Toxoplasma gondii infection supports the infiltration of T cells into brain tumors.

Yen Thi Minh Nguyen
Alameda, CA

Bachelor of Arts, Major of Integrative Biology, University of California, Berkeley, 2013

A Dissertation presented to the Graduate Faculty of the University of Virginia in Candidacy for the Degree of Doctor of Philosophy.

Department of Microbiology, Immunology, and Cancer Biology

University of Virginia, School of Medicine
July, 2024

TABLE OF CONTENTS

Abstract

Acknowledgements

Chapter 1: Introduction

1.1. Overview of treatment approaches for primary brain tumors.

1.1.1. Current standard of care for malignant primary brain tumors exampled by glioblastoma and medulloblastoma.

1.1.2. Targeted therapies for primary brain tumors.

1.1.3. Challenges with targeted therapies.

1.2. Immunotherapy – the new frontier against cancers.

1.2.1. Discovery of cancer immune surveillance

1.2.2. Major Immunotherapy approaches.

a) Non-cellular therapies.

b) Cellular therapies.

c) Microbial therapies.

1.2.3. Results from notable clinical trials that test the efficacy of immunotherapies for primary brain cancers.

1.3. Hurdles for T cell therapies in primary brain cancers.

1.3.1. The immune landscape in the brain at physiological state.

a) The Blood-Brain-Barrier (BBB).

b) Microglia.

c) The brain meningeal borders.

1.3.2. The immune landscape of primary brain tumors.

a) The BBB of brain tumors.

b) At baseline, primary brain tumors are T cell-quiescent and potentially lack active myeloid cell infiltration.

1.3.3. The immunosuppressive barriers present in primary brain cancers.

a) Brain tumors lack support for T cell recruitment and entry.

b) Brain tumors lack support for T cell activity.

c) The brain is sensitive to inflammatory changes.

1.4. *T. gondii* as a potential biological agent to remodel brain tumor environment for T cell therapies.

1.4.1. Overview of *T. gondii* immune biology in intermediate mammalian hosts.

a) *T. gondii* life cycle in intermediate hosts.

b) Type II *T. gondii*, the most prevalent strain in humans, induces mild to asymptomatic infection.

c) *T. gondii* infection is controlled by Th1 immune response.

1.4.2. *T. gondii* are genetically tractable.

1.4.3. *T. gondii* infection induces T cell response to the brain.

a) *T. gondii* naturally disseminate to the brain.

b) In the brain, while *T. gondii* cysts are immune-quiescent, T cell recruitment is observed during early chronic infection.

c) Concerns about *T. gondii* as in brain cancer context.

1.5. Overview of this dissertation.

Chapter 2: *T. gondii* infection transformed the T-cell deserted Shh-Medulloblastoma into a T-cell supportive state with robust infiltration of functional T cells into TME.

2.1. Introduction

2.2. Results

2.2.1. *T. gondii* infection was well-controlled in Medulloblastoma-bearing mice.

2.2.2. *T. gondii* infection efficiently recruited functional T cells into Medulloblastoma.

2.2.3. *T. gondii* infection's locally elevated IFNγ remodeled Medulloblastoma into a T-cell accessible and supportive state.

2.3. Discussion

2.4. Methods

Chapter 3: Future Directions.

3.1. Immune activities of myeloid cells in the tumor could be further augmented to support T cell response in brain tumors during *T. gondii* infection model.

3.1.1 Hypothesis, rationales, and preliminary data.

3.1.2 Proposed experiments.

3.2. Evaluate whether a self-terminating *T. gondii* upon bradyzoite differentiation could induce a similar degree of T cell entry in brain tumors.

3.2.1 Hypothesis, rationales, and preliminary data.

3.2.2 Proposed experiments.

3.3. Additional future directions for consideration.

Concluding Remarks

References

Abstract

T cell presence in the tumor microenvironment (TME) is recognized as a major prerequisite to enact the efficacy and clinical benefits of T cell-based immunotherapies. However, few T cells infiltrate into primary brain tumors hampering the effectiveness of immunotherapy. Notable reasons contribute to the poor T cell presence in primary brain tumors include the lack of molecular cues to recruit and retain T cells into the tumors as well as an inhospitable environment to sustain T cell activity. But the presence of the blood-brain-barrier hinders effective delivery of immune-adjuvant such as pro-inflammatory cytokines and CAR-Ts into tumor parenchyma. Furthermore, the brain's sensitivity to inflammatory alterations presents additional complications to building an effective and tolerable Th1 immune response in brain tumors.

