) on the neutrophil surface will interact with ICAM-2 on the endothelium, firming adhesion and allowing for crawling of the neutrophil along the endothelial cells to a site optimal for transmigration. CD31 (platelet-endothelial cell adhesion molecule-1 – PECAM-1) will then interact with its ligands at the endothelial cell junctions to mediate extravasation of the neutrophil into the tissue.

This general process of neutrophil transmigration in response to inflammation can vary depending on the tissue type. As noted earlier, the brain is unique in that tighter cell junctions between endothelial cells and pericyte coverage surrounding the endothelial cells lead to decreased permeability, a characteristic referred to as the blood brain barrier. Unique mechanisms of adhesion and transmigration have been described for T cell and monocyte trafficking across the blood brain barrier, however the process of neutrophil transmigration in the context of the BBB is less understood (116). Most studies evaluating neutrophil BBB migration have been performed in vitro and suggest the involvement of the same adhesion molecules normally involved in normal neutrophil transmigration (238, 239).

One mechanism that is unique to travel through the BBB is the necessity to also migrate through pericytes surrounding the endothelium. This process involves interactions between Mac-1 and LFA-1 on the neutrophil with ICAM-1 on pericytes (240). Neutrophils use these interactions to crawl along the pericyte to gaps between pericyte cells. Under inflammatory conditions, pericytes change shape, allowing for these gaps to
increase in size and further support the migration of neutrophils through the pericyte layer (240). In addition, pericytes also contribute to neutrophil migration by releasing the neutrophil chemoattractant IL-8 under inflammatory conditions (239). To facilitate crossing of the pericyte layer, neutrophils also use MMP-2 and MMP-9 to decrease adhesion to pericytes and further support complete transmigration (239).

Once neutrophils have undergone successful transmigration into the inflammed tissue, they will move through the tissue to the source of inflammation by following a chemotactic gradient. Chemoattractants such as bacterial byproducts and cell wall constituents, complement factors, and chemokines are produced by tissue damage or infection and guide neutrophils to the source of inflammation. Chemokines specific to neutrophil chemotaxis include IL-8, epithelial cell derived neutrophil activating peptide-78 (ENA-78), neutrophil activating peptide-2 (NAP-2), growth-related oncogene (GRO)-α, GRO-β, GRO-δ, macrophage inflammatory protein (MIP)-2α, and MIP-2β (236), with IL-8 considered to be the primary factor (241). Along the way, neutrophils will release proteases such as elastase, matrix metalloproteinase (MMP) 8, MMP9, and MMP25 to aid in the breakdown of the basal membrane and allow for further migration into the tissue (242).

Once neutrophils have reached the source of inflammation, interactions between cell surface receptors on the neutrophil and factors within the inflammatory environment will initiate phagocytosis of micro-organisms and neutrophil activation, leading to release of anti-microbial and tissue damaging molecules. Phagocytosis is initiated by the interaction of opsonized particles with receptors on the neutrophil surface. Opsonization occurs through binding of complement fragments (243) or antibodies (244) to the infecting micro-organism. Once initiated, pseudopodia will be extended from the neutrophil and engulf the particulate with plasma membrane, forming a phagocytic
vacuole. Cytosolic granules will fuse with the vacuole to release oxidants and enzymes that will destroy the ingested material (245).

Neutrophils can also be activated by soluble stimuli, such as cytokines and chemokines, which will lead to the release of granular components in the extracellular space through a process called degranulation. Deposition is initiated when a soluble stimulus interacts with its specific receptor on the neutrophil surface, leading to calcium mobilization and actin remodeling that induces movement of preformed granules within the neutrophil cytosol to the plasma membrane (246). Here, the granules dock with the plasma membrane through interactions between soluble NEM-sensitive-factor attachment protein (SNAP) receptors (SNAREs). The vesicular SNAREs (v-SNAREs) vesicle-associated membrane protein (VAMP)-7 and VAMP-2 on the granule bind to the target SNARE (t-SNARE), a complex between SNAP-23 and syntaxin-4, on the plasma membrane (247, 248). This interaction initiates fusion of the granule membrane with the plasma membrane leading to exocytosis of the granule contents.

There are four types of granules found within neutrophils that are formed during different stages of the neutrophil maturation in the bone marrow [reviewed in (237, 249)]. Azurophilic (primary) granules are the first to form, early in neutrophil maturation at the promyelocytic stage. Next, specific (secondary) granules are formed during the myelocytic stage. Gelatinase (tertiary) granules are formed during the metamyelocyte stage, just prior to transformation into a mature band cell neutrophil. The fourth type of granule, secretory vesicles, is formed after neutrophil maturation by endocytosis of surrounding proteins. The distinction between granules is important not only because the cargo they contain is based on the type of proteins synthesized during the stage of their formation, but also because there are differences in the mobilization of the different types of granules. Granules formed early in the maturation process, such as the azurophil and specific granules, are more difficult to mobilize and require stronger
activation signals than granules formed at later stages of maturation, such as the gelatinase granules and secretory vesicles (250-252).

Contained within these preformed granules are a multitude of peptides, proteases, enzymes, and membrane proteins that are released during degranulation. The membrane proteins are moved from the interior of the granule to the exterior of the cell as the membranes fuse together, allowing for interactions with their ligands in the extracellular environment. Included in these membrane proteins are adhesion molecules, receptors that detect damage or pathogen patterns, and reducing or oxidative enzymes. Soluble factors within the granules such as neutrophil elastase, cathepsin G, protease 3, myeloperoxidase, lactoferrin, lipocalin, and MMPs are released during degranulation to have effects on the surrounding extracellular environment. Together, these granule contents have powerful anti-microbial and tissue damaging effects [reviewed in (249, 253)].

Activated neutrophils within the tissue also release factors involved in the recruitment and activation of APCs. Cytokines such as MIP-1α, MIP-1β, TNF-α, IL-8, and IFN-γ are released during neutrophil activation and act to recruit and activate monocytes and DCs at the site of inflammation (254-257). Once activated, macrophages will also act as phagocytic cells to remove the invading pathogen and release factors to promote further inflammation. DCs are also highly influenced by alarmins (molecules that signal cell damage such as defensins, cathelicidins, high-mobility group proteins, granulysin, and lactoferrin) released from neutrophils that induce DC maturation and recruitment. Upon activation, DCs will display co-stimulatory molecules and release IL-12 that will activate a T cell mediated immune response (258, 259). Through this indirect mechanism, neutrophils help initiate the transition from an innate to adaptive immune response.
In addition to releasing their granular contents, neutrophils also form neutrophil extracellular traps (NETs) to prevent the spread of infection. NETs are formed when either an intact or apoptosing neutrophil releases its nuclear content into the extracellular environment through a process called netosis. By doing so, DNA is extruded from the cell along with proteins from the cytosol and granules. A complex of decondensed chromatin and granular contents (such as serine proteases) is formed that traps and kills various microorganisms extracellularly (260, 261). After performing these multiple mechanisms in response to inflammation, if neutrophils do not release their nuclear content to form NETs they will undergo apoptosis. Apoptotic neutrophils are then cleared by macrophages and the process of wound healing can begin (262).

While their role in response to tissue damage and infection has clearly been defined, the role that neutrophils play in tumor immunology and pathology has only recently begun to be explored. As mentioned previously, neutrophils have been shown to suppress immune function in renal cell carcinoma through activation and the release of Arg I (215), which is found within neutrophil azurophilic granules (263). Neutrophil activation in breast, lung, and prostate cancer has been linked to suppression of T cell function through the production of hydrogen peroxide (216).

Neutrophil activation represents one of several methods tumors can use to induce immunosuppression. The method of choice clearly depends on the type of tumor. Therefore, identification of the immunosuppressive mechanisms utilized by each tumor type is needed in order to target immunosuppression and reverse its effects.

**Therapies to Target Immunosuppression**

To be effective, immunotherapies have to overcome the immunosuppression induced by the tumor. Therefore, recent research has focused on ways to target immunosuppression and reverse its effects. The ideal antitumor treatment would be a
combination of an immunosuppressive target with therapies such as vaccines aimed to activate the immune system against the tumor. The most successful of these therapies has been the targeting of the immune checkpoint molecules CTLA-4 and PD-1. CTLA-4 is targeted with the monoclonal antibody ipilimumab, which binds CTLA4 and blocks interaction with CD80 and CD86 (264). By doing so it enhances T cell activation by allowing CD80 and CD86 to bind CD28 without a regulatory signal from CTLA-4. This uninhibited interaction enhances the endogenous antitumor response.

There was initial concern that this would also lead to autoimmunity, which in early clinical trials was observed in 25-30% of patients, with colitis being the most common adverse event (265-267). However, correct dosing strategies in terms of the amount given and timing of distribution have mitigated these side effects and allowed for the safe use of ipilimumab in clinical trials (268). Ipilimumab has been shown to be particularly effective in patients with metastatic melanoma. When combined with a peptide vaccine, it provided a 3.5 month survival advantage over the vaccine alone (264). In addition, the combination of ipilimumab with the melanoma-specific vaccine provided for an increase in long-term survival, with a two-year survival rate of 18% compared to 5% in patients receiving the vaccine alone (269). These studies demonstrated the effectiveness of combining therapies targeted at preventing immune suppression with therapies to stimulate an immune response. As a result, ipilimumab was approved for use in these patients by the FDA in 2010.

PD-1 has also been targeted with a monoclonal antibody. It has been used in Phase I clinical trials of several different cancer types including colon, renal, lung and melanoma (270). In contrast to CTLA-4, PD-1 ligands are expressed by tumor cells. Therefore PD-1 blockade not only allows for increased T cell activation but also counteracts one of the tumor’s mechanisms of immunosuppression. In these initial
studies, the level of PD-1L expression correlated with the effectiveness of the patient response to the treatment (270).

Antibodies targeting PD-1 and CTLA-4 have the added effect of also inhibiting regulatory T cell function, as both are highly expressed on Tregs (271-273). Tregs can similarly be targeted with an antibody to CD25, which is also expressed at high levels on Tregs. The anti-CD25 monoclonal antibody daclizumab is currently approved for use in organ transplant patients to prevent rejection and has been used in clinical trial for the treatment of metastatic breast cancer in combination with a peptide vaccine. The antibody showed a 7 month improvement in survival compared to vaccination alone (274). A similar target of Tregs is the immunotoxin denileukin, which is a fusion protein between human IL-2 and diphtheria toxin. IL-2 will bind to CD25 on Tregs and induce internalization, delivering the toxin and inducing apoptosis. While denileukin has also been used in clinical trials, it has not been as successful as daclizumab (275).

There have been multiple mechanisms proposed to target MDSCs in cancer, depending on the type of MDSC found to be present within the specific tumor type [reviewed in (276)]. In cancers where immature MDSCs have been described, treatments are targeted at forcing their differentiation into mature myeloid cells. Differentiation can be achieved with Vitamin A or retinoic acid receptor (RAR) antagonists (213, 277, 278). Another strategy is to prevent MDSC formation from hematopoietic precursors. While many tumor derived factors have been described in the formation of MDSCs, and finding an individual to target would prove challenging, many of these factors share a common downstream pathway involved in the differentiation of the MDSC phenotype, the singal transducer and activator of transcription 3 (STAT3) pathway. Inhibitors of STAT3 are being explored as a possible mechanism of preventing MDSC formation (279). Targeting their inhibitory functions can also reverse immunosuppression induced by MDSCs. As described above, MDSCs primarily act
through the enzymes ArgI and NOS2. Inhibitors of ArgI and NOS2 have been explored, but need to be approached with caution. Inhibitors of NOS2 have shown adverse side effects and ArgI is necessary for the urea cycle, making it difficult to target without interrupting this physiologic process (280, 281).

It is clear that the mechanisms utilized for immunosuppression differ between tumor types. Understanding the immunosuppressive mechanisms exploited by specific tumors will allow for specialized therapy targeting these specific mechanisms. When used in combination with immune stimulatory therapies directed at tumor antigens this may provide for powerful anti-tumor effects.

**Immunosuppression in Glioblastoma**

The identification of peripheral immunosuppressive mechanisms is imperative for the development of successful immunotherapies in GBM patients. Despite this notion, there are very few clinical trials aimed at targeting immunosuppression. In fact, an analysis done by Xu and Heimberger (282) showed that as of November 2012 there were 120 open or completed clinical trials for GBM immunotherapy and only 7 of these targeted GBM immunosuppression. There is a desperate need for the investigation of targetable immunosuppressive mechanisms in GBM patients.

Immunosuppression is a particular problem in glioblastoma due to extension of immunosuppression beyond the tumor microenvironment to the periphery. Suppression of peripheral cellular immunity has long been observed in GBM patients. It is apparent by the limited activation of peripheral T cells in response to mitogenic stimulation *in vitro* (283, 284) and defects in signaling pathways downstream of the T cell receptor, including decreased phosphorylation of phospholipase Cγ1 (PLCγ1), pp100 and reduced expression of p56lck (285). There is a selective depletion of CD4 T cells in peripheral circulation, as most patients have CD4:CD8 ratios close to 1:1, rather than the normal
The degree of immunosuppression varies between GBM patients and, in fact, the degree of immunosuppression correlated with survival (287). This extra level of immunosuppression represents an additional challenge in the successful activation of an antitumor immune response.

In the tumor microenvironment, GBM has been shown to express immunosuppressive cytokines, including TGF-β, IL-10 and PGE₂ (288, 289). As discussed above, these molecules can prevent immune activation by suppressing the function of multiple types of immune cells and similar roles have been described in GBM. The expression of IL-10 by tumor cells themselves has recently been challenged. Recent studies have indicated IL-10 production is limited to tumor associated macrophages or microglia (290). In addition, VEGF, which is expressed in abundance in GBM, has also been linked to the suppression of immune function. It does so by preventing the maturation of DCs, a critical step following antigen recognition in their process to activate T cells (170).

While many tumors downregulate MHC expression to avoid immune destruction, brain tissue naturally maintains a lower expression of MHC compared to other peripheral tissues (291). Therefore, it's not that surprising the GBM also expresses low levels of MHC, reducing their antigen presentation (292). Likewise, APCs within GBM tend to have defects in antigen presentation, with a decreased ability to upregulate MHC expression when activated. When microglia and macrophages were isolated from tumor and normal brain, those found in the tumor compartment had significant impairments in MHC II induction compared to those from normal brain (293). Though this lack of MHC expression also contributes to immunosuppression due to their inability to initiate an immune response, macrophages/microglia in GBM were not thought to have an active role in immunosuppression, as described in other tumor types, until recently. Microglia stimulated in the presence of tumor cells in vitro gained an immunosuppressive
phenotype, with increased expression of IL-10 (294). It has also very recently been shown that tumor-associated macrophages in GBM produce IL-10 which leads to an increased expression of PD-1L on their surface and could influence immunosuppression within the tumor microenvironment (295).

GBM tumor cells themselves have also been shown to express PD-1L (296). In fact, its overexpression is associated with immunoresistant phenotypes. GBM tumor cells expressing increased PD-1L showed decreased susceptibility to cytotoxic T cell targeting (297). In addition, the regulatory molecule FasL has also been shown to be expressed on GBM tumor cell surface. This expression has been correlated with decreased presence of TILs in corresponding tissue samples from GBM patients.

T regulatory cells have been implicated in GBM patient immunosuppression. Tregs have been identified in GBM, though to varying degrees. Two groups found significant Treg populations within GBM; however the markers used to identify Tregs were not exclusive to regulatory T cell populations and could also be identifying other cell types (298, 299). Despite these discrepancies, the advantage of targeting Tregs in GBM was demonstrated in a mouse model of GBM. Depletion of Tregs using a CD25 antibody resulted in increased survival. A clinical trial involving the anti-CD25 monoclonal antibody daclizumab in combination with temozolamide demonstrated decreased number of T cells in GBM patient peripheral blood and correlated with an increase in the number of antigen specific T cells after vaccination (300). These preliminary results show promise for the use of daclizumab to treat GBM patient immunosuppression but clearly need further testing in GBM patients.

The mechanisms described so far account for immunosuppression within the tumor microenvironment, but do not account for the global suppression of peripheral T cell function. The mechanisms responsible for this phenomenon are much less understood. It is unlikely that immusuppressive molecules expressed by tumor cells or
immune cells within the tumor microenvironment accumulate enough within the peripheral blood to effect T cells there. Likewise, suppressor molecules on tumor cell surfaces are not accessible by T cells in the periphery. These observations suggest that immunosuppression within peripheral circulation is most likely induced by a cellular source.

Tregs have been shown to exist within GBM patient blood, though they represent a very small proportion of the circulating cells (301). Secondary to the decrease in CD4+ cells within GBM patient peripheral blood, absolute counts of Tregs have actually been found to be lower in GBM patients than in normal normal donors (298). However, the proportion of CD4+ cells represented by the Treg population was much higher in GBM patients than in normal donors. When Tregs were removed from T cell cultures, an increase in the function of the remaining T cells was observed, suggesting that though they are present at low levels they have powerful immunosuppressive effects (298). This finding provides further evidence that a benefit may be gained from targeting Tregs in GBM patients.

While these results are promising, similar to the tumor microenvironment, the mechanism of peripheral immunosuppression is likely multifactorial. Though MDSCs have not been described within GBM tumor tissue, they may have a role in peripheral immunosuppression. Prior to the initiation of the work described in this thesis, only two studies existed looking at the role of MDSCs in human GBM. A study by Rodrigues et al identified a population of Lin−HLA-DR−CD33+ immature MDSCs in GBM patients (302). Although the immunosuppressive abilities of these cells were not directly confirmed, they did show that normal donor monocytes mixed with human GBM cells lines induced an immunosuppressive phenotype with increased expression of IL-10, TGF-β and PD-L1, decreased phagocytic ability and increased ability to induce apoptosis of lymphocytes. While this demonstrated the ability of GBM cell lines to induce phenotypes similar to
those observed on monocytes in GBM tissue and circulation, it did not directly correlate this phenotype with immunosuppressive abilities.

