Enhancing Local Delivery of Macrophage Checkpoint Inhibitors with Chemokine Gradients to Lure and Destroy Pediatric Brain Tumor Cells

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Abstract

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Pediatric brain tumors (PBTs) are the leading cause of cancer-related death in children. These malignancies tend to occur in locations of the brain where complete resection and adjuvant therapy could lead to an impaired quality of life. Residual cells from incompletely resected tumors may invade nearby areas of the brain where they can cause recurrence and ultimately lead to patient death. This thesis explores whether hydrogel-based delivery chemokine gradients and macrophage checkpoint inhibitors (MCIs) into orthotopic xenograft PBTs can result in enhanced elimination of tumor cells. Tumor cells lured to an immunotherapy "trap" by chemokines would then become exposed to MCIs they would have otherwise avoided, and targeted for elimination by phagocytic cells in the brain. Our data confirms that gradients of CXCL12 are effective at eliciting chemotaxis of patient-derived, pediatric high grade glioma (pHGG) tumor cells *in vitro*. Use of CD47 mAb as a single agent was effective at promoting pHGG tumor cell phagocytosis in co-cultures with murine macrophages. Therefore, we engineered slow-release hydrogels to locally deliver CXCL12 and CD47mAb to mice bearing orthotopic xenograft pHGGs for timescales up to one month. Mice receiving intratumorally injected gels containing this combination demonstrated attenuated tumor growth compared to mice receiving gels containing CD47mAb alone. These pre-clinical results suggest a combination of chemokines and MCIs could be a safe and effective means to promote recruitment and immunological clearance of remnant PBT cells in patients.

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DEDICATION

This thesis is dedicated to my Mom and Dad, and my brother, Jack.

Mom, your battle with breast cancer turned a young kid's interest in science class into a career. I didn't want anyone else to suffer from debilitating chemotherapy and radiation treatments like you had to endure. I am lucky to still have you, and I hope I have lived up to the greatness you see in me. You put in a lot of hard work to keep Jack and me out of the streets and in the books. I hope my success is seen as a direct reflection of your commitment to your children. You deserve every bit of praise in these words.

Dad, though you lost your battle with cancer, I picked up your sword and shield and used them to help me finish this fight. Going through my 20s in grad school has been one hell of a marathon and I've wanted to stop many times over the years. Before you passed, you told me if I quit, then "all of that time would have been for nothing." You were right. So, I crossed the finish line with your words pushing me forward. As you know, until mom got sick with cancer, I wanted to be just like you and follow in your footsteps on the railroad. Now it's time for me to lay the tracks of my own destiny, so to speak, as I continue this journey of lifelong scientific discovery. I know you are proud of the man I've become, and I'll carry your last name with honor as Dr. Nealy.

Jack, as your little brother, you have always been someone I have looked up to. Whether it was playing sports or going to college, I knew I could succeed at these things because I watched you do them first. Life isn't so scary when you have someone laying the path for you, and I modeled myself after your example. I'll always appreciate you for that. Now that I'll be the first in the family with an advanced degree, I hope I can inspire *you* to push beyond your boundaries and succeed beyond your wildest dreams.

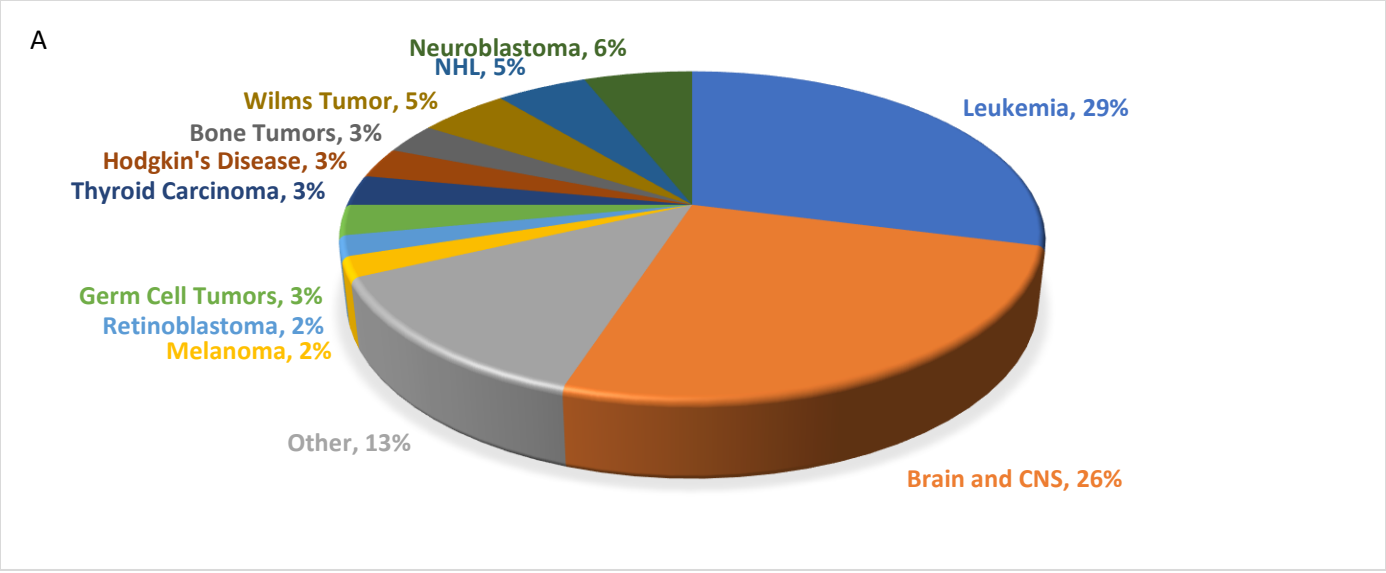
To my family: this Ph.D. is as much yours as it is mine. I love all of you dearly.

CHAPTER 1

Gaps in Our Knowledge Become Opportunities for Novel Pediatric Brain Tumor Treatments

Overview of Pediatric Brain Tumors

Pediatric brain tumors (PBTs) are a collection of primary CNS neoplasms that arise in patients between 0-18 years of age. PBT incidence varies by country, but from 2012-2016, PBTs occurred at a rate of over 6 cases per 100,000 persons per year in the United States [1]. PBTs represent the most commonly diagnosed solid tumors in children and the leading cause of childhood cancer-related deaths [2-5] **(Figure 1)**. A growing tumor pressing against the brain, as well as the damage incurred from removing the tumor may result in acute cognitive, neurological and behavioral defects. In the long term, surgery and radiation within a child's developing brain may result in lifelong neurological deficits and radiation-induced brain tumors [2]. Depending on the age of the patient, PBTs may preferentially arise in certain locations of the brain and manifest from different cell types. Supratentorial PBTs are more common in children 0-3 years old and again after age 10. Between these years, infratentorial tumors are more common [6, 7]. Younger children have a higher incidence of tumors of embryonal origin, whereas older patients tend to have tumors of glial origin (gliomas) [6]. Patient age and the location of these tumors with respect to vital nervous tissues affects clinical treatment options, patient morbidity, and survival. Over the course of this chapter, I will present a high-level overview of some of the most common PBT types, how they are classified and treated in the clinic, and the challenges scientists and clinicians must overcome to improve survival rates while reducing lifelong neurological deficits.



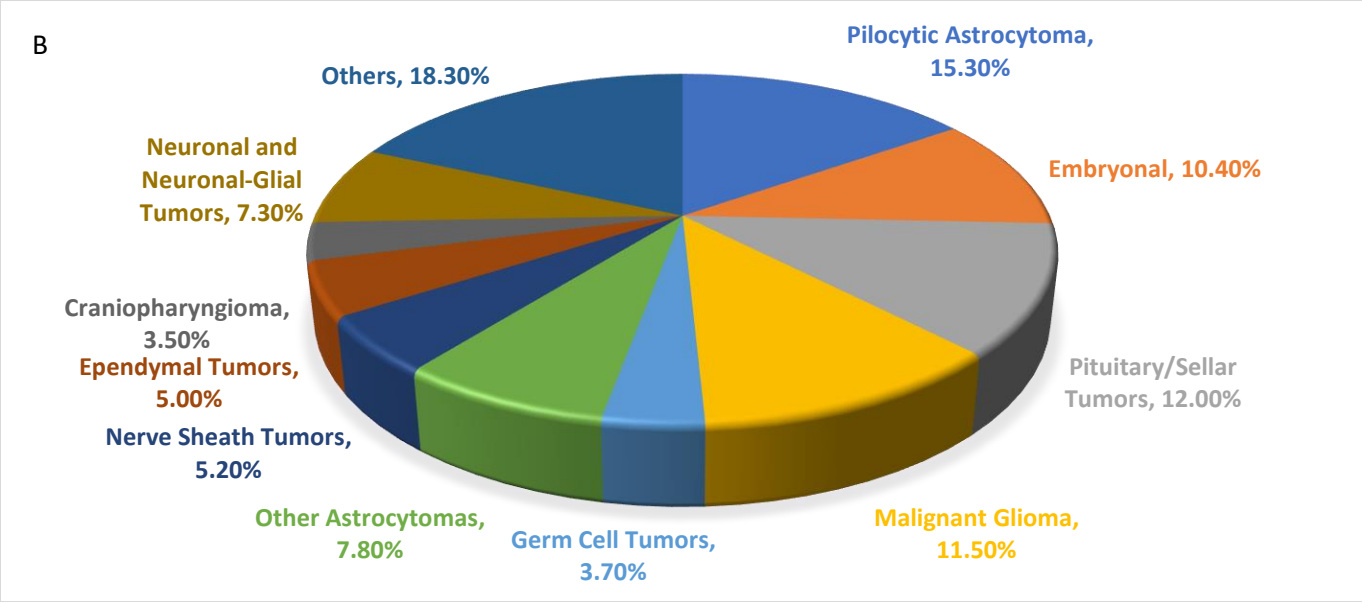


Figure 1: **Relative Proportions of Cancer Types in Pediatric Patients**. A: PBTs are the most common solid tumor in children and 2nd only to leukemia as the most common childhood cancer. B: PBTs are a heterogeneous collection of CNS tumors. Most PBTs are a form of LGG, though HGGs are common and usually fatal. Adapted from [3].

Embryonal Brain Tumors: Biology and Challenges in their Treatment

Embryonal tumors are a major subset of PBTs that, as their name suggests, are believed to arise from undifferentiated embryonic tissue in the brain [2, 4]. These tumors are more common in younger children whose brains are still undergoing rapid development and growth. Two common types of embryonal tumor types that I will cover in detail are: Medulloblastoma and Atypical teratoid/ rhabdoid tumors (ATRTs). Medulloblastoma is a tumor type that exclusively arises in the cerebellum. These tumors comprise up to 20% of all PBT diagnoses and are currently the most common malignant PBT [3, 6, 8, 9]. This tumor type is believed to arise from neural progenitor cells found within the cerebellum and often has the capacity to protrude into the 4th ventricle and invade surrounding nervous tissues [9-11]. While primarily diagnosed in children, some medulloblastomas uncommonly occur in young adults up to age 30. Medulloblastomas are classified into four major subgroups determined by histology and molecular markers: Group 1 (WNT), Group 2 (SHH), Group 3 and Group 4. These subgroups have different driver mutations, may occur in different patient demographics, and have differing survival outcomes [2, 9-11] **(Figure 2)**.

Group 1 tumors are the least common medulloblastoma subtypes, comprising about 10-15% of all cases, but it also has the most favorable prognosis [2, 9-11]. This subtype is characterized by over-activation of the WNT pathway and often contains mutations in *CTTNB1*, coding for Beta Catenin, and partial loss of chromosome 6. These tumors do not typically spread and patients with these tumors have an expected 5-year survival rate of around 95% [2, 9-11]. Group 2 tumors constitute about 30% of all medulloblastoma cases and are characterized by aberrant signaling within the SHH pathway, usually with mutations in *PTCH1, SUFU* and *GLI3*. Patients presenting with Group 2 tumors and *TP53* mutations have a 5- year overall survival rate of 40%, while *TP53* wildtype tumors have better 5-year survival at around 80% [2, 9-11]. Group 3 medulloblastoma comprises approximately 25% of all cases and is characterized by high levels of *MYC,* usually concomitant with *Notch* and *TGF-β* mutations. Group 3 tumors have the worst prognosis of all medulloblastoma subtypes –50% 5-year survival– and have the highest level of metastatic recurrence [2, 6, 9-12]. Group 4 medulloblastoma represents ~35% of all cases and has a strong 3:1 male predominance. These tumors exhibit amplifications in *MYCN* and *CDK6,* usually with accompanying mutations in chromosome 17q and loss of one copy of the X-chromosome in female patients. Group 4 tumors have similar prognoses as the SHH subgroup with 5-year survival at around 75-80% [2, 9-11]. Current treatment strategies across all medulloblastoma categories consists of a combination of surgical resection followed by radiation and/or chemotherapy to eliminate remnant tumor cells [2, 6, 9-12]. Resection of the bulk tumor is the initial treatment step for most brain tumor types. Total, or near-total resection of medulloblastomas is critical for promising outcomes, particularly in patients without metastatic disease [2, 6, 9-12]. However, around 20% of patients undergoing surgery may develop a collection of neurological deficits known as Posterior Fossa Syndrome (PFS) [8]. This syndrome is characterized as a reduction or absence of a patient's ability to speak [8]. PFS is often accompanied by other neurological deficits such as: ataxia, hypotonia, emotional lability, and other neurobehavioral abnormalities. Surgeons must sometimes choose between aggressive resections at the risk of PFS and more conservative, incomplete resections which may lead to recurrence at the tumor margins. Patients older than 3 may receive radiation therapy, followed by cisplatin-based chemotherapy to eliminate

remnant tumor cells. For patients younger than 3 years old, radiation therapy poses a challenge due to the long-lasting damage that can occur in the developing brain. For these patients, radiation is often forgone and postsurgical treatment is usually accompanied by chemotherapy alone [2, 6, 9-12]. Between 10-40% of patients with medulloblastoma have disseminated disease at diagnosis, further complicating treatment. Infants have a higher incidence of metastasis --up to 40% have metastatic disease-- while adolescents and young adults have the lowest. Five-year disease-free survival for patients with medulloblastoma is stratified by risk factors including: presence of metastases and the amount of residual disease remaining after surgery [2, 6, 9-12]. More than 80% in those with "average-risk" survive treatment, while 60-65% for those with "high-risk" survive after 5 years [2, 6, 9-12]. Although survival has been improving over the past 30 years for the treatment of medulloblastoma, successful treatment may result in significant neurocognitive deficits, often for life [2, 6, 9-12].

Another major type of embryonal tumor is ATRT. This is an uncommon, highly malignant brain tumor that most often occurs in infants and young children under 3 years old [6, 12-14]. ATRTs occur far less frequently than medulloblastomas, comprising only about 1-2% of all PBTs, but make up about 10% of

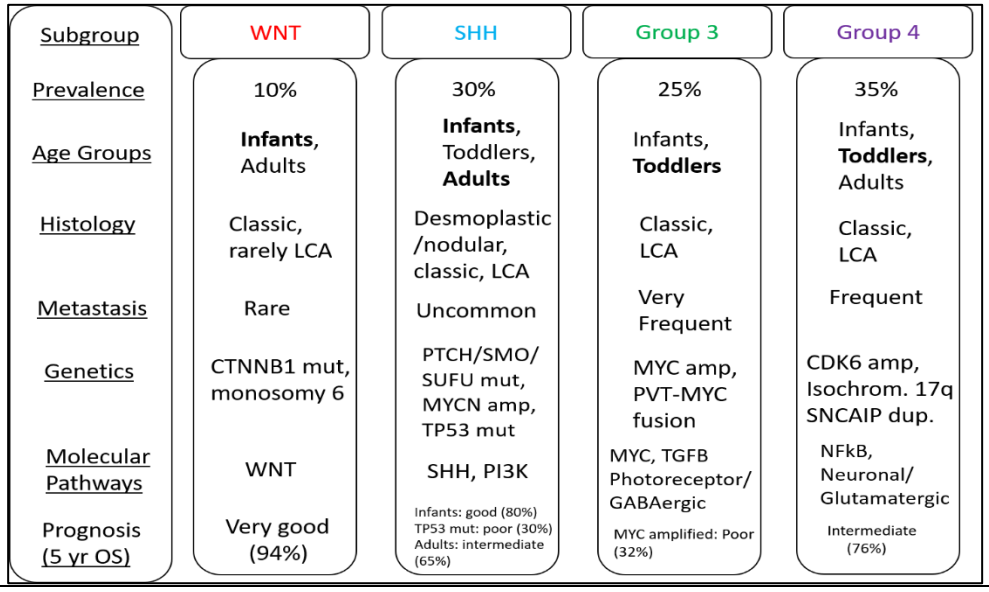


Figure 2: **Overview of Medulloblastoma Subtypes:** Medulloblastoma is the most common malignant PBT. It is divided into 4 subtypes based on histological and genetic markers. Wnt activated tumors are the least common, but have the best prognosis. Group 3 tumors are one of the more common types, but have the worst prognosis by far. Adapted from [13]

brain tumors in patients under 3 years old [6, 12-14]. ATRTs are composed of rhabdoid (rod-shaped) cells, and they tend to co-express neural, epithelial, and mesenchymal molecular markers based on histological staining. These findings suggest ATRTs may be derived from pluripotent embryonic cells within the brain [13]. Unlike medulloblastomas, which form exclusively in the cerebellum, ATRTs can occur in all brain regions. However, supratentorial ATRTs are the most common overall and spinal ATRTs are the rarest [6, 12-14]. The genetic hallmark shared across all ATRTs is commonly inactivation or bi-allelic loss of *SMARCB1* and, rarely, *SMARCA4* [6, 12-14]. These genes are key players in chromatin regulation and are known tumor suppressors. DNA methylation profiling has identified 3 distinct molecular subgroups of ATRT which contain mutations in various biochemical pathways in addition to the characteristic *SMARCB1/A4* mutations: ATRT-TYR, ATRT-SHH, and ATRT-MYC [6, 12-14].

Like Medulloblastoma, the ATRT subgroups also have a predilection for occurring in specific locations, and differing ages of onset **(Figure 3)**. ATRT-TYR tumors are named after the increased expression of tyrosinase, a protein involved in the development of the neural tube. These tumors are predominantly infratentorial and occur in the youngest patients at a median age of 12 months [14]. ATRT-SHH tumors are characterized by overexpression of genes that are components of the SHH and Notch pathways. Unlike the SHH driven medulloblastoma subtype, there do not appear to be any mutations within SHH pathway genes like *PTCH1* and *SMO* in ATRT-SHH tumors, suggesting over-expression of the pathway's gene products may be caused by the *SMARCB1/A4* mutation itself. These tumors are primarily supratentorial and the median age group is about 20 months old [14]. ATRT-MYC tumors are characterized by elevated levels of *MYC*, but unlike Myc driven Medulloblastoma, there are no amplifying mutations within the *MYC* gene itself in these tumors. Around 50% of all ATRT-MYC tumors are supratentorial, but they are unique from the other ATRT subgroups in that ~12% develop in the spinal cord. ATRT-MYC tumors occur in the oldest patient group, with the median age around 27 months [14].

Standard treatment for ATRTs consists of surgery, multi-agent chemotherapy and radiotherapy [12-15]. Given the young age of patients who often present with ATRT, use of radiation therapy may be excluded entirely due to the risk of long-term neurological deficits caused by radiation damage. Attractive chemotherapeutic targets for ATRT include inhibitors of EZH2, Aurora Kinase, Notch and the PDGFR pathway [13, 15]. Overall, the prognosis of ATRTs is worse than Medulloblastoma. However, survival rates have improved in recent years for patients receiving multiple avenues of treatment. The median lifespan ranges from 12-24 months and in recent years, 5-year disease free survival has increased from 10% to 50% in those with non-disseminated disease [13, 15].

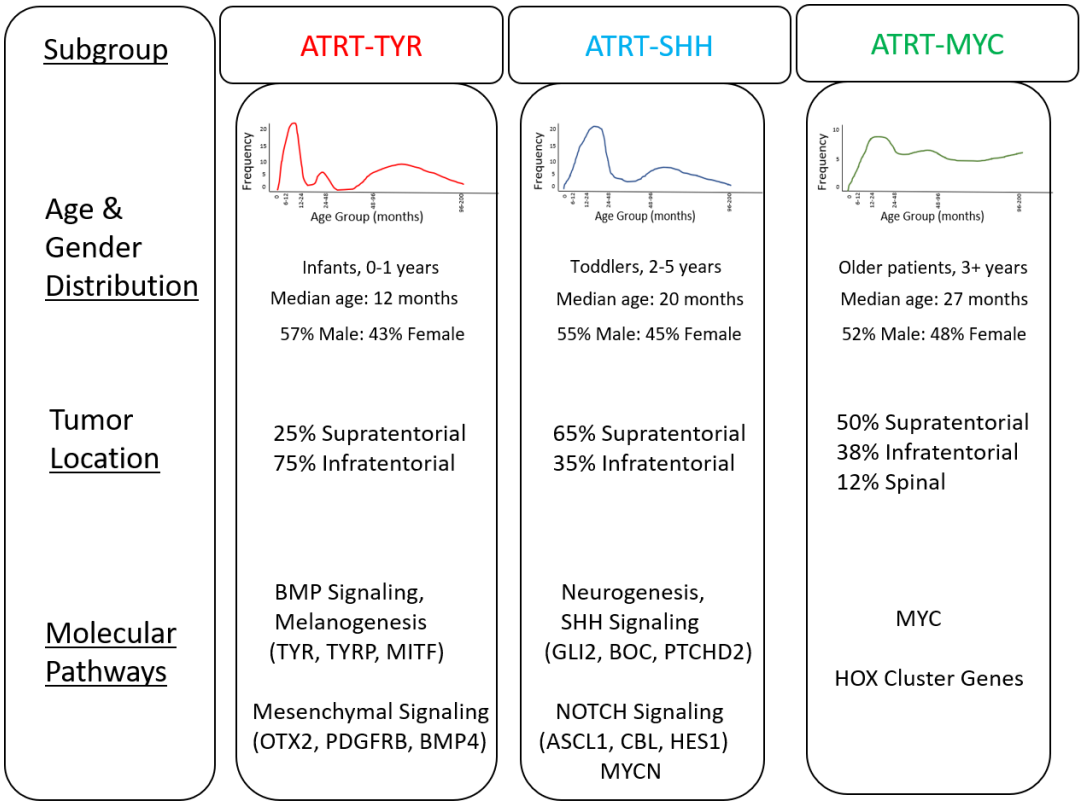


Figure 3: **Overview of ATRT subtypes:** ATRTs are rare PBTs, but make up over 10% of tumors in patients under 3. It is divided into 3 subtypes based on overactive signaling pathways. These subgroups have differing ages of onset, occur in different locations of the brain, and are correlated with differing survival outcomes. Adapted from [15].