Microbial based therapies have played an instrumental role in the history of cancer immunotherapy, from the discovery of Coley's Toxins to the development of Bacillus Calmette–Guérin intravesical therapy for non-muscle invasive bladder cancer. Among the currently explored microbial agents is *Toxoplasma gondii (T. gondii)* with intratumoral injection of engineered short-lived *T. gondii* strains yielded clinical benefits in several preclinical solid tumor models. However, in addition to the ability to enact Th1 immune response as an intracellular pathogen, *T. gondii* can naturally disseminate to the brain and establish long-term residence in the mammalian intermediate hosts (including humans and murine) as part of the as the long host-pathogen co-evolution. In the brains of the immune-competent hosts, the pathogenic form of *T. gondii* are quickly cleared and controlled by the same Th1 immunity as in the peripheral tissues, forcing *T. gondii* entering a slow-growing form within the immune-quiescent cysts. Given the ability of *T. gondii* to recruit Th1 T cells in a well-tolerated manner in the brain of infected hosts, I hypothesize that *T. gondii*'s immune-modulatory capabilities in the brain could be harnessed to address the immune desert challenge of primary brain tumors.

As a proof-of-principle study on *T. gondii*'s potential as a "bug-as-drug" candidate in the context of primary brain tumors, I set out to address three fundamental questions. First, given that brain tumors have been shown to induce systemic immune suppression, would brain tumor presence severely hinder the host's ability to control infection with intracellular microbe such as *T. gondii*? Second, considered that the TME is much more immune-suppressive than normal brain environment, can *T. gondii* infection overcome this barrier and induce effective T cell response? Thirdly, how does *T. gondii* infection make the TME more amenable for T cell arrival and activity?

Using a mouse genetic model for medulloblastoma that confers intact immune system and brain TME, we found that *T. gondii* infection did not cause severe health issues in tumor-bearing mice. TME suppressiveness posed minimal barrier to *T. gondii* induced T cell response with the number of functional T cells detected the tumor mass similar to normal cerebellar regions of infected wild-type mice. Lastly, *T. gondii* infection led to myeloid cell reprogramming toward a T cell-supportive state. The study provides a concrete foundation for future studies to take advantage of the immune modulatory capacity of *T. gondii* to facilitate brain tumor immunotherapy. Overall, this first-of-a-kind in-vivo study demonstrates that *T. gondii* challenge can remodel brain tumors into a T-cells accessible state without causing overt damage to the brain tumor-bearing hosts.

Acknowledgements

I am incredibly fortunate to receive my doctoral training in the Biomedical Sciences Graduate Program (BIMS) at the University of Virginia (UVA) School of Medicine. From the early days of entering the umbrella program to the rotations and finding my thesis lab, the faculty and administrative staff demonstrated their dedication to supporting trainees. I appreciate the institutional Cancer Training Grant for helping me substantiate my scientific knowledge about cancer immunology through courses such as Tumors and the Immune System. I also appreciate the collegial environment at BIMS, which encourages students to pursue interdisciplinary research. The opportunities to interact with the research peers from the Center for Brain Immunology and Glia (BIG) or other cancer researchers at the Cancer Center seminar and Cancer Immunology group meetings were essential for my scientific growth. I also would like to thank the Department of Microbiology, Immunology, and Cancer Biology for their support toward students. The department retreat and the seminars were great opportunities for students to exchange scientific ideas and build professional bonds with department members. To my thesis committee – Dr. Roger Abounader (chairperson), Dr. Melanie Rutkowski, Dr. Tajie Harris, and Dr. Sarah Ewald – I'm grateful for their time and input in each meeting.

Most importantly, I would like to thank my mentor, Dr. Hui Zong, for supporting me throughout the past years. You taught me the values of critical thinking, resilience, kindness, and wisdom to lead a research project from its inception to its publication. From designing a concrete experiment to embracing a rejected hypothesis, from seeking peer critique to building a reasonable scope of work – the valuable skills would transcend other aspects of my life. I'm filled with gratitude for the opportunity to perform my thesis work under your guidance. Beyond the scientific and professional development, I appreciate your caring about the student's well-being and personal growth. Thank you for the time and effort you invested in me during my Ph.D. journey.

I also feel blessed to have met and worked with Dr. Sarah Ewald