A study by Gustafson et al demonstrated the presence of a monocytic MDSC population in GBM patients defined by positive expression of CD14 and low or negative expression of HLA-DR, fitting the canonical monocytic MDSC phenotype (220). These cells were found at higher frequencies in GBM patients compared to normal donors and were increased even higher with dexamethasone treatment. They confirmed the immunosuppressive abilities of these cells by mixing them at increasing concentration with normal donor T cells. Monocytic MDSCs from GBM patients were not able to activate T cells as well as normal donor monocytes from mismatched controls.

Given the lack of exploration of MDSCs in GBM patients, the goal of this study was to analyze myeloid cells in GBM patient peripheral blood and identify immunosuppressive populations capable of inducing peripheral T cell dysfunction. In addition, we aimed to identify the mechanism of immunosuppression used by these cells to provide for a target to improve T cell function.
CHAPTER II

NEUTROPHIL DEGRANULATION AND IMMUNOSUPPRESSION IN PATIENTS WITH GLIOBLASTOMA: RESTORATION OF CELLULAR IMMUNE FUNCTION BY TARGETING ARGINASE I

Introduction

Immunotherapy has become a focus of recent research in GBM due to the potential for combined target specificity and sensitivity. Numerous groups have tested immunotherapeutic strategies in patients with GBM. Unfortunately these efforts have been in large part unsuccessful (289). A major potential pitfall for immunotherapy in GBM patients is the known suppression of cellular immunity, which has been well described over the past few decades. Many groups have reported on the variety of functional defects seen in the circulating pool of T cells from these individuals (286). We, and others, have documented the exceedingly rare and ultimately ineffectual T cell infiltrates found within GBM (301). In spite of these apparently local as well as global aberrations in cellular immunity, patients with GBM are generally not systemically immunocompromised prior to the growth of their tumor. This fact, combined with the potential for recovery of cellular immune function following surgical resection (283), has implicated a tumor-derived factor in the suppression of cell-mediated immune responses. It is likely that tumor-associated immunosuppressive factors will similarly affect clinical attempts to augment anti-tumor responses. Therefore, targeting tumor-associated immunosuppression in patients with GBM will be critical for the development of meaningful immunotherapeutic strategies.

1 Published with permission from Clinical Cancer Research 2011;17:6992-7002
Cells of myeloid lineage have been increasingly associated with immunosuppression in a number of systems, including various forms of cancer. Myeloid derived cells at different states of maturation have been studied as potent inactivators of both CD4+ and CD8+ T cells (303). Populations of immature myeloid cells as well as more mature, differentiated monocytes and granulocytes have been previously shown to possess immunosuppressive abilities (222, 226, 230). Given prior observations of T cell dysfunction in GBM patients and the documentation of myeloid cells with immunosuppressive characteristics in patients with other cancers, the current study attempted to identify a myeloid-derived source of peripheral immunosuppression in GBM patients.

**Materials and Methods**

**Patient and Sample Collection**

Peripheral blood was collected from patients undergoing neurosurgical resection of intracranial tumors (GBM, anaplastic glioma, meningioma, and pituitary tumor) at the University of Colorado Hospital with appropriate Institutional Review Board approval. Patient age and gender did not vary significantly between groups. Preoperative steroid treatment was taken into consideration; however no statistically significant differences were found between pre-operative steroid use and the presence of activated neutrophils (reviewed in Table 2.1). Normal donor blood was collected from anonymous donors from the blood bank at the University of Colorado.

Within one hour from harvest, plasma was removed from peripheral blood samples and stored at -70°C. Peripheral blood mononuclear cells (PBMC) were purified by centrifugation over a Ficoll Histopaque (Sigma) density gradient according to the manufacturer’s protocol. PBMC were used immediately, without freezing, for T cell
functional assays or staining by flow cytometry. Normal donor granulocytes used for staining by flow cytometry were collected within the flow-through fraction of the Ficoll prep; red blood cells were lysed via brief incubation in 0.84% ammonium chloride.

**T cell Functional Assays**

Mitogenic stimulations were performed using bulk PBMC or isolated T cells from normal donors or patients cultured in RPMI 1640 medium with 10% FBS and 1% penicillin-streptomycin. T cells were isolated using CD3 positive selection magnetic beads per manufacturer’s protocol (Miltenyi Biotec). Cells were plated at 1x10^5 cells per well in a 48 well plate with 500 μL of medium. Cells were stimulated with either 1x10^5 Dynabeads (Invitrogen) or 5 μg/mL Phytohemagglutinin (PHA) (Sigma) per well and incubated for 48 and 72 hours post-stimulation. Medium interferon-γ (IFN-γ) levels were assayed by ELISA (Thermo Scientific) according to the manufacturer’s protocol. In functional assays where proliferation was measured by carboxyfluorescein succinimidyl ester (CFSE) staining, isolated PBMC at a concentration of 6x10^5 cells/mL were mixed with 5 mM CSFE (BD Pharmingen) for 5 min and washed with medium. Cells were then stimulated with 5 μg/mL PHA and incubated for 72 hours prior to flow cytometry. For flow cytometric analysis, samples were stained with anti-CD3-APC and CD3+ T cells were gated upon for subsequent evaluation of CFSE fluorescence. For comparative measurement of T cell proliferation as measured by CFSE dilution, the proliferation index (PI) was calculated based on the proportion of proliferating cells over total T cells.

Mixed lymphoid reactions (MLR) were carried out using bulk PBMC collected from patients and normal donors. “Modified” MLR utilized purified CD11b+ myeloid cells and CD3+ T cells from patients and normal donors, again isolated using positive bead selection. Cells from two different normal donors or a normal donor and a tumor patient were mixed at 1.0x10^5 cells/well of each cell type in 200 μL medium in 96 well plates.
MLR were incubated for 48 and 72 hours. Medium IFN-γ levels were assayed by ELISA as described above.

**Flow Cytometry**

Directly after isolation, 1x10^6 cells were resuspended in 200 μL FACS buffer (PBS + 20% FBS). Cells were incubated with antibodies against CD11b, CD33, CD14, HLA-DR, CD15, or CD66 (BD Biosciences) for 45 minutes at 4°C prior to measuring expression on a FACSCalibur flow cytometer. Intracellular staining for CD3ζ was performed by resuspending CD3 stained PBMC in 100 μL of Cytofix/Cytoperm solution (BD Biosciences) for 20 min at 4 °C, washing with Perm/Wash Buffer (BD Biosciences) and staining with anti-CD3ζ (CD247) (BD Biosciences) for 45 min at 4 °C prior to measurement. Flow data was analyzed using the FlowJo software program (Treestar).

**Flow Sorting, Cytospin, and GBM Histopathological Analysis**

CD11b+CD33lo and CD11b+CD33hi populations within PBMC from GBM patients were sorted using a FACSaria flow sorter. In parallel experiments, putative granulocytes within PBMC were isolated using magnetic bead separation by positive CD66 selection (Miltenyi Biotec). The flow-through fraction from the CD66+ selection was collected and incubated with CD11b positive selection beads to collect CD11b+CD66- monocytes. Sorted populations were spun onto Superfrost microscope slides (Fisher) by centrifuging at 750 rpm for 2 min. Slides were stained with Wright-Giemsa stain and visualized at high power. For evaluation of actively necrotic GBM samples, formalin-fixed specimens were prepared as per standard procedures and stained with hematoxylin and eosin. Representative sections were also subject to immunohistochemical analysis for CD15 (Ventana Medical Systems) and
myeloperoxidase (Dako) using standard techniques. Pathological slides were reviewed by the neuropathologist on the study (BKD).

**Arginase I Measurement**

Plasma samples and medium from T cell functional assays described above were subject to Arginase I ELISA (Hycult Biotechnology) according to the manufacturer’s protocol. Samples were diluted 1:1 with kit dilution buffer. For evaluation of ArgI levels within necrotic material from GBM, necrotic tissue was weighed and diluted in unsupplemented RPMI medium to a ratio of 60 μL per mg of tissue. The tissue was then disbanded and vortexed to suspend extracellular contents into the medium. The resulting samples were centrifuged at 5000 rpm for 10 min to remove excess tissue and supernatants were collected. Supernatant from necrotic GBM samples were diluted over a range of 1:1 to 1:100 and used for ArgI ELISA as above.

**Induction of Degranulation Using fMLP**

Formyl-Methionyl-Leucyl-Phenylalanine (fMLP) (Sigma) was added to whole blood at a concentration of 1 μM and incubated at room temperature for one hour. Whole bloods without fMLP were used as controls. Following incubation, PBMC were collected using a Ficoll density gradient as described above. Bulk PBMC were used to assess T cell function through PHA stimulation and stained for flow cytometry as described previously.

For detection of dose-dependent neutrophilic suppression of T cell proliferation, neutrophils were sorted from normal donor whole blood using density centrifugation purification over a 42%/51% Percoll gradient, followed by CD66 positive bead separation. Purified neutrophils were activated with 1 μM fMLP. T cells were purified using CD3 positive separation. Cultures were then prepared with varying T
cell/neutrophil ratios and stimulated with 5 μg/mL PHA for 72 hours. Bromodeoxyuridine (BrdU) was added to cultures at a concentration of 1 μM for the final 20 hours and cells were then harvested for flow cytometric quantification of proliferation. Medium Arg I levels from these cultures analyzed by ELISA as described above.

**Assays to Overcome Arginase Activity in vitro**

T cell functional assays (PHA stimulation and MLR) using bulk PMBC were performed as above. Groups of samples were treated with 7.81 mg/mL L-arginine (Sigma) or 40 μM N-ω-Hydroxy-L-norarginine (nor-NOHA) (Cayman Chemicals) at the time of plating and PHA addition or cell mixing. Dose-response profiles for each compound were developed prior to testing on patient samples in order to identify the highest possible dose that did not affect baseline T cell functional response (i.e. toxicity or augmented functional response) in normal donor samples. Cells were incubated for 48 and 72 hours and medium IFN-γ levels were tested by ELISA as described above.

**Statistical Analysis**

Data are represented as mean ± SEM. Multigroup analysis was performed using ANOVA. Differences between two variables were determined using Student’s t test. P values less than 0.05 were considered significant.

**Results**

**Direct ex vivo T cells from Patients with GBM are Functionally Suppressed in vitro**

To confirm prior reports describing decreased proliferative responses of T cells from patients with GBM, PBMC were purified, stained with CFSE, and stimulated with PHA directly ex vivo. Flow cytometric analysis of stimulated T cells from patients with
GBM indeed demonstrated significantly lower levels of proliferation than seen from normal donors or patients with other intracranial tumors (Figure 2.1A). To evaluate stimulation-induced cytokine production, levels of IFN-γ within medium from PHA-stimulated PBMC cultures were assayed by ELISA. Cultures from patients with GBM generated significantly less IFN-γ at both 48 and 72 hours than did matched samples from normal donors or patients with other intracranial tumors (Figure 2.1B). Taken together, these results corroborate prior experimental data documenting the hyporesponsive nature of T cells in GBM patients.

**PBMC and Purified CD11b+ cells from GBM Patients Suppress Normal Donor T cell Function**

To confirm the presumptive cellular source of peripheral T cell suppression in GBM patients, we explored the possibility that GBM-associated immunosuppression could be transferred to normal donor T cells. Mixed-lymphoid reactions (MLR) were prepared using PBMC from normal donors (ND) and patients with various intracranial tumors. T cell alloresponses were confirmed by measuring IFN-γ production at 48 and 72 hours by ELISA. IFN-γ production in MLR using ND PBMC with PBMC purified from patients with pituitary tumor or meningioma demonstrated no reduction in alloresponse compared to MLR using two different ND. In contrast, T cell responses within GBM-associated MLR were markedly suppressed, producing only 20-30% of the IFN-γ as seen by ND or other intracranial tumor MLR (Figure 2.1C).

To further investigate the possibility that a myeloid-lineage cell within GBM PBMC was responsible for suppression of T cell activity in these assays, “modified MLR” were prepared using purified CD11b+ myeloid cells and sorted CD3+ T cells. Again, no decrease in ND T cell alloresponse was observed when cultured with CD11b+ cells from alternate ND, as measured by IFN-γ production. However, purified CD11b+ cells from
GBM patients exerted a robust suppressive effect on ND T cells, resulting in similar levels of IFN-γ production as was seen in MLR using bulk PBMC (Figure 2.1D).

Together, these results confirm that peripheral GBM-associated immunosuppression is in part associated with a CD11b+ myeloid-lineage population and that the suppressive effect can be transferred to normal donors.

Figure 2.1: GBM patient T cell function is suppressed; myeloid cells from GBM patients can transfer suppression to normal donors. (A) Representative (left panel) and averaged (right panel) flow cytometric analysis of gated CD3+ CFSE-stained T cells from GBM patients (orange line, n=5) when compared to normal donors (ND – blue line, n=6) or T cells from patients with pituitary tumors (PIT – black line, n=6) (p=0.006). Averaged numbers are reported as a proliferation index (PI) calculated by the ratio of proliferating CD3+ T cells to the total number of T cells. (B) ELISA measurement of IFN-γ within culture medium after PBMC stimulation with PHA in patients with GBM (n=10), meningioma (MEN, n=5), pituitary tumor (n=9) or ND (n=20) (p<0.0001). (C) Measurement of IFN-γ production in MLR using bulk PBMC (ND n=5, MEN n=5, PIT n=8, GBM n=10) (p<0.0001). (D) IFN-γ production in “modified” MLR using isolated CD3+ T cells (T) from ND and isolated CD11b+ myeloid cells (M) from alternate ND (n=8) or GBM patients (n=9) at both 48 (p<0.0001) and 72 hrs (p<0.0001).
PBMC from Patients with GBM Harbor Increased Numbers of CD11b+CD33loCD14-HLA-DR- Myeloid-lineage Cells

Subsequent experiments were designed to further identify the CD11b+ myeloid-derived population responsible for the transferable immunosuppressive effect. Prior studies of myeloid-related immunosuppression have identified both monocytic- and granulocytic-lineage cells within the PBMC fraction as potentially capable of suppressing T cell function (304). To further characterize CD11b+ cells associated with immunosuppression in GBM patients, we evaluated the PBMC fraction from GBM patients in comparison to other intracranial tumors and normal donors. The common myeloid markers CD11b and CD33, along with more specific monocytic markers CD14 and HLA-DR, were initially used to evaluate the frequency and phenotype of monocyte-lineage cells within PBMC. Evaluation of CD11b staining patterns revealed that patients with primary GBM harbor significantly increased percentages of circulating CD11b+ myeloid cells (as a proportion of total PBMC) than do patients with meningioma, pituitary tumor, or anaplastic glioma (Figure 2.2C). Subsequent marker analysis demonstrated that the expanded CD11b+ population within GBM patients was almost entirely composed of a distinct population expressing lower levels of CD33 (CD33lo) that segregated away from the CD14+ monocytyc population expressing high levels of CD33 (CD33hi) (Figure 2.2A). To further confirm a non-monocytic phenotype, CD11b+CD33lo cells were shown to be negative for staining with antibodies against CD14 or HLA-DR (Figure 2.2B).

We next attempted to correlate the frequency of CD11b+CD33lo cells within PBMC from brain tumor patients with demographic and clinical data, including age, gender, tumor location, presence of pre-operative steroids, and imaging characteristics, such as extent of edema, tumor location and tumor size. There was no statistically significant correlation between the presence of CD11b+CD33lo cells and any of the
factors listed above. Most notably, although trending towards positive correlation, we did not observe a statistically significant correlation between presence of CD11b+CD33lo cells within PBMC from patients with intracranial tumors and pre-operative steroid therapy (p=0.10, data outlined in Table 2.1) which has been previously implicated in the generation of suppressive monocytes in GBM (220).

Figure 2.2: GBM patients harbor an expanded population of circulating CD11b+CD33lo cells that appear within the PBMC fraction. (A) PBMC from normal donors and patients with meningioma or GBM were stained for the myeloid markers CD11b and CD33. (B) Gated CD11b+CD33hi and CD11b+CD33lo cells within GBM PBMC were stained for the prototypic monocytic markers CD14 and HLA-DR. (C) Analysis of average CD11b+CD33hi and CD11b+CD33lo population frequency between all patients sampled (outlined in Table 2.1, anaplastic glioma – APG) (p= 0.016).
Table 2.1: Demographics of patients used to analyze the relationship between pre-operative steroids and presence of CD11b+CD33lo suppressive cells. All patients undergoing neurosurgical resection were treated with peri-operative steroids 1-2 hours prior to blood acquisition. Patients noted to have received pre-operative steroids (Y) were treated with dexamethasone for varying time periods at varying doses prior to the day of surgery. Normal donor demographics are not reported, as blood was collected from anonymous donors at the blood bank of the University of Colorado.
In our evaluation of the monocyte population, we did observe a slight increase in the percentage of CD14+ monocytes within PBMCs from GBM when compared to PBMCs from normal donors. However, CD14+ monocyte frequency in GBM did not vary significantly from the percentage of CD14+ monocytes within PBMCs from patients with benign meningioma, pituitary tumor, or anaplastic glioma (Figure 2.2C).

**CD11b+CD33lo Myeloid-lineage Cells within GBM Patient PBMC are Degranulated Neutrophils**

As the expanded population of CD11b+CD33lo cells within PBMC from GBM patients did not appear to be of monocytic lineage, we next investigated if these cells arise from granulocytic origin. Baseline phenotypic analysis using flow cytometric scatter data demonstrated that CD11b+CD33lo cells within PBMC from GBM were smaller and more granular than characteristic monocytes seen in our prior experience (Fig 2.3A). In contrast, CD11b+CD33lo cells from GBM patients closely paralleled scatter characteristics exhibited by normal donor granulocytes (Figure 2.3A). To further verify the potential granulocytic phenotype, GBM patient PBMC were stained for the neutrophil markers CD15 and CD66; expression of these markers was similar to patterns seen on normal donor neutrophils (Figure 2.3B).