Gliomas: Biology and Challenges in their Treatment

Gliomas are a category of brain tumors that originate from glia; cells physiologically responsible for neuronal support, initiating immune responses, and regulation of synaptic transmission.[16] Collectively, gliomas are the most common type of PBT [17, 18]. The most common types of glioma are: astrocytomas, ependymomas, and oligodendrogliomas. Astrocytomas are tumors derived from the astrocyte lineage and >75% of these tumors present as infiltrative, indicating advanced grade [2]. Ependymomas originate from the ependymal lineage, which lines the ventricular cavities and the central canal of the spinal cord. Ependymomas usually present as highly demarcated lesions, with <10% infiltrative at diagnosis [19]. Oligodendrogliomas are derived from the oligodendrocyte lineage. These tumors are more rare than either astrocytomas or ependymomas and are often well-demarcated [20]. Determining glioma lineage was historically determined based on their phenotypic similarities to normal glial cells. However, some gliomas present phenotypically different from their actual cell of origin, which would often lead to misdiagnoses and ineffective treatments. As of 2016, the WHO began classifying the lineage of these tumors based on the expression of molecular markers, which may reveal a cell type of origin different from their observed phenotype [21].Gliomas are further defined by the WHO on a I-IV grading scale based on molecular markers, cell morphology and mitotic activity [21]. On this grading scale, gliomas can be divided into: Low grade gliomas (LGG), Diffuse Midline Glioma (DMG), and high-grade (HGG) gliomas [12, 17, 22]. Treatment strategies and patient outcomes differ wildly across these categorizations -- pediatric LGGs are rarely life-threatening, but DMG and HGGs are often fatal. Collectively, LGGs are the most common category of PBT and represent approximately 30% of all PBT diagnoses [17, 22, 23]. Within the LGG umbrella, the most common diagnosis is pilocytic astrocytoma, though other common LGGs include: pilomyxoid astrocytoma, oligoastrocytoma, oligodendroglioma, and gangliоglioma [6, 17, 18, 23]. LGGs are most commonly found in the cerebellum, but brainstem and supratentorial LGGs can occur [6, 17, 18]. Although the LGG subtypes are regionally and genetically distinct from each other, these tumors tend to have mutations within the RAS/MAPK pathway [12, 22, 23] **(Figure 4).** Unlike adult LGGs, the pediatric variety rarely progresses to HGG and patient survival is

very high, with a 5-year disease-free survival above 90% [22, 23]. Therefore, treatment of these tumors mostly aims to reduce long-term patient deficits. Surgical resection is the recommended first-line therapy for pediatric LGGs and may be curative if total resection is achieved. Unfortunately, many pediatric LGGs arise in areas of the brain that are very sensitive to mechanical destruction, such as the brain stem and optic pathway, where only a biopsy or incomplete resection is possible [17, 23]. In areas of the brain where subtotal (or no) resection is possible, the chances of progression or relapse are increased. Many patients undergoing subtotal tumor resection may require adjuvant therapy. Radiation is an effective adjuvant therapy for the majority of pediatric LGGs, but often results in unintended adverse effects including cognitive deficits and radiation-induced secondary malignancies [17]. Therefore, radiation is currently recommended only when all other therapy options have been exhausted, especially in younger patients. Chemotherapy using carboplatin and vincristine has been shown to be an effective treatment option of some pediatric LGGs [3, 12, 17]. This line of therapy has an overall tumor reduction rate of 85% in patients with neurofibromatosis type 1 (NF1) and 67% in non-NF1 amplified tumors [3, 12, 17]. Targeted inhibitors against MEK and BRAF have also shown clinical promise in some patients. [23]

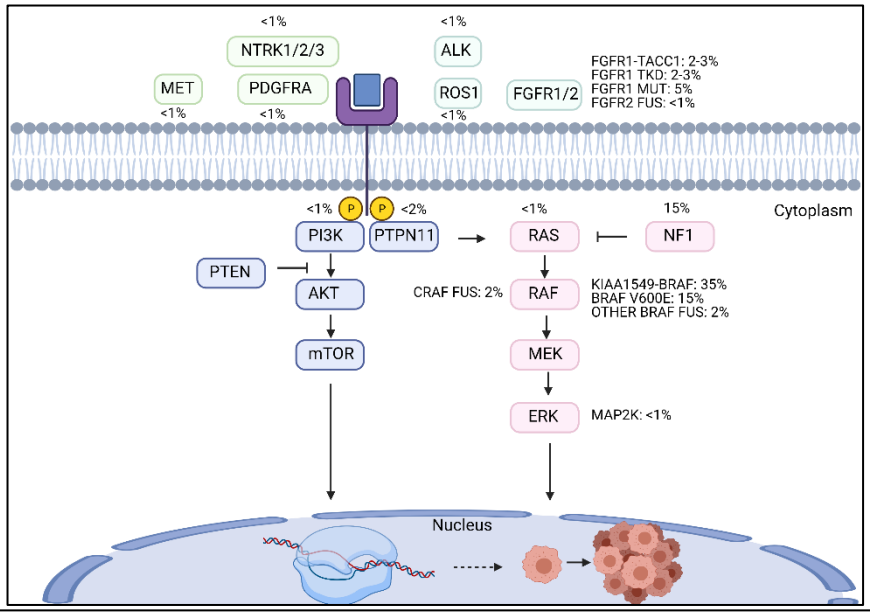


Figure 4: **Common Genetic Mutations Across Pediatric LGGs:** Collectively, LGGs are the most common PBT. These tumors can occur in all brain regions and tend to possess hallmark mutations in the RAS/MAPK pathway -- commonly NF1 and BRAF. Adapted from [23].

Pediatric high-grade gliomas (pHGG) and diffuse midline gliomas (DMGs), formerly known as diffuse intrinsic pontine glioma (DIPG), are two distinct gliomas that are sometimes grouped together due to their similar histologic appearance and very poor prognoses. pHGGs have a median survival of 12–24 months whereas DMGs have a median survival of less than 12 months [6, 12, 17, 22, 24]. pHGGs are routinely diagnosed as WHO grade III or IV based on mitosis, necrosis, and/or microvascular proliferation [17, 21, 24]. Respectively, these are classified as Anaplastic Astrocytoma and Glioblastoma (GB). pHGGs can manifest across all ages and in all regions of the brain and are among the most common malignant PBTs after Medulloblastoma. Most cortical pHGGs arise in older children and tend to have driver mutations in H3.3 G34, BRAF V600E or IDH1/2 [24]. Frequently accompanying these driver mutations are mutated chromosome remodeling genes, like ATRX [24] **(Figure 5)**. Loss of ATRX has been shown to be associated with a distinct mechanism of telomere elongation, promotion of tumor growth, and DNA instability[17, 22]. On the other hand, DMGs tend to arise in younger children and often possess a point mutation in H3.3 or H3.1 [6, 12, 17, 22, 24, 25]. This histone mutation inhibits the polycomb repressive complex 2 (PRC2) activity via sequestration of EZH2, resulting in globally decreased H3 K27 trimethylation (H3K27me3) [25]. Functionally, this mutation appears to lock these cells in a stem cell-like transcription phenotype, encouraging unrestricted growth and malignant transformation. Frequently accompanying these H3.3/3.1 mutations are regionally restricted mutations in genes like ATRX, found in Thalamic DMGs, and ACVR1 which is unique to Pontine DMGs. [24] **(Figure 5)**.

Standard of care treatment for HGG begins with maximally tolerable resection, if possible, depending upon the location of tumor onset [6, 17]. Surgical resection for DMGs and HGGs forming within the midline is not tolerated due to the tumor's proximity to vital nervous tissues and the infiltrative nature of the tumor itself [6, 17, 18]. Adjuvant radiotherapy typically follows surgical resection with exceptions made for infants and younger children due to concern for unacceptable neurological deficits. Chemotherapy after radiation has shown very little survival benefit in patients with HGG [6, 17, 18]. Temozolomide, an alkylating agent used in the treatment of adult GB, has not shown a clear benefit in

pediatric HGG [22]. With respect to patients with DMG, no chemotherapeutic regimen has added lifespan extension beyond radiation [6, 17, 18]. Due to the diffusely infiltrative nature of these tumors around vital nervous tissues, surgical resection is not possible. Despite advances in radio-sensitizing agents and multiagent chemotherapeutic regiments, before and after radiation, overall survival for DMG has remained dismal.

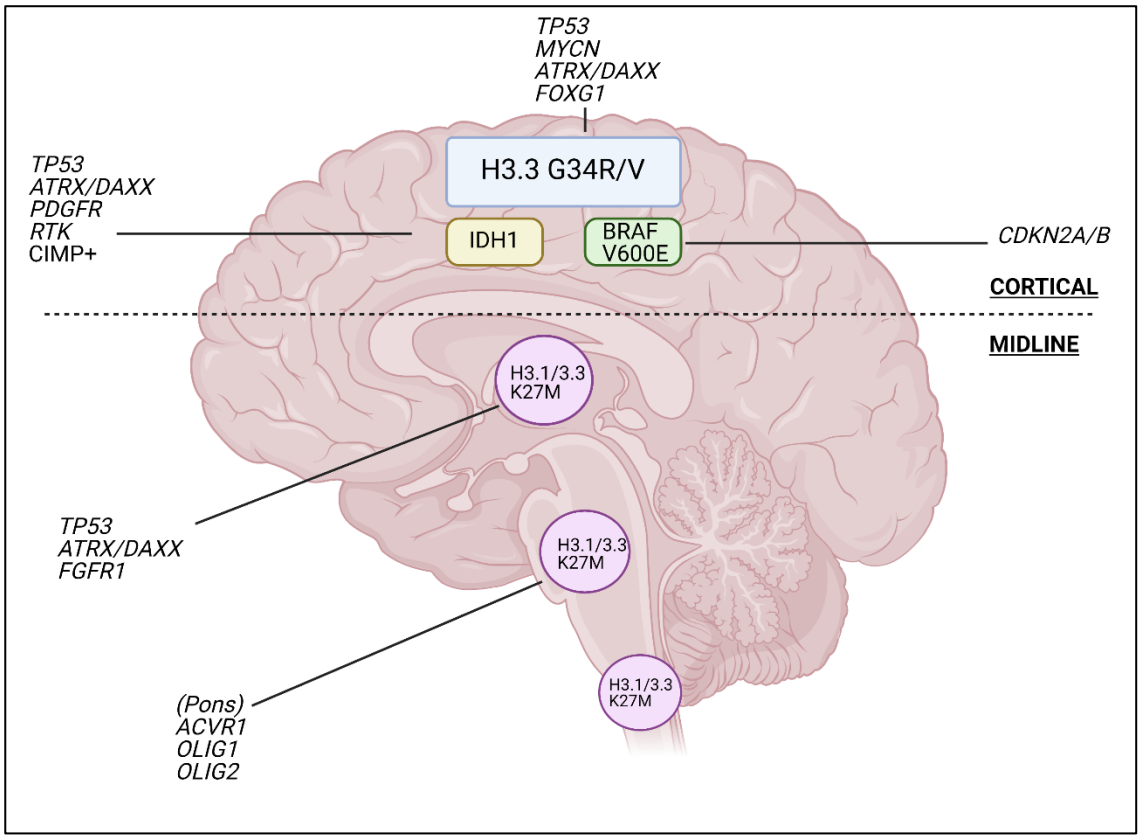


Figure 5: **Genetic and Regional Distribution of Pediatric HGGs/DMG:** Pediatric HGG and DMGs are typically poorly prognostic and highly metastatic. These glial-based tumors can occur in all regions of the brain and are genetically categorized based on driver mutations, like H3.3 G34V/R and H3.1/3.3 K27M, as either pHGG or DMG respectively. These tumors often present with accessory mutations, like ACVR1, that are specific to certain regions of the brain. Adapted from [24].

CHAPTER 2

Adapting Immunotherapy for the Treatment of Pediatric Brain Tumors

Shifting Paradigms in Oncology: Immunotherapy

As described in the previous chapter, standard of care treatment options across all PBT types includes a combination of: surgical resection to remove as much of the tumor mass (if possible), followed by radiation and/or chemotherapy to eliminate remnant, neoplastic cells. These treatment avenues are not perfect and are all associated with varying degrees of neurological harm to the patient. PBTs are often incompletely resected and adjuvant therapies like radiation may be forgone if the risk of intolerable neurological damage is too high (e.g., infants and toddlers). Some types of PBTs are inherently radiation and chemotherapy resistant to begin with, so any leftover tumor tissue presents a risk for relapse and metastasis. As a result, pediatric neuro-oncologists are now moving towards immunotherapy as a safer, more effective adjuvant treatment. One of the most distinguished hallmarks of cancer is its ability to evade the immune system and this is particularly true for PBTs [26]. Within the sensitive environment of the developing brain, therapies that target the immune system's role in cancer, rather than direct targeting of neoplastic cells, may be a safer avenue of treating PBTs when compared to chemotherapy and radiation [27]. Over the course of this chapter, I will present a high-level overview of the immune system's physiological roles, strategies cancer cells employ to manipulate the immune system, and immunotherapy approaches that may have the most success for PBT treatment.

Overview of the Mammalian Immune System

Our immune system is a heterogenous collection of organs, cell types, proteins, and soluble signaling molecules working in concert to maintain tissue homeostasis and provide host protection against infection and foreign bodies. Vertebrates evolved two separate, but mutually dependent systems that fulfill this broad role: innate immunity and adaptive immunity [28, 29]. Innate immunity is the evolutionarily older form of immune protection and it consists of rapid, nonspecific responses towards pathogens and non-self antigens [28, 29]. Major players in this system consist of physical barriers like the skin and mucous

membranes, in addition to hematopoietic-derived cells known as leukocytes including: natural killer cells (NK cells), granulocytes like eosinophils and phagocytic cells like macrophages [28, 29] **(Figure 6)**. These cells are considered "first responders" to the sites of infection and tissue injury and participate in a process called inflammation: increased vascular leakage and a substantial increase in white blood cell trafficking from blood vessels into the affected tissue [28, 29]. Innate immune cells detect the molecular signals released from pathogens and damaged cells (PAMPS & DAMPs) via cell-surface Toll-like receptors (TLRs). TLR agonism prompts these cell to secrete a variety of soluble molecules into their environment including: pro-inflammatory cytokines, cell recruitment molecules known as chemokines, and anti-microbial molecules like nitric oxide to nonspecifically destroy foreign bodies [28, 29]. Inflammation, complement activation, non-specific destruction of foreign material, and phagocytosis are among the primary effector mechanisms of the innate immune system [28, 29]. These processes are tightly regulated as systemic inflammation and accompanying non-specific destruction can result in potentially life threatening syndromes, including cytokine storm [28, 29].

Adaptive immunity is "younger" in evolutionary terms -- existing only in vertebrates -- and consists of cell types with the capability of specifically recognizing and forming memory of foreign antigens [28, 29]. This allows for a faster immune response and pathogen clearance from the body upon repeated exposure to a particular antigen. Cell types involved in this process are of hematopoietic origin, called lymphocytes: B- cells and T-cells [28, 29] **(Figure 6)**. In contrast to the rapid activation and recruitment of innate immune cells, adaptive immunity can take up to a few days before it is fully engaged. The characteristic slow response time of adaptive immunity is due to the prerequisite differentiation and maturation of naïve B-cells and T-cells once antigens dock with their respective B-cell receptors (BCRs) and T-cell receptors (TCRs) [28, 29]. Individual lymphocytes can identify a unique antigen and, upon recognition, will mature and multiply into clones that will traffic towards the site of infection where they exhibit a variety of effector functions [28, 29]. Many of these are shared with the innate immune system, including inflammation and complement activation. Unique to B-cells is the shedding of BCRs as soluble

immunoglobulins (IgGs). These large glycoproteins, also known as antibodies, can deactivate a target antigen by preventing interactions with its substrate, or via opsonization of the target. Opsonization is the coating of a foreign object with antibodies, whose exposed Fc domains can then trigger phagocytosis when docked with an FcR on phagocytic cells [28-30]. T-cells are divided into two categories with differing effector functions: CD4+ and CD8+. Mature CD4+ T-cells serve regulatory roles ranging from: the secretion of opposing pro/anti inflammatory cytokines, to disabling other T-cells via cell surface ligands like CTLA and PDL-1 [28, 29]. Mature CD8+ T-cells dock with a target cell and form an immunological synapse, culminating with the release of perforin and granzymes directly into their target to induce cell death [28, 29]. Many CD8+ T-cells die off once an infection is cleared, but a fraction of these cells further differentiates into long-lived memory T-cells. These cells remain alert to a particular antigen and remain ready to rapidly activate and proliferate if reinfection occurs in the future [28, 29].

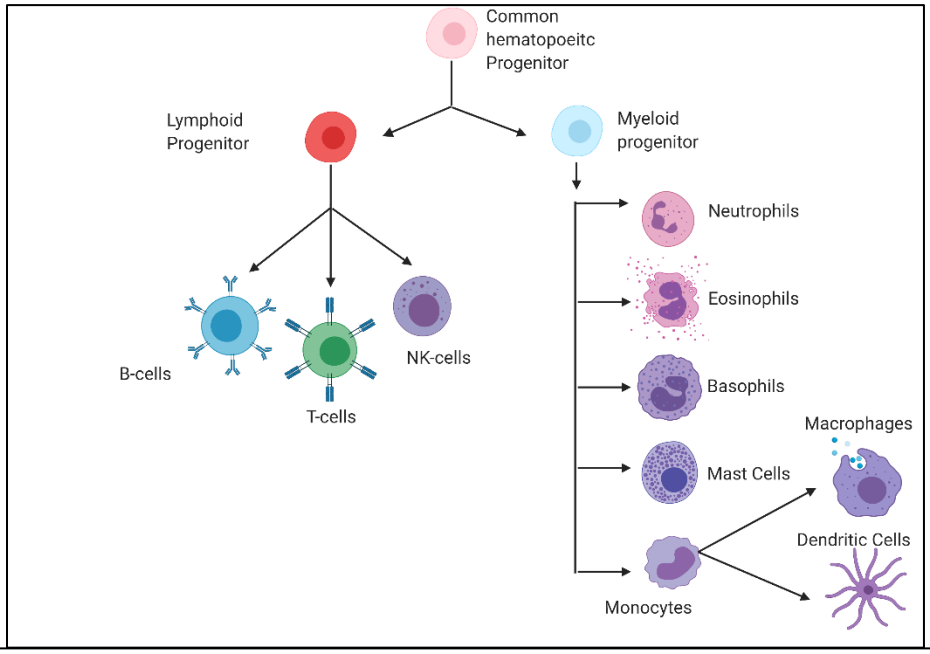


Figure 6: **The Hematopoietic Family of Cells:** All white blood cells are derived from a common hematopoietic progenitor stem cell. This cell can pursue two major pathways of cell fate: myeloid and lymphoid. Myeloid cells make up the bulk of the innate immune cells and employ non-specific effector functions. Lymphoid cells comprise the adaptive immune cells and employ antigen-specific effector functions.

The Immune System and Cancer: Friends *and* Foes

Historically, the immune system was viewed primarily for its aforementioned physiological roles as a homeostatic regulator and a pathogen defense mechanism. But over the last century, we've developed a clearer understanding of the immune system's paradoxical role in cancer prevention *and* cancer promotion. In the late 1800s, German physicians, Busch and Fehleisen, first made the connection between the immune system and cancer when they observed tumors spontaneously regressing in patients who had developed a bacterial skin infection [31]. Shortly after, Dr. William Coley reported in 1891 the spontaneous regression of sarcomas in some patients recovering from an *S.pyogenes* infection [32]. In the 1950s, Thomas and Burnett postulated that a healthy immune system had the ability to recognize and destroy cancer cells as they arose, a process called immunosurveillance [28, 29, 31, 33]. However, the immunosurveillance theory was met with skepticism as there were no models to definitively link the immune system as a major regulator of cancer protection at the time [28, 29, 31, 33]. Recent advances in mouse models with severe immunodeficiency, particularly NK, T and B cell development, provided the definitive link that had been missing for many years. Tumor transplant experiments in immunocompromised mice demonstrated rapid colonization and growth of tumors that were not observed in immunocompetent mice. Additionally, tumors that grew in immunodeficient mice were qualitatively different in their expression of antigen when compared to immunocompetent mice [28, 29, 31, 33] **(Figure 7)**. Today, cancer immunosurveillance is now broadly accepted and has been further refined as cancer immunoediting. This concept describes the immune system's role in regulating tumor quantity *and* their qualities (immunogenicity). [28, 29, 31, 33].

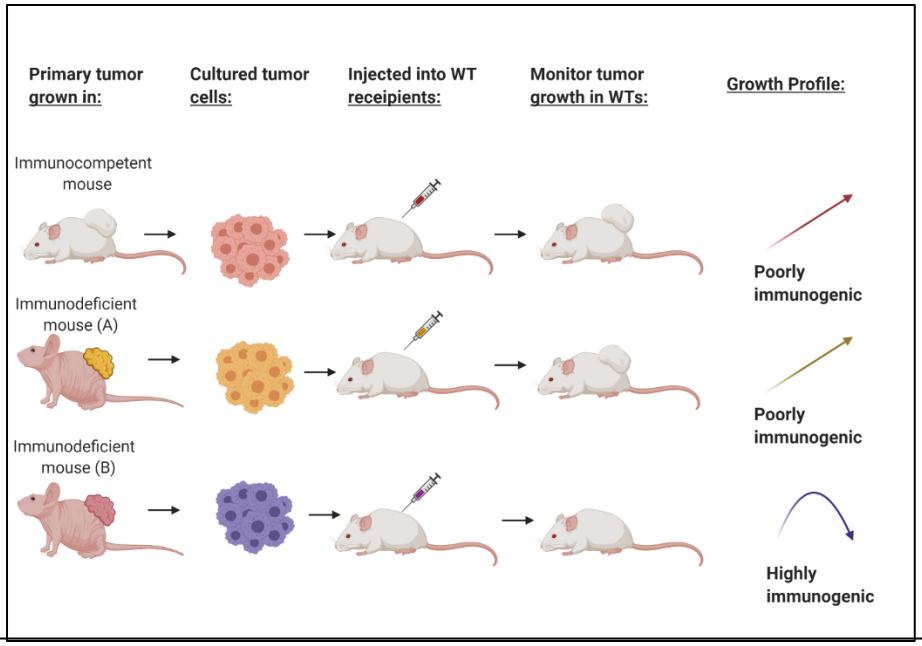


Figure 7: **Demonstrating the concept of Immunoediting:** A healthy immune system has the ability to quantitatively and qualitatively regulate tumors in the body. Tumor cell transplant experiments utilizing severely immunodeficient mice grew tumors that were recognized and destroyed by immunocompetent mice after transplant. These landmark experiments proved the connection between a healthy immune system and immunoediting. Adapted from [32].