To provide final confirmation that CD11b+CD33lo cells within PBMC fractions from GBM patients represent neutrophils, the CD33lo and CD33hi populations were purified for histological analysis. Initial attempts at purification using flow-sorting demonstrated the CD33lo population to be physically fragile, as membranes of sorted cells were disrupted to the point where they could not be phenotypically identified following cytospin. In an attempt to provide a more gentle sorting process, magnetic bead separation was used to isolate CD11b+CD66+ and CD11b+CD66- populations.
Figure 2.3: CD11b+CD33lo cells within GBM PBMC express granulocyte markers and demonstrate histological characteristics of neutrophils. (A) Representative forward (FSC) and side scatter (SSC) analysis of flow cytometry data from ND and GBM PBMC as well as the flow-through fraction after Ficoll centrifugation of ND blood; circular gates represent cells with scatter characteristics of granulocytes. (B) ND granulocytes, collected from the flow-through fraction after Ficoll centrifugation, and gated CD11b+CD33lo cells from GBM PBMC stained with the granulocytic markers CD15 and CD66. (C) Wright-Giemsa stain of sorted CD11b+CD66+ and CD11b+CD66- cells from GBM PBMC.

Subsequent histological analysis confirmed the presence of typical monocytes in the CD11b+CD66- population, while the CD11b+CD66+ population demonstrated the morphology of neutrophils (Figure 2.3C).

As neutrophils should normally segregate to the flow-through fraction following Ficoll density separation of whole blood, their presence within the PBMC fraction from GBM patients was somewhat puzzling. We hypothesized that the shift to the PBMC fraction could potentially arise from two sources: 1) an atypical, de novo granulocytic cell
population generated from circulating myeloid precursors segregating with other mononuclear cells during density centrifugation, or 2) reduced density of mature circulating neutrophils, possibly secondary to degranulation, resulting in a shift to the PBMC fraction on Ficoll density gradient. As flow-sorting experiments had suggested that CD11b+CD33lo cells within PBMC from GBM patients possessed relatively weak cell membranes, we proposed to further evaluate the potential that these cells were, in fact, neutrophils in a “degranulated” state.

To first confirm the theoretical possibility that degranulation could induce a shift of neutrophils to the PBMC fraction, whole blood from normal donors was stimulated with fMLP and subject to Ficoll density centrifugation. We confirmed that fMLP-induced degranulation of normal donor neutrophils resulted in decreased density, corresponding with a shift to the PBMC fraction (Figure 2.4A), and that patterns of CD11b, CD33, CD14, and HLA-DR expression on degranulated normal donor neutrophils matched those seen in the CD11b+CD33lo population from patients with GBM (Figure 2.4B). To provide additional physiological confirmation for potential in vivo degranulation of neutrophils in GBM patients, we attempted to document increased circulating levels of Arginase I (ArgI), a factor known to be present within neutrophilic primary granules and possess immunosuppressive activity (263). We evaluated patient plasma samples for increased levels of ArgI, and found direct ex vivo plasma ArgI levels were indeed significantly higher in patients with GBM than in normal donors or patients with other intracranial tumors (Figure 2.4C).
Figure 2.4: CD11b+CD33lo cells within GBM PBMC are degranulated neutrophils. (A) Degranulation was induced in normal donor whole blood using fMLP; PBMC were collected from stimulated blood and matched unstimulated controls and subject to flow cytometry; circular gates represent cells with scatter characteristics of granulocytes. (B) Flow cytometric analysis of gated CD11b+CD33lo cells within fMLP-degranulated normal donor PBMC, stained with CD14, HLA-DR, CD15, and CD66. (C) Arginase I ELISA analysis of plasma samples from GBM patients (n=6) when compared to ND (n=10) or patients with meningioma (MEN, n=14) or metastatic tumors (MET, n=5) (p<0.0001). (D) 40X (i)- and 100X (ii)-magnification images as well as immunohistochemical staining for CD15 (iii) and myeloperoxidase (iv) from a representative sample of GBM undergoing active necrosis; arrows mark an area of active necrosis in (i).
**Neutrophilic Infiltrates are Increased in GBM Undergoing Active Necrosis**

Although there has been limited prior documentation of the presence of neutrophils within human GBM (305), neuropathological association of neutrophilic infiltration within necrotic tissue is well known. In prior analysis we identified limited neutrophilic infiltrates within active and infiltrative components of GBM tissue (BK DeMasters, unpublished data). However, as neurosurgeons typically provide infiltrative (i.e. “active”) tumor specimens for the purposes of pathological analysis, and necrosis in these lesions is likely a time-limited event, pathological evaluation of actively necrotic regions is often not possible. However, in isolated cases where tumors demonstrate evidence of widespread active necrosis, it is possible to evaluate the cellular infiltrate involved with the ongoing process of necrosis. We obtained several specimens of human GBM containing robust regions of active necrosis. Evaluation of hemotoxylin and eosin (H&E) stained specimens from these tumors demonstrated profound neutrophilic infiltrates within the regions of acute necrosis, as would be expected with any acutely necrotic tissue (Figure 2.4D). Examples of GBM with more advanced coagulative necrosis and paucity of residual inflammatory cells were not examined.

**Arginase I Expression Correlates with T cell Dysfunction in GBM Patients**

ArgI has been shown to exert immunosuppressive effects through the consumption of L-arginine, a critical cofactor for sustained T cell activation due to its central role in the re-expression of the T cell co-receptor CD3ζ (224). When released into the extracellular environment, ArgI can potently and rapidly deplete extracellular L-arginine, resulting in T cell anergy and immune dysfunction. Neutrophil degranulation and subsequent release of ArgI have previously been linked to immunosuppression in renal cell carcinoma (215, 218) and non-small cell lung cancer (306). We therefore
Figure 2.5: Arginase I levels correlate with GBM T cell dysfunction in vitro and in vivo.
Figure 2.5: Arginase I levels correlate with GBM T cell dysfunction in vitro and in vivo. (A) Representative (left panel) and averaged (right panel) flow cytometric analysis of intracellular CD3ζ expression in direct ex vivo T cells from PIT (blue, n=6), MEN (green, n=6), and GBM (orange, n=5) patients compared to isotype (black) (p=0.024). (B) ELISA measurement of PHA-induced IFN-γ production by normal donor PBMC, following neutrophil degranulation by fMLP in whole blood prior to PBMC purification, when compared to matched PBMC from unstimulated blood at 48 (p=0.0007) and 72 hrs (p=0.015). (C) Purified neutrophils from normal donors (n=5) were activated with fMLP and mixed at varying concentrations with sorted T cells. Cultures were stimulated with PHA for 72 hrs and proliferation was measured by BrdU incorporation in gated CD3+ populations (p<0.05). (D) Medium from cultures was collected and ArgI was measured by ELISA (p<0.0001). (E) Comparative ELISA-based measurements of ArgI levels in immunofunctional assays (PHA stimulation and MLR) using bulk PBMC from normal donors (n=4) or patients with meningioma (n=4) or GBM (n=5) (p<0.0001). (F) The ArgI inhibitor nor-NOHA (n=4) or supplemental L-arginine (n=5) were added to (left panel) PHA stimulated bulk PBMC or (right panel) MLR developed from the aforementioned groups. IFN-γ production was measured by ELISA at 48 hrs. (p<0.0001 in each case).
hypothesized that \textit{in vivo} degranulation of neutrophils in GBM patients would result in increased levels of serum ArgI and may be a source of cellular immunosuppression.

As ArgI is known to regulate CD3\(\zeta\) expression T cells, we initially explored levels of this marker on unmanipulated, direct \textit{ex vivo} T cells from GBM patients. Flow cytometric analysis demonstrated that baseline CD3\(\zeta\) levels were modestly lower upon circulating T cells from GBM patients than T cells from patients with pituitary tumors or meningioma (Figure 2.5A).

To demonstrate that the immunosuppressive effect of ArgI release could be recapitulated \textit{in vitro}, PBMC were collected from fMLP-treated normal donor whole blood and stimulated with PHA in culture. IFN-\(\gamma\) levels in these cultures were compared to PHA stimulated PBMC collected from untreated normal donor whole blood. PBMC from fMLP-stimulated samples demonstrated significantly less IFN-\(\gamma\) production at both 48 and 72 hours compared to controls (Figure 2.5B). In addition, sorted normal donor T cells stimulated in the presence of increasing numbers of purified, activated neutrophils resulted in a concentration-dependent suppression of T cell proliferation (Figure 2.5C) confirming that neutrophil degranulation (and ArgI release, Figure 2.5D) results in functional T cell suppression \textit{in vitro}. To provide further evidence for an association of neutrophil degranulation with GBM-induced immunosuppression, levels of ArgI within medium from \textit{in vitro} GBM T cell functional cultures described previously were measured and compared to ArgI levels within medium from normal donor and control patient cultures. Again, levels of ArgI were markedly elevated within medium harvested from PHA-stimulated GBM PBMC, as well as within medium from MLR containing GBM PBMC, when compared to normal donors or tumor controls (Figure 2.5E).

As mentioned above, neutrophils can be found within regions of active necrosis within GBM specimens. To demonstrate the potential for an ArgI mediated immunosuppressive effect within the tumor microenvironment, we selectively harvested
necrotic tissue during neurosurgical resection of GBM and the resulting material was subject to ArgI ELISA. Although there was significant variance between the tumors tested, all necrotic samples demonstrated ArgI levels that were manifold higher than levels observed in matched plasma samples, ranging from 171-2946 pg/mL.

**GBM T cell Function can be Restored by Targeting ArgI in vitro**

To provide pre-clinical evidence that targeting ArgI may provide benefit for restoring cellular immune function in GBM patients, we utilized several approaches to restore arginine levels within functional *in vitro* cultures. The ArgI enzymatic pathway was first targeted using the specific ArgI inhibitor nor-NOHA, which was added at a concentration of 40 μM to the medium of T cell functional assays (PHA stimulation or MLR prepared as described previously). The addition of nor-NOHA to the medium of functional cultures containing GBM T cells within bulk PBMC (which under normal conditions did not produce IFN-γ) resulted in a significant increase in functional response after stimulation with PHA (Figure 2.5F), restoring IFN-γ production to levels comparable with T cells from normal donors or patients with other intracranial tumors. A similar restitution of T cell functional response was seen in GBM-suppressed MLR in the presence of nor-NOHA (Figure 2.5F).

In an attempt to provide an immediately translatable mechanism for targeting neutrophil-derived ArgI activity, we explored the use of L-arginine supplementation to restore GBM T cell function *in vitro*. As seen in experiments using nor-NOHA, L-arginine supplementation significantly increased *in vitro* IFN-γ production by PHA-stimulated GBM T cells and similarly reversed suppression of normal donor T cells within MLR containing GBM PBMC (Figure 2.5F). Together, these data confirm that 1) ArgI exerts a central and reversible role in the suppression of cellular immune function in patients with GBM, and 2) that reversal of ArgI-mediated effects through either pharmacological
inhibition or addition of exogenous L-Arg can restore GBM T cell function to levels equivalent with normal controls.

**Discussion**

In this study, we confirmed prior findings that GBM patient T cell functions are suppressed directly *ex vivo* compared to normal donors. We demonstrated that myeloid cells from PBMCs of GBM patients suppress normal donor T cell function. This suppressive ability correlates with increased levels of Arginase I expression in the medium of these cultures. Further analysis of the myeloid populations found in GBM patient PBMC identified an expanded population of cells found specifically in GBM patients, which we identified as activated neutrophils due to their expression of CD11b, CD33, CD66 and CD15, lack of expression of CD14 and HLA-DR, and presence within the PBMC fraction. In addition, when isolated from the PBMC fraction they had the morphologic appearance of mature neutrophils.

These findings identified a novel population of myeloid cells capable of inducing T cell dysfunction in GBM patients. Prior reports of MDSCs in GBM patients described cells with a monocytic phenotype (220). As noted in Chapter I, some groups might consider the cells we describe to be activated neutrophils to be granulocytic MDSCs based on their morphology, marker expression, and ability to suppress T cell function *in vitro*. However, we argue that our parallel studies with normal donor neutrophils demonstrating their activation leads to ArgI release, a shift to the PBMC fraction of blood, and ability to suppress T cell proliferation suggests they are activated neutrophils rather than a unique population of myeloid cells programmed to suppress immune function. This notion is further supported by our studies presented later, in Chapter III,
that further characterize these cells and indicate that circulating activated neutrophils in GBM patients express markers of activation.

In addition to the identification of activated neutrophils within GBM patient circulation, we also demonstrated the presence of neutrophils within GBM patient tissue, a phenomenon that has not been well described in GBM. Neutrophils tended to be associated with areas of necrosis within the tissue; consistent with their role in wound healing. Also of note was the high levels of ArgI found in GBM tissue conditioned medium. One caveat to this finding is that some of the tissue samples used to generate this conditioned medium contained more red blood cells than others. As red blood cells also express ArgI (307), if any were lysed in the process of generating the TCM, this would cause the ArgI level to be deceptively high and would not accurately represent the levels of ArgI found within the tumor microenvironment. Arginase release from red blood cells most likely accounts for the high variability in the amount of ArgI detected within GBM samples. Even so, these two observations suggest a role for neutrophils and ArgI in intratumoral immunosuppression, further expanding the possibilities of targeting ArgI. Continued exploration of the role of neutrophils and ArgI in intratumoral immunosuppression is needed.

Importantly, we demonstrated that suppression of T cell function could be rescued in vitro by targeting ArgI with the ArgI inhibitor nor-NOHA or by supplementation with L-arginine. This result demonstrated a novel mechanism of immunosuppression that has never been described in the context of GBM and also provides for a possible therapeutic target to reverse immunosuppression in GBM patients, a commodity that is lacking in the field of GBM immunotherapy. As a direct result of these studies, our group initiated a clinical trial to determine the effect of L-arginine supplementation on GBM patient T cell function.
CHAPTER III

TUMOR-SPECIFIC DISRUPTION OF NEUTROPHIL TRANSMIGRATION IN HUMAN GLIOBLASTOMA

Introduction

We recently identified a pathway of peripheral cellular immunosuppression in patients with GBM, whereby activated circulating neutrophils release the immunosuppressive enzyme Arginase I which induces a reversible hypofunction of T cells. While providing a targetable pathway for improving anti-tumor immunity in affected patients, the presence of activated neutrophils within the peripheral blood of patients with GBM remains of unclear etiology.

Following initial stages of activation within the vasculature of inflamed tissues, neutrophils are classically thought to undergo extravasation through a well-defined mechanism of rolling, adhesion and transmigration [reviewed in (236, 237)]. This process is regulated through modulated interactions of neutrophilic adhesion molecules with corresponding ligands on the luminal surface of the activated endothelium. Following extravasation, chemotactic gradients further motivate neutrophils to complete their activation process and release a large of number of pre-formed, biologically active factors through degranulation. Under normal conditions, neutrophil activation, transmigration, and degranulation are highly regulated to avoid inappropriate release of potentially damaging granular contents. The consequence of a significant disturbance in this process can be seen in sepsis (308), where severe infection leads to systemic activation of neutrophils, multiorgan failure, and death. Although seen to a lesser extent, immunosuppressive consequences of systemic neutrophil activation are similarly evident in patients with GBM.
The process of adhesion and transmigration involves a complex interaction of multiple adhesion molecules present on the surface of the neutrophil that interact with their corresponding ligands on the inflamed endothelium. Through analysis of these adhesion markers, we sought to gain insight into which phase of this process might be disrupted, allowing for activated neutrophils in circulation. In addition, an imperative aspect of this system is the factor(s) responsible for activation itself. There are several inflammatory cytokines, along with other factors released during inflammation and tissue damage, which are known to activate neutrophils. Glioblastoma has been shown to express several of these cytokines (122, 123). While the role of these cytokines have been well described in aspects of tumor growth, such as proliferation, migration, invasion, and angiogenesis, their involvement in immune cell regulation, and more specifically neutrophil activation, has yet to be explored. Here we examine the milieu of neutrophil activating cytokines present within GBM tissue and attempt to identify the factor(s) responsible for neutrophil activation by GBM.

We hypothesized that a detailed analysis of the phenotypic and functional characteristics of neutrophils within various biological compartments in GBM patients, along with investigation into inflammatory factors and pathological features of GBM influencing neutrophil activation, would shed light into the formation of activated neutrophils within GBM patient circulation. Understanding this mechanism might identify targetable aspects of this system to prevent systemic neutrophil activation and the resulting immunosuppression in these patients.
Methods

Sample Acquisition and Collection of Clinical Data

All tissue acquisition was performed under the auspices of an active Institutional Review Board protocol. Briefly, peripheral blood samples were acquired at the time of surgery from patients undergoing resection of either primary or recurrent GBM and were immediately transported to the laboratory for processing. Tumor specimens, including areas of viable tumor as well as necrotic tissue (as determined by image guidance as well as microscopic evaluation by the operative neurosurgeon), were flash frozen in the operating room or were transferred in medium for preparation of single cell suspensions. Samples from patients undergoing hemispherectomy for epileptic events were similarly collected and flash frozen in the operating room to be used as normal brain controls.

Clinical and radiographic data were collected in retrospective fashion. Patients were stratified according to the length of corticosteroid treatment prior to their surgical procedure (the time at which peripheral blood samples were collected) into short (less than two days), intermediate (two to seven days), and long-term (greater than one week) duration. For comparison of adhesion marker expression between patients with different stages of disease, patients were defined as having receiving steroid treatment or not, regardless of duration. For evaluation of extent of edema, pre-operative T2-weighted magnetic resonance images (MRI) were used to classify patients into three strata including mild (trace surrounding fluid signal), moderate (surrounding fluid signal equal to or less than volume of enhancing tumor), or significant (surrounding fluid signal greater than volume of enhancing tumor mass) brain edema. Extent of necrosis was similarly evaluated using pre-operative T1-weighted MRI, stratified into categories of mild (minimal central necrosis in respect to enhancing disease), moderate (balanced central necrosis in respect to enhancing disease), or significant (tumor mass largely consistent with central necrosis).
Isolation of Resting, Circulating Activated, and Intratumoral Neutrophils

To obtain resting neutrophils, peripheral blood samples collected from normal donors or GBM patients were drawn into a 3.8% citrate solution and the plasma fraction was separated by low-speed centrifugation. Cell pellets were resuspended and red blood cells were subsequently removed through sedimentation with a 6% Dextran solution. Isolated white blood cells were then resuspended into autologous platelet-poor plasma and layered on top of 42/51% Percoll (GE Healthcare) bilayer gradients. The pure population of resting neutrophils located between the Percoll layers was collected and washed with phosphate buffered saline (PBS) prior to further testing.

For analysis of circulating activated neutrophils, separate blood samples were used for Ficoll Histopaque (Sigma) density gradient centrifugation as previously described. The traditional PBMC layer, previously shown to contain the fraction of activated (low-density) neutrophils within peripheral blood from GBM patients, was harvested from the gradient and washed with PBS for further analysis.

For isolation and analysis of intratumoral neutrophils, freshly resected specimens of human GBM were minced and gently digested with Accutase (Invitrogen) for 30 minutes. Tumor specimens were subsequently triturated and passed through a 70 micron filter to generate a single cell suspension. The resulting preparations were washed with PBS prior to further analysis. Neutrophils within tumor single cell suspension were identified according to CD66 expression by flow cytometry.

Flow Cytometry

Samples of interest were resuspended in 200 μL FACS buffer (20% FBS in PBS) at a concentration of 2.5x10^6 cells/mL and stained for analysis. All flow cytometry utilized a FACSCalibur machine equipped for four-color analysis. Mouse anti-human antibodies against surface markers and corresponding isotypes included FITC anti-CD66, PE anti-
CD62L, PE anti-CD11a, PerCP-Cy5.5 anti-CD18, APC anti-CD11b, and AlexaFluor-687 anti-CD31 (BD Biosciences). Resulting flow data were analyzed using the FlowJo software program (Treestar). Comparative expression levels for each receptor were calculated by subtracting the mean fluorescence intensity (MFI) of the corresponding isotype from the MFI for the marker of interest.

**Quantitative Analysis of in vitro Neutrophil Activation**

For controlled analysis of activation, resting neutrophils were isolated from normal donors and GBM patients as described above. Cells in RPMI at a concentration of 1x10^6 cells/mL and were activated with 1 μM fMLP for 30 minutes prior to staining and flow cytometry.

**Assessment of in vitro Neutrophil Adhesion**

Adhesion was measured as previously described by Louis et al (309). Briefly, human microvascular endothelial cells (HMEC) were grown to confluence in 24 well plates and activated overnight with 40 ng/mL tumor necrosis factor-α (TNF-α). Resting neutrophils were isolated from normal donors or GBM patients as described above and fluorescently labeled with 1 μM BCECF (2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein) (Invitrogen) for 15 min at 37 °C. Labeled neutrophils were layered onto activated HMEC cultures in Hanks Balanced Salt solution (HBSS) supplemented with HEPES and 200 nM fMLP. An initial fluorescence reading was taken at a wavelength of 450 nm using a Hidex Chameleon V plate reader immediately after neutrophil addition to acquire the baseline fluorescence. Neutrophils were then allowed to adhere during a five-minute incubation period at 37°C, after which plates were gently washed with warm HBSS and analyzed for remaining fluorescence. Subsequent serial washes and fluorescence readings were completed at five-minute intervals. Adhesion
over time was calculated by plotting the change in fluorescence associated with each wash cycle compared to baseline.

**Activation of Normal Donor Neutrophils with Tumor, Necrosis or Normal Brain Conditioned Medium**

Conditioned medium was generated using snap frozen samples of tumor, necrotic tumor or normal brain tissue. Samples were thawed and suspended in unsupplemented RPMI at a ratio of 30 μL medium per 10 mg of tissue. The tissue was disbanded and the solution vortexed prior to centrifugation at 5000 rpm for 10 min to remove solid tissue. Supernatant was collected and used for subsequent activation of normal donor neutrophils.

Resting neutrophils isolated from normal donors as described above were mixed with 100 μL of tumor conditioned medium at a concentration of 5x10^6 cells/mL and incubated for 30 min at 37˚C. Cells were then harvested and stained for flow cytometric analysis of marker expression associated with neutrophil activation.

**Cytokine Array Acquisition and Analysis**

A custom cytokine array was designed to analyze the 16 most common neutrophil activating cytokines as identified by an extensive literature search. The array, manufactured by Ray Biotech, contained the following cytokines: IL-8, epithelial-derived neutrophil activating peptide 78 (ENA-78), granulocyte chemotactic protein 2 (GCP-2), granulocyte colony-stimulating factor (G-CSF), GM-CSF, growth-regulated protein α (GRO-α), IFN-γ, IL-1α, IL-1β, IL-17, IL-2, IL-6, macrophage migration inhibitory factor (MIF), neutrophil activating peptide 2 (NAP-2), TGF-β1, and TNF-α. The cytokine array was performed as described by the manufacture’s protocol using 100 μL of conditioned medium from 8 tumor, 8 necrosis and 6 normal brain tissue samples. Fluorescent signal
intensities for each array was then obtained using a Perkin Elmer ScanArray Express HT microarray stage scanner. Each glass chip was scanned using the Cy3 channel at an excitation frequency of 543 nm. Resulting images were analyzed using the ScanArray Express protein array analysis software to obtain median fluorescence intensity signals for each spot on the array. Relative expression for each cytokine was determined by subtracting the median background fluorescence and normalizing the resulting signal intensity to the signal intensity of the positive control of a chosen sub-array defined as the reference.

**Cytokine Depletion from Tumor Conditioned Medium**

Comparing the relative expressions of each cytokine within the different conditioned media allowed us to identify the two most highly expressed cytokines (IL-8 and IL-6) within tumor and necrosis conditioned medium compared to normal brain. To deplete IL-8 and IL-6 from conditioned medium, new tumor conditioned medium was generated from 6 tumor samples (as there was no difference in expression between tumor and necrosis according to the array) and expression of IL-8 and IL-6 were confirmed by ELISA (Thermo Scientific). IL-8 and IL-6 were depleted from conditioned medium separately and in combination using biotinylated goat anti-human antibodies specific to the cytokine (R & D Systems). Biotinylated goat IgG was used as a control. A total of 10 μg antibody was added to 300 μL of conditioned medium and incubated for 15 min at 4 °C. Anti-biotin microbeads (Miltenyi Biotec) were added at a 1:4 volumetric ratio of beads to conditioned medium and incubated for 20 min at 4 °C. Antibody-bead solutions were poured over a pre-washed magnetic column (Miltenyi Biotec) and flow through depleted of cytokine was collected. Depletion of each cytokine from the conditioned medium was confirmed by ELISA.
Statistical Analysis

Data are represented as mean + SEM. Multigroup analysis was performed using ANOVA. Differences between two variables were determined using Student’s $t$ test. P values less than 0.05 were considered significant.

Results

Circulating Resting Neutrophils and Intratumoral Neutrophils from GBM Patients Express Predicted Levels of Surface Markers

To explore the possibility that circulating activated neutrophils in patients with GBM are indicative of a more global abnormality, we began by comparing the expression patterns of a range of critical adhesion markers between resting neutrophils from GBM patients and those from normal donors. Resting neutrophils were isolated from GBM patient and normal donor peripheral blood (as described in the materials and methods) and adhesion marker expression was analyzed by flow cytometry. As previously described (310, 311), expression of CD62L (L-selectin), CD11a, and CD31 (PECAM-1, platelet-endothelial cell adhesion moledule-1) was high on resting neutrophils from normal donors, with correspondingly low levels of CD11b, CD66, and CD18 (Figure 3.1A,C). In our analysis of the expression patterns of these markers on resting neutrophils from GBM patients, we found no significant differences for any of the markers tested when compared to normal donor neutrophils (Figure 3.1C).

The patterns of change in these markers in response to biological stimuli have been well described, either increasing or decreasing in abundance on the neutrophil surface in association with activation and transmigration status [reviewed in (236, 237)]. At the site of inflammation, neutrophils will first tether to the activated endothelium
Figure 3.1: GBM patient resting neutrophils have the same phenotype as normal donor resting neutrophils and intratumoral neutrophils have a transmigrated phenotype.
Figure 3.1: GBM patient resting neutrophils have the same phenotype as normal donor resting neutrophils and intratumoral neutrophils have a transmigrated phenotype. Adhesion marker expression was measured by flow cytometry of normal donor (ND) resting, GBM resting and GBM intratumoral neutrophil populations isolated as described in the material and methods. (A) Representative flow plot of resting neutrophils isolated from a GBM patient. The neutrophil population was gated around based on forward and side scatter (far left panel), followed by CD66 expression (middle left panels). Levels of adhesion marker expression were then determined based on mean fluorescence intensity (MFI) of the gated CD66+ population (black, right panels) compared to MFI of the isotype control (grey, right panels) found using the forward and side scatter gate. (B) Intratumoral neutrophils within tumor single cell suspensions were identified based on CD66 expression (far left panels). Forward and side scatter gate of the CD66 population was then used to identify neutrophils within the isotype controls (middle left panels). Adhesion marker expression was again quantified by comparing the CD66+ population (black, right panels) to the isotype control (grey, right panels). (C) Average MFI of adhesion markers on ND resting (n=19), GBM resting (n=17) and GBM intratumoral neutrophils (n=). Marker expression is reported as mean fluorescence intensity (MFI) calculated by subtracting the mean intensity of the isotype from the mean intensity of the specific marker. (**p<0.001, *p<0.05).
through an interaction between CD62L and its corresponding ligands. This interaction will initiate rolling of the neutrophil along the endothelium. Further stimulation by inflammatory cytokines present on the activated endothelium will induce shedding of CD62L from the neutrophil surface and allow for the interaction of lymphocyte function-associated antigen 1 (LFA-1 – a complex formed between CD11a and CD18) with intracellular adhesion molecule 1 (ICAM-1) on the endothelium to start the process of adhesion. Next, macrophage-1 antigen (MAC-1 – a complex formed between CD11b and CD18) will interact with ICAM-2, firming adhesion and allowing for crawling of the neutrophil along the endothelial cells to a site optimal for transmigration. CD31 (PECAM-1) will then interact with its ligands at the endothelial cell junctions to mediate extravasation of the neutrophil.

Following neutrophil transmigration, predictable changes in adhesion marker expression occur. Once on the basal side of the endothelium, LFA-1 and MAC-1 are cleaved from the neutrophil’s surface to allow for migration away from the endothelium toward the source of inflammation (312, 313). It has also been suggested that CD31 is similarly cleaved from the neutrophil surface (314). As the neutrophil moves along the chemotactic gradient radiating from the source of inflammation there is increased mobilization of preformed receptors from neutrophilic granules to the surface (CD11b, CD66, and CD18). Therefore, despite being cleaved upon migration away from the endothelium, CD11b and CD18 will increase on a neutrophil further activated within the tissue. In contrast, CD11a and CD31 are not thought to be found in neutrophil granules (310) and therefore will remain at low levels even as the neutrophil becomes further activated within the tissue.

These predicted changes in adhesion marker expression were examined on neutrophils that had undergone transmigration in GBM patients by analyzing neutrophils
within tumor tissue. Direct ex vivo analysis of intratumoral neutrophils confirmed the expected phenotype (Figure 3.1 B,C). Levels of CD66, CD11b and CD18 were increased on intratumoral neutrophils, suggesting a fully activated state. Levels of CD62L and CD11a were correspondingly decreased, as would be expected on neutrophils that had undergone endothelial transmigration. Levels of CD31, however, were similar to that of resting neutrophils.

Together, these data show that GBM patients have a population of phenotypically normal circulating neutrophils, as well as a population of neutrophils which have successfully migrated into the tumor tissue and have a fully activated/transmigrated phenotype.

**Resting Neutrophils from GBM Patients Parallel Normal Donor Neutrophils in Their Functional Capacity for in vitro Activation and Endothelial Adhesion**

To evaluate the functional capacity of resting neutrophils from GBM patients, cells were stimulated for activation by a known stimulus, fMLP, in parallel with resting neutrophils from normal donors. Stimulation with fMLP in solution will induce similar changes in adhesion marker expression as described above for neutrophils activated in tissue – shedding of CD62L and increased expression of CD66, CD11b, and CD18. However, without the presence of endothelium to attach to, there is no change in CD11a or CD31. Analysis of surface marker expression again confirmed predictable changes in activation-induced marker expression for normal donor neutrophils (Figure 3.2A,B). Notably, activation-induced changes in marker expression on GBM neutrophils mirrored those of normal donors in both detail and extent of change, suggesting that these cells are not handicapped in their ability to respond to an activating stimulus (Figure 3.2B).
Figure 3.2: GBM patient resting neutrophils do not functionally differ from normal donors.
Figure 3.2: GBM patient resting neutrophils do not functionally differ from normal donors. (A) Representative flow plot of resting neutrophil isolated from a GBM patient and stimulated with fMLP. Neutrophils were again gated around based on forward and side scatter (far left panel) followed by CD66 expression (middle left panels). Levels of adhesion marker expression were then determined based on mean fluorescence intensity (MFI) of the gated CD66+ population (black, right panels) compared to MFI of the isotype control (grey, right panels) found using the forward and side scatter gate. (B) Average adhesion marker expression on resting neutrophils (white) and neutrophils activated in vitro with fMLP (black) from normal donors (ND, n=19) and GBM patients (n= 17). (***p<0.001) (C) Adhesion was measured using fluorescently labeled neutrophils binding to a monolayer of HMECs over a series of washes. Relative fluorescence was calculated by the fluorescence reading compared to the baseline reading immediately after labeled neutrophils were added to the HMECs (wash zero).
GBM and normal donor neutrophils were tested for adhesion to an activated endothelial lining in vitro. Adhesion was measured by quantitating the persistence of adherence of fluorescently labeled neutrophils to a monolayer of human microvascular endothelial cells (HMECs). GBM patient resting neutrophils did not differ from normal donor resting neutrophils in adherence to endothelial cells (Figure 3.2C).

Collectively, these results indicate that GBM patients have a population of normal, resting neutrophils that do not phenotypically or functionally vary from those of normal donors. This finding also indicates that the presence of activated neutrophils in GBM circulation is not due to a global abnormality in the general neutrophil population.

**Circulating Activated Neutrophils in GBM Patients Exhibit an Altered Pattern of Adhesion Marker Expression Indicative of Disrupted Transmigration**

Previously, we documented the presence of a subpopulation of activated neutrophils within the peripheral circulation of GBM patients. This conclusion was driven by data confirming decreased cellular density secondary to degranulation (resulting in a shift to the PBMC fraction following Ficoll density centrifugation) and confirmation of in vitro and in vivo release of granular protein. To further explore the phenotypic characteristics of these cells, we evaluated the adhesion molecule expression of activated neutrophils in GBM patient circulation to determine whether their phenotype is consistent with that of activation. Flow cytometric analysis of neutrophils within GBM patient PBMCs revealed that circulating activated neutrophils do indeed have an activated phenotype in terms of CD66 and CD62L expression, with increased and decreased expression respectively compared to resting neutrophils (Figure 3.3A,B).

Interestingly, the level of CD11a, which remains unchanged with activation but decreases only after interaction with the endothelium and transmigration into the tissue,
Figure 3.3: Circulating activated neutrophils in GBM patients have an altered adhesion molecule expression and are capable of further activation.
Figure 3.3: Circulating activated neutrophils in GBM patients have an altered adhesion molecule expression and are capable of further activation. (A) Activated neutrophils within GBM patient PBMC were identified by forward and side scatter (far left panel) and further gated on based on CD66 expression (middle left panels). Levels of adhesion marker expression were then determined based on mean fluorescence intensity (MFI) of the gated CD66+ population (black, right panels) compared to MFI of the isotype control (grey, right panels) found using the forward and side scatter gate. (B) Average MFI of adhesion molecules on GBM patient circulating activated neutrophils compared to GBM resting and fMLP activated. (C) Circulating activated neutrophils within GBM patient PBMC fractions were further activated with fMLP and adhesion marker expression was measured as described in (A) (***p<0.001, **p<0.01, *p<0.05).
was lower on circulating activated neutrophils compared to resting neutrophils.
Consistent with this observation, levels of CD11b and CD18, which are also cleaved
upon adhesion and transmigration, were lower than would be expected on activated
neutrophils. In fact, the expression level of CD11b on circulating activated neutrophils
was lower than that of resting neutrophils (Figure 3.3A,B). PECAM-1 (CD31) has also
been shown to be down-regulated from the surface of neutrophils after transmigration,
however we did not observe a difference in the levels of CD31 on circulating activated
neutrophils in GBM patients compared to resting neutrophils (Figure 3.3A,B) – consistent
with our observations of intratumoral neutrophils. The low levels of CD11a, CD11b, and
CD18 suggest that activated neutrophils in circulation have interacted with the
endothelium, but the process of transmigration was disrupted, allowing for them to
remain in circulation rather than moving further into the tissue.

As previously described, because CD11b and CD18 are found within neutrophil
granules, it is possible for them to once more be upregulated on the neutrophil surface
following cleavage upon transmigration and further activation by cytokines within the
tissue. To determine if circulating activated neutrophils retain the ability to be further
activated (and increase CD11b and CD18 as they would upon successful transmigration
into tissue), circulating activated neutrophils within GBM patient PBMCs were stimulated
with fMLP and change in adhesion marker expression was assessed by flow cytometry.
Circulating activated neutrophils did indeed retain the ability to be further activated as
was demonstrated by a further increase in CD66 and decrease in CD62L, along with an
increase in CD11b and CD18, while CD11a and CD31 remain unchanged (Figure 3.3C).

Together, these data suggest that the presence of activated neutrophils in GBM
patient circulation is attributable to initial intratumoral intravascular activation and
endothelial binding that is followed by a disruption in the transmigration process,
allowing for their presence in peripheral circulation.
GBM soluble factors are capable of activating neutrophils but do not alter the adhesion molecule expression in the same manner as neutrophils activated in GBM patient circulation.