Under certain circumstances, the immune system can also play a major role in promoting tumor growth. For some cancer patients, immune cell activity may not bode well for their outcomes. In many patients with solid tumors, high levels of tumor associated macrophages (TAMs) within and around the tumor is generally poorly prognostic for survival and treatment response [34-37]. Furthermore, the activation status of the immune cells within the tumor microenvironment plays a larger role than their presence [27, 28, 35-38]. Some tumors are known to recruit Tregs and myeloid-derived suppressor cells (MDSCs) via chemokine secretion to suppress the activation of cytotoxic tumor infiltrating lymphocytes (TILs) [27, 28, 33, 35-38]. Tumor cells themselves can also express cell-surface ligands commonly found on Tregs and MDSCs, such as PDL-1 and CTLA-4, to directly deactivate and kill TILs [27, 31, 37, 39, 40]. Tumors are known to secrete cytokines such as IL-10, TGF-β and other anti-inflammatory cytokines to coax infiltrating immune cells to secrete pro-growth and pro-angiogenic factors onto the tumor bed [33, 35, 40, 41]. Thus, immunotherapy as a cancer treatment seeks to maximize the cytotoxic effects of our immune system against cancer while suppressing or reversing its potential pro-tumorigenic aspects.

Successes and Failures of Immunotherapy for Cancer Patients

One of the first recorded examples of immunotherapy as a cancer treatment can be traced to Dr. William Coley, whose observations of spontaneously regressing tumors in patients infected with *S.pyogenes* led him to inject a cocktail of killed bacteria into inoperable tumors [42]. He observed a regression in size of some of the tumors receiving "Coley's toxin", mimicking his initial observations in patients [42]. Although the mechanism was unknown back then, these results were most likely the result of an inflammatory response by immune cells within the tumor that non-specifically destroyed the tumor cells. In the 1970s, researchers reported the regression of metastatic melanoma in a patient receiving injections of bacillus Calmette–Guerin (BCG) directly into their tumor [28]. Biopsies of the tumor revealed the presence of activated macrophages, neutrophils and dendritic cells, confirming an approach like this induces a non-specific response that destroyed the tumor [28]. Since these experiments, more contemporary approaches to immunotherapy have relied heavily upon prompting a tumor-specific immune response.

In the 1990s, the FDA approved monoclonal antibody (mAb) targeting CD20, Rituximab, demonstrated great efficacy in the elimination of CD20+ B-cell lymphomas [28]. By utilizing antibody-based therapies, the immune system can target antigens on the cell surface without requiring presentation in MHC molecules. This kicked off the market for mAb-based immunotherapy and since then, over a dozen mAb therapeutics have been FDA approved for targets including: HER2, VEGF and GD2 expressed in breast cancer, gastric cancer and neuroblastoma, respectively [28] **(Figure 8)**. Some mAbs are not targeted at cancer-specific antigens, but rather immune checkpoint molecules like PDL-1 and CTLA-4 displayed on the tumor, with the goal of reversing the suppression of TILs [27, 28, 38]. Antibody-based cancer treatment has since advanced towards bi-specific antibodies (BsAb) to further enhance the T-cell mediated cytotoxicity of this approach [43]. These multi-functional molecules are designed to bind to the target tumor cell, a T-cell and a phagocytic cell via its FcR, forgoing the need for MHC complex, co-stimulation, and education from an APC [43]. Advances in protein engineering techniques have enabled a

variety of scaffolds to construct BsAbs. One example involves stripping the antibody down to its antigen binding domains alone (scFV BsAbs), which alleviated much of the systemic toxicity observed with full-sized BsAb-based therapeutics. [43] The smaller scFV-BsAb molecules induce greater tumor cell cytotoxicity than full sized BsAbs and their smaller profile can more easily access antigens out of reach of large, IgG-like molecules [43]. However, these benefits are counter-balanced by poorer serum half-life, requiring constant infusion of scFV-BsAbs into the patient. Today, there is only one FDA approved bi-specific for cancer treatment, Blinatumomab, which targets CD3 on T-cells and CD19 on B-cell ALL [44].

Name	Target	Indication	Year
Rituximab	CD20	N.H. Lymphoma	1997
Trastuzumab	HER2	Breast Cancer	1998
Ibritumomab	CD20	N.H. Lymphoma	2002
Cetuximab	EGFR	Colorectal Cancer	2004
Bevacizumab	VEGF	Colorectal Cancer	2004
Panitumumab	EGFR	Colorectal Cancer	2006
Ofatumumab	CD20	CLL	2009
Ipilimumab	CTLA-4	Metastatic Melanoma	2011
Brentuximab	CD30	Hodgkins Lymphoma A.L.C. Lymphoma	2011
Pertuzumab	HER2	Breast Cancer	2012
Obinutuzumab	CD20	CLL	2013
Ramucirumab	VEGFR2	Gastric Cancer	2014
Pembrolizumab	PD1	Melanoma	2014
Blinatumomab	CD19 + CD3	ALL	2014
Nivolumab	PD1	Melanoma Non-S.C Lung Cancer	2014
Dinutuximab	GD2	Neuroblastoma	2015

Figure 8: **Landscape of mAb-Based Immunotherapy:** The use of mAbs to specifically induce destruction of human tumor cells began with Rituximab, which targets CD20 on lymphoma cells. Since then, over a dozen antibodies have been FDA approved for targets across multiple cancers, mostly hematological.

T-cell infusion as a therapy has also shown promise in the treatment of some cancers. Adoptive T-cell therapy (ACT) is an approach that involves removing tumor infiltrating T-cells from a patient's tumor, expanding and exposing them to activation factors *in vitro*, then re-implanting them into the patient [45, 46]. One 2018 study has shown ACT to be effective in the treatment of metastatic melanoma [47]. A more advanced variant of ACT involves engineering chimeric antigen receptor (CAR)-T cells [48]. Some

tumor cells down-regulate MHC expression, which would prevent engagement by a wildtype TCR. CAR-T cells possess a modified TCR that possesses the same internal signaling components, but the antigen binding domains of an antibody on its extracellular domain [48]. This allows it to bypass the need to identify peptides loaded in an MHC and can directly engage a cell-surface antigen. So far, CAR-T-cells targeting CD19 on B-cell lymphomas have been FDA approved [48]. Different generations of CAR T-cells specific for CD19, CD20, CD22 and other targets have since been developed that show increasing efficacy and longevity over previous iterations [48].

While the multitude of immunotherapy avenues have seen promising outcomes in clinical settings, there are also many occasions where these approaches have failed. Some cancer patients, particularly those with PBTs, have what is known as an immunologically "cold" tumor [41, 49]. This type of tumor is defined as the presence but poor infiltration of lymphocytes into the tumor bulk (immune excluded), or an absence of lymphocytes around the tumor (immune desert) [41, 49]. A cold tumor could have many causes, including low antigen presentation, poor trafficking of immune cells to the tumor and/or suppressed activation of the immune cells that are present [41, 49]. Because the effector cells are absent or inactive in cold tumors, they are difficult to treat with immunotherapy without additional stimulatory factors to turn the tumors "hot" [41, 49] **(Figure 9)**. Furthermore, tumors that are poorly immunogenic through downregulation of MHC and other cell surface antigens are not easily targetable by antibodies or T-cells [41, 49]. Assuming a targetable antigen is present, there is a risk of on-target, off-tumor toxicity. In 2010, one patient receiving CAR-T cells for HER2+ breast cancer died when the CAR T-cells instead targeted healthy lung epithelium expressing HER2 [50]. Some tumors are heterogenous in their expression of an antigen, like EGFRvIII on adult GB. CAR-T cells targeting EGFRvIII resulted in the selection of GB cells that don't express that EGFR variant, necessitating combinatorial therapy to increase the efficacy of this approach [49, 51]. Lastly, antibody and T cell therapies have notoriously poor

penetrance into solid tumors and are not as effective as those that target free-floating hematological cancers [49]. Many of these factors would play a role in the use of immunotherapy for PBTs.

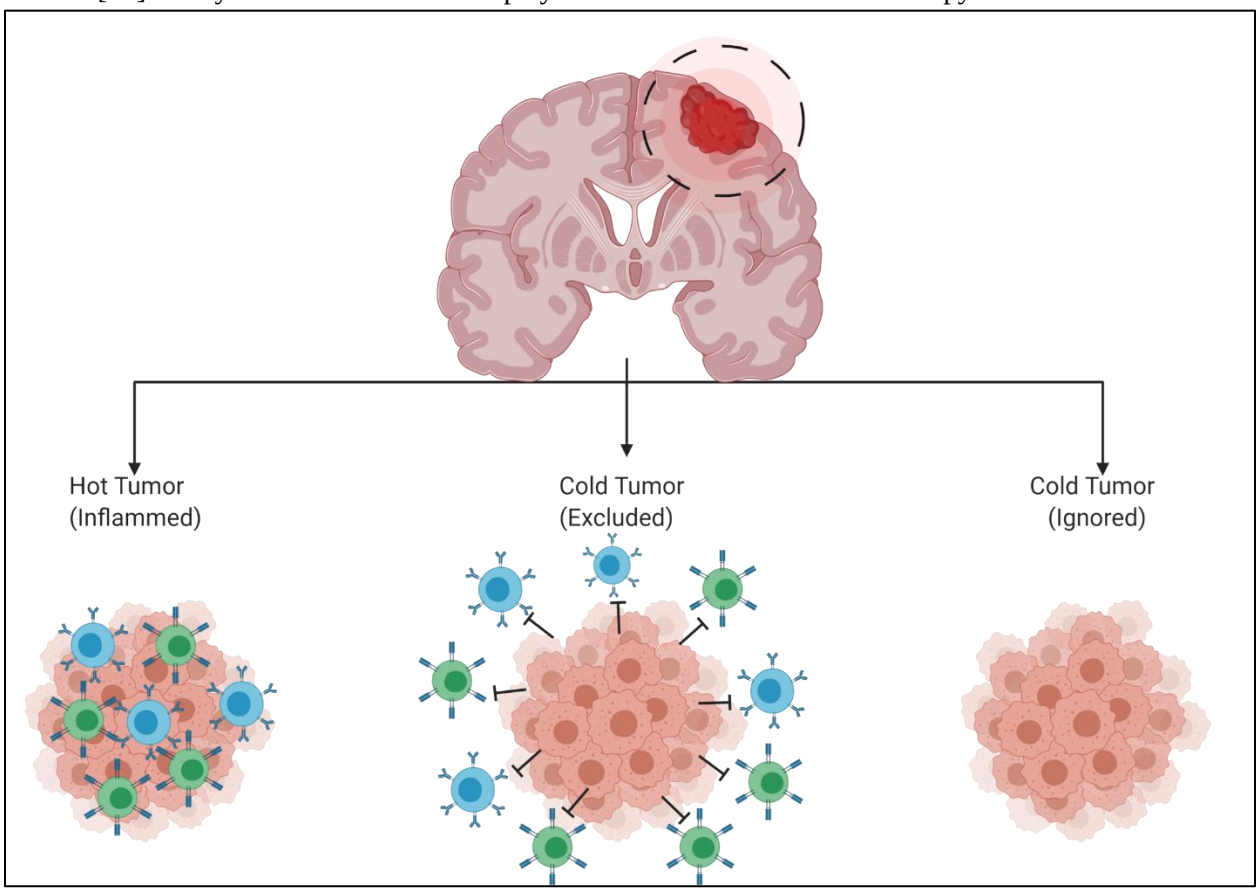


Figure 9: **Tumor Microenvironment Predicts Immunotherapy Outcomes:** Tumors can be categorized as either "hot" or "cold", which describes how the abundance of infiltrated immune cells within a tumor. Hot tumors are generally highly immunogenic and have good prognosis for immunotherapy. Cold tumors are generally highly immunosuppressive and/or not very immunogenic. These tumors are major challenges for immunotherapy.

Maximizing Success of Immunotherapy Against PBTs

Treatment of PBTs with immunotherapy faces many unique physiological and patient-intrinsic challenges when compared to other tumor types. The CNS is an organ system that selectively tolerates immune system activity [52]. Swelling and rapid cellular infiltrate may cause structural harm to the brain as it presses against the rigid skull. Unchecked inflammation within the brain due to activated immune cells can cause encephalitis, resulting in the deterioration of cognitive functions and possibly death [52]. Immune cell trafficking across the BBB is tightly regulated under physiological condition unless there is

injury or insult to the surrounding nervous tissue [52]. Systemically administered therapeutic antibodies also have difficulty crossing the BBB, necessitating other methods of delivery [53]. Furthermore, these patients are very young, so their tumor mutational burden is often small. Consequently, PBTs do not usually display many tumor-specific antigens to target on their surfaces [12]. Young children also do not have fully-matured immune systems until around age 12, so immunotherapeutic routes applicable for adult brain tumors may not be effective in this context [54]. Despite these challenges, there are still viable immunotherapy avenues for PBTs to explore.

The brain is not an immune cell-barren site -- macrophages and microglia are present in abundance and may be attractive targets for immunotherapy against PBTs [5, 55, 56]. Macrophages and microglial cells perform similar physiological roles but have diverging origins in the body. Macrophages are bone marrow derived (BMD), originally as monocytes, and easily cross the BBB from circulation where they will mature into macrophages [36]. Microglia cells, on the other hand, are derived from yolk sac hematopoietic progenitors [35]. Together, BMD macrophages and microglia jointly play major physiological roles in the CNS: active surveillance for pathogens, phagocytosis of foreign bodies, and homeostatic maintenance like dendrite pruning [55]. Beyond their physiological roles, BMD macrophages and microglia cells are also believed to be play a role in the growth and maintenance of PBTs [35, 57, 58]. Depending on the brain tumor type, BMD macrophages and microglia can comprise upwards of 30% of a solid brain tumor's bulk [35, 57]. Within this context, they are known as tumor associated macrophages/microglia (TAMs) **(Figure 10)**. The degree of TAM activation status correlates with brain tumor grade, suggesting not only the presence but the activity of TAMs can promote brain tumor progression and spread [35, 57, 58]. MMPs produced by TAMs break down the local ECM, permitting

tumor cell spread, and secretion of pro-growth cytokines can activate pro-growth and pro-angiogenic biochemical pathways in tumor cells like PI3K/Akt [57, 58].

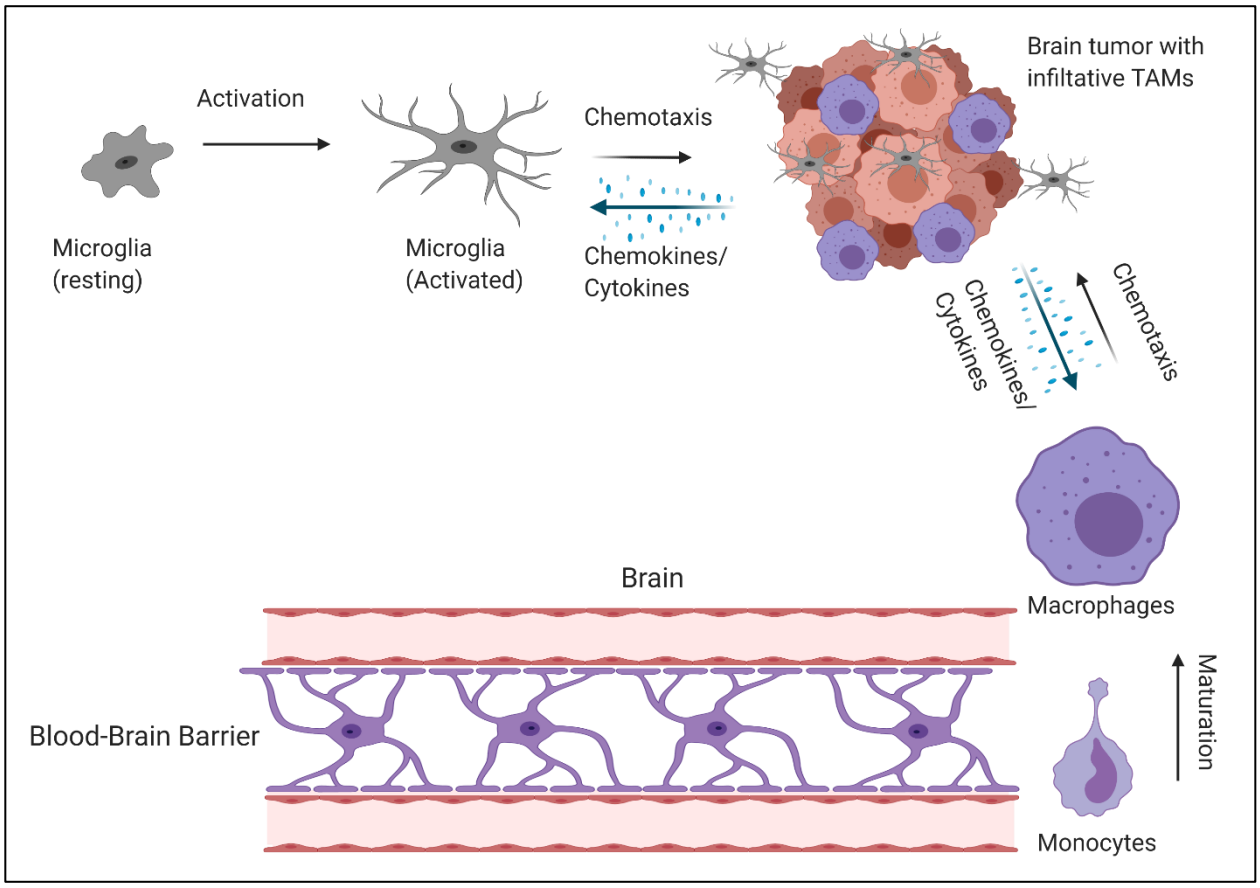


Figure 10: **TAMs are Recruited to Brain Tumors to Assist in Growth** Tumor associated microglia/macrophages have diverging origins, but play similar protective roles in the brain. Brain tumors are known to recruit these cells, now known as TAMs, and coax them to secrete pro-tumorigenic, anti-inflammatory environments.

The pro-tumorigenic aspects of TAMs are influenced by a multitude of tumor cell-intrinsic factors. Brain tumor cells in general are known to participate in autocrine signaling via secretion of cytokines and chemokines [27, 57]. These soluble molecules, and their cognate receptors on TAMs, are believed to play a role in the trafficking and activation of TAMs to support tumor cell spread and proliferation within the brain. For example, chemokines like CCL2 and CX3CL1, are upregulated within brain tumors, encouraging the recruitment of macrophages and microglia expressing their cognate receptors, CCR2 and CX3CR1 respectively [35, 36, 57]. Brain tumors are capable of altering the activation status of TAMs

away from a cytotoxic profile towards one that favors tumor growth through the secretion of soluble molecules like IL-10 and TGF-β [35, 36, 57]. PBTs, with the assistance of TAMs, may also participate in more direct methods of evading immunological destruction. Some high grade gliomas, and TAMs themselves, are known to express cell surface T-cell checkpoint ligands like PDL-1 [59, 60]. Brain tumor cells also employ mechanisms to escape destruction from TAMs themselves by over-expressing macrophage checkpoint ligands like CD47, CD24 and the LILRs on their surfaces [61-64]. These molecules, once docked with their respective targets on TAMs, prevent the phagocytosis of the tumor cell [65].

Given the abundance of macrophages/microglia in the brain, as well as the multitude of ways they assist in tumor growth and immunological escape, therapeutic interventions that seek to reverse their pro-tumorigenic capabilities have been investigated extensively. Delivery of pro-inflammatory molecules like as INF-γ and TLR agonists demonstrated increased TAM phagocytic activity and reduced tumor cell burden in the brain [35, 57]. One major caveat to this approach is that unchecked inflammation caused by activated TAMs can non-specifically damage the sensitive nervous tissue of the developing brain [52]. Drugs like minocycline hydrochloride and amphotericin B have also showed some indirect efficacy through the downregulation MMPs, slowing the physical spread of the brain tumor by preventing breakdown of the brain's ECM [57]. Other methods to attenuate tumor cell spread include blocking chemokine receptors. HOE 140 (Icatibant), a B_2R agonist, was investigated as a potential therapy for metastatic brain tumor cells to prevent their chemotaxis towards gradients of Bradykinin released by endothelial cells [66]. Administration of Icatibant decreased recruitment of malignant glioma cells to blood vessels in brain slices, but not a complete absence of invasion [66]. This is probably because endothelial vessels secrete multiple chemokines that can recruit tumor cells, including CXCL12, so it would be incumbent to block all of the signals to completely stop tumor cell motility. PBTs that over-express macrophage checkpoint ligands like CD47 may be especially vulnerable to therapeutics that target these signaling molecules. Recent publications have shown monoclonal antibody blockade of

CD47, which binds SIRPα on phagocytic cells, promoted extensive phagocytosis of human brain tumor xenographs [61, 62, 67, 68]. Like PD-1 on T-cells, SIRPα on macrophages/microglia contains inhibitory ITIM-domains in its cytoplasmic region **(Figure 11)** [69]. The resulting downstream signaling cascade through SIRPα prevents phagocytosis [65]. By blocking the CD47-SIRPα signaling axis with monoclonal antibodies, activating signals are able to propagate in the macrophage [65]. Opsonization of the tumor cell by the Fc domain of the blocking antibody itself, or another IgG, allows "pro-eat me" signaling to cascade through the FcR, whose activating ITAM domains work in opposition of ITIM-signaling **(Figure 12)**.

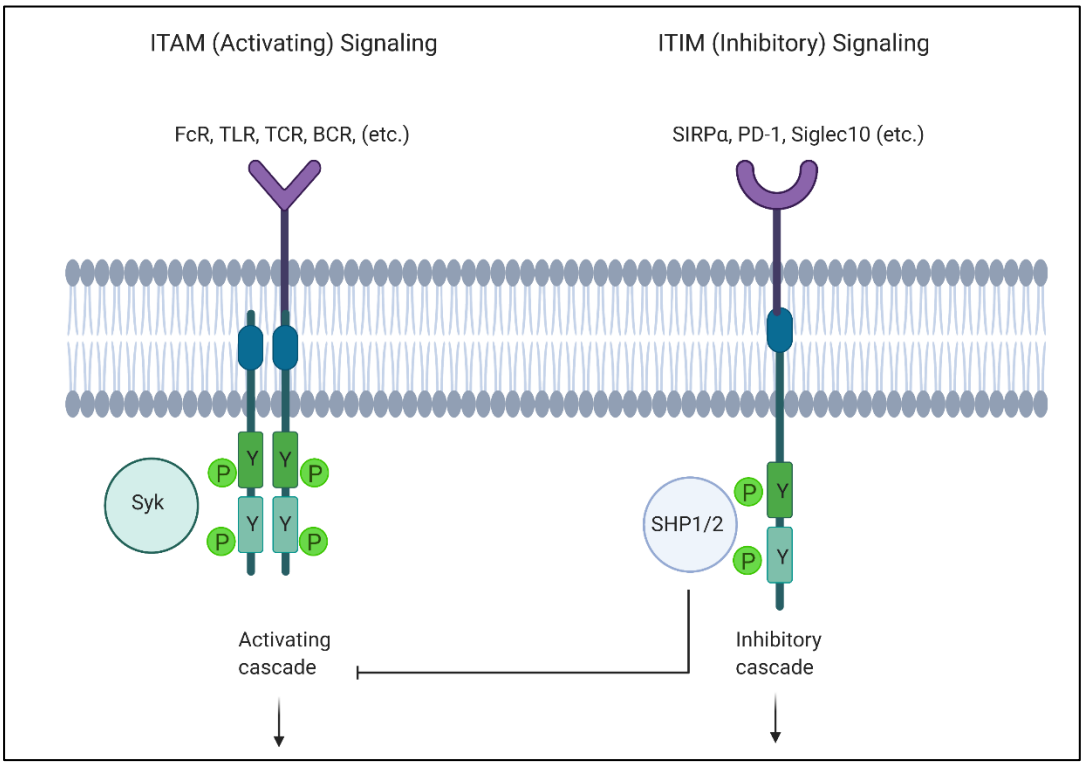


Figure 11: **ITIMs and ITAMs are the Gas and Brake Pedals of the Immune System** Both innate and adaptive immune cells require external activations signals binding to their ITAM-domain containing receptors like the FcR and TCR. Receptors with ITIM domains, like PD-1 and SIRPα, are dominant in this dynamic and provide inhibitory signals to the immune cell to prevent activation. Adapted from [68].