To explore the possibility that tumor related factors released from GBM are capable of inducing the phenotype observed on activated neutrophils in GBM patient circulation, we assayed the influence of tumor conditioned medium on changes in adhesion marker expression of normal donor neutrophils. Since we have previously noted neutrophil infiltrates are increased in necrotic areas of the tumor, we hypothesized that necrotic tumor tissue is likely to contain a higher concentration of factors influencing neutrophil activity. During tissue collection, the surgeon distinguished areas of healthy tumor and necrotic tumor tissue using image guided assistance and microscopic analysis. Tissue collected from epileptic patients undergoing hemispherectomy was considered a normal brain tissue control. Conditioned medium was generated by disbanding tissue into RPMI medium, centrifuging, and collecting the supernatant. Supernatant was then mixed with isolated normal donor neutrophils and change in adhesion marker expression was measured by flow cytometry to determine the status of neutrophil activation.

Normal donor neutrophils treated with all three conditioned media reflected an activated phenotype, as was observed by increased CD11b, CD66 and CD18 and decreased CD62L (Figure 3.4). In this series of experiments, CD11a also increased on activated neutrophils, whether activated by conditioned medium or fMLP. As seen previously, CD33 decreased with activation by fMLP and was similarly decreased by conditioned medium. Surprisingly, normal brain conditioned medium was capable of activating neutrophils and did not differ from tumor or necrotic tumor in its ability to activate as observed by similar changes in most of the evaluated adhesion markers.
Activation induced by normal brain may be influenced by inflammatory environments also existing within epileptic brain or differences in tissue collection methods for hemispherectomy versus tumor removal. Importantly, tumor and necrotic tumor did not differ in their ability to activate neutrophils.

Figure 3.4: GBM soluble factors are capable of activating neutrophils but not inducing the circulating activated neutrophil phenotype. Normal donor neutrophils were mixed with tumor, necrosis or normal brain conditioned medium and change in adhesion marker expression was measured by flow cytometry. Marker expression is reported as fold change of the mean fluorescence intensity as compared to the medium control.

These data demonstrated that GBM tissue contains soluble factors which are capable of activating neutrophils, but these factors alone are not responsible for the decreased in CD11b, CD18 and CD11a observed on circulating activated neutrophils in GBM patients. Instead, these findings further supports the hypothesis that an interaction
with the endothelium is required for the formation of circulating activated neutrophils with altered adhesion marker expression.

**Tumor Conditioned Medium Expresses High Levels of the Cytokines IL-8 and IL-6 but Neither are Necessary for Activation of Neutrophils by TCM**

To determine factors present in GBM that induce neutrophil activation, a cytokine array was performed on the conditioned medium. A custom cytokine array was designed to measure the 16 most commonly identified cytokines known to directly induce neutrophil activation (236, 237, 253, 315-328). Though they did not differ in their ability to activate neutrophils, differences in neutrophil activating cytokines present within normal brain and necrosis or tumor conditioned medium was observed. Necrosis and tumor conditioned medium contained significantly higher levels of IL-8, ENA-78, IL-1α, IL-1β, and IL-6 compared to normal brain conditioned medium, with IL-8 and IL-6 having the highest expression (Figure 3.5A).

To determine if the high level of IL-8 and IL-6 are necessary for tumor conditioned medium to activate neutrophils, both cytokines were depleted separately and in combination from tumor conditioned medium and the ability to activate normal donor neutrophils was re-assessed. Cyokine depletion was accomplished using biotinylated antibodies specific to the cytokine followed by magnetic bead separation with anti-biotin microbeads. IL-8 and IL-6 were depleted from tumor conditioned medium to the point where neither was detectable by an ELISA with a detection level of 10 pg/mL (Figure 3.5B). The ability of depleted medium to activated neutrophils was compared to untreated tumor conditioned medium or tumor conditioned medium diluted with PBS to account for volumetric changes from addition of the antibodies and microbeads. A goat IgG antibody was also used as a control.
Figure 3.5: Tumor and necrosis conditioned medium have high levels of IL-8 and IL-6 but neither are necessary for neutrophil activation.
Figure 3.5: Tumor and necrosis conditioned medium have high levels of IL-8 and IL-6 but neither are necessary for neutrophil activation. (A) Cytokine array results from conditioned medium, reported as relative expression to array positive control. (B) Concentration of IL-8 and IL-6 in conditioned medium as measured by ELISA. (C) Adhesion marker expression of normal donor neutrophils mixed with conditioned medium or cytokine depleted medium.
When depleted medium was mixed with normal donor neutrophils there was no change in the ability of the conditioned medium to activate neutrophils as measured by increased CD66, CD18 and decreased CD62L (Figure 3.5C). All variations of tumor conditioned medium activated normal donor neutrophils and there was no difference between depleted and control antibody treated medium. This finding indicated that neither IL-8 nor IL-6 is necessary for GBM conditioned medium to activated neutrophils. Instead, it suggests there are multiple factors present in GBM conditioned medium that are responsible for neutrophil activation.

**Presence of Activated Neutrophils in GBM Patient Circulation does not Correlate with Extent of Edema or Necrosis**

The presence of a number of inflammatory cytokines within GMB conditioned medium demonstrates the highly inflammatory environment that exists within GBM. It is likely that the release of such inflammatory cytokines by tumor tissue also influences the activation of neutrophils in circulation. There are several pathological features of GBM that can be associated with this inflammatory environment. As mentioned above, we have previously shown that neutrophils tend to be associated with areas of necrosis within GBM. Furthermore, inflammation and tissue damage leads to edema within the brain, and it is likely that the leakiness of the endothelium associated with edema could contribute to the presence of activated neutrophils in circulation. Since necrosis and edema can both be observed by MRI, we determined whether these pathological conditions associated with neutrophil activation in circulation.

We estimated the amount of necrosis or extent of edema for each tumor, based on the T1 weighted or T2 FLAIR MR images respectively, and correlated each with the prevalence of activated neutrophils within the same patient. Each tumor was classified as mild, moderate, or significant in terms of extent of necrosis or edema, as shown in
Figure 3.6A&B. Stratifying tumors based on extent of necrosis or edema showed no correlation with prevalence of activated neutrophils in peripheral circulation (Figure 3.6C&D).

**Figure 3.6**: Extent of edema and necrosis do not correlate with presence of activated neutrophils in GBM patient circulation. (A) The extent of GBM patient tumor necrosis was classified according to T1 weighted MR images. Necrosis was considered mild if most of the tumor contained enhancement, moderate if there were scattered areas of hypointensive necrotic areas and significant if most of the tumor was hypointensive with only an enhancing rim. (B) The extent of GBM patient tumor edema was classified according to T2 FLAIR MR images. Edema was considered mild if it was confined within the tumor, moderate if it extended slightly beyond the tumor or significant if there was extensive expansion. The percent of GBM patient PBMC made up of CD66+ neutrophils was measured by flow cytometry and stratified according to tumor extent of necrosis (C) or edema (D).
Steroid Treatment is Not Associated with Alteration of Neutrophil Adhesion Molecule Expression but Does Augment Transmigration Failure in GBM

We previously demonstrated that frequency of activated neutrophils in GBM patient circulation was significantly higher compared to other intracranial tumors. In this study we also found no correlation between steroid treatment and the presence of activated neutrophils in GBM patient circulation despite previous reports of steroids influencing myeloid suppressive cells in GBM patients. However, this cohort included only six primary GBM patients. Since then, we have expanded our data set to 48 GBM patients and 19 recurrent GBM patients. Therefore, we sought to reevaluate the influence of steroids on the presence of activated neutrophils in GBM patient circulation.

Interestingly, recurrent GBM patients also had an increased prevalence of activated neutrophils in their PBMC, though not to the same extent as primary GBM patients (Figure 3.7B). When primary and recurrent GBM patients were stratified according to dexamethasone treatment, there was no difference with steroid treatment in recurrent GBM patients. However, in primary GBM patients, there was an increased prevalence of activated neutrophils with steroid treatment. Consistent with the overall data, GBM patients on steroids had an increased prevalence of activated neutrophils compared to recurrent patients on steroids. Together, these data confirm that activated neutrophils in circulation correlate with tumor presence, but also suggest that steroids augment the otherwise tumor induced formation of activated neutrophils in the circulation of primary GBM patients.

Steroids also alter adhesion molecule levels on neutrophils, including reducing levels CD11b, CD18 and CD62L (329-333). To investigate whether steroid treatment could be influencing the altered levels of CD11b and CD18 we observed on GBM patient circulating activated neutrophils, we sought to explore the effect of steroid treatment on neutrophil adhesion marker expression. We stratified our GBM patient population
Figure 3.7: Steroids have no effect on adhesion marker expression, but augment the presence of circulating activated neutrophils in primary GBM patients. (A) GBM patient samples were stratified according to treatment with dexamethasone prior to sample collection. Adhesion molecule expression on resting and in vitro fMLP activated neutrophils was compared between the two groups. (B) The percent of GBM patient and recurrent GBM patient PBMC made up of CD66+ neutrophils was measured by flow cytometry and stratified according to dexamethasone treatment (C) (*p<0.05).
according to their steroid treatment and measured the level of baseline adhesion molecule expression on their resting neutrophils. We found that regardless of steroid treatment, none of the adhesion molecules measured previously varied in expression between the treated and untreated populations (Figure 3.7A). In addition, neutrophils from GBM patients treated with steroids activated normally in vitro as observed by the same change in marker expression as neutrophils from GBM patients that were not on steroids (Figure 3.7A). This finding suggested that while steroids appear to increase the frequency of circulating activated neutrophils, steroid treatment alone is not inducing the altered phenotype observed on these neutrophils.

**Discussion**

We previously described a mechanism by which activated neutrophils in circulation correlate with peripheral immunosuppression in GBM patients. The activation of neutrophils in circulation is in contrast to the conventional sequence of neutrophil migration into the tissue before degranulation. In our current study we analyzed the population of activated neutrophils found within GBM patient circulation in attempt to understand the basis for their existence in circulation. We found they have an activated phenotype, but low levels of two molecules (CD11b and CD18: MAC-1) which are necessary for transmigration but are also cleaved from the neutrophil surface following transmigration. We hypothesize that activated neutrophils within circulation are binding to the endothelium and cleaving MAC-1, but re-entering circulation rather than moving into the tissue. CD11a has also been shown to decrease after transmigration and was found at lower levels on circulating activated neutrophils, further supporting this hypothesis. Even so, this finding is still circumstantial evidence, and further investigation using an in vivo model is necessary to determine if this hypothesis is correct.
Of note, activated neutrophils in circulation have been described in other pathologies, such as traumatic injury (334-337), and myocardial infarction (338, 339), however these were defined by a global change in adhesion marker expression across the entire neutrophil population. To our knowledge, we are the first group to use Ficoll Histopaque separation to isolate the activated neutrophil population from the resting neutrophils. By doing so we analyzed the unique aspects of the activated neutrophils themselves rather than observing global changes in the neutrophil population as a whole, which might mask the extent of change in phenotype induced by the specific pathology.

To explore the inflammatory signals present in GBM that are capable of inducing neutrophil activation, we generated tumor conditioned medium and assayed its ability to activate normal donor neutrophils. Tumor conditioned medium activated neutrophils and did so to the same extent as necrosis and normal brain conditioned medium. “Normal” brain was collected from epileptic patients undergoing hemispherectomy and also activated neutrophils. This activation is likely due to previous observations that epileptic events, which each of these patients would have been undergoing in the weeks prior to their surgery, can cause inflammation within the brain (340). Notably, the activated phenotype induced by conditioned medium was similar to that of fMLP activation in vitro rather then the phenotype of activated neutrophils found in GBM patient circulation. This finding further suggests that the phenotype observed on neutrophils activated in circulation results from an interaction occurring within the tumor.

The most highly expressed of these cytokines were IL-8 and IL-6. This result is consistent with previous findings that IL-8 and IL-6 are present in GBM (123). Both IL-8 and IL-6 have demonstrated tumor promoting activating in GBM, influencing GBM proliferation, migration, invasion and angiogenesis, however their role in immune cell function in GBM has never been explored. Here we found that despite their high
expression, neither cytokine was necessary for GBM activation of neutrophils as depletion of each separately and in combination did not prevent conditioned medium from activating neutrophils. This finding was not that surprising considering normal brain conditioned medium, which did not express high levels of IL-8 and IL-6, but had similar levels of the other neutrophil activating cytokines analyzed on the array, was also able to activate neutrophils to the same extent. Further analysis of the effect of depleting the other cytokines found to be present in GBM conditioned medium would need to be explored to narrow down the cytokine(s) required for neutrophil activation. However, the ability of GBM to activate neutrophils is most likely multifactorial. It is not likely that targeting one or two factors will prevent GBM from activating neutrophils as we had originally predicted.

In addition to inflammatory factors influencing neutrophil activation in GBM patients, we explored several clinical factors that could be associated with neutrophil activation. In other pathologies where systemic activation of neutrophils has been observed, the extent of tissue damage also correlated with an increased presence in circulation, suggesting that a stronger activating signal could lead to increased systemic activation (341). As necrosis represents tissue damage in GBM, we attempted to correlate the extent necrosis with the presence of activated neutrophils. Similarly, the break down of the blood brain barrier leading to increased leakiness of the vasculature (which is represented by the presence of edema) has been described to prevent T cell migration into GBM (124), and could similarly influence the ability of neutrophils to migrate into the tumor. However, using MRI to quantify the extent of edema or necrosis, we found no correlation between the extent of edema or necrosis and the presence of activated neutrophils in circulation.

Dexamethasone is commonly used in brain tumor patients to reduce swelling of the brain from increased edema associated with the presence of tumor. In several in
vitro and in vivo systems, dexamethasone has been shown to cause a decrease in the expression of CD11b, CD18 or CD62L on circulating neutrophils (329-333). However, in our study, there was no difference in adhesion marker expression with dexamethasone treatment on GBM patient neutrophils analyzed directly ex vivo. While dexamethasone had no effect on adhesion marker expression, we did observe an increase in the presence of activated neutrophils in primary GBM patient circulation with dexamethasone treatment. This difference was not found in recurrent GBM patients, suggesting in primary GBM dexamethasone treatment augments the tumor-induced production of activated neutrophils in circulation.

In this chapter we described several important aspects of GBM patient neutrophil biology. As activated neutrophils are associated with immunosuppression in these patients, these observations are important to keep in mind when considering options to treat GBM immunosuppression.
CHAPTER IV

ARGINASE I RELEASED FROM ACTIVATED NEUTROPHILS INDUCES PERIPHERAL IMMUNOSUPPRESSION IN A MOUSE MODEL OF STROKE

Introduction

GBM is a highly necrotic tumor, with massive areas of necrosis visible by MRI. In fact, central necrosis with an enhancing rim is the classical distinguishing feature of GBM on an MRI. This massive amount of tissue damage is accompanied by break down of the blood brain barrier leading to increased edema and intracranial pressure. As a result of the amount of tissue damage observed in GBM, it is no surprise that neutrophils would be attracted to these tumors as they play an essential role in wound healing and are the first responders to tissue injury. However, this begs the question as to whether neutrophils are specifically recruited by tumor associated factors or if they are simply responding to inflammatory signals released during tissue damage. Likewise, which of these signals are responsible for the activation of neutrophils observed in the circulation of GBM patients?

Stroke represents an opportunity to delineate this difference. Stroke is induced by hemorrhage or vessel occlusion within the brain leading to severe hypoxia and glucose deprivation that induces rapid necrotic cell death in the infracted area (342, 343). Similar to GBM, this tissue damage is associated with blood brain barrier breakdown and edema (344). The local inflammatory response to this tissue damage has been well studied in animal models of stroke. Inflammation is induced by the release of inflammatory cytokines such as IL-1β, TNF-α, IL-6, IL-1Ra, IL-8, IP-10, and MCP-1 from microglia, perivascular macrophages, astrocytes, and endothelial cells surrounding the hemorrhage or occlusion (345-353). As a result, adhesion molecules
to recruit inflammatory cells, such as ICAM-1 and E-selectin, are upregulated on the apical surface of endothelial cells (354). This induces an influx of inflammatory cells to the infarcted area.

As described in other types of tissue injury, neutrophils are one of the first immune cells to migrate to the infarcted area. In a rat model of stroke, neutrophil infiltration is dominant for the first 3 days of stroke, followed by a massive infiltration of macrophages from days 2-7 post stroke (355). Neutrophils also rapidly enter the infarcted area in human stroke, within 6-24 hours, and remain at high levels for 6-9 days (356). Mononuclear cell infiltration in human stroke is also delayed, taking over at 7 days post stroke and persisting for many weeks (357). This mononuclear cell infiltration correlates with the completion of necrosis within the infarcted tissue. Unlike the rat model, the subtypes of mononuclear cells making up this infiltration in the human disease have not been distinguished.

The critical time period for tissue damage from stroke appears to be within 12 hours of onset (358). As neutrophils have been found to infiltrate the infarct area during this time period, several studies have examined the influence of neutrophil infiltration on the extent of tissue damage. Using a rat model, inhibition of neutrophil migration to the region of infarction was achieved by treatment with neutrophil inhibitory factor (rNIF). Rats treated with rNIF during reperfusion had a significant reduction in the number of neutrophils infiltrating the infarcted area, which correlated with a 48% reduction in the infarct size (359). Similarly, extended neutrophil infiltration correlates with a worse outcome in human stroke. Patients with severe neutrophil infiltration that persisted for 28 to 30 days had worse neurological outcome as measured by the Mathew Scale for neurological assessment and larger infarction volume as measured by CT scan (356).

Similar to GBM, stroke can induce changes in peripheral immune cells. Following stroke, there is a marked increase in peripheral leukocyte counts, with neutrophils
representing the cell type most responsible for this increase. Interestingly, increase in peripheral neutrophil count also correlates with the size of infarction, with an increase in infarction size correlating to increased peripheral neutrophil counts (360). On the other hand, peripheral lymphocyte counts are decreased following following stroke. Within 12 hours of stroke onset, there is a dramatic decrease in peripheral T cell count (361). In addition, T cell function is suppressed as \textit{ex vivo} stimulation results in decreased cytokine production and proliferation (362-365). Once again, the degree of suppression correlated with infarct size (366). Larger stroke volumes correlated with decreased T cell function in stroke patients (362, 363).