In my view, blocking macrophage checkpoints like CD47 as a treatment for PBTs has an advantage over other types of immune cell – enhancing therapies mentioned in this chapter. This may be safer for the developing brain because it requires direct cell-cell engagement instead of nonspecific secretion of cytotoxic molecules. Off-target effects when using CD47mAb systemically in clinical trials was most

commonly anemia, given the over-expression of CD47 on the surface of reticulocytes and platelets [70]. This was overcome by delivering priming dosages of antibody systemically prior to a therapeutic dose to get rid of "old" reticulocytes and to promote the differentiation of new red blood cells with low CD47 on their surfaces [70]. In totality, delivery of macrophage checkpoint inhibitors (MCIs) like blocking antibodies to CD47, have demonstrated macrophage-mediated human brain tumor cell clearance. Given the sheer abundance of phagocytic immune cells already residing within the brain, an immunotherapy approach that delivers MCIs may be the most promising avenue towards successful treatment of PBTs.

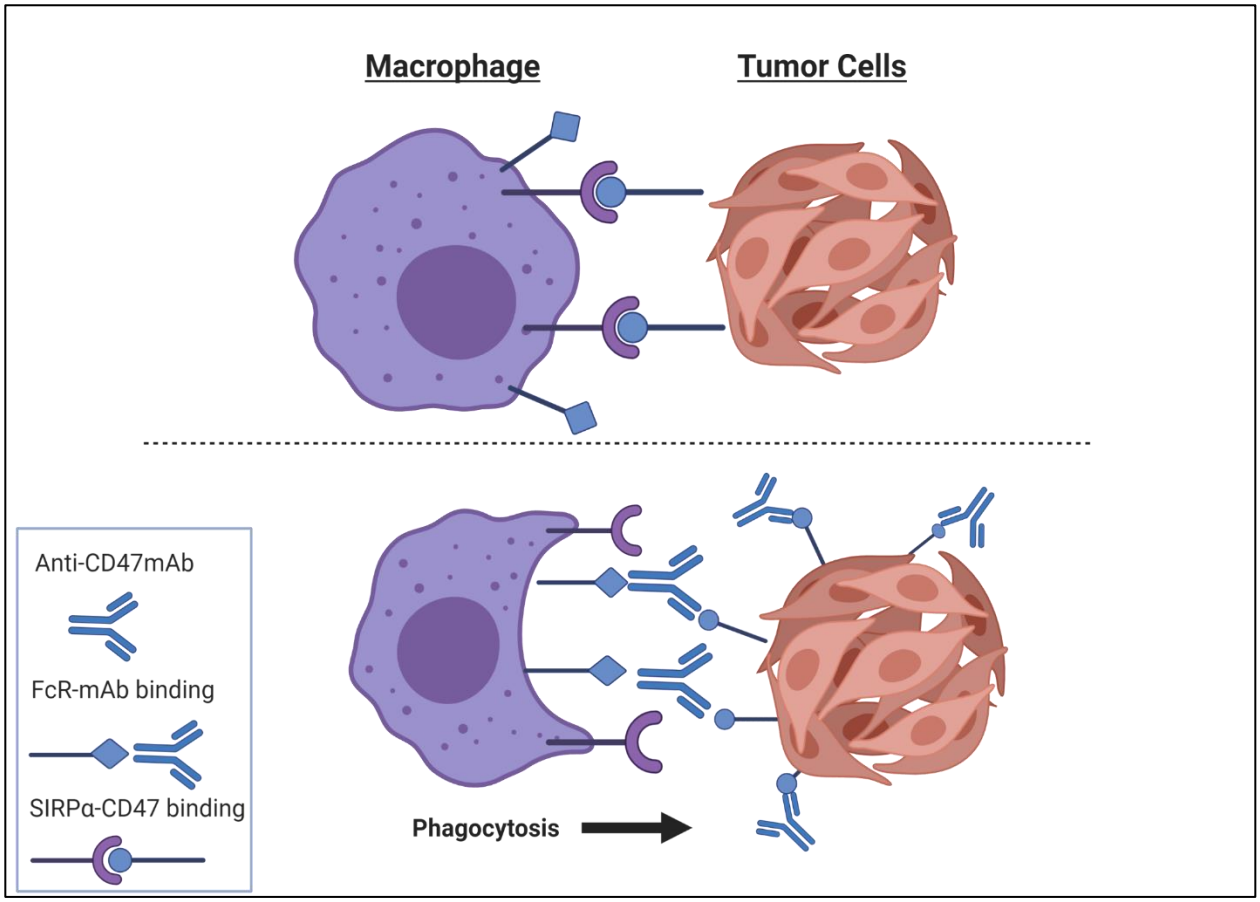


Figure 12: **Macrophage Checkpoint Blockade Promotes Tumor Phagocytosis** Tumor cells, including PBTs, prevent their destruction by TAMs by over-expressing macrophage checkpoint ligands such as CD47. Pharmacological blockade of CD47-SIRPα interactions permits tumor cell phagocytosis via activating signals, such as FcR-Fc binding.

Chapter 3:

Local Delivery of Immunotherapy into the Brain from Biomaterials

Local Delivery May Circumvent Many Issues of Immunotherapy

Immunotherapy as a treatment of remnant PBT cells presents many advantages over pharmacological and radiological methods currently employed as adjuvant therapies. Two key advantages in particular are: 1) Tumor specific cytotoxicity and 2) Reduction of unintended damage to the patient's developing brain. However, there are additional risks involved with this route that must be considered. Systemic administration of blocking antibodies to "don't phagocytose me" signals have been shown to have varying degrees of systemic toxicity. Blocking antibodies to CD47, for example, can result in patients developing anemias and/or thrombocytopenia, as this ligand is often over-expressed on reticulocytes and platelets. Furthermore, uncontrolled release of cytokines can result in catastrophic inflammatory states like cytokine storm which can cause unintended harm, or even patient death. And due to the stringent nature of what the BBB permits, many therapeutic avenues, including some antibody therapies, cannot be administered systemically to reach the brain **(Figure 13)** [71]. I hypothesize that local delivery of immunotherapy within the brain can circumvent many of these issues. In this chapter, I will cover a brief history of local therapeutic delivery for brain tumors, recent advances in materials science, and how to utilize these new materials to better delivery immunotherapy than what was possible in the past.

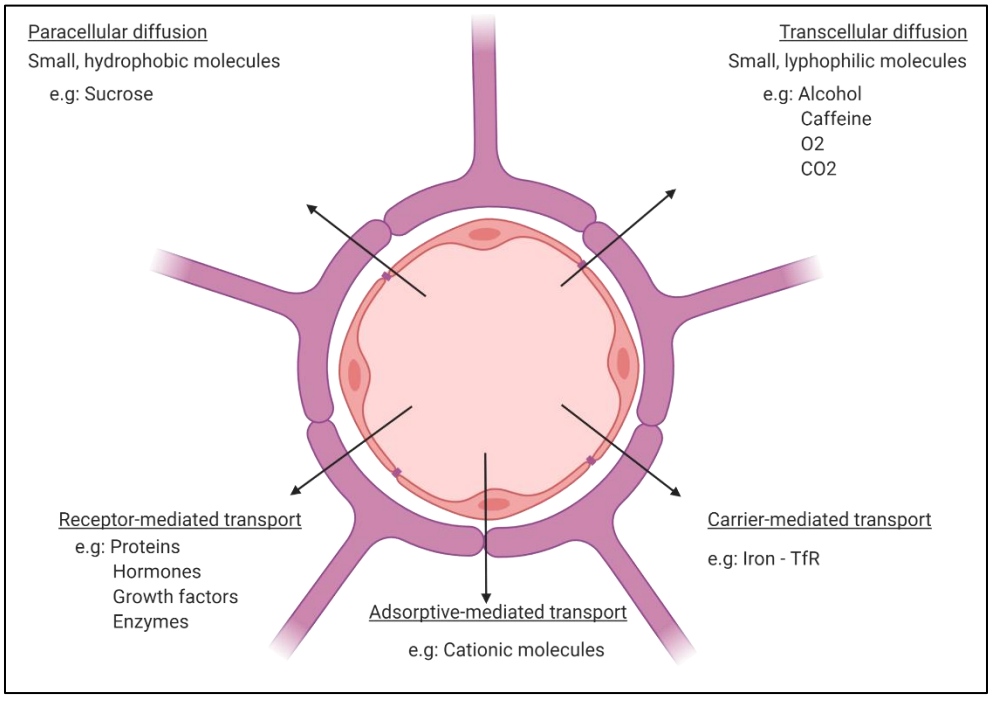


Figure 13: **The BBB Restricts Systemic Delivery of Drugs to the Brain** Molecules crossing into the brain from the peripheral blood is tightly regulated by the BBB. Many chemotherapies and antibodies with efficacy against brain tumors cannot enter the brain on their own. Strategies to overcome the BBB restriction include using molecules that can bind to transmembrane receptors for entry or local delivery directly into the parenchyma. Adapted from [70]

Lessons Learned from Local Chemotherapy Delivery into the Brain

Local delivery of therapeutic molecules to treat brain tumors began decades ago to improve the delivery of chemotherapy to the brain. The stringent nature of the BBB prohibited many drugs that show *in vitro* activity from ever entering the brain [72-76]. Molecules capable of crossing the BBB were restricted to those with low molecular weight, high hydrophobicity, low ionization and little/no protein binding ability [77]. For therapeutic agents that met this criteria, like Temozolomide, acquiring effective concentrations within the brain tumor environment would require dosages much higher than the tolerance thresholds of other systemic organs [72-76]. Clinical trials using these systemically administered chemotherapeutics resulted in insignificant changes in 5 year outcomes of children with highly infiltrative brain tumors [72-76]. By opting for local delivery of drugs into the brain, investigators sought to overcome these limitations and experienced variable success in that endeavor. The physical and chemical properties of the therapeutic agents chosen could be much broader now that the BBB was being bypassed. As was demonstrated with the BCNU wafers, Gliadel, much higher concentrations of drug could be introduced in the brain than could ever be administered systemically [78-80]. The slow degradation of the polymers housing BCNU created a slow-release profile of the drug into the brain, attenuating the toxicities associated with the burst release of drug [78-80]. Although local delivery of chemotherapy ultimately had a significant effect on the lifespan of patients with metastatic GB, the efficacy of this approach was limited to a few extra months of life and no change in 5 year outcomes [78-80]. Studies looking into this method of delivery revealed two major flaws in this approach [78]. The physical degradation of the BCNU wafers meant that, over time, smaller and smaller surfaces of the brain came in contact with the BCNU wafers to receive chemotherapy [78]. And, more importantly, the effective concentration of

chemotherapy to trigger tumor cell death did not penetrate more than a few millimeters beyond the resection cavity. Meanwhile, highly infiltrative brain tumors like GB tended to recur up to multiple centimeters away [78] **(Figure 14)**.

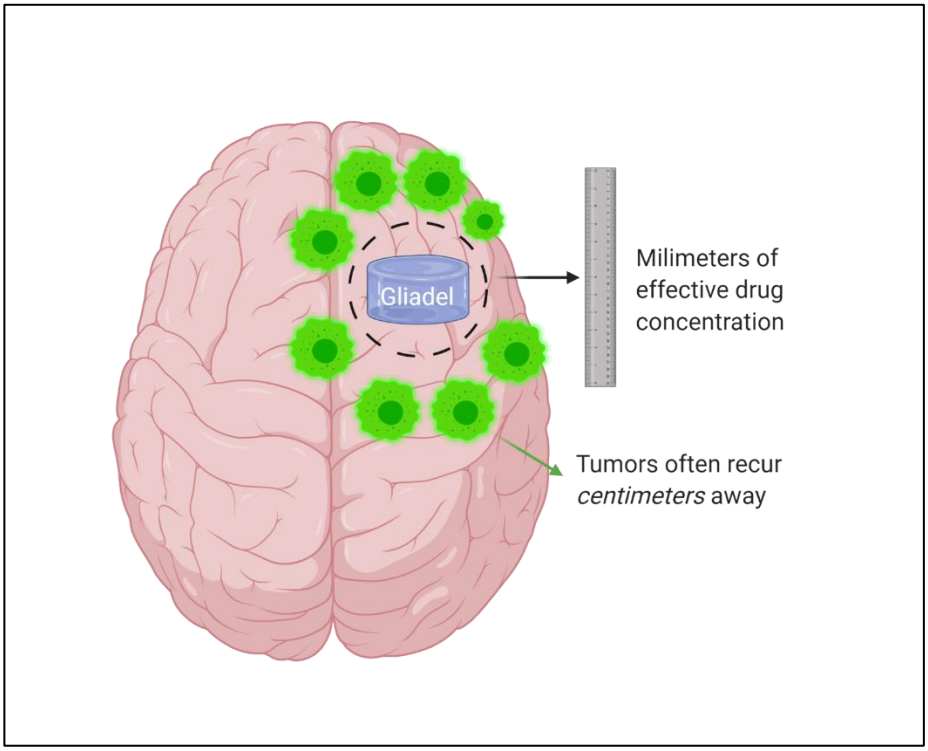


Figure 14: **Therapeutic Molecules have Limited Diffusion within the Brain** Data investigating the efficacy of the Gliadel wafer in brains revealed delivered payloads were at sufficiently high concentrations to induce tumor cell toxicity a few millimeters away. Meanwhile, metastatic tumors like GB recurred centimeters away. Adapted from [77].

Convection enhanced delivery of drugs into the brain is an approach that attempted to overcome the issue with shallow penetration of therapeutic agents into the brain [80-82]. Utilizing small catheters inserted through the skull and into a brain tumor, clinicians sought to pump therapeutics further distances through the brain using a pressure gradient, rather than simple diffusion from a static implant [80-82]. Utilizing this technique, therapeutics could be delivered up to a few centimeters away from the catheter [81, 82]. While the issue of therapeutic penetrance was addressed, new problems emerged. Backflow of the drugs being infused into the brain allowed them to exit the target area and into unintended areas of the brain. This is worsened at high rates of flow into the tissue [81]. Air bubbles within the catheter line caused unpredictable flow rates and often resulted in the backflow of drugs and tissue disruption, altering the

flow pattern of the drugs being introduced [81]. Tumor biology also played a role in interfering with CED. Some brain tumors have high levels of interstitial pressure, disrupting the pressure gradients driving the drugs into the tissue [81]. Necrosis in the tumor can cause the drugs to pool in some areas over others, causing unequal drug distribution and payload loss [81]. Ultimately, while this technique demonstrated tumor reduction and generally low toxicity in clinical trials, it failed to truly move the needle in terms of survival for patients with aggressive brain tumors. Nevertheless, local delivery of therapeutics into the brain have demonstrated proven advantages over systemic therapy that we can further build upon for immunotherapy. Today, we have a plethora of implantable biomaterials at our disposal. Each with customizable properties, including release rates and degradation that can potentially deliver a selected cocktail of immunotherapy in a more efficacious manner than what was available in the past.

Biomaterials as Delivery Platforms for Immunotherapy in the Brain

Biomaterials are implantable substances intended to harmlessly coexist within biological settings. Their potential functions can vary dramatically, including but not limited to: serving as prostheses, three-dimensional scaffolds for tissue engineering, restoring function to damaged organs, and serving as delivery vehicles for therapeutics [83-90]. As such, the family of biomaterials is as diverse as their potential applications **(Figure 15)**. Materials currently employed for these purposes can be of: metallic, alloy, polymeric, or ceramic origins [83, 84]. Given the complex nature of the human body and the wide range of potential applications these devices are used for, no single material can be considered the “best” biomaterial for all purposes. Therefore, choosing the optimal material tailored for delivering immunotherapy into the brain is a necessary step. In my view, materials suited for this environment should have properties that reduce the risk of chronic inflammation and pose little risk of mechanically harming sensitive nervous tissue of the developing brain.

Biomaterial	Advantages	Disadvantages
Naturally derived polymers: Collagen Chitosan	Good biocompatibility Bioactive Biodegradable	Low mechanical, thermal, chemical stability Immunogenic responses
Synthetic polymers: Polylactic acid (PLA) Polyglycolic acid (PGA) Polycaprolactone Polyethylene glycol (PEG) Co-block polymers	Good biocompatibility Highly modular backbone Low immunogenicity	No natural bioactivity Little/no biodegradability
Synthetic elastomers: Polyglycerolsebacate	Soft elasticity Good biocompatibility Tunable degradability	Mild cytotoxicity
Calcium phosphates	Excellent biocompatibility Supports cell activity Osteoconductive	Brittle structure Slow degradation
Silicate glass	Excellent biocompatibility Supports cell activity Osteoconductive Vascularization	Brittle structure
Composites	Excellent biocompatibility Supports cell activity	Inferior to real bone matrix

Figure 15: **The Greater Biomaterial Family** Biomaterials are derived from a variety of sources, both natural and synthetic, to fit a wide variety of biomedical needs. Every material has its own strengths and weaknesses, making their usage application specific.

One specific type of biomaterial that may fit such a role is a hydrogel [91, 92]. These materials are crosslinked polymers that possess the ability to swell upwards of 90% their total volume with fluid, allowing for the diffusion of nutrients and molecules between the tissue and the gel itself [91, 92]. Due to this nature, they have often been used to assist in the regeneration of organs, drug delivery, and as tissue engineering scaffolds [83-86, 89-93]. Hydrogels exist as two major types: natural and synthetic. Natural hydrogels are composed primarily of biomolecules such as proteins and polysaccharides, like fibrin, chitosan and hyaluronan [89, 91-93]. Some advantages of this type of gel include the ability to perform biological functions like cell adhesion and degradation, making them great candidates for tissue scaffolds. On the other hand, these gels are known to have poor mechanical properties and high immunogenic

activity, which may not be tolerated in the brain where unchecked or chronic inflammation should be avoided [91, 92].

Alternatively, synthetic hydrogels possess many properties that may make them better suited than natural hydrogels for use as drug delivery depots in the brain. These gels are composed of synthetic chains of molecules, including but not limited to: Poly-ethylene glycol (PEG), polyacrylamide (PAAm) and poly vinyl alcohol (PVA). [91, 92] These gels can be polymerized using a wide variety of techniques such as photopolymerization, enzymatic reactions and bio-orthogonal click chemistry [86, 91, 92, 94-96]. Synthetic hydrogels may contain adjustable mechanical properties and stiffness, allowing the user to tune the physical stress the gels impart on the surrounding nervous tissue. While they are naturally less immunogenic than their natural cousins, synthetic gels are not naturally biodegradable in the body [86, 91, 92, 97]. However, synthetic gels are versatile enough to allow for the functionalization of degradable peptide sequences within its structure, rendering it sensitive to chemical, pH and light cues -- at the scientist or clinician's control [86, 95, 98, 99].

PEG is a particular type of synthetic polymer that is an attractive candidate for use as a drug delivery depot in the brain [86]. There are currently FDA approved PEG-ylated drugs, so this polymer is known to be safe for human use [100]. PEG is used for a variety of biomedical applications, including surface modification, bioconjugation, drug delivery and tissue engineering [86, 101]. PEG hydrogels may be attractive options for drug delivery in the brain because they typically exhibit minimal or no intrinsic biological activity due to the non-adhesive and non-immunogenic nature of PEG chains [86, 100, 101]. These chains, whether linear and branched, can be modified at some (or all) of their hydroxyl termini with a wide variety of functional groups to alter their biological activity and chemical/enzymatic reactivity [86] **(Figure 16)**. If desired, large biomolecules like antibodies, growth factors, integrin binding sites, and degradation sites can be introduced into the backbone of these otherwise-inert gels to bestow a tailored biological effect [86].

Like other synthetic hydrogels, PEG gels can be polymerized by several chemistries, but only a few are considered bio-orthogonal, which defines a reaction whose components have no endogenous representation in biological molecules, cells, or organs [86]. One such bio-orthogonal chemistry is Strain Promoted Azide Alkyne Cyclo Addition (SPAAC) click chemistry [96, 102, 103]. This type of reaction allows for bio-orthogonal PEG hydrogel polymerization under aqueous conditions at neutral pH without the use of a catalyst. This reaction takes advantage of the spontaneous covalent bonding of azide groups to molecules possessing strained rings, such as cyclo-octyne and bi-cyclononyne (BCN) [103, 104]. By functionalizing the hydroxyl ends of a PEG chain with BCN (PEG-BCN) for example, azide-functionalized molecules can covalently bond with the ends of the PEG chain itself [95, 98, 99, 104]. As azides are not naturally found in the human body, this click reaction can take place in the presence of tissue and cells with no risk of toxic side reactions and by products [103, 104]. In my view, the versatility of PEG-based hydrogels in terms of molecule functionalization, bio-orthogonal polymerization and their established tolerance in the human body makes these gels an ideal platform for immunotherapy delivery in the brain.

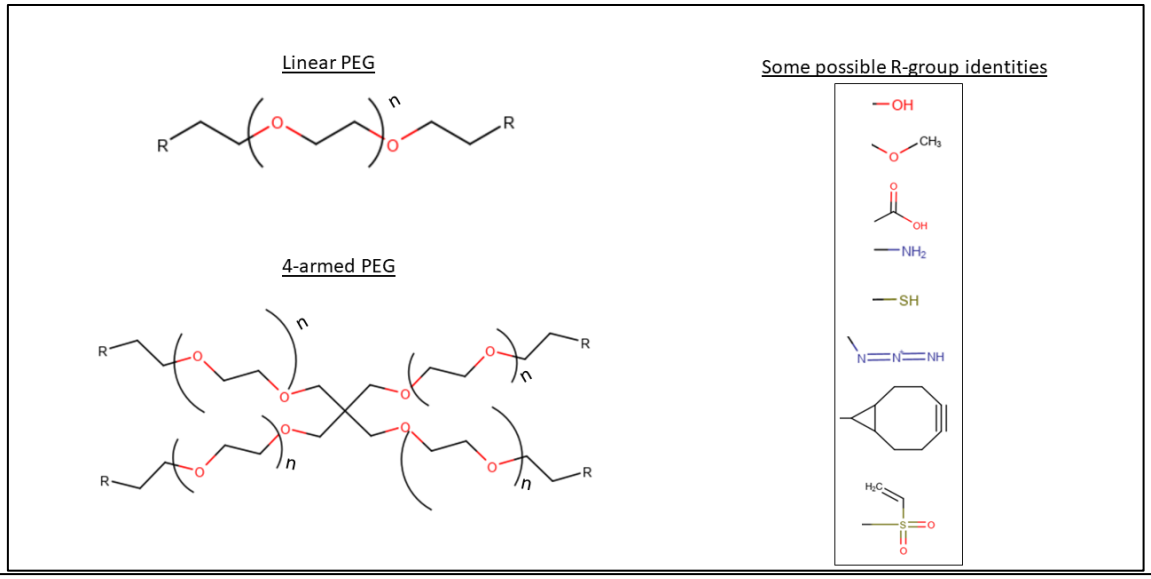


Figure 16: **PEG Chains are Versatile Backbones for Hydrogel Formation** PEG is a synthetic polymer commonly used in drug delivery applications. PEG has low inherent immunogenicity and can be functionalized at one or multiple arms with chemical handles to make it amenable to bio-orthogonal chemistries and polymerization strategies.

Tailoring Release Rates of Therapeutic Payloads to Hydrogel Depots

If hydrogel-based delivery of therapeutics into the brain is to be attempted, it should have extended release capabilities so multiple surgeries can be avoided [105]. Slow release also helps attenuate toxicities associated with burst release of drug into tissue [78, 79]. Release rates of payloads from hydrogel drug depots can be altered in a number of ways. Changing the size of the pores formed between polymer crosslinks, or adjusting crosslink density itself will alter the diffusion characteristics of the hydrogel [106]. Physical degradation of the polymer to release drug encapsulated in the polymer is a strategy employed by the Gliadel wafers. However, as those polymers degrade, they physically interface with a smaller surface of tissue, impacting the drug distribution profile [78, 79]. One strategy to achieve long term release rates from polymers while maintaining their structural integrity is to tether a given therapeutic payload to the backbone of the gel via cleavable linkers, as seen in antibody-drug conjugates [107]. Further taking advantage of SPAAC click chemistry, one may employ a type of azide-functionalized fatty acid (azidoacids) as either permanent or transient linkers between a therapeutic payload and the PEG gel.

These azidoacid linkers may covalently attach to the alkynes of a PEG-BCN gel and, depending on the functional groups present, can form either an ester or amide with the payload [108, 109]. As previously demonstrated by Hubbell's group, esters formed between a payload and these linkers hydrolyze in an aqueous solution and release an attached payload at a rate determined by the length of the acid's carbon chain [110] **(Figure 17)**. This can bestow a degree of tunability to this release system not possible with unlinked drugs, particularly if multiple are to be used at once. Alternatively, molecules conjugated to the linker as an amide are permanently linked to the gel without the assistance of an enzyme or the use of non-physiological pH conditions [109].

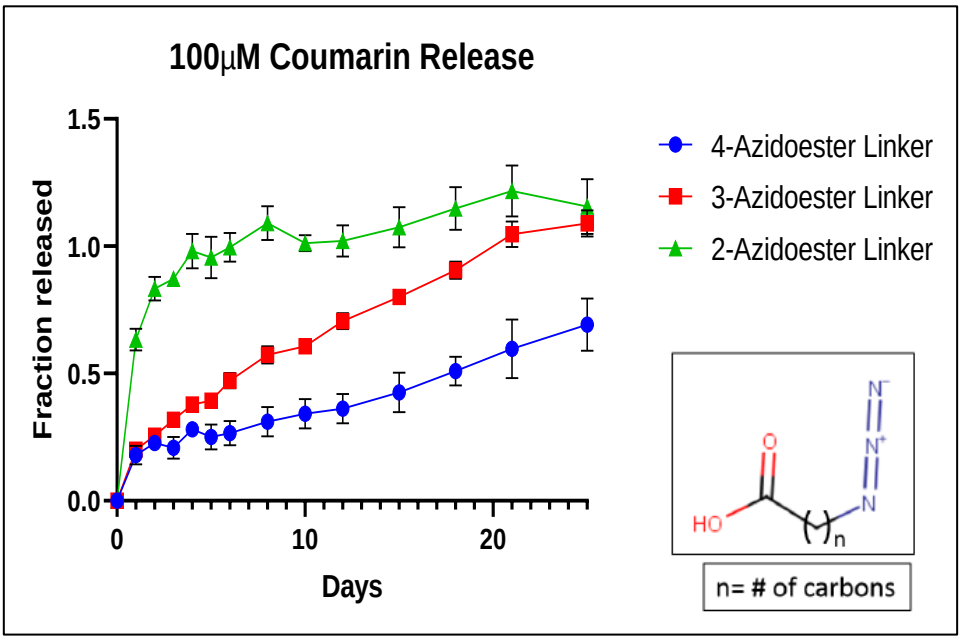


Figure 17: **Variable Hydrolysis Rates of Payloads Conjugated to Azidoacids** Attaching therapeutic payloads to a cleavable linker can enhance its longevity in tissue while reducing the toxicity of burst release. Fatty acid linkers like azidoacids form hydrolysable esters with a target payload. By altering the number of carbons in the linker (2-4 in this example), one can tailor the hydrolysis of the linker and therefore payload release.

Sortagging – A Universal Attachment Strategy for Coupling Therapeutics to a Hydrogel

Some large biomolecules, such as antibodies, may possess multiple sites where methods of chemically adding a linker molecule or drug payload could result in disrupted biological activity and prevention of release altogether [111]. Large proteins may have multiple serines, threonines, and tyrosines, whose free hydroxyl groups can form esters with a cleavable azidoacid linker, resulting in a stochastic release rate of a payload from the gel. Alternatively, the primary amines at the N-terminus or lysines can generate amide bonds with an azidoacid, permanently linking a payload to the gel and preventing its release within the body [109]. In the interest of maintaining control of a payload-to-linker ratio and biological activity, there exists a simple enzymatic method of site-specifically modifying a particular biomolecule of interest with these azidoacids. Using the process called “sortagging” one may use the bacterial transpeptidase, sortase,

to achieve this goal [112, 113]. By recombinantly expressing a biomolecule with a N- or C- terminal sortase recognition sequences, such as “LPXTG”, the sortase enzyme will conjugate triglycine (GGG)-containing molecules to this tag [112, 113] **(Figure 18)**. Utilizing this strategy, one may couple a wide variety of molecular tags, including azidoacids, to a specific terminus of a biomolecule. Thus, we have a method of bridging an immunotherapeutic biomolecule and a PEG-BCN hydrogel via cleavable linkers to tunably control its release over time within the body.

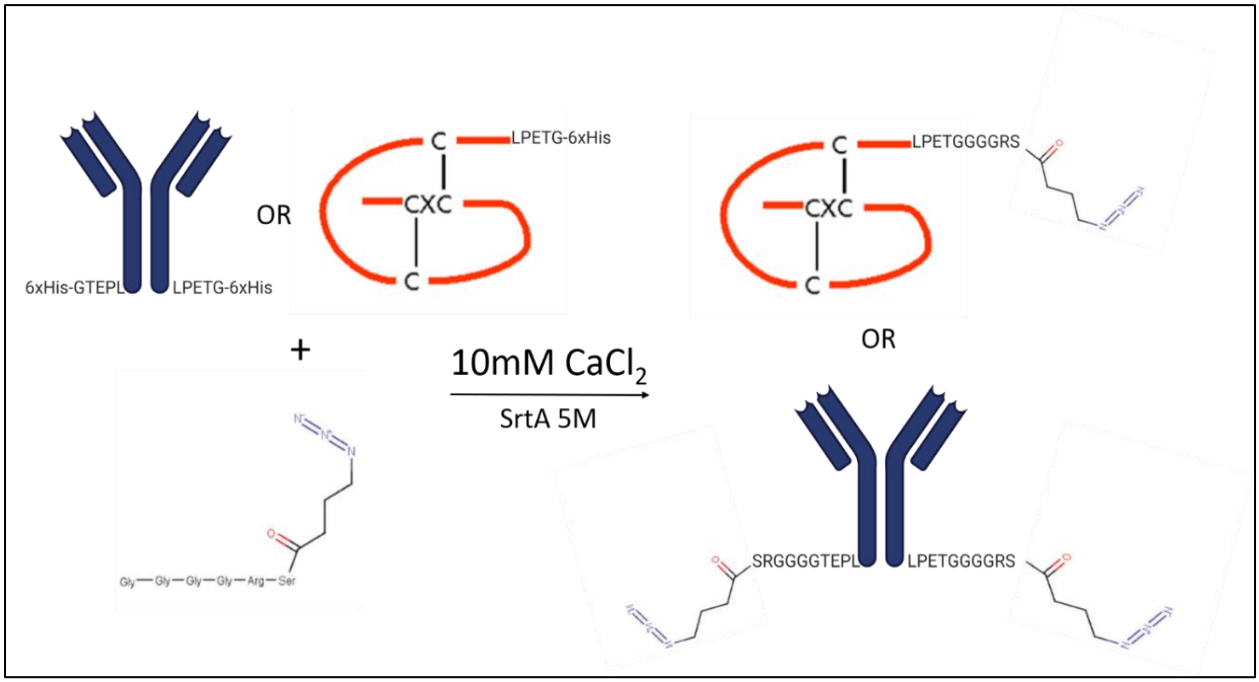


Figure 18: **Sortase Tagging is an Efficient Enzymatic Method to Site Specifically Modify Biomolecules**. Site specific modifications of biomolecules is necessary to ensure continued biological function and release properties from a linker. The bacterial transpeptidase, sortase, recognizes “LPXTG” recognition site genetically encoded into recombinant proteins N or C termini. GGG-containing molecular probes can be seamlessly attached to the recognition site on recombinant biomolecules like chemokines and antibodies.

Chapter 4:

Exploiting Cancer Cell Motility to Improve Localized Immunotherapy Treatments

Casting a "Lure" to Attract Migratory Brain Tumor Cells

Localized, slow release of therapeutic agents into or around the tumor bed has a number of advantages. Higher concentrations of active agents can be delivered to the brain than can ever be achieved systemically. And the slow release aspect of the implanted biomaterial attenuates toxicities related to burst release of drugs into tissue. However, this strategy has a major disadvantage: area of effect. Due to the brain's very small intracellular spaces and active transport of molecules out of the parenchyma, therapeutics delivered locally from a static source may only have a small range where they're at high concentrations [114]. As has been demonstrated with the Gliadel system, though drugs may physically reach migratory tumor cells a few centimeters away, they may not do so at concentrations high enough to cause cell death [78]. The same could be said about delivery of blocking antibodies to macrophage checkpoints: sufficient antibody concentrations to enact a phagocytic response on brain tumor cells may only exist close to a static source of therapeutics. What I seek to investigate in this body of work is whether artificially introduced chemotactic signals within the brain will encourage migratory tumor cells to come within the effective range of a static source of anti-tumor therapeutics. Throughout this final introductory chapter, I will present a high-level overview of chemokines and their functions, how chemotaxis may play a role in tumor biology, and how we may exploit tumor cell chemotaxis as a therapeutic approach.

Chemokines and Their Receptors

Chemokines are soluble cytokines that elicit directed migration patterns of a cells and organisms –a process known as chemotaxis [115, 116]. These biomolecules usually fall between 7-15 kDa in molecular weight and, in totality, there are over 50 different types of chemokines conserved between humans and mice. Chemokines are grouped into sub-divisions based on the position of the first cysteine amino acids within their primary structure. Thus, chemokines fall into the C, CC, CX3C and CXC varieties [115, 116]. Physiologically, chemokines play a number of roles in mammals, including: guiding cells through the developing embryo, axon motility, wound healing and immune cells trafficking. Chemokines are

known to be physiologically secreted by a wide variety of cell types in the body, including astrocytes, immune cells, and endothelial cells. Once secreted, chemokines can form physical and chemical gradients for cells to follow, either through binding to glycosaminoglycans on cell surfaces or dispersing through the fluids of the extracellular space [115, 116]. Cell types responsive to these chemical cues express chemokine receptors and travel up the concentration gradient of a particular chemokine to their destination.

There are approximately 25 chemokine receptors responsible for chemokine binding. 20 receptors are G-protein coupled receptors (GPCRs) and are known for downstream signaling responses. The remaining 5 chemokine receptors are known to be non-signaling and are referred to as "Atypical" [115-117]. While GPCRs can induce movement of a particular cell type, atypical chemokine receptors are usually used for chemokine scavenging from the microenvironment to prevent GPCR over-saturation. The structure of the GPCR consists of 7 transmembrane domains, a glycosylated N-terminus and a phosphorylated C terminus that's used for recruitment of a family of proteins called arrestins [116, 117]. The current model for chemokine binding to their receptors describes a two-step, two-site process, where each site individually imparts specificity and receptor activation [116, 118]. The main body each chemokine binds to the N-terminus and transmembrane loops of the chemokine receptor. These interactions are specific for each family of receptor. The N-terminus of the chemokine then binds inside of the transmembrane domain on the receptor, activating downstream signaling [116, 118]. Ligand binding to the chemokine receptor is the first of four major events leading to chemotaxis. The other three are: signal transduction from the GPCR, cytoskeleton re-arrangement, and establishing polarity of the cell. Transduction of the signal is a central figure in this process as it receives and relays input from the G-protein network, cytoskeletal and polarity biochemical pathways. These factors work in concert to ultimately result in the cell's cytoskeleton forming pseudopodia in the direction of the gradient, the leading edge, and contraction of the cytoskeleton at the lagging edge.

Chemotaxis' Role in Cancer

In addition to the many physiological roles of chemotaxis, (e.g: immune cell trafficking, embryonic development, etc.) it is also believed to play a major role in the metastasis of various cancer types [119, 120]. Many metastatic cancers originating from a particular organ system tend to spread to the same locations in the body, regardless of the patient. For example, metastatic breast cancer tends to spread to the bones, lymph nodes, the brain and the lungs. Some researchers believe this is not by accident, and these tumor cells may be homing in on chemokine gradients expressed endogenously at these sites [119, 120]. Over 20 different cancer types are known to express chemokine receptors at levels higher than those found in the surrounding healthy parenchyma [119]. Some researchers believe this increased receptor expression may play a contributing role in the "homing" properties these metastatic cancers have for their respective niches [119, 120]. Returning to the breast cancer example, breast tumor cells often over-express CXCR4, the receptor for CXCL12. This chemokine is naturally secreted in areas of the body where this tumor type tends to metastasize, such as: brain, lungs, lymph nodes and bones [119, 120].

Brain tumor cells, of both adult and pediatric origins, also tend to over-express chemokine receptors whose natural ligands are found within known patterns throughout the brain [119, 121-125]. For example, GB migration patterns, known as "Scherer's structures", tend to follow paths of physiological chemokine secretion within the brain, usually CXCL12 [125] **(Figure 19)**. As the WHO grade of brain tumors increase, so too does their expression of chemokine receptors like CXCR4 [126]. In many cases, this chemokine's most prominent effects on brain tumor cells appears to be primarily a migration signal, but secondarily perhaps a mitogen. As such, many pre-clinical therapeutic strategies targeting the activity of chemokine receptors in brain tumor cells are designed to block their function. There is ample evidence that the motility of brain tumor cells expressing CXCR4 towards gradients of CXCL12, is reduced, if not ablated, when deploying competitive agonists against the receptor. For example, CTCE-9908 is a competitive inhibitor of CXCR4 with reduced agonist and signaling capabilities compared to CXCL12 [122]. This drug was shown in a mouse model of osteosarcoma to reduce tumor cell growth and adhesion,

but most importantly reduced metastatic spread. AMD3100 is another inhibitor of CXCR4 that has been investigated against a variety of tumor types, including brain. In one study, blocking this receptor demonstrated reduced chemotaxis of a human GB line towards gradients of CXCL12 [122].

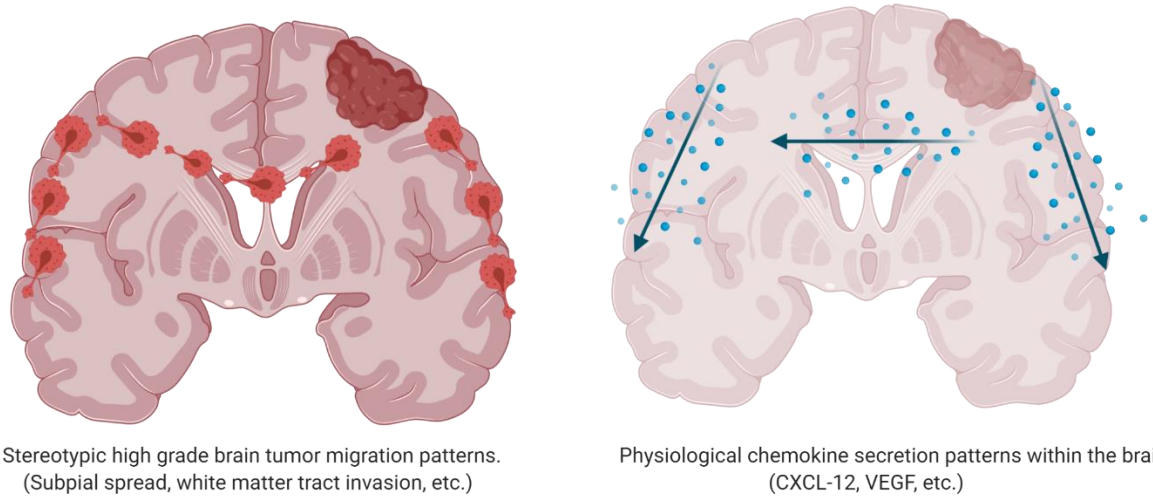


Figure 19: **Migratory Brain Tumor Patterns Mirror Physiological Chemokine Gradients**. Invasive tumor cells tend to over-express chemokine receptors for gradients found in their environment. HGGs in particular have shown a tendency to migrate along physiological gradients of chemokines in the brain, such as CXCL12 (SDF-1). Adapted from [123]

Weaponizing Chemotaxis against Pediatric Brain Tumors

Exploiting brain tumor cell migration, as opposed to antagonizing it, has gained some attention in recent years. In one publication, which became the foundation for a commercialized product, researchers employed polarized fibers to create tracks for brain tumor cells to travel upon, leading them into an extra-cortical chemotherapy "trap" [127]. Although the study showed brain tumor cells will travel on these fibrous tracks out of the brain and into the trap, I find major drawbacks with this approach. It necessitates direct contact between the implanted fibers and a bulky tumor, which may not be possible in circumstances where there only remains locally disseminated disease in areas surgeons cannot physically

access. Any tumor tissue not touching the device would have no incentive to enter the trap, nor would they be affected by the extra-cortical chemotherapy reservoir. In my mind, a superior method of luring brain tumor cells into a therapeutic trap employ chemokine gradients to act on tumor cells whether they are in direct contact or a distance away from a localized therapeutic source.

Unlike chemotherapy, antibodies, and other cytotoxic molecules, chemokines have activity at extremely low concentrations over long range in a gradient fashion [115]. Furthermore, unlike a bundle of fibrous tracks, a therapeutic depot that secretes chemokine gradients through the brain may not have to make direct contact with a bulky tumor, nor disseminated tumor cells in the parenchyma to attract them to the implant. Indeed, a recent publication demonstrates subcutaneous nanoparticles secreting CXCL12 can act as a "lure" to catch circulating prostate cancer cells in a mouse model [128]. Another group recently used an implanted biomaterial impregnated with chemokine-secreting cells to "lure" CTCs in a mouse model as well [129]. If such a chemokine lure system could be combined with a cytotoxic mechanism, then chemokine gradients may be able to lure migratory tumor cells into a localized therapeutic source.

Given the shared sensitivity of immune cells and tumor cells to the same chemokine gradients, the incorporation of potent chemokines into a biomaterial platform loaded with immune cell-enhancing payloads would make an ideal combination, in my view. There may be a therapeutic window where potent chemokines, at low enough concentrations, can recruit tumor cells toward a localized immunotherapeutic "trap" while minimizing any boosts to their proliferation. It may be possible that a clinician could tailor the types of immune cells present in the microenvironment surrounding the implant, thus increasing the likelihood of successful treatment and the generation of an abscopal effect. Within the context of the PBTs, combining chemokine gradients with macrophage checkpoint inhibitors could enhance the effective cytotoxic range of an implanted material as a safe adjuvant treatment to eliminate remnant PBT cells. **(Figure 20)**

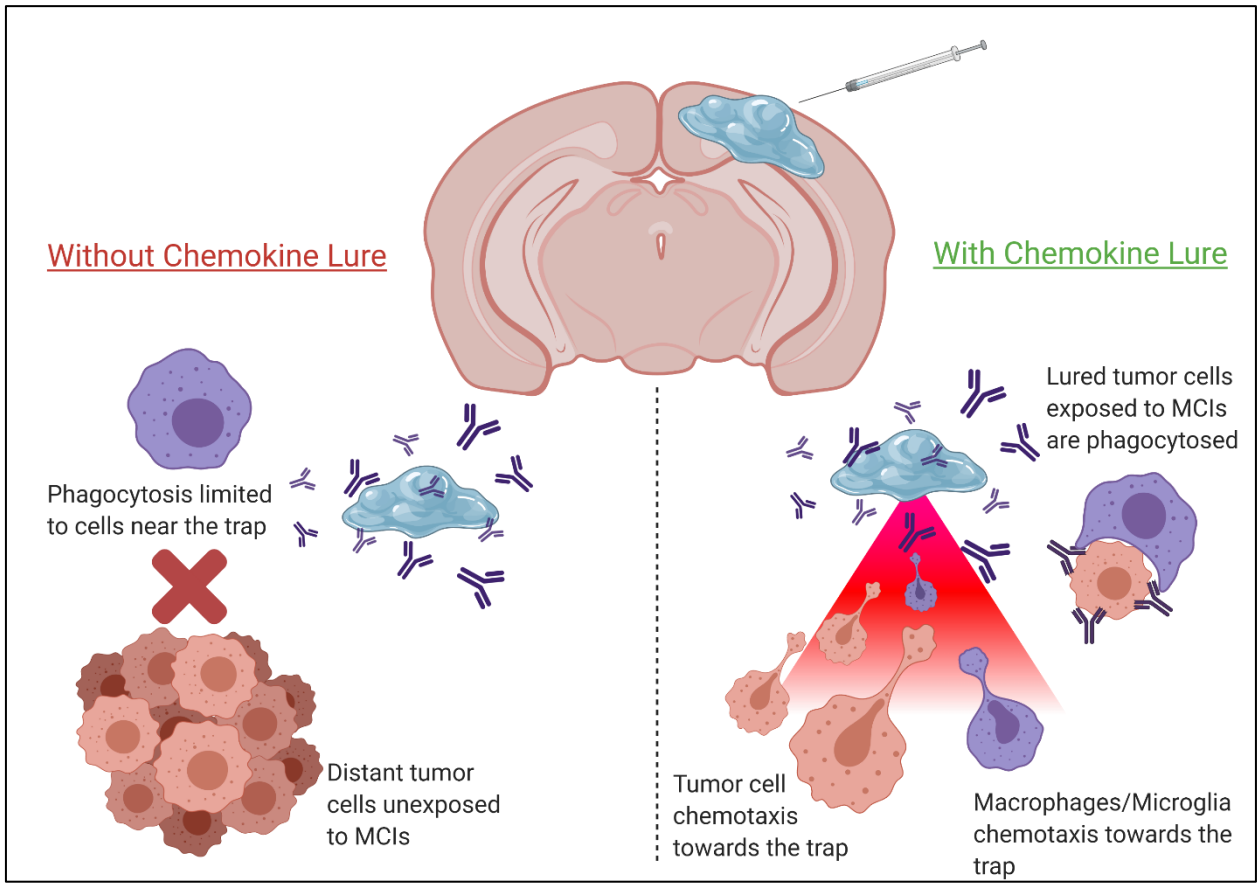


Figure 20: **Luring Pediatric Brain Tumor Cells into an Immunotherapy Trap** . Invasive tumor cells may be out of range of a local source of macrophage-enhancing therapeutics. The addition of a chemokine "lure" may attract migratory PBT cells (and immune cells) into the effective range of these therapeutics, facilitating their elimination by immune cells near the site.