This data suggests that similar to GBM patients, stroke patients are peripherally immunosuppressed following their ischemic event. This immunosuppression appears to contribute to infections that are often lethal in stroke patients. Infection is a major source of secondary complication post-stroke and the main cause of morbidity and mortality in stroke survivors (367, 368). In fact, infection has been identified as an independent risk factor for poor outcome following ischemic stroke (369). Post-stroke infection occurs in 25-65% of patients (367, 370, 371), with the most common infections including urinary tract infections and pneumonia (372, 373). Similar to the changes in immune cells described above, infection correlates highly with the severity of stroke (367).

In addition to prolonging ICU stays and reducing overall outcome, infection may also contribute to exacerbation of stroke (369, 373). Therefore, identification and targeting of stroke-associated factors that induce suppression of immunity may be critical for improving outcomes. Specific mechanisms leading to stroke induced immunosuppression remain to be determined. Based on the similarities observed between peripheral immunosuppression in stroke and GBM, we hypothesized that neutrophil activation and subsequent release of Arg I may play a role in stroke-related suppression of cellular immunity. To test this hypothesis, we utilized the middle cerebral
artery occlusion (MCAO) model of murine stroke to explore phenotypic and functional changes in cellular immunity potentially associated with Arg I activity.

**Methods**

**Mouse Model of Stroke**

All experimental protocols were approved by the Institutional Animal Care and Use Committee and conformed to the National Institutes of Health guidelines for the care and use of animals in research. Following isoflourane anesthesia, male C57Bl/6 mice at 8-10 weeks of age were subjected to middle cerebral artery occlusion via the intraluminal suture method as described previously (374). Adequacy of MCAO was confirmed by laser Doppler flowmetry measured (>70% drop required for inclusion) over the ipsilateral parietal cortex in all mice. Groups of animals were sacrificed at 4 and 10 days following MCAO and tissues were harvested for analysis. Successful occlusion was confirmed by slicing each brain into five 2 mm-thick coronal sections and placing each slice in a 1.2% solution of 2,3,5-triphenyltetrazolium chloride (TTC, Sigma) for 30 min at 37°C to identify infarcted tissue. Sham surgeries were performed by exposing the external carotid artery without insertion of the filament. Tissues from sham mice were collected four days post-surgery. All experiments were performed at least in triplicate.

**Isolation of Splenic Leukocytes and Purification of Low-Density Cells**

Spleens collected from MCAO or sham mice were placed in unsupplemented RPMI (Invitrogen), mechanically disrupted into single cell suspensions, and filtered through a 70 μm strainer. Splenic cells were resuspended into phosphate buffered saline (PBS, Invitrogen) and poured over an equal volume of Ficoll Histopaque 1077 (Sigma). The low-density cell layer (which typically includes only mononuclear cells) was collected
after centrifugation at 1650 rpm for 30 min. The resulting splenic mononuclear cell (SMC) layer was subsequently used for cell marker analysis by flow cytometry and T cell functional assays.

**Flow Cytometry of Immune Cell Populations**

SMC were stained for myeloid lineage and T cell markers, along with the appropriate isotypes, to identify immune cell populations. Murine myeloid cell-specific antibodies included FITC-anti-Ly6C, PE-anti-Ly6G, and PerCP-Cy5.5-anti-CD11b from BD Biosciences and PE-Cy5-anti-MHCII and APC-anti-F4/80 from eBioscience. Myeloid cell populations were delineated using the following phenotypes: CD11b+Ly6GloLy6Chi for monocytes, CD11b+Ly6GhiLy6Clo for granulocytes, and MHCII+F4/80+ for macrophages. T cell markers included FITC-anti-CD8, PE-anti-CD3 and PE-Cy5-anti-CD4 from BD Biosciences. The fluorescently labeled antibodies were added to 2.5x10^5 cells in Stain Buffer (BD Biosciences) and incubated on ice at 4 °C for 30 min prior to washing and analysis on a FACS Calibur flow cytometer.

Intracellular staining for CD3ζ was performed by resuspending SMC into 100 μL of Stain Buffer, staining with FITC-anti-CD3 (BD Biosciences) for 20 min, transferring to 100 μL of Cytofix/Cytoperm buffer (BD Biosciences) for 25 min, and staining with PE-anti-CD3ζ (CD247) (eBioscience) in 100 μL of Stain Buffer for 20 min prior to flow cytometry.

Flow data were analyzed using the FlowJo software program (Treestar). Positivity for the various markers was determined based on comparison with isotype controls.
**T cell Functional Assays**

SMC were plated at a concentration of 1x10^5 cells per well in 96 well round-bottom plates with 200 μL of RPMI supplemented with 10% fetal bovine serum (FBS, Invitrogen) and 1% penicillin-streptomycin (Invitrogen). T cells were stimulated using 1x10^5 CD3/CD28 microbeads (Dynabeads, Invitrogen). Medium were collected at 48 and 72 hours and tested for IFN-γ concentration by ELISA (ThermoScientific). To evaluate mitogen-induced T cell proliferation, BrdU (BD Biosciences) was added to mitogen-stimulated SMC cultures at a concentration of 10 μM. Following 24 hours of stimulation in the presence of BrdU, SMC were harvested, washed, and stained with PE-anti-CD3 for 20 min, fixed and permeabilized for 25 min with Cytofix/Cytoperm buffer, treated with DNAase (Sigma) for 1 hour to expose incorporated BrdU, and stained with APC-anti-BrdU (BD Biosciences) for 20 min prior to analysis by flow cytometry. The T cell population was initially identified by positive expression of CD3 and the percent of BrdU+ cells within that gated population was recorded.

**Mouse Neutrophil Degranulation and Measurement of Argl Release**

To confirm the potential for murine neutrophils to shift to the low-density layer of a Ficoll density gradient following activation and degranulation, single-cell splenocyte preps were generated (as above) from unmanipulated animals. These preps were mixed with 1 μM N-formyl-methionyl-leucyl-phenylalanine (fMLP, Sigma) or left untreated for 20 min. Cells were then resuspended in PBS and poured over an equal volume of Ficoll Histopaque 1077. SMC were collected and analyzed by flow cytometry as above to detect an increased frequency of neutrophils within the low-density fraction following *in vitro* activation.

To explore the potential that Arginase I could be released from preformed neutrophilic granules in the murine system, neutrophils were isolated from bone marrow...
of unmanipulated animals as described by Siemsen et al (375). Briefly, marrow was collected from the fibia and tibia of three mice by flushing the bone with mouse PMN buffer (Hanks Balanced Salt Solution without calcium or magnesium (HBSS-) with 0.1% bovine serum albumin and 1% glucose). Bone marrow cell solutions were filtered through a 70 μm strainer, centrifuged and washed, and then resuspended into a 45% Percoll (GE healthcare) solution. A multilayer gradient was prepared with 81%, 62%, 55% and 50% Percoll and the bone marrow preps were layered over the gradient. Following centrifugation at 1650 rpm for 30 min, the band of cells between the 62% and 81% layers was collected. To further purify the indwelling neutrophil population, a Ly6G+ magnetic bead selection kit (Miltenyi Biotec) was used according to the manufacture’s protocol. The purified neutrophils (95% pure by flow cytometric analysis) were placed in 1mL of RPMI at a concentration of 5x10^5 cells/mL and stimulated with 1 μM fMLP or left untreated for 30 min at 37 °C. Cell suspensions were then centrifuged and supernatant was collected for measurement of Arginase I.

Arginase I was measured in medium from T cell functional cultures, splenic mononuclear cell fractions, or purified bone marrow neutrophil cultures by ELISA (USCN Life Science Inc)

**L-arginine Supplementation in vitro**

SMC preps from MCAO-treated animals were used for T cell functional analysis as described above. Culture medium was supplemented with 4 μM L-arginine. Cultures were incubated for 48 or 72 hours before cells were harvested for BrdU proliferation analysis and medium was collected for IFN-γ measurement as outlined above.
Statistical Analysis

All data are presented as mean ± SEM. Student’s t-test was used to compare Sham to MCAO day 4 or day 10 animals. P values less than 0.05 were considered statistically significant.

Results

Experimental Stroke Induces Splenic Atrophy and T cell Dysfunction

As has been previously reported (365, 376), we noted a significant decrease in the splenic mass of animals following MCAO (Figure 4.1A). Splenic atrophy appeared to be temporally associated with the onset of stroke, as we observed partial reconstitution of weight by day 10 (Figure 4.1A).

T cell function in animals following experimental stroke was quantitatively evaluated through in vitro mitogenic stimulation. In vitro stimulation of T cells harvested from mice following MCAO demonstrated significantly lower levels of proliferation (Figure 4.1B-D) and IFN-γ production (Figure 4.1E) than control T cells from sham-operated mice. This functional effect was sustained through day 10 following experimental stroke, in spite of the reconstitution of splenic mass. Taken together, these data confirm prior observations linking stroke with cellular immunosuppression.

Stroke Results in Accumulation of Activated Neutrophils in the Spleen

To test our hypothesis that experimental stroke is associated with an increase in activated peripheral neutrophils, we purified SMC as described above and analyzed the frequency of various myeloid lineage populations using flow cytometry. We observed a significant relative expansion of CD11b+ cells within atrophic spleens by 4 days following
Figure 4.1: MCAO is associated with splenic atrophy and T cell hypofunction

(A) Mean splenic weight for tissues collected from MCAO mice or sham surgery controls (sham n=12, MCAO day 4 n=25 and MCAO day 10 n=6, *p<0.01). (B,C) Representative flow plots of BrdU incorporation for SMC preparations from sham (B) or MCAO (C) mice stimulated with CD3/CD28 beads in vitro. T cells were first identified based on CD3 expression (left panels) and gated CD3 populations were analyzed for BrdU expression (right panels) with average BrdU incorporation reported in (D) (Sham n=9, MCAO day 4 n=7, MCAO day 10 n=5, *p<0.05). (E) Measurement of SMC IFN-γ production following in vitro stimulation with CD3/CD28 beads (Sham n=9, MCAO day 4 n=7, MCAO day 10 n=5, *p<0.05).

MCAO (Figure 4.2A&B, left panels). Critically, further investigation of the expression of myeloid lineage markers demonstrated that the CD11b+ population was predominantly Ly6GhiLy6Clo (Figure 4.2A&B, right panels), confirming a neutrophilic phenotype. The expansion of this population appeared to be temporally correlated with the induction of MCAO, as the frequency of intrasplenic neutrophils approached baseline levels by 10
days following stroke (Figure 4.2C). In contrast to the expansion of intrasplenic neutrophils, we observed no change in the frequency of other myeloid-lineage cells that have previously been associated with immunosuppression (Figure 4.2C).

**Figure 4.2: Spleens from MCAO mice harbor increased numbers of activated neutrophils.** (A-B) Representative flow plots of myeloid cell populations in SMC from sham (A) and MCAO (B) mice as identified by initial gating of CD11b+ expression (left panels) followed by analysis of staining for Ly6C or Ly6G expression (right panels). (C) Frequency of CD11b+Ly6ChiLy6Glo monocytes, CD11b+Ly6CloLy6Ghi neutrophils and MHCII+F4/80+ macrophages within total SMC (Sham n=14, MCAO day 4 n=16, MCAO day 10 n=6, *p<0.05). (D) Representative flow plots (left panels) and overall frequency (right panel) of neutrophils within splenic cell preparations at rest or following activation with fMLP (n=3, *p<0.05).
It is generally expected that Ficoll purification of leukocytes should separate high-density neutrophils from the lower density mononuclear cell populations. We, and others, have previously reported that human neutrophils can “shift” to the lower-density population following activation and degranulation. However, this functional change has not been previously demonstrated in the murine system. To explore this possibility, neutrophils within splenic single-cell suspensions from unmanipulated animals were activated \textit{in vitro} using fMLP. Following activation, preps were subject to Ficoll density separation in parallel with unstimulated controls and the resulting low-density fractions were evaluated with flow cytometry. As we have previously seen with human neutrophils, \textit{in vitro} activation of resting murine neutrophils resulted in a significant shift to the low-density SMC fraction following Ficoll purification (Figure 4.2D).

Together, these data suggest that the increased frequency of neutrophils in the SMC fraction from animals following MCAO is due to \textit{in vivo} activation, degranulation, and subsequent decrease in density.

**Activated Murine Neutrophils can Release ArgI; Increased Levels of ArgI are Associated with Experimental Stroke and Reversible T cell Hypofunction**

ArgI release from pre-formed granules following neutrophil activation has been widely described in humans (231, 235). However, to our knowledge a similar phenomenon has not been previously described in the murine system. To explore this possibility, murine neutrophils were isolated from bone marrow of unmanipulated mice (Figure 4.3B) and stimulated \textit{in vitro} with fMLP. ELISA analysis of medium from \textit{in vitro} activated mouse neutrophils revealed a stimulation-induced release of ArgI (Figure 4.3B, right panel), confirming that this enzyme exists within pre-formed neutrophilic granules in the mouse and can be released upon activation.
Figure 4.3: Release of ArgI from activated murine neutrophils and correlation with the frequency of cells within spleens following MCAO

(A) Arginase I levels in medium from SMC cultures harvested from animals following MCAO (Sham n=14, MCAO day 4 n=16, MCAO day 10 n=6, *p<0.05). (B) Representative data from neutrophil populations isolated from mouse bone marrow (right panel demonstrates relative purity) stimulated in vitro with fMLP and analyzed for ArgI release into the medium. Average ArgI release is shown in the left panel (n=4, *p<0.05). (C) Correlation between the relative percentage of Ly6G+ cells within SMC from sham and MCAO animals compared to ArgI levels in medium from matched SMC cultures.
To further support the hypothesis that activated neutrophils in post-MCAO animals release ArgI, we measured levels of the relevant protein in the medium of SMC cultures from study animals. Figure 4.3A illustrates our seminal finding that experimental stroke is associated with a significant increase in the level of ArgI release from splenic leukocytes at day 4 following MCAO. Critically, we noted a return to baseline levels of ArgI by day 10, providing a temporal relationship to the onset of stroke, the extent of splenic atrophy, and most notably the relative accumulation of activated neutrophils within post-stroke spleens. The association between ArgI and neutrophil activation following experimental stroke was further strengthened through quantitative correlation of ArgI protein levels with the frequency of Ly6G+ cells within SMC from animals at day 4 following MCAO. As predicted, we noted an extremely strong correlation between ArgI protein concentration and the frequency of activated neutrophils (Figure 4.3C).

To provide indirect mechanistic confirmation for the immunosuppressive effects of ArgI in stroke, we evaluated levels of CD3ζ expression on direct ex vivo T cells from animals following MCAO. As predicted, we observed a significant decrease in CD3ζ expression on splenic T cells following experimental stroke (Figure 4.4A). Final preclinical data regarding the potential benefit of targeting ArgI activity in stroke was generated by supplementing T cell functional cultures with exogenous L-arg, an approach that we have previously used with success in targeting neutrophil-mediated immunosuppression in GBM. As seen in our prior experiments, L-arg supplementation of T cell cultures from animals following MCAO resulted in rescue of proliferative response to mitogenic stimulation (Figure 4.4B).
Figure 4.4: T cell dysfunction correlates with L-arginine deprivation

(A) Flow cytometric evaluation of intracellular CD3ζ expression on splenic T cells within SMC (Sham n=6, MCAO day 4 n=11, MCAO day 10 n=6, *p<0.05). To restore T cell function in vitro, SMC cultures were supplemented with L-arginine throughout stimulation and restoration of response was confirmed through BrdU incorporation as a measurement of proliferation (B) (Sham n=9, MCAO day 4 n=4, MCAO day 4 + L-arg n=4, *p<0.05) and IFN-γ production (C) (Sham n=9, MCAO day 4 n=4, MCAO day 4 + L-arg n=4).

Discussion

Here we describe a novel pathway of stroke-associated immunosuppression, through which splenic atrophy and T cell hypofunction are associated with the accumulation of activated neutrophils and associated ArgI release. Critically, post-stroke T cell function can be restored in vitro through L-arg supplementation, providing confirmatory evidence for the functional relevance of this pathway and defining a novel
translational target for improving immune function in patients suffering from ischemic stroke. In addition to describing the neutrophil-ArgI axis as a novel mechanism of stroke-induced immunosuppression, to our knowledge we are the first to describe the capacity for murine neutrophils to release ArgI into the extracellular environment following activation and degranulation.

The mechanistic link between stroke and the accumulation of activated neutrophils within the spleens of affected animals remains to be determined. Whether this phenomenon is the result of direct causation, or is simply the effect of epiphenomenon, is the subject of future experimentation. As we have recently shown that the increased frequency of circulating activated neutrophils in GBM patients likely results from disrupted endothelial transmigration following intravascular activation, it is possible a similar event could be inducing the activation of systemic neutrophil in these stroke mice. Further experimentation in the MCAO model is needed to explore a similar pathology-specific effect on peripheral neutrophils.

There are several caveats that should to be taken into consideration when considering these findings. As has been consistently demonstrated in many systems, immunosuppression is generally a multifactorial process; ArgI-mediated immunosuppression likely represents only one arm of a more global effect. Therefore, isolated therapeutic intervention targeting this pathway may offer only partial benefit, mandating continued exploration of additional sources of immunosuppression in ischemic stroke. In addition, it remains unclear whether L-arg depletion confers additional effects outside of the immune system, and whether these effects may in fact provide some benefit in the setting of ischemic stroke. The potential for addition effects could be particularly important when considering clinical interventions, as increased levels of L-arg may have result in deleterious effects depending on the timing of administration.
CHAPTER V

DISCUSSION

Glioblastoma remains one of the deadliest of all tumor types. Despite decades of research to develop treatment options for these patients, only a moderate improvement in patient survival has been achieved. With encouraging new developments in immunotherapeutic strategies for other types of tumors, immunotherapy represents a promising option for the treatment of GBM given its pathological features and mechanisms of recurrence. Initial attempts at harnessing the immune response to target GBM have essentially failed. One theory for this disconnect is the presence of immunosuppression in GBM patients, not only within the tumor microenvironment but also within peripheral circulation. T cell dysfunction has long been described in GBM and is likely the reason for failure of clinical responses from immunotherapies attempted thus far. While mechanisms have been described that induce immunosuppression in the tumor microenvironment, few explain the tumor’s ability to expand beyond the tumor to induce peripheral immunosuppression.

One such mechanism that hasn’t been explored in GBM but could account for peripheral immunosuppression is the induction of suppression by cells of the myeloid lineage. Within this thesis we have described a novel mechanism of peripheral immunosuppression in GBM patients involving the activation of neutrophils within peripheral circulation. In addition, we have provided a treatment strategy to possibly overcome the immunosuppression induced by GBM. This study extended beyond these original results to findings to attempt to define the origination of this mechanism and even expanded into stroke, an ailment that initiates pathological features in the brain that are also found in GBM, though in the absence of tumor. This finding suggests an
overarching immunosuppressive ability of brain inflammation but needs to be confirmed in human patients. It also provides for a possible treatment option to overcome immunosuppression observed in stroke patients that leads to increased risk of infection.

**Neutrophils and Arginase I Mediated Immunosuppression in GBM**

To our knowledge, the current study represents the first documentation of increased numbers of degranulated neutrophils within the peripheral circulation of GBM patients. This phenomenon has been previously described in several other human cancers (215, 231, 306), perhaps first outlined by Rodriguez et al in their analysis of patients with renal cell cancer. We observed similar expression patterns of myeloid-lineage markers within the cell population of interest and confirmed the neutrophilic phenotype through histopathological analysis. As in the renal cell cancer cohort, we observed increased ArgI in plasma samples from GBM patients. We have further expanded upon the hypothetical effect of increased ArgI release *in vivo* by confirming a concomitant decrease in surface CD3ζ expression on T cells from GBM patients analyzed directly *ex vivo*.

Importantly, we provide the first evidence that neutrophil-mediated suppression of T cell function in cancer patients can be reversed *in vitro* using either selective pharmacological inhibition of ArgI or, more simply, through the addition of exogenous L-arginine. In addition, the identification of increased frequency of neutrophils and massively elevated ArgI levels within actively necrotic GBM specimens offers not only potential insight into the ontological source of degranulated neutrophils in these patients, but also a potential mechanism through which cellular immunity may be disrupted within the tumor microenvironment.

Recent studies have explored immunosuppressive qualities of monocytic populations within GBM patients. Gustafson et al. recently identified an expanded
population of MDSC within steroid-treated patients with GBM, phenotypically defined as CD14+HLA-DRlo/neg (220). We were unable to document a similar monocytic population within our cohort and could not identify a difference in monocytic expression patterns associated with steroid treatment. A potential explanation for this discrepancy could derive from differing methods for tissue handling; most notably, all analyses in our study were performed with fresh (1-4 hours post-resection) PBMC and frozen samples were never utilized. It has been our experience that monocytic expression patterns can change rapidly with freeze-thaw cycles, and neutrophils from patients or normal donors do not survive the freeze-thaw process.

Though it is likely that monocytic populations within GBM patients may contribute to the observed immunosuppressive effect, our studies have shown a strong correlation between the presence of degranulated neutrophils and T cell dysfunction. The ability of normal granulocytes to suppress T cell function has been previously described (302) and has also been linked to immunosuppression in non-small cell lung, pancreatic, colon and breast cancer (231, 306). Populations of granulocytic MDSC have also been described in renal cell carcinoma (218) and non-small cell lung cancer (214). Whether described as MDSC or normal neutrophils, the mechanism by which granulocytic cells induce immunosuppression is commonly linked to the release of ArgI into the extracellular environment. While monocytic MDSC-derived immunosuppression has also been linked to ArgI expression, human monocytes tend to deplete extracellular L-arginine via increased CAT2B expression and intracellular transport. Transported L-arginine is subsequently metabolized by intracellular ArgI (a biological characteristic demonstrated by alternatively activated (M2) macrophages) (225). The fact that ArgI is found at increased extracellular levels within the plasma of GBM patients suggests that the enzyme is released from the expressing cell, consistent with a granulocytic mechanism of ArgI-mediated immunoregulation.
In the current study, we have identified two possible mechanisms through which ArgI-mediated T cell dysfunction may be reversed in vitro. Perhaps most importantly, we have confirmed that the simple supplementation of extracellular L-arginine to T cell functional assays can reverse the immunosuppressive phenotype. Intriguingly, L-arginine supplementation has been previously utilized within non-cancer clinical settings. On an initially empiric basis, oral arginine supplementation was explored and found to demonstrate efficacy for improving immune function in patients suffering major trauma or undergoing extensive surgical procedures (377). It was subsequently confirmed that ArgI is transiently found at increased levels in these patients (378, 379), supporting the clinical utility for dietary L-arginine supplementation in the reversal of immunosuppression.

These clinical results encourage parallel translation to cancer patients. Oral L-arginine supplementation is clinically attractive due to low cost, ease of delivery, and negligible toxicity. Although augmentation of T cell function by targeting ArgI in vivo may not, in isolation, confer significant clinical benefit in regards to tumor clearance, we predict that reversal of ArgI-mediated suppression of cellular immunity may offer a critical therapeutic adjuvant for the development of effective immunotherapy in patients with GBM.

**Disruption of Neutrophil Transmigration in GBM**

As an extension to this study, we further analyzed populations of neutrophils within GBM patient blood and tissue to attempt to determine how neutrophils become activated in the circulation of these patients, since this is generally not an accepted phenomenon. We predicted that analysis of a subset of adhesion molecules on the surface of these neutrophils that are necessary for varying stages of transmigration would allow for a better understanding of the interactions that had occurred in vivo.
between the neutrophil and the endothelium. The pattern of adhesion markers expressed on activated neutrophils in GBM patient circulation suggests that these cells form as a result of a disruption in transmigration. However, this finding is only circumstantial evidence, and further exploration of the steps involved in their formation is needed to confirm this hypothesis.

Recently, in some experimental systems, neutrophils have been described to undergo reverse transmigration from within the tissue back into circulation (380-382). While reverse transmigration could be a possible explanation for the existence of activated neutrophils in GBM patient circulation, we hypothesize that reverse transmigrated neutrophils would have a phenotype similar to those found within the tissue. Since activated neutrophils found in GBM patient circulation differ from those found within the tumor, it is more likely these neutrophils are failing to migrate into the tissue in the first place. In addition, we found that circulating activated neutrophils from GBM patients are capable of further activation, suggesting failure to enter the tissue and fully degranulate in response to inflammatory signals there.

Dexamethasone has been implicated in inducing defects in neutrophil adhesion marker expression and migration. While we did not find a correlation between dexamethasone treatment and altered neutrophil adhesion molecule expression, dexamethasone treatment correlated with an increase in the presence of activated neutrophils within GBM patient circulation. This observation is of important significance since most GBM patients are treated with dexamethasone at some point during the course of their treatment. If dexamethasone is increasing the formation of neutrophil activation in circulation, which is associated with immunosuppression, its possible dexamethasone treatment is contributing to increased immunosuppression. This information would be vital for physicians to know when treating a patient with dexamethasone. In addition it would be an important detail to note during
immunotherapy development and clinical trials testing these immunotherapies when considering patient response to the treatment.

Interestingly, dexamethasone has been shown to have significant effects on the endothelium. Dexamethasone increases endothelial cell junction strength by increasing junctional proteins (383, 384), which decreases the permeability of the endothelium, thus preventing edema. As a result, this has been associated with a decrease in neutrophil transmigration with dexamethasone treatment, without an effect on adhesion to the endothelium (385). If this phenomenon is likewise occurring in GBM patients on dexamethasone treatment, the decreased permeability of neutrophils through the endothelium could in part explain their presence in circulation. Thus, the increased presence of activated neutrophils in circulation with dexamethasone treatment supports our hypothesis that neutrophils are adhering to the endothelium and getting activated without undergoing transmigration. Again, this result needs further exploration to confirm the role of dexamethasone on altering the endothelium and affecting neutrophil transmigration.

We attempted to identify the factor(s) responsible for neutrophil activation by GBM through the analysis of several cytokines known to activate neutrophils. This study identified several cytokines present in GBM conditioned medium, with IL-6 and IL-8 demonstrating the highest expression. However, despite depletion of one or both cytokines to levels undetectable by ELISA, there was no change in the ability of GBM conditioned medium to activate neutrophils. This result suggests there are multiple factors expressed by GBM that are capable of activating neutrophils. These factors could be the cytokines found to be expressed at lower concentrations working together to induce neutrophil activation, or it could be another factor all together that was not analyzed by the custom array. There are other molecules capable of activating neutrophils which are not cytokines, such as the bioactive lipids, leukotriene B4, and
platelet activating factor (284), that could not be detected by the array but still may be present and influencing neutrophil activation by GBM conditioned medium. From a therapeutic standpoint however, it appears that targeting a single cytokine will not be effective in preventing neutrophil activation. In contrast, to prevent neutrophil activation in circulation and therefore the resulting immunosuppression, it would be more advantageous to directly inhibit neutrophil activation.

**Neutrophils and Arginase I Mediated Immunosuppression in Stroke**

Given the similarities of GBM and stroke pathology, in that both induce tissue damage within the brain, it was particularly interesting that a similar mechanism of immunosuppression was observed in mice following ischemic stroke as recently observed in human GBM patients. We confirmed the presence of T cell dysfunction following stroke by decreased response of splenic T cells to mitogenic stimuli in vitro. This suppressed function correlated with an increased presence of activated neutrophils and increased levels of ArgI within stimulation culture medium. When L-arginine was supplemented to T cell functional cultures, T cell proliferation in response to mitogenic stimuli significantly improved.

While this mechanism needs to be confirmed in human stroke patients, the striking similarities between a mouse model of stroke and human GBM are suggestive that brain inflammation as a result of tissue damage is capable of inducing this mechanism of immunosuppression. It would be interesting to test this theory in other brain inflammatory states, such as traumatic brain injury. Considering that L-arginine has been used clinically for the improvement of immune function in patients with major trauma or undergoing major surgery, the implications of this mechanism could extent
beyond GBM and stroke. Our findings have demonstrated potential for the role of
neutrophils in immunosuppression in multiple disease states.

These findings may also have huge implications for the treatment of
immunosuppression in stroke patients. Systemic infection in stroke patients is an
independent risk factor for poor clinical outcome and may contribute to increased
mortality rates. In surviving patients, prolonged intensive care remains a significant
source of health care cost. Therefore, efforts to improve immune function in the days to
weeks following stroke may provide for significant gains in both time to discharge as well
as overall outcome.

Also of significance in this body of work was the demonstration that mouse
neutrophils are capable of releasing ArgI into the extracellular environment. In mice,
ArgI expression has been described in a range of myeloid cells, most notably MDSCs
(226). In humans, ArgI appears to be constitutively expressed in neutrophil granules
(from which it is liberated during inflammation) and can be induced in a range of
monocyte-lineage cells (263). In contrast, ArgI expression in murine MDSC has been
primarily described to be restricted to the intracellular compartment (386).

To our knowledge this is the first evidence that isolated neutrophils from mouse
bone marrow relase ArgI in a similar manner to human neutrophils. Previous reports
have suggested the complete absence of ArgI from mouse neutrophils, unless
stimulated, or presence of ArgI within the cytosol of granulocytic myeloid derived
suppressor cells. In parallel, mouse neutrophils shifted to the PMBC fraction once
activated, a phenomenon commonly described for human neutrophils. These similarities
provide evidence for the benefit of studying neutrophil related immunosuppression in
mouse models.

The mouse model of stroke also represents a prime opportunity to study the
pathophysiology behind neutrophil activation in circulation. The accumulation of
activated neutrophils within the spleen of stroked animals suggests a similar systemic activation as observed in GBM patients. While rodent models of GBM failed to recapitulate this phenomenon, it appears to be present in this stroke model. Therefore this model could be used to further understand the interaction of neutrophils and the inflammatory stimulus within the brain that leads to their activation but failure to migrate into the tissue.
CHAPTER VI

FUTURE DIRECTIONS

L-arginine Supplementation as a Treatment for GBM Patient Immunosuppression

As a direct result of our finding that GBM patient T cell function can be rescued in vitro, our group initiated a clinical trial to determine if oral L-arginine supplementation can similarly improve GBM patient T cell function. We chose L-arginine supplementation over the inhibitor nor-NOHA due to the ease of translation into clinical use. L-arginine has also been used effectively for the improvement of immune function in other pathologies, demonstrating its general tolerance by patients.

The trial was designed to provide evidence of improved immune function with L-arginine supplementation. Patients enrolled in the trial were given L-arginine or placebo in the form of powder and asked to consume a total of 24 mg of L-arginine per day for seven days prior to surgical resection. Blood was drawn prior to L-arginine dosing and again at the time of surgery. T cell function and CD3ζ expression was measured at both time points for comparison of baseline to post-treatment effects in addition to comparison of patients receiving placebo control. Unfortunately, due to low accrual rate this trial had to be prematurely closed and we were not able to determine if L-arginine supplementation effected GBM patient T cell function. However, we did find that the few patients who took L-arginine tolerated the dose without adverse effects.

We hope to re-open this trial, with slight adjustments to its design. We propose to eliminate the placebo arm from the trial and use baseline T cell functional data from each patient as an internal control. By treating each patient with L-arginine we will cut in half the number of patients required to determine it’s efficacy. In doing so, we will determine more quickly whether L-arginine has the potential for improving GBM patient
T cell function and provide the evidence needed to initiate a larger clinical trial where the effect of improved T cell function on other clinical factors could be analyzed.

While L-arginine has potential for an easy translation to the clinic, there are potential risks to its use that would need to be considered when using it as a treatment for GBM. It is unclear what effect the increased concentration of L-arginine might have on the tumor itself. As described in Chapter I, L-arginine is not only a substrate for ArgI, but is also metabolized by NOS2 to produce NO. NOS2 is expressed by brain tumor stem cells in GBM and its activity contributes to their tumorigenic functions (40). It is possible that increased L-arginine feeding into this pathway could be beneficial for these stem cells and lead to increased tumor growth. In addition, L-arginine has also been shown to directly contribute to tumor growth in other types of cancer (387). The influence of L-arginine supplementation on GBM tumor growth would therefore need to be explored. These effects were not considered an issue in our trial, due to the short duration of treatment and completion of treatment prior to surgical resection; however, these possible adverse events would need to be considered if L-arginine supplementation were to be used for extended treatment.

Additional effects of L-arginine excess could theoretically be seen. For example, increased production of NO from increasing L-arginine availability through L-arginine supplementation may result in increased reactive nitrogen species (RNS) production, which could have tissue-damaging effects (388). While tissue damage within the tumor may be beneficial, increasing NO production systemically could have damaging effects. Similarly, NO plays a role in a variety of pathways that have other systemic effects, including smooth muscle relaxation, regulation of blood pressure and inhibition of platelet aggregation (389), that could be affected if peripheral NO levels were increased with L-arginine treatment. However, the dose used in our study has previously been used safely, without such side effects, in patients following major surgery (377).
Following initial trials to demonstrate the safety and effectiveness of L-arginine supplementation on improving T cell function in GBM patients, this study will hopefully be extended further to examine the influence of L-arginine on endpoints such as progression free survival and overall survival. If L-arginine improves baseline cellular immunity in GBM patients and this reversal of immunosuppression is enough to activate the endogenous anti-tumor response, it is possible increased survival could be observed. However, as described in Chapter I, there are multiple other mechanisms of immunosuppression that have been observed in GBM. While most of these are primarily found within the tumor microenvironment, it is likely that improvement of peripheral T cell function might still be suppressed once entering the tumor. Therefore, targeting multiple aspects of immunosuppression might be warranted to rescue endogenous antitumor responses.

Even so, the endogenous antitumor response of the baseline functioning immune system may not be enough to fight off the tumor. To improve this, treatments targeting immunosuppression have been combined with tumor vaccines. Likewise, L-arginine may be an effective adjuvant to vaccines targeting GBM patient antigens. EGFRvIII, tumor lysate-DC or heat shock protein vaccines have shown little or no improvement in GBM patient survival in early clinical trials; however it is possible that combination of these vaccines with a therapy targeted at improving immunosuppression, such as L-arginine, could improve their efficacy.

Besides the use of L-arginine supplementation, Arginase I mediated immunosuppression could be targeted through other modalities. As demonstrated in our in vitro experiments, the Arginase I inhibiting drug nor-NOHA was also capable of rescuing GBM patient T cell function. However, this drug has not been tested in clinical trials and therefore the process of determining its safety and efficacy would be a far
more extensive process than L-arginine supplementation and would require approval by the FDA.

Similar to L-arginine supplementation, it is possible nor-NOHA treatment would have adverse effects. Just as with L-arginine supplementation, inhibiting Arg I activity would make more L-arginine available to feed into the nitric oxide pathway. Therefore, similar side effects as described above would be a concern. In addition, inhibiting Arg I would also inhibit the urea cycle. This cycle is important for nitrogen metabolism. Inhibition of this cycle could lead to the build nitrogenous waste, which can be toxic (377).

Besides directly targeting Arginase I, another option for the prevention of this immunosuppressive mechanism would be to target the cell that is inducing its prevalence within GBM patient circulation: the neutrophil. Therefore understanding the mechanism utilized by GBM to induce the activation of neutrophils within circulation might identify additional targetable aspects of this system to prevent peripheral immunosuppression in these patients. This notion was the basis of our continued analysis of activated neutrophils within GBM patient peripheral blood and possible factors released from GBM that could induce neutrophil activation.