Chapter 5:

Aim of Dissertation Research

In my dissertation research, I investigated whether intertumoral, concurrent release of chemokine gradients and macrophage checkpoint inhibitors (MCIs) would induce greater patient-derived, pediatric high grade glioma (pHGG) reduction compared to MCI treatment alone. Towards this goal, I executed experiments that covered four major areas of study. 1) I first identified potent chemokines that induced chemotaxis of the pHGG cells used throughout this study. 2) I next sought to discover which combination of macrophage-enhancing drugs described in literature was effective at eliciting destruction of these pHGG cells while reducing the possibility of non-specific destruction of healthy CNS tissue. 3) Next, I engineered a non-toxic, PEG-based hydrogel that could be modified to serve as a slow-release delivery depot for recombinantly expressed chemokines and antibodies once tethered to the gel with hydrolysable linkers. 4) Lastly, I implanted gels containing this therapeutic combination into mouse brains bearing pHGG tumors and assessed their effect on tumor growth via IVIS luminescence tracking. These experiments, and their results, are described in greater detail in chapter 6 of this dissertation.

Chapter 6

Enhancing Local Delivery of Macrophage Checkpoint Inhibitors with Chemokine Gradients to Lure and Destroy Pediatric Brain Tumor Cells

INTRODUCTION Pediatric brain tumors (PBTs) are the most commonly diagnosed solid tumor in children [5, 130-132]. These malignancies rank among the leading causes of pediatric cancer-related death, rivaled only by leukemia [5, 130-132]. Standard of care surgery and radiation for these tumors is often complicated by the tumor location [133-135]. The majority of PBTs manifest in areas of the brain where complete tumor removal and radiation therapy could permanently impair a patient's cognitive, behavioral and motor functions [136-138]. Surgeons may choose to leave tumor tissue behind to reduce these adverse effects [133-135]. However, residual brain tumor cells may regrow and metastasize, ultimately leading to patient death.

Many systemically administered immunotherapeutic approaches utilized for adults are unlikely to be successful for PBTs. These tumors have notoriously low mutation burdens and often do not express tumor-specific markers to target with T-cell mediated approaches. Furthermore, trafficking of immune cells across the BBB is tightly regulated. However, many PBTs are composed upwards of ~30% tumor-associated macrophages/microglia (TAMs) and non-clinical studies showed that antibody blockade of macrophage checkpoints, like CD47, induced macrophages to effectively kill brain tumor cells [61, 62, 139]. Effective CD47 blockade in human brain tumors through intravenous administration may be hampered by poor penetration of antibodies across the BBB, coupled with the "sink effect" of CD47 on red blood cells and platelets throughout the body [140, 141]. For this reason, local delivery of macrophage enhancing-therapeutics warrants consideration.

Current studies in mouse models demonstrate the efficacy of locally delivered immuno-stimulatory molecules within the perioperative cavity of incompletely removed tumors [89, 142]. Many groups modeling this approach in the brain often utilize bulky osmotic pumps and biomaterials that either protrude from the skull or do not afford prolonged release rates of their payloads, possibly necessitating multiple surgeries when translated to human patients. Furthermore, tumor cells that migrate more than a few millimeters away from the tumor cavity may be out of reach from an implanted drug depot due to poor diffusion of therapeutics through the brain's parenchyma [78, 79, 143-145]. In contrast to therapeutic

agents, literature demonstrates chemokines need only be present in trace amounts, ideally in a gradient, to chemoattract neural progenitors, macrophages and microglia to a specific location in the brain [56, 146-148]. Interestingly, several studies suggest that the same chemotactic signals known for physiological recruitment of immune cells may *also* have chemotactic activity on brain tumor cells [125, 149-157]. Brain tumor cells expressing chemokine receptors exhibit directional migration when exposed to concentration gradients of their appropriate ligands *in vitro* and *in vivo* [125, 149-157]. Thus, it may be possible to exploit tumor cell biology to lure PBT cells and phagocytic immune cells to a shared location within the brain where sufficiently high concentrations of macrophage checkpoint inhibitors allow for maximal immunotherapeutic engagement.

We investigated whether simultaneous delivery of chemokines and macrophage checkpoint inhibitors from a slow-release hydrogel depot within an infiltrative xenograft pHGG tumor can create a "trap" capable of enhanced phagocytic elimination of tumor cells. Our data confirms gradients of classical immune cell chemokines, like CXCL12, are effective at eliciting chemotaxis of patient-derived pediatric high grade glioma (pHGG) tumor cells *in vitro*. CD47 mAb blockade and subsequent opsonization was an effective single agent to promote tumor cell elimination in co-cultures of murine and human macrophages *in vitro*. Intratumoral delivery of chemokines and CD47mAbs together from slow-release hydrogels within mouse brains attenuated xenograph tumor growth based on bioluminescence data, better than antibody delivery alone. These results suggest macrophage checkpoint blockade could be enhanced by chemokine gradients to safely promote recruitment and immunological clearance of remnant brain tumor cells.

RESULTS

Classical chemokines elicit migration of pHGG cells *in vitro*

Locally delivered therapeutics in the brain may need to account for the tendency of remnant brain tumor cells to migrate away from the tumor margins -- potentially out of reach locally delivered therapeutics [78,

79, 143-145]. We hypothesized that we may be able to employ artificial gradients of chemokines to lure tumor cells and phagocytic cells in the brain towards a static "trap" of macrophage-enhancing therapeutics to facilitate tumor cell elimination. To model this, we're employing a patient-derived, pediatric high grade glioma (pHGG) line, PBT-05. This line demonstrates a similar infiltrative phenotype when engrafted into the brains of immunodeficient mouse models, as it did in the patient from whom it is derived. We conducted *in vitro* chemotaxis assays with PBT-05 against a panel of classical chemokines cited in literature as potentially being potent attractants for migratory brain tumor cells [125, 149-157]. Our results demonstrate that CCL2 and CXCL12 induced varying degrees of chemotaxis in PBT-05 cells. Human CCL2 (hCCL2) had the greatest potency of the chemokines tested, but PBT-05 was unresponsive to the murine version of this chemokine (mCCL2). Both human and murine CXCL12 (hCXCL12 and mCXCL12, respectively) had similar chemotactic potencies against PBT-05, though mCXCL12 showed reduced potency. Flow analysis of PBT-05 reveals no expression of CCR2, the canonical receptor for CCL2, but very high expression of CXCR4, the canonical receptor of CXCL12. This suggests PBT-05's attraction towards hCCL2 gradients may be mediated by another receptor that is promiscuous with this chemokine. However, its chemotactic sensitivity to human and murine CXCL12 may be mediated by the canonical pathway. Literature precedence suggests human chemokines are not very effective against murine immune cells. We opted to move forward with mCXCL12 because our murine models bear human tumors and we wanted to maximize the number of murine macrophages and microglia we engaged along with human PBT cells **(Figure 1).**

Monoclonal antibody blockade of macrophage checkpoints promotes the elimination of pHGG cells by human and murine macrophages *in vitro*

To determine the most potent factors to induce elimination of any lured brain tumor cells by macrophages and microglia near the immunotherapy "trap", we conducted co-culture assays of PBT-05 cells with either murine bone marrow derived macrophages (BMDMs) or human macrophages derived from peripheral blood monocytes (PBMCs). These cultures were challenged with combinations of macrophage checkpoint inhibitors (MCIs) and immune cell polarizing factors described by literature as capable of promoting an

immune response against tumor cells [63, 158-161]. Flow analysis of PBT-05 line shows high expression of the macrophage checkpoint, CD47, and moderate levels of CD24, another recently described macrophage checkpoint [63]. Blockade of CD47 with as little as 1ug/mL of monoclonal antibody was the most effective single agent at eliciting phagocytosis of PBT-05 cells by murine *and* human macrophages, although the effect was nearly maximized with murine macrophages. Inclusion of anti-tumor macrophage polarizing agents, R848 and/or IFNγ did not significantly contribute to tumor cell cytotoxicity over CD47 blockade alone in murine or human macrophage co-cultures. However, combinatorial blockade of CD47 and CD24 showed greater phagocytosis of human tumor cells in human macrophage co-cultures than either antibody alone. Compared to a media control, no combination of MCIs and polarizing factors caused significant cytotoxicity of PBT-05 cells cultured alone, confirming the cytotoxic effect we observed is mediated by the macrophages, not the drugs themselves. Although our data suggests human patients may require combinatorial MCI blockade, we opted to utilize just CD47 blockade for future mouse experiments as it was potent on its own without polarizing factors within the murine context **(Figure 2).**

Engineering user-programmable hydrogel depots for long term molecule release *in vivo*

Local delivery of therapeutics into the brain should have long-lasting release capabilities to avoid the need for repeat surgeries. To this end, we engineered PEG-tetraBCN hydrogels to act as an implantable "trap" capable of extended release of biomolecules like mCXCL12 and CD47mAbs within the brain's parenchyma [94, 95, 162]. PEG chains are known to elicit very little immune activity, which is necessary for limiting inflammation in the brain. The arms of the PEG chains can be modified with a variety of functional handles to bestow customizable properties to an otherwise-inert polymer. Conjugating therapeutic payloads to the PEG-tBCN backbone via hydrolysable azidoester linkers affords tunable, prolonged molecule release compared to diffusion alone [110]. We demonstrate the programmability of this linker system in our gels by sustaining the release of 100μM of a small fluorescent molecule, coumarin *in vitro*. Time until complete release of this molecule determined by the length of the azidoacid linker to which it was conjugated. Complete release was achieved within 5 days using the 2-carbon linker, 21 days for the 3-carbon linker,

and at the conclusion of this 28 day study, only 69% was released using the 4-carbon linker. The 4-carbon azidoester linker was chosen going forward due to its long-acting capabilities compared to diffusion alone. The SPAAC reaction involved in the polymerization of our gels can be slowed by chilling the solution to 4ºC. With the reaction rate slowed, this complete gel solution was loaded into a pre-silanized Hamilton syringe. Using the Hamilton needle, a lateral incision was created in the parenchyma at the tumor's location and 2µL of the still-liquid hydrogel solution was injected into this cavity. The mouse's body temperature facilitated the polymerization process *in situ* **(Figure 3).**

Generation of "sortaggable" mCXCL12 and CD47mAb variants for extended release

Recombinant biomolecules of interest, like CXCL12 and CD47mAbs, can be site-specifically conjugated to the azidoester linkers for extended hydrogel release via sortase-mediated conjugation [112, 113]. Sortase recognizes "LPETG" sites on proteins of interest and can be used to conjugate GGG-containing molecular tags to the N- or C- termini of a protein. We recombinantly expressed mCXCL12 with LPETG sites at the C-terminus and "sortagged" it with a GGGGRS polypeptide that was pre-esterified to the 4-carbon azidoacid at its serine. We also recombinantly expressed "sortaggable" variants of the 2.3D11 clone of the CD47mAb with LPETG recognition sites at both of its heavy chain C-termini and subsequently conjugated them onto our GGGRS-azidoester linkers. These "sortaggable" variants maintained equal biological activity as commercial comparators (BE0019-1, BioXcell). Linking them to our PEG-tetraBCN gels demonstrated similarly prolonged release rates as coumarin *in vitro* **(Figure 4).**

Intratumoral release of CD47mAb and CXCL12 attenuates pHGG xenograft growth *in vivo*

To characterize the tumoricidal capability of co-delivered CD47mAb and mCXCL12, we injected untreated gels, gels with linked and unlinked CD47mAb, gels with linked mCXCL12 or a combination of the two intratumorally within the brains of athymic *Nu-/Nu-* mice harboring Luciferase-expressing PBT-05 tumors. These mice are deficient in B, T, and NK cells to allow for tumor cell engraftment, but have a fully intact innate immune system which permits us to study in phagocytic cell activity alone. The gels were implanted

for 4 weeks and bioluminescence time course data showed steady growth in tumors receiving untreated gels, CXCL12-containing gels, and CD47mAb antibody-alone gels. In contrast, the group of mice receiving gels that contained the combination of CD47mAb + mCXCL12 resulted in arrested tumor growth relative to the other treatments for just over two weeks before the effect began tapering off. **(Figure 5)**

DISCUSSION

Complete elimination of pediatric brain tumor cells is fraught with challenges. Surgical resection of tumors in certain locations of the brain carries the risk of causing long-lasting neurological deficits in the patient. In many cases, surgeons know that it is necessary to leave microscopic or macroscopic disease behind.[133-138] In this proof-of-concept demonstration, hydrogel based delivery of chemokines and blocking antibodies to macrophage checkpoint ligands on the tumor cell surface shows potential to be a viable strategy for safely luring pediatric brain tumor cells into an immunotherapy "trap" for their elimination by phagocytic cells within the brain. While our *in vivo* model demonstrated positive results when delivering these factors into fully-intact bulky tumors, we believe this strategy would be most effective at eliminating microscopic disease cells in patients after much of the tumor was debulked.

Delivery of any soluble factors that stimulate the immune system must be carefully controlled, particularly in the brain. Cytokines and CD47 mAbs have been shown clinically to elicit systemic toxicity on their own [61, 163-166]. Delivery of these factors from slow-release depots would allow a surgeon to safely deliver higher concentrations of therapeutics than would be possible with systemic drug administration [78, 79, 143-145]. To this end, we engineered PEG-based hydrogels to act as a simultaneous *in vivo* delivery system of chemoattractants and MCIs [94, 95, 162]. PEG is nontoxic, and the click chemistry involved in the polymerization of these gels allows for either permanent or labile coupling of cytokines and other therapeutic agents to the gel backbone in a cyto-compatible manner [96, 101, 102, 104]. To demonstrate the tunability of this system for releasing therapeutics, we showed variable release rates of small molecules

as well as biomolecules like CXCL12. Gels coupled to these payloads on hydrolysable linkers demonstrated release profiles spanning a few days to beyond an entire month [110]. This flexibility could allow controlled release of a cocktail of molecules at different rates, if needed. As demonstrated, recombinantly expressed biomolecules can be C-terminally ligated to these linkers via sortase tagging and maintain their biological function [167]. Thus, this system can be adapted for a wide variety of uses, not limited to chemokines and antibodies.

Antibody-mediated phagocytosis of tumor cells relies on both tumor and immune cell types residing in close proximity to each other [30]. Some high-grade brain tumor cells are known to migrate away from the tumor cavity, potentially further than the reach of locally administered mAbs. This was discovered to limit the efficacy of localized chemotherapy, like the FDA approved GLIADEL, to eliminate residual brain tumor cells [78, 79, 143-145]. We hypothesized this problem could be overcome if nearby brain tumor cells could be coaxed closer to the therapeutic source. Some tumor cells express receptors for chemokines physiologically produced in metastatic niches such as the leptomeningeal space of the brain [125, 149-157]. Although some therapeutic strategies seek to block chemokine receptors on tumor cells to restrict their movement, we instead attempt to exploit this behavior to bring brain tumor cells closer to immune cells and immunotherapeutic agents [151, 168]. We found that gradients of classical immune cell chemokines such as CXCL12 were effective at eliciting migration of brain tumor cells *in vitro*. Additional studies may be necessary prior to clinical development to identify chemokine receptors that are near-universally expressed on the tumor types of interest. This technique has flexibility and potential to become a universal method to attract migratory tumor cells out of nearby, unreachable locations of the brain without causing additional physical disruption to the tissue to gain access to them.

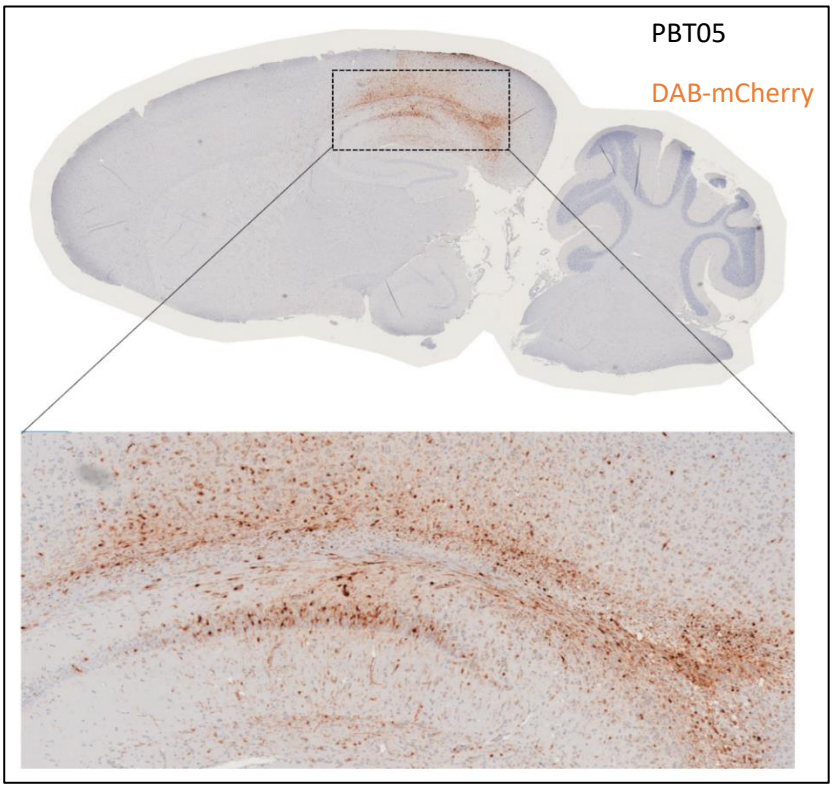
Monoclonal antibody blockade of the cell-surface “don’t eat me” ligand, CD47, was by far the most effective single agent at eliciting tumor cell elimination by both human and murine macrophages, even at low concentrations of antibody [61, 62]. The differences in magnitude of the CD47mAb effect between murine and human macrophages suggests there may be multiple “don’t eat me” axes besides CD47-SIRPα

impeding the phagocytic activity of human macrophages. Indeed, we found that monoclonal antibody blockade of CD47 *and* CD24, another macrophage checkpoint, afforded greater tumor cell destruction by human macrophages than either antibody alone [63]. Additionally, our results suggest that macrophages and microglia within the brain may not need to be polarized for maximum tumor cell consumption when using these antibodies. The TLR agonists R848 and IFNγ, which induce anti-tumor phenotypes in macrophages, had mild effects on the viability of tumor cells in co-culture and did not significantly increase cytotoxicity when combined with CD47mAbs. This may be clinically relevant because activated macrophages can non-selectively damage cells around them, something to consider avoiding while working within the space of very sensitive nervous tissue [158, 160, 161].

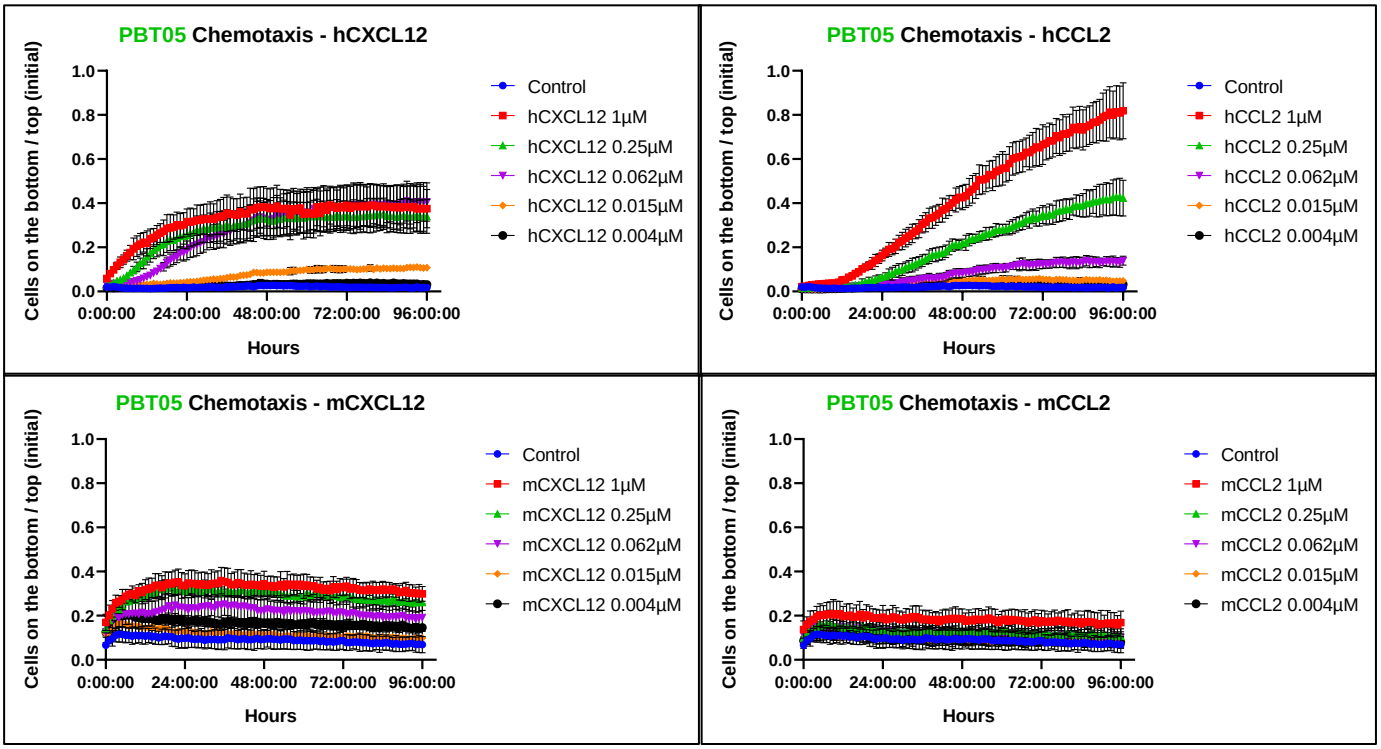
In summary, we've created a novel therapeutic combination and delivery strategy to eliminate pediatric brain tumor cells. We demonstrated classical immune cell chemokines, like CXCL12, effectively chemoattract a pediatric HGG brain tumor line. This approach synergizes with already established methods of antibody-mediated opsonization and blockade of macrophage checkpoint signals, like CD47, that are often over-expressed on the surface of tumor cells. Delivering these factors directly into the brain via an implantable, slow release hydrogel can recruit both immune cells and brain tumor cells into a tumoricidal environment that would have otherwise not favored immunological engagement. Our data not only demonstrate the safety and efficacy of this combinatorial approach when used in mice, but also highlights the sheer customizability of this system for a variety of uses. The modularity of the PEG-tetraBCN hydrogel chemistry could enable clinical development of multiple products to address cancers with different tumor microenvironments and/or chemokine receptor expression. Overall, these results demonstrate the feasibility of combining chemokines, MCIs and other forms of immunomodulatory factors to create a tumor cell "trap" within tissue. Given that chemokine receptors are present on many cancers and MCIs are largely universally expressed on cancers, it is possible that many tumor types could be addressed with a small number of hydrogel varieties. [150, 169, 170].