**Targeting Neutrophil Activation in GBM Patients**

In Chapter III, we showed that GBM conditioned medium expressed several cytokines known to activate neutrophils. Depletion of the most highly expressed of these, IL-8 and IL-6, did not prevent conditioned medium from activating normal donor neutrophils. The expression of several other neutrophil activating cytokines within conditioned medium that was still capable of neutrophil activation demonstrated that this process is likely multifactorial. It is unlikely that targeting a single agent will prevent
neutrophil activation. Thus, to prevent the activation of neutrophils in GBM patient circulation and their immunosuppressive effects, the best approach would be to prevent their activation altogether. There are several inhibitors that would be capable of doing this, but the most logical one to use would be pentoxiphylline due to its current clinical use in other pathologies (390, 391).

One caveat to this approach is the presence of activated neutrophils within GBM tissue. Despite inducing activation of circulating neutrophils, there also exist neutrophils that have successful migrated into and are retained within the tumor tissue. In addition, we have demonstrated that neutrophils within the tumor reflect an activated phenotype. Therefore targeting neutrophil activation would similarly affect the activation of neutrophils within GBM tissue. Before this could be done, the influence of these neutrophils on tumorigenicity would need to be determined.

Neutrophils have increasingly been studied in the context of tumor growth. In most cases neutrophils have been described to act in a protumoral manner, though antitumoral effects have also been described (233, 392, 393). Neutrophils within the tumor microenvironment release cytokines, proteases and reactive oxygen species that act to initiate angiogenesis, aid in invasion and metastasis, enhance tumor growth, and induce immunosuppression. On the other hand, these products can also induce cell death and recruit other immune cells that mediate tumor rejection. As neutrophils have been observed within GBM tissue and have an activated phenotype, the role neutrophils play in the pathology of GBM would need to be determined before a treatment preventing their activation could be pursued.

To determine the anti- vs protumoral effect of neutrophils within GBM, a rodent model of GBM would need to be utilized. While most rodent models of GBM do not accurately recapitulate every aspect of GBM pathology observed in humans, some models demonstrate clinical features better than others. For the purpose of this
experiment we would obviously want to use an immunocompetent model, since we have already identified one of the roles of neutrophils in GBM pathology to be immunosuppression. Since neutrophils have also been implicated in tumor angiogenesis and invasion, the model should also demonstrate the ability to carry out these aspects of GBM.

One model that has been explored by our lab is the rat model of GBM induced by intracranial injection of a retrovirus engineered to express PDGF into Fisher rats (394). This model demonstrates the invasive, necrotic and angiogenic aspects of human glioblastoma while also being present in an immunocompetent animal. Before the role of neutrophils in this model could be assessed, we would first have to confirm the presence of neutrophils within the tumor. If neutrophils are present, their effect on tumor growth could be determined by depleting neutrophils in these animals. This depletion could be done prior to virus injection, to determine the role of neutrophils in tumor initiation, or after tumor establishment to determine their role in tumor growth and invasion.

Neutrophils could be depleted in this model using a polyclonal antibody specific to the Rat neutrophil marker RP-3 (395). When used at the right dose, this antibody specifically depletes neutrophils for up to 48 hours without affecting other white blood cells. Tumors grown in neutrophil depleted animals could be analyzed for variations in tumor growth rate and overall survival as well as more specific factors influencing tumor promotions such as proliferative markers, tumor cell invasion into the surrounding parenchyma, and markers of angiogenesis.

Given their presence within GBM tissue (in an activated state), the ability of GBM to express several neutrophil activating cytokines and their established role in tumorigenicity in other tumor models, neutrophils are potentially playing a vital role in the progression of GBM. Somewhat surprisingly, these cells have been virtually ignored in
the study of GBM and GBM immunology. A better understanding of their function in GBM will no doubt lead to new targetable aspects for the treatment of GBM.

**Role of the Endothelium in Peripheral Neutrophil Activation**

Based on the adhesion marker expression of circulating activated neutrophils in GBM patients, we predict these cells are interacting with the endothelium but a disruption in the transmigration process occurs which allows for their presence in circulation rather than retention in the tumor tissue. A further analysis of the mechanism behind this phenomenon might identify targetable aspects of their formation and provide yet another option for the treatments targeting immunosuppression in GBM patients.

To continue this work, a parallel study of the adhesion molecules expressed by GBM endothelium would allow for a better understanding of the interaction between neutrophils and GBM vasculature. If GBM endothelium is found to lack certain adhesion molecules necessary for the transmigration of neutrophils into the tissue, this could help explain their existence in circulation. Adhesion molecule expression could be analyzed by immunohistochemistry (IHC) and compared to the expression in lower grade brain tumors where a peripheral activation of neutrophils is not observed. IHC would provide information about their expression patterns in addition to identifying their presence or absence.

Secondary to this study, the physical interaction of neutrophils with GBM endothelium could be analyzed using an *in vitro* transmigration system. This system would involve the isolation of endothelial cells from GBM patient tumor tissue and growth of these cells on trans-well inserts. This set up is commonly used with human endothelial cell lines for the study of neutrophil transmigration and has also been used to study the blood brain barrier *in vitro* (396). Using these inserts, once the cells have
grown to confluence, a neutrophil attracting chemokine is placed in the lower well, while isolated neutrophils are placed in the upper well. The amount of neutrophil transmigration over time can be quantified by collecting the medium from the lower chamber and using the myeloperoxidase (MPO) assay to quantify the number of neutrophils that have transmigrated (309). In addition, the number of neutrophils remaining in the upper well that have failed to transmigration can also be quantified. Endothelial cells from GBM patients could be compared to endothelial cells isolated from lower grade brain tumor patients or normal brain in their ability to allow or prevent neutrophil transmigration.

A caveat of this experiment is that GBM endothelial cells may change their marker expression upon removal from the tumor microenvironment and placement into culture. In addition, culture for extended periods of time, as would be needed to grow up enough endothelial cells to use in these assays, would likely result in a change in endothelial properties. Another option to use in this system would be human endothelial cells lines cultured in tumor conditioned medium to attempt to recreate the inflammatory factors present in GBM that would influence endothelial adhesion marker expression and permeability.

An advantage of this system is that GBM endothelial cells could be manipulated to determine the effect on neutrophil transmigration or the disruption of transmigration. For example, we found an association between dexamethasone treatment and the presence of circulating activated neutrophils in GBM patients. We hypothesize that this effect may be result from the known mechanism of dexamethasone to strengthen endothelial cell junctions. We could directly demonstrate the ability of dexamethasone to inhibit neutrophil transmigration across GBM endothelium in this culture system.

Of course the ideal system to study the influence of the endothelium on disrupted neutrophil transmigration would be to use a rodent model. However, there is not
currently an animal model available which recapitulates the activation of neutrophils within peripheral circulation. We initially examined this phenomenon in the PDGF rat model of GBM, but did not find a change in the peripheral neutrophil populations even with the presence of large tumors.

**Influence of Tumor Presence on Neutrophil Activation in Circulation**

During the correlation of steroid treatment to presence of activated neutrophils in circulation it was also noted that recurrent GBM patients had far fewer circulating activated neutrophils compared to primary GBM patients. The reasons for this are no doubt numerous, however it raises the question as to whether there is a correlation between tumor presence and the activation of circulating neutrophils and in parallel, does this presence correlate with decreased immune function?

To study this relationship, during my time in Dr. Waziri’s lab, I followed seven GBM patients over the course of their treatment. Blood was initially drawn and analyzed at the time of surgical resection and serial blood draws were collected at follow-up appointments typically occurring every 3 months. The abundance of activated neutrophils in circulation at each time point for each patient was documented as a percentage of the total PBMC fraction.

Initial results demonstrated a decrease in the presence of circulating activated neutrophils following tumor resection. On average patients had high percentages of neutrophils within their PBMC prior to surgery, but this percentage dropped to less than one percent as soon as 3 months following resection and continues to be suppressed on subsequent visits. Interestingly, two patients showed increases in the abundance of circulating activated neutrophils at 10-12 months post-resection that correlated with recurrence. These increases did not amount to the same level as observed with their
primary GBM, prior to resection, consistent with the overall trend observed in our steroid analysis.

While these finding are extremely intriguing, there are multiple factors besides tumor presence that could be influencing the presence of these cells within circulation. For instance, the treatment regimen the patient is undergoing could likely effect neutrophil activity. Chemotherapy can induce a dramatic decrease in the number of white blood cells. Every GBM patient will receive temozolamide, which can induce lymphopenia. The decrease in activated neutrophils within GBM patient circulation could simply be a result of a decrease in the neutrophil population as a whole. Likewise, GBM patients are often treated with dexamethasone, which has effects on the presence of activated neutrophils in circulation as described within this study. It would be important to identify whether or not the patient is receiving dexamethasone treatment.

To draw solid correlations between neutrophil activation in circulation and the presence of tumor, a much larger cohort of patients would need to be analyzed and other factors that could be influencing changes in the activated neutrophil population frequency would also need to be considered.

**Targeting Arginase I Induced Immunosuppression in Stroke**

We initially set out to explore immunosuppression in stroke with the perspective that it represented another source of brain tissue damage, only lacking the presence of tumor cells. Consequently we have identified an important mechanism essential to stroke biology. The potential for targeting immunosuppression in stroke patients could have profound impacts on post-stroke secondary care. While these results need to be verified in human patients before therapies targeting immunosuppression could be
pursued, the mouse model of stroke presents the opportunity to further investigate nature of the neutrophil/Arginase axis in association with stroke.

When considering the possibility of targeting Arginase I to improve cellular immunity in stroke patients, it would be beneficial to understand the temporal relationship between the onset of stroke, induction of neutrophil activation, suppression of T cell function and resolution of these events. Targeting ArgI too early in this cascade of events might augment tissue damage resulting from the ischemic event, while waiting too long would allow for the onset of immunosuppression that might be harder to overcome. Therefore, it would be beneficial to study the temporal nature of stroke-associated neutrophil activation and the associated suppression of cellular immunity to identify a therapeutic window.

Our initial investigations have demonstrated T cell suppression and increased neutrophil activation as early as 24 hours post stroke (data now shown). This effect was sustained through to 10 days post stroke. While T cells function was still suppressed in these animals, the activation of systemic neutrophils was resolved. Correspondingly, the level of Arg I returned to baseline levels. This finding suggests that early immunosuppressive events are sustained in the absence of immunosuppressive cells. Earlier time points would be required to determine the exact onset of immunosuppression in relation to stroke initiation. Also, later time points would be required to determine if immunosuppression eventually resolves on its own or if these animals remain immunosuppressed for extended periods of time.

Targeting ArgI to improve baseline immune function at each of these time points should be also assessed. This evaluation would allow for a correlation between the presence of suppressive neutrophils and the efficacy of targeting their immunosuppressive effects. It would also demonstrate if rescuing immune function by targeting ArgI is more efficient in the absence of this neutrophil population but continued
observance of T cell suppression. Targeting ArgI could be achieved through L-arginine supplementation or use of the ArgI inhibitor nor-NOHA. As L-arginine is most efficiently delivered to mice by oral solution and stroke mice tend to become too ill to consistently drink water, nor-NOHA would represent a more manageable option.

As with our studies in GBM patients, the effect that targeting Arg I has on stroke pathology would also need to be considered. As described above, L-arginine supplementation or inhibition of Arg I would increase the availability of L-arginine for metabolism by NOS to produce NO. In stroke, NO has tissue damaging effects that lead to increased infarct volume and cerebral edema, which are detrimental to final outcomes (397-399). Increased nNOS activity has also been shown to decrease neurogenesis following stroke (400, 401), a process important for recovery. Given the known activities of NO in stroke, the effect of targeting Arg I on stroke volume and overall outcomes would need to be carefully considered. It is possible that targeting both Arg I and NOS could be beneficial in the setting of stroke.

In addition to gaining further insight into endogenous effects on baseline immune function, we could explore the effects of the ArgI-suppressive axis in the setting of systemic infection. While the demonstration of ex vivo T cell suppression suggests stroke mice are immunocompromised, it does not directly demonstrate this concept. To further demonstrate that stroke mice are immunosuppressed, we could examine their susceptibility to secondary infection. By comparing the sensitivity of stroke mice to infection with S pneumonia to sham controls, we could determine if stroke mice are more likely to become infected with S pneumoniae. In parallel we could use this system to demonstrate in vivo rescue of T cell function by targeting ArgI.
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APPENDIX A

COLORADO CLINICAL AND TRANSLATIONAL SCIENCES INSTITUTE TL1 FELLOWSHIP CLINICAL EXPERIENCE

Since 2010, following completion of my first year as a PhD graduate student, I have had the opportunity to participate in the Colorado Clinical and Translational Sciences Institute TL1 program. During this time, I received one year of funding for my glioblastoma research project. This program is designed to facilitate interaction between PhD students and the clinical community while also giving them exposure to the clinical relevance of their research projects. Students in the program gain expertise in the area of translational research. This training includes seminars on the logistics of clinical trials, T-club meetings discussing the recent achievements in clinical translational research, and forums on a variety of topics including the process of grant submission and lessons on how to give scientific presentation.

As part of the program, I also attended the national meeting for TL1 fellows, once in combination with the Doris Duke research fellows at Washington University in St. Louis, MO and once at the Mayo Clinic in Rochester, MN. I presented a poster at each of these meetings. The first poster, presented in St. Louis, was entitled “Neutrophil suppression of glioblastoma patient T cell function is rescued by L-arginine” and discussed findings described in the first chapter of this thesis, which identified a novel mechanism of immunosuppression in GBM patients. The second poster, presented at the Mayo Clinic, was entitled “Activation of Circulating Neutrophils in Glioblastoma Patients” and described the initial findings in the second chapter of this thesis that
analyzed the etiology of circulating activated neutrophils in GBM patient peripheral blood.

My clinical experience for the TL1 program involved many different aspects of interactions with patient that have GBM and clinicians who are helping them fight it. For my clinical experience, I shadowed Dr. Waziri to tumor board discussions, observed surgical resections of tumors in the OR, interacted with patients in the clinic and even saw the planning that goes into the initiation of a clinical trial. The University of Colorado Hospital’s brain tumor board is comprised of neurosurgeons, oncologists, radiologist and pathologists who all meet once a week to discuss their challenging cases. I attended this tumor board many times throughout my participation in the program.

I was continually amazed at the collaboration that goes into the treatment of a single patient. It was a great experience to see the challenges I have read about in the GBM literature play out before me in the discussions of the tumor boards over the course of a few months. For example, at one meeting a patient was presented that had been diagnosed with GBM six months prior, had undergone chemotherapy and radiation and had stable disease since, but showed a slight enhancement on his recent MRI. They decided it could be radiation necrosis or psuedoprogression and decided to wait until the next month to get another MRI and see if the enhancement changed. A month later they found the enhancement did change and decided to put the patient on Avastin, the anti-VEGF antibody. However the enhancement got worse with Avastin treatment, which they hypothesized was from the increased invasion that is sometimes seen with anti-VEGFR treatment. The patient was taked off Avastin and unfortunately succumbed to his disease two months later.

This experience, of following the patient over the course of their treatment, thought it was only through the eyes of the treating physicians, brought the disease to life for me. I got to experience first hand what pseudoprogression looks like and the
effects of treatment with Avastin. These are things I had read about many times, but to actually see a patient experiencing them was an invaluable experience. As a scientist working in the lab it is easy to forget the impact such treatments can have on the patient themselves and the difficulties they will encounter throughout the course of treatment for their disease.

I also had the opportunity to attend several surgeries with Dr. Waziri. There were two in particular that stuck out in my mind. One was when I observed for the entire surgery, from prepping the patient, to lining up the intraoperative imaging system, to the cutting of the skin, to the sawing of the bone and the removal of the bone flap. I'll never forget the first time I saw the intact brain from under that bone flap. It was an amazing sight. In previous surgeries by the time I arrived to collect tissue from the patient the brain had already been cut into. This was also the first time that I saw an intact tumor. Dr. Waziri called me over to take a closer look and you could actually see the increased blood vessel density in the tumor tissue. The invasiveness of GBM was clearer than ever to me, as it was very hard to distinguish the borders of the tumor.

The other surgery that really stood out to me was one where the patient was awake and Dr. Waziri was performing brain mapping to distinguish areas of the tumor that were removable from those that were not. The patient was asked to read a card with a sentence on it while Dr. Waziri simultaneously stimulated part of the tumor. It was amazing to see the reaction of the patient when the simulation was in an area required for speech. The patient would be reading the card and then suddenly go into a stutter and would not be able to speak. This experience demonstrated to me the utility of this technique to prevent loss of important brain functions.

I also attended clinic with Dr. Waziri a few times and met some of the patients from which we collected samples. It was very interesting to watch Dr. Waziri perform different neurological tests on the patients to assess their level of health either prior to or
post resection of their tumor. It was very humbling to hear their stories and learn about the side effects they experienced from their treatment. Most of the patients would also have family members with them, sometimes a whole room full. It was great to see the support they offered each other as they made decisions on what course of treatment to take next. It really brought my research to life for me and showed me that it really is important and will eventually help patients just like the ones I met some day. As Dr. Waziri says, it’s always the nicest people that get brain tumors.

In addition to these beneficial clinical experiences with GBM patients and their physicians, I was involved with a clinical trial. As a result of our work demonstrating that GBM patient immunosuppression could be targeted by L-arginine supplementation in vitro, we initiated a clinical trial to assess the efficacy of oral L-arginine supplementation on GBM patient T cell function. I observed this process from the collection of the initial data, to the writing of the COMIRB protocol, to the initiation of patient accrual and collection of samples to be used in the trial. It was a great experience and will be very beneficial for me as I hope to initiate clinical trials in the future based on my research.

Overall my experience in the CCTSI TL1 program has been a beneficial one. I’ve gotten to experience the true clinical side of my research and understand the complications that come with treating humans, not just cells in a dish. I’ve made a lot of connections, not only with clinicians and scientist in my field, but also scientist outside my field that I probably would have never met without this program. It allowed me to experience the investigations going on in all disciplines of science and get a clearer understanding of the importance of clinical translational research. I have had valuable training on the ins and outs of clinical trials that will be imperative as I move forward as a translational scientist.