FIGURES

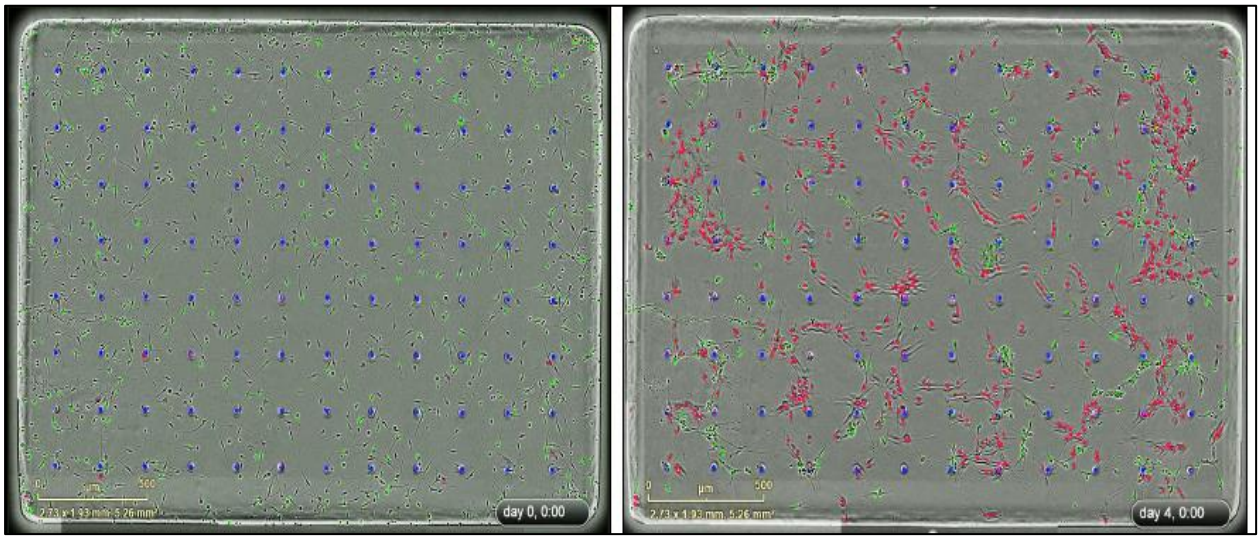
A


PBT05
DAB-mCherry

B


PBT05 Chemotaxis - hCXCL12
Cells on the bottom / top (initial)
1.0
0.8
0.6
0.4
0.2
0.0
0:00:00
24:00:00
48:00:00
72:00:00
96:00:00
Hours
Control
hCXCL12 1µM
hCXCL12 0.25µM
hCXCL12 0.062µM
hCXCL12 0.015µM
hCXCL12 0.004µM
PBT05 Chemotaxis - hCCL2
Cells on the bottom / top (initial)
Hours
Control
hCCL2 1µM
hCCL2 0.25µM
hCCL2 0.062µM
hCCL2 0.015µM
hCCL2 0.004µM
PBT05 Chemotaxis - mCXCL12
Cells on the bottom / top (initial)
Hours
Control
mCXCL12 1µM
mCXCL12 0.25µM
mCXCL12 0.062µM
mCXCL12 0.015µM
mCXCL12 0.004µM
PBT05 Chemotaxis - mCCL2
Cells on the bottom / top (initial)
Hours
Control
mCCL2 1µM
mCCL2 0.25µM
mCCL2 0.062µM
mCCL2 0.015µM
mCCL2 0.004µM

C



D

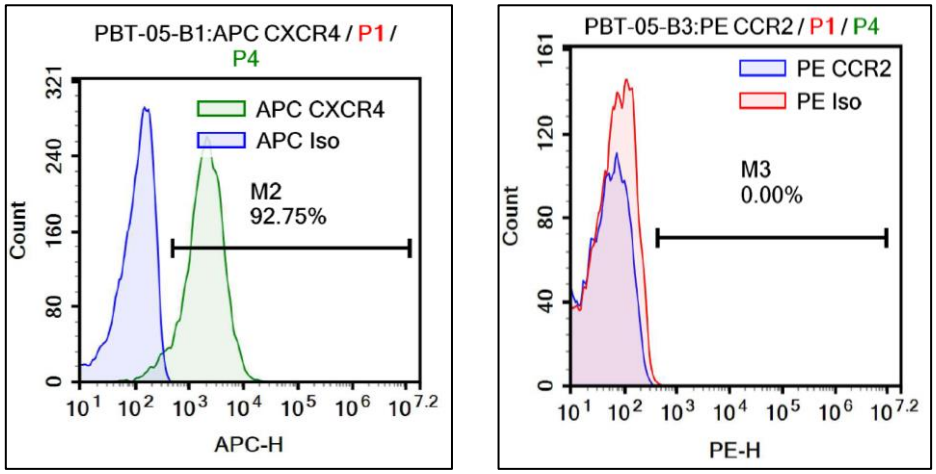
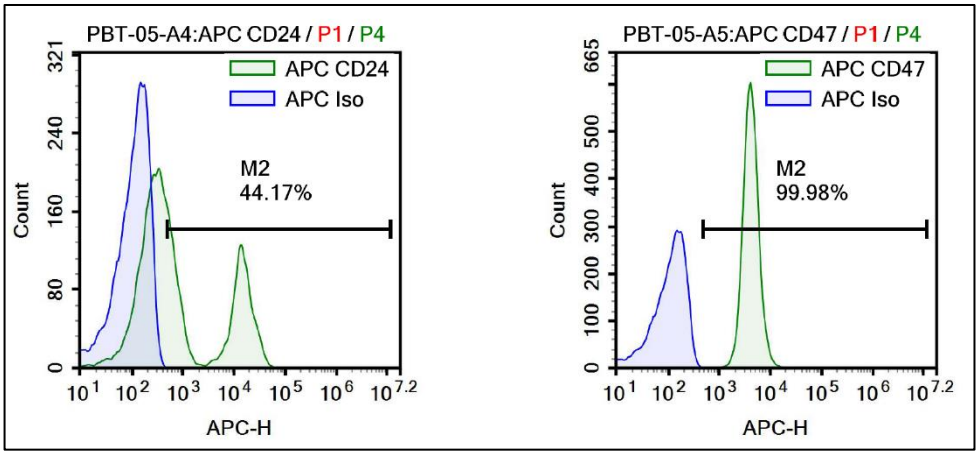


Figure 1: **Human pHGG cells are sensitive to chemokines. A**: IHC images of xenografted PBT-05, a patient derived pediatric high-grade glioma (pHGG). This tumor type is highly infiltrative in the brains of a mouse model as it was in human patients. **B**: Chemotaxis assays using PBT05 demonstrate varying sensitivity to the classical chemokines: CCL2 and CXCL12. Human variants were more potent than their murine homologs. **C:** GFP+ PBT05 cells seeded on a transwell were exposed to chemoattractants diffusing from a lower chamber. After 96 hours, many cells seeded on top migrated to the bottom, where the Incucyte software masks them as red. **D**: Flow cytometry data for the canonical receptors of CCL2 and CXCL12 revealed no expression of CCR2, but high levels of CXCR4, respectively.

A


PBT-05-A4:APC CD24 / P1 / P4
APC CD24
APC Iso
M2
44.17%
Count
APC-H
PBT-05-A5:APC CD47 / P1 / P4
APC CD47
APC Iso
M2
99.98%
Count
APC-H

B

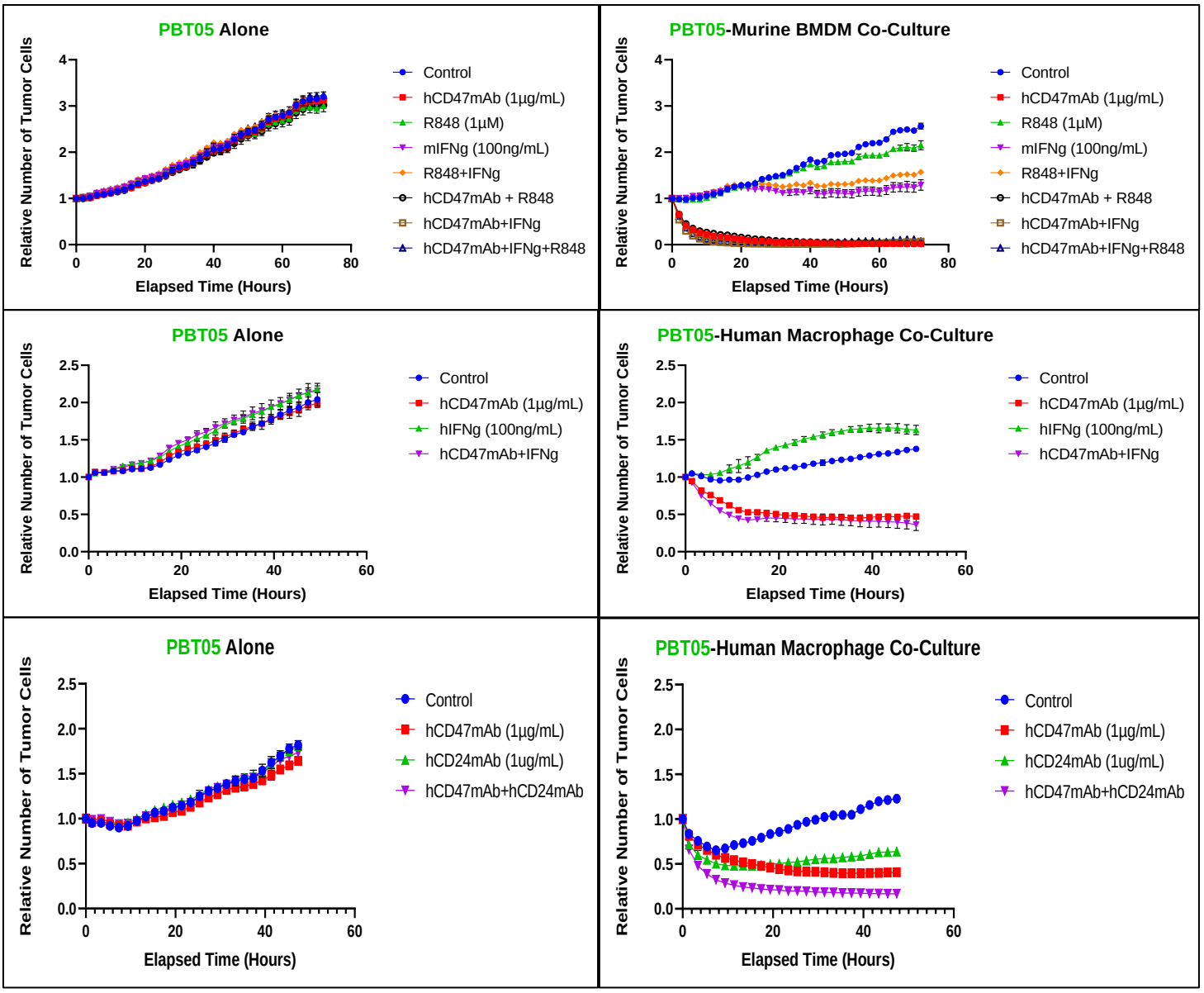
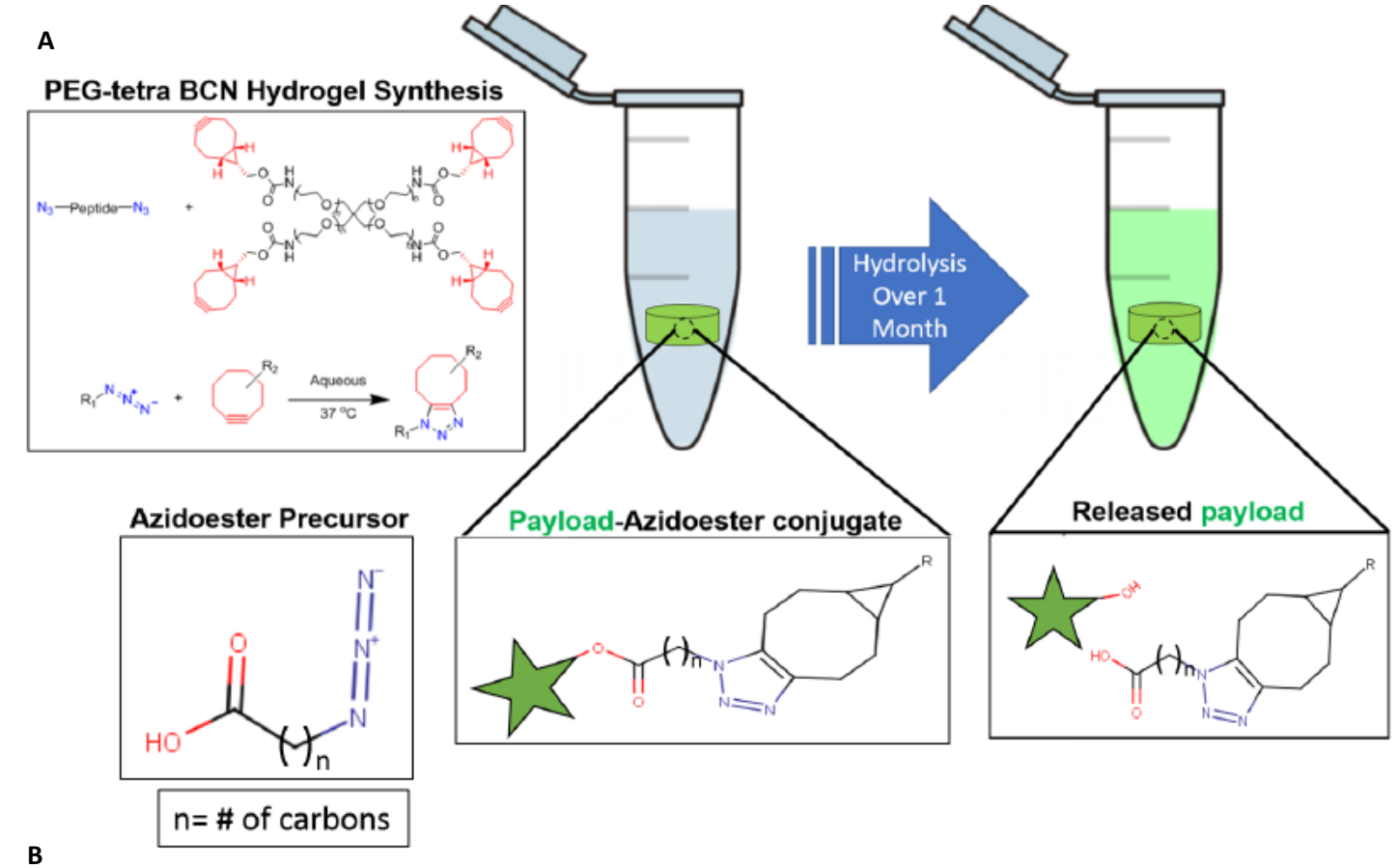

PBT05 Alone
Relative Number of Tumor Cells
Elapsed Time (Hours)
Control
hCD47mAb (1µg/mL)
R848 (1µM)
mIFNg (100ng/mL)
R848+IFNg
hCD47mAb + R848
hCD47mAb+IFNg
hCD47mAb+IFNg+R848
PBT05-Murine BMDM Co-Culture
PBT05 Alone
hIFNg (100ng/mL)
PBT05-Human Macrophage Co-Culture
PBT05 Alone
hCD24mAb (1ug/mL)
hCD47mAb+hCD24mAb
PBT05-Human Macrophage Co-Culture

Figure 2: **Blockade of macrophage checkpoints on pHGG cells was an effective strategy to induce phagocytosis by murine and human macrophages. A:** Flow cytometry of PBT05 showed high levels of the macrophage checkpoint, CD47, and moderate expression of CD24, another macrophage checkpoint. **B:** PBT05 cells co-cultured with murine bone marrow derived macrophages (BMDM) or human macrophages derived from PBMCs. Co-cultures or PBT05 alone were challenged with various immunomodulators. CD47 mAb blockade was the most effective single agent in both groups, though to a higher degree with murine macrophages. Combinations of CD47mAb and CD24mAb elicited higher degrees of phagocytosis in human macrophage co-cultures than CD47mAb alone No toxicity was observed in tumor cells cultured alone.



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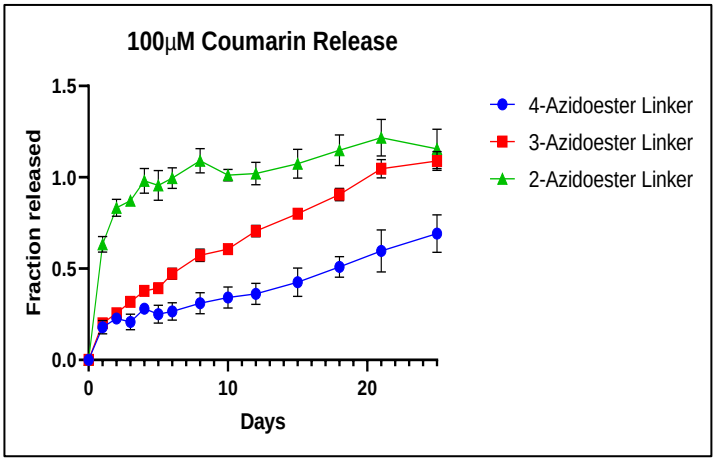
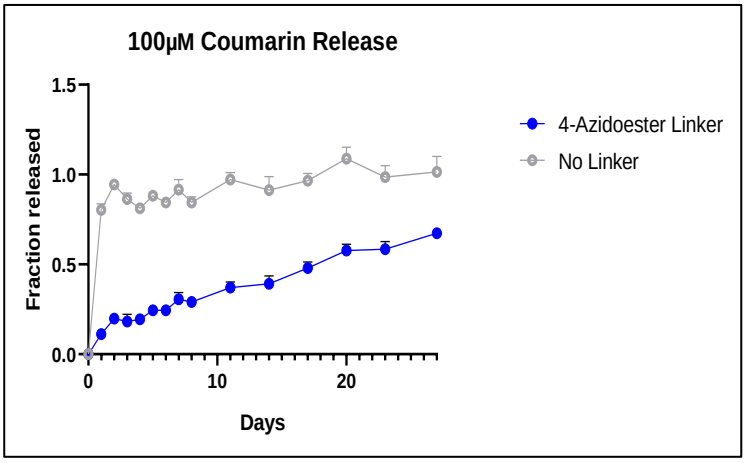




C

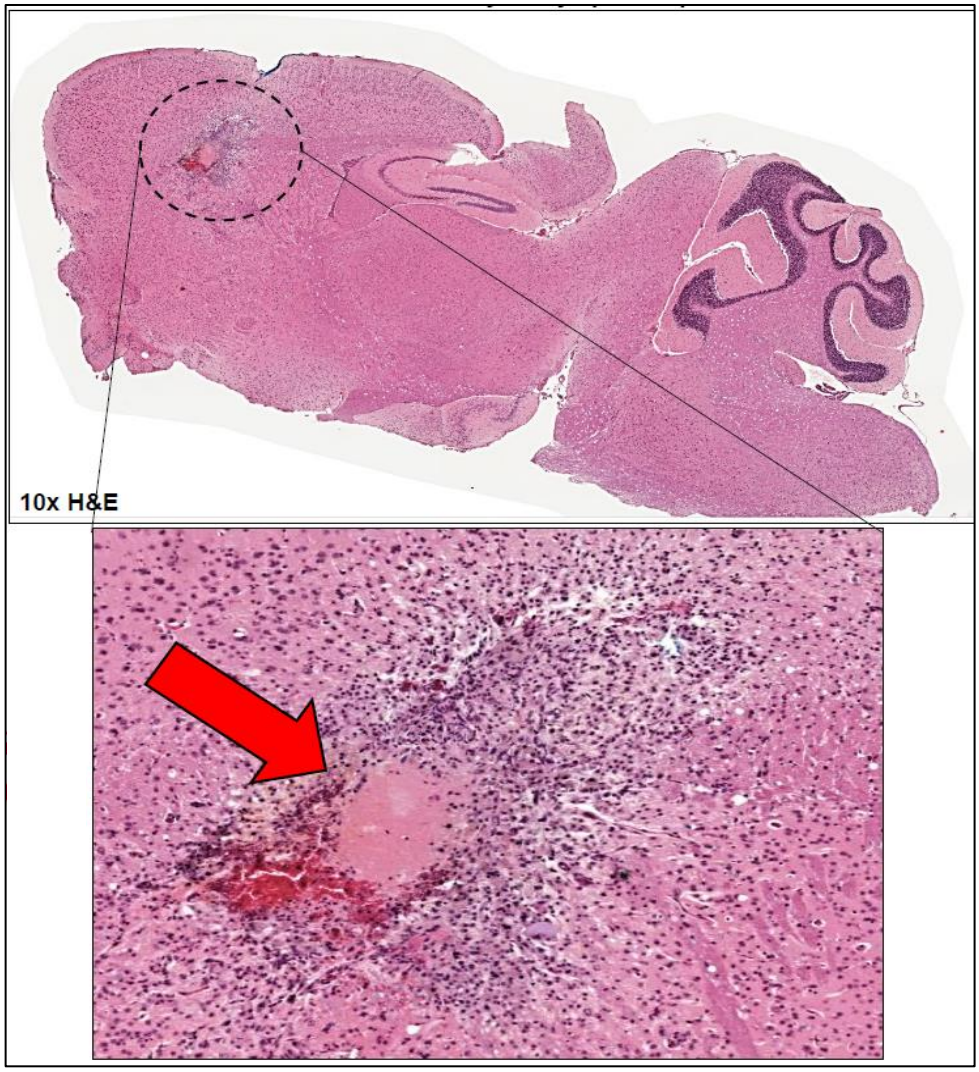
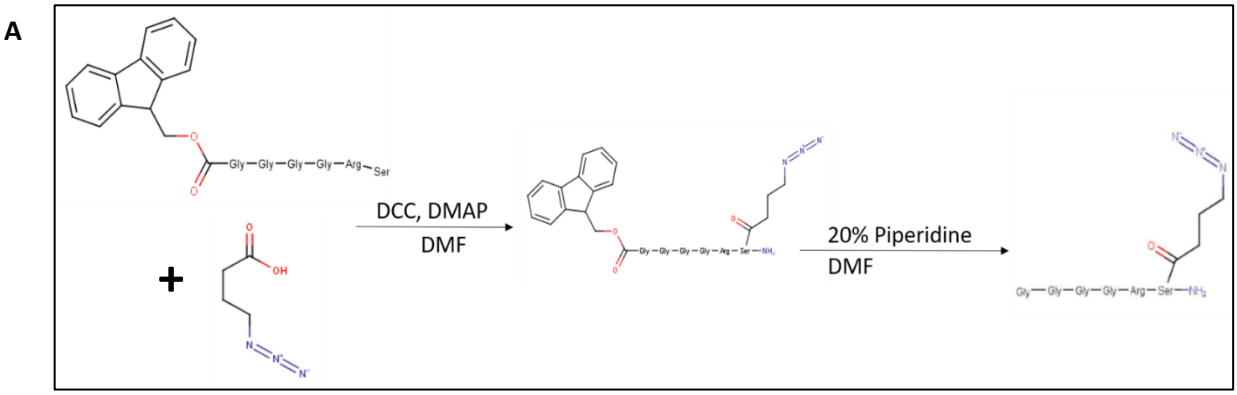


Figure 3: **Synthesizing a highly customizable hydrogel to serve as an *in vivo* delivery depot. A:** Schematic summarizing payload release from a PEG-tetraBCN hydrogel into release media. Hydrolysable azidoesters are employed as linkers to tune molecule release rates from the gel. **B:** Quantified release of a small fluorescent molecule, coumarin, over 4 weeks using various azidoester linkers. The 4-carbon linker was chosen for further experiments and significantly slows release of coumarin compared to diffusion alone. **C:** H&E stain of a murine brain containing a polymerized PEG-tetraBCN gel after being injected into the parenchyma.



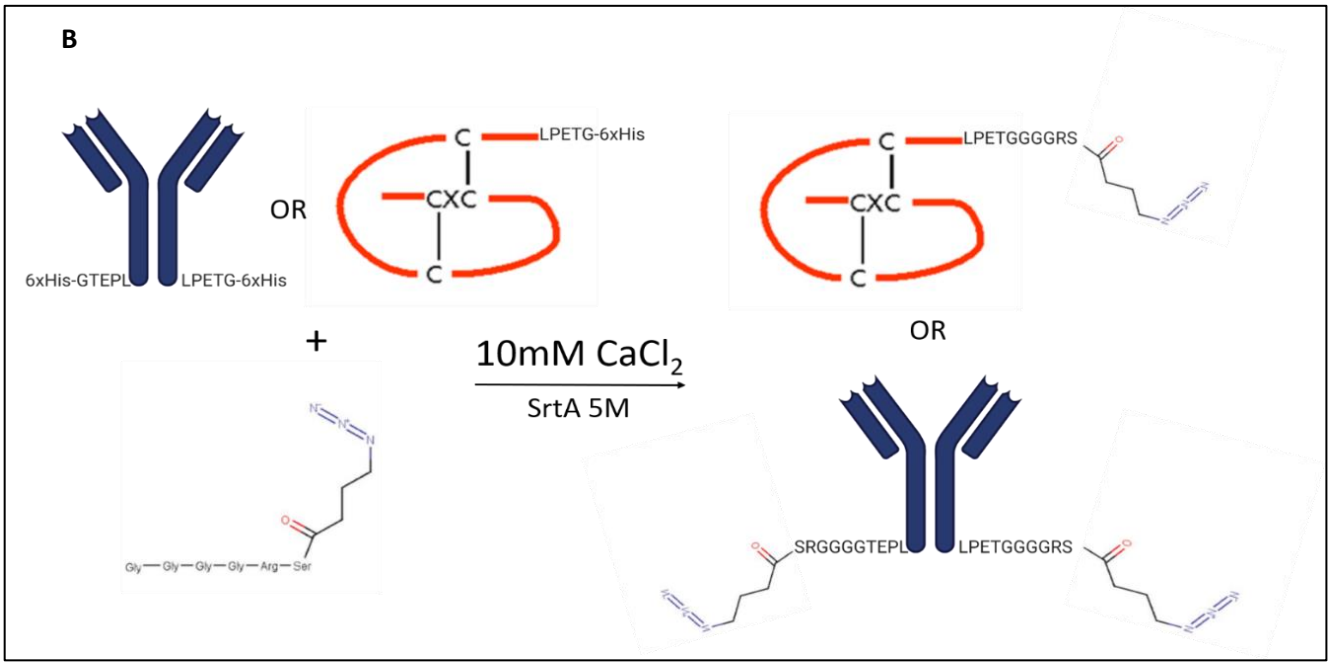
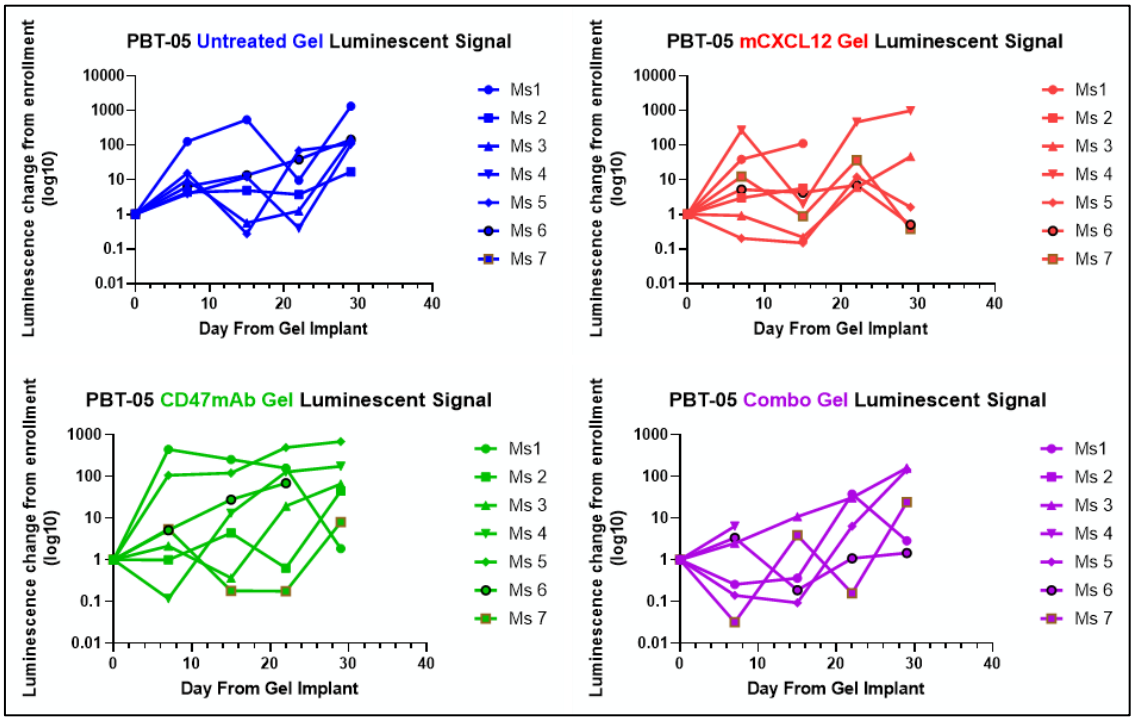


Figure 4: **Sortagging CD47mAbs and CXCL12 to hydrolysable azidoester Linkers.** Biomolecules of interest can be site-specifically sortagged to azidoester linkers for extended release while retaining biological activity. **A:** Using Steglich esterification, the prerequisite GGG-containing polypeptide needed for sortase tagging can be conjugated to the azidoacid linker. **B:** Recombinantly expressed CXCL12 and CD47mAbs can be produced with LPETG sortase recognition sites at their C-termini. Running the sortase reaction with these molecules and the Polypeptide-azidoacid conjugate will C-terminally label the biomolecule of interest with a hydrolysable linker, allowing conjugation to gels via SPAAC click chemistry.

A



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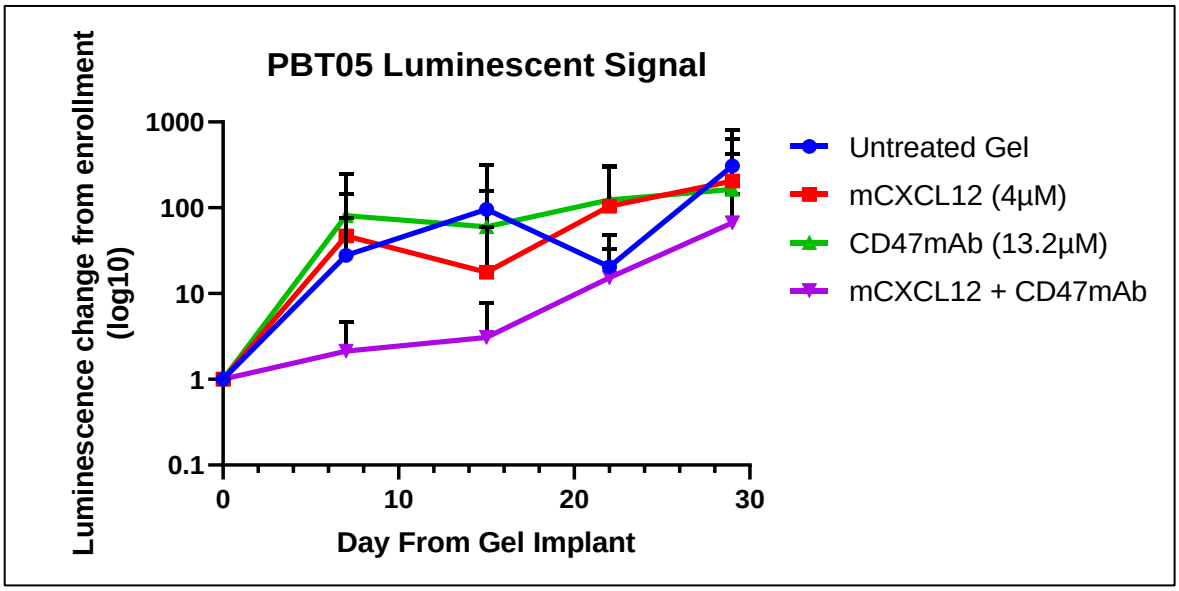

Figure 5: **Hydrogel delivery of mCXCL12 and CD47mAb into mouse brains attenuates pHGG xenograft tumor growth *in vivo.*** **A**: Quantified changes in tumor bioluminescence of individual mice receiving hydrogels from different treatment groups. Values are presented in $\log_{10}$. **B**: Averaged tumor bioluminescence values of all 4 groups of mice from part **A**. Gels treated with CD47mAb in combination with CXCL12 show arrested tumor growth over the course of two weeks. All other conditions continue growing at the same pace as mice receiving untreated gels. Error bar = SD.

MATERIALS AND METHODS

Animal and Tumor Cell Line Preparation

Patient-derived xenograft (PDX) cells were obtained from autopsy or biopsy (SCH/COG). PDX lines were cultured in Neuralcult NS-A Basal Medium (Stem Cell) with Proliferation supplement supplement (Stem Cell, 05753), PenStrep (Thermofisher), Glutamax (Thermofisher) EGF (Peperotech, AF-100-15) and FGF (Peperotech, 100-18B). Cells were grown adherent on tissue-culture treated plates after at least 2 hours of Laminin coating (Sigma-Aldrich) in an incubator at 37°C in 5% CO_2. All PDX lines were lentivirally transduced with H2b-GFP, mCherry and Luciferase to assist in cell counting and tumor size visualization via IVIS. Xenograft tumors were established in the cortex of female Athymic *Nu-/Nu-* (Harlan) mice (age). Tumors were allowed to grow to z flux value of $1e^6$ before study enrollment. All mouse studies were carried out following protocols approved by the IACUC at FHCRC (protocol 1457) and complied with all relevant ethical regulations.

In vitro chemotaxis Assays

Chemokines were purchased from: Sigma-Aldrich (CCL2: SRP3109) and RnD Systems (CXCL12: 350-NS/CF). Cell migration assays were performed using the chemotaxis module on the Incucyte Zoom 2016 and S3 (Essen Bio). Specialized 96 well transwell plates and reservoir dishes were supplied by the manufacturer (cat no's 4582, 4600). H2B-GFP+ PBT05 lines cells were cultured in the Neuralcult media + supplement without EGF/FGF to reduce background mobility caused by these growth factors. The Incucyte software was tailored to identify GFP+ nuclei to count the number of tumor cells on top and bottom of the transwell membranes.

Isolation of murine macrophages from mouse bone marrow and human macrophages from PBMCs

Murine monocytes were harvested and cultured from femurs of C57BL/6 mice using RPMI (Thermofisher, 11875093) containing 10% heat deactivated FBS and 100ng/mL mCSF1 for 7 days. Mature macrophages from these cultures were later harvested for experiments. Human monocytes were isolated from human PBMCs (Bloodworks) and purified by EasySep monocyte depletion kit (StemCell, 19355). Monocytes were cultured in RPMI containing 10% heat deactivated FBS and 25ng/mL human CSF1 for 10 days. 50ng/mL IL-10 was added to the cultures at day 5 to induce M2 phenotypes. Mature macrophages were later harvested for use in experiments.

In vitro phagocytosis Assays

Monoclonal antibodies were purchased from Bioxcell (LEAF hCD47 , BE0019) and Biolegend (LEAF hCD24mAb: 101810, MsIgG1: 400153). Phagocytosis assays were performed using the Basic Analyzer software on EssenBio's Incucyte Zoom and Incucyte S3. 12 or 24 well plates were seeded 1:1 with GFP+ tumor lines and macrophages (murine/human) in fully supplemented Neuralcult plus various MCIs and immunomodulators described previously. Using the aforementioned definitions, the Incucyte calculated tumor cell counts based on the number of GFP+ nuclei in the wells over time.

Release of Coumarin-1-OH from PEG-tetraBCN hydrogels

Coumarin-1-OH was synthesized in house and conjugated to: 2-azidoacetic acid (Click chemistry tools, 1081) 3-azidopropionic acid (Click chemistry tools, 1082) and 4-azidobutyric acid (made in house) using Steglich esterification in DCM [108]. Conjugates were then incorporated into the backbone of PEG-tetraBCN hydrogels and 25μL gels were plated in triplicate into a 12 well plate

containing 500µL PBS/well. The plate was left in an incubator at 37°C, 5% CO_2. Supernatants were taken from each well according to the experimental timepoints, read on a plate reader for fluorescence (Molecular Devices VersaMax) and added back to each well. Fluorescence of released coumarin was related to concentration using linear regression of a coumarin standard in PBS.

Synthesis of GGGGRS-4azidoesters

Fmoc-GGGGRS, was synthesized using the Liberty peptide synthesizer (CEM) with amino acids purchased from ChemImpex (Glycine: 02416, Arginine: 01964, Serine: 02454). The peptide was then HPLC purified in 70/30 H_20/Acetonitrile and lyophilized. 1.06mmol 4-Azidobutyric acid (Synthonix, A1941), 0.75mmol Fmoc-GGGGRS, 2.28mmol DMAP (Sigma-Aldrich, 8.51055) and 1.06mmol DCC (Sigma-Aldrich, D80002) were stirred for 3 hours at 40°C in minimal DMF (Sigma-Aldrich, 319937) [108]. Upon completion, the reaction was Fmoc-deprotected with the addition of Piperidine (ChemImpex, 02351) to a final concentration of 20%, stirring for 1 minute. The peptide was then precipitated in di-ethyl ether, HPLC purified using 95/5 H_20/Acetonitrile and lyophilized. Purified GGGGRS-4Azidoesters was stored at -20°C for later use under a nitrogen atmosphere.

Expression of "sortaggable" mCXCL12 and 2.3D11 CD47mAbs

The mature amino acid sequence for mCXCL12 was obtained from NCBI, GeneID 20315. The VH and VL sequences for the 2.3D11 CD47mAb was obtained from US patent 9,650,441 B2. Gblocks (IDT) were created for these sequences, modified with "LPETG" sortase recognition sites and a 6x his tag at the C-terminus of mCXCL12 and the VH C-terminus of the 2.3D11 CD47mAb. Gblocks containing these sequences were ligated into double digested expression plasmids using Gibson assembly (NEB). Assembled plasmids were transformed into chemically competent STBL3 E.coli (Thermofisher) and sequenced for accuracy. Plasmids were subsequently maxiprepped (Qiagen, 12162) and transduced into HEK293F for protein expression. Supernatants containing the molecules of interest were collected and purified via Ni-NTA pulldown and AKTA FPLC (Cytiva). See [171] for greater detail of the expression process.

Conjugation of mCXCL12 and 2.3D11 CD47mAb to GGGGRS-4azidoesters

Purified chemokines and antibodies were buffer exchanged into sortase reaction buffer (50mM Tris, 125mM NaCl, 10mM CaCl2 pH 7.5). 50µM mCXCL12 or CD47mAb was reacted with 2.5µM Sortase 5M and 1mM GGGGRS-4azidoester in 500µL SrtA reaction buffer for 2hr at 37°C in a shaking incubator [113]. 500µL of Ni-NTA (Thermofisher, 88221) beads were added to the completed reaction for 1 hour at 4°C to sequester any un-reacted antibody, chemokine and Sortase 5M. The reaction was spun at 10k G's for 2 minutes and the supernatant containing the linked chemokine/antibody conjugates was removed and buffer exchanged back into PBS for downstream use.

Bioluminescence imaging

PBT05 lines were transduced by lentivirus to express a cytoplasmic Luciferase-mCherry construct. Mice harboring Luc+ tumors were injected with D-Luciferin (Xenolight) at concentrations of 3mg/100µL PBS per mouse. 3 minutes post injection of D-Luciferin, mice were anesthetized using isoflurane for an additional 7 minutes. 10 minutes post Luciferin injection, anesthetized mice were placed in the IVIS (Perkin Elmer) chamber and bioluminescence imaging was obtained with 1 minute exposure time, F/stop 1 and 8, field D. Luminescent photos and total flux ROIs was analyzed using Living Image software (PerkinElmer).

IHC and tissue imaging

Xenograft mouse brains were harvested, formalin fixed and paraffin embedded. Brain block were then sliced and stained for DAB-GFP and DAB-F4/80. IHC sections were imaged using a TISSUEFAX slide scanner (Gnosis) in the imaging core at FHCRC.

Preparation and cortical injection of PEG-tetra BCN hydrogel solutions into Athymic Nu-/Nu- mice bearing H2bGFP+/Luciferase+/mCherry+ tumors

80uL hydrogel mastermixes were created for each treatment group at a final concentration of 3.25mM (6.5%) PEG-tetraBCN (20kDa) and 6.5mM PEG-diazide (3.5kDa). For treatment groups containing mCXCL12-4azidoester and/or CD47-4azidoester, solutions of PEG-tBCN and and the payloads were pre-reacted at 37°C for 2 hours in a shaking incubator to have time for backbone incorporation before crosslinker addition. PEG-diazide and PEGtBCN mastermixes (+/-mCXCL12-4azido, +/-CD47mAb-4azido) were placed on ice in separate tubes. When ready to be used, both tubes were combined, vortexed, and brought up to a final volume of 80µL in PBS and placed back on ice to slow the polymerization rate of the now-forming gel network. 3µL of complete hydrogel mastermix was quickly loaded into a pre-silanized 10µL Hamilton Neuros syringe (Hamilton, #65460-05) and injected cortically into isoflurane - anesthetized mice. The needle stop was set to 2mm of depth and the still-liquid master mix was administered into a cavity created by the lateral movement of the needle within the brain. Gels were targeted to the same location in the brain as the original tumor implant, as indicated by a depression in the skull from the initial implant surgery.

Chapter 7

Conclusions and Future Directions

Over the course of this thesis project, I have demonstrated the combinatorial efficacy of locally released chemokine gradients and blocking antibodies to CD47 into the brains of nude mice bearing pediatric HGG tumors from a hydrogel depot. Based on IVIS luminescence data, the combination of mCXCL12 and CD47mAb demonstrated slowed tumor growth for multiple weeks when compared to antibody treatment alone. This novel therapeutic combination and delivery strategy creates an immunotherapy "trap" that may be useful for safely recruiting and eliminating residual cancer cells in patients with incompletely resected brain tumors. There are several follow up questions these studies do not address that could further enhance the effect we currently see.

One of the first expeirments to conduct would be to determine how far the chemokine gradient is extending the tumoricidal effect of CD47mAb released from implanted hydrogels. One method of answering this question would be to implant these chemokine-bearing gels into mouse brains as we've previously done and allowing the payload to release over the course of a few weeks, then staining the fixed brain with an anti-CXCL12 mAb. From there, we can determine roughly the distance the chemokine is traveling away from the static gel source. Along with those studies, we would also want to know how far a pocket of migratory tumor cells will travel to reach the gels. Migratory tumor cells would need to be placed in a pre-determined location in a mouse brain (or higher order animal) and, using stereotactic apparatus, chemokine-laden gels would need to be placed at varying distances away from the tumor implant site. From there, IHC of the brains should reveal roughly how far tumor cells will travel in between the two implant sites. Combined, these two pieces of data may allow us to form an idea of how large of an effective radius these gels have at a particular chemokine concentration. I can imagine using this data to strategically place multiple hydrogels within the brain whose combined effective radii may lure a far greater infiltrative tumor cells distances than one single implant.

Another experiment to conduct would be identifying the complete profile immune cell infiltrate that can be co-recruited towards the gel site in addition to tumor cells and macrophages. CXCL12 and other chemokines are known as broad immune cell recruitment signals. Using this to our advantage may create a microenvironment around the gel site that can cause a complete innate and adaptive immune response to the tumor. As this study utilized nude mice, which lack adaptive immunity, to study the efficacy phagocytic macrophages and microglia in the brain, we have no way of knowing whether cells of the adaptive immune system can participate in this technique to generate a more robust tumor clearing response, and possibly memory. Repeat studies would need to be performed with syngeneic models of mouse brain tumors with fully intact immune systems. Dosing of the CD47mAb would need to be adjusted as the entire mouse brain will become a sink for the delivered antibody, unlike our model where we are using an anti-human CD47mAb against human tumors within a mouse. With that solved, histochemical staining for B-cells, T-cells and other immune infiltrate should be performed on brains receiving combination chemokine and mAb-laden gels. Our use of an antibody that recognizes CD47 on human tumor cells, but not on mouse brain cells could also underestimate toxicity to normal brain, so additional studies to assess potential on-target/off-cancer toxicity will also be necessary.

As mentioned within the introductory chapters, brain tumors are but one of many tumor types that are responsive towards chemokine gradients. As there are over 20 different types in total, this concept of luring tumor cells into an immunological “trap” may extend to many other cancer types. Just to name a few, breast, lung and melanoma metastases also tend to over-express chemokine receptors for ligands found at their pre-metastatic niches. We may be able to utilize this system to capture and eliminate those cells if we strategically implant gels around the surgical cavities that secrete the appropriate chemokine gradient for the tumor cells we’re trying to capture. As the body cavity is not nearly as sensitive to immune cell infiltrate and activation, we may also add other immunomodulatory factors, such as TLR agonists, to further enhance the tumoricidal effect of CD47 mAb blockade.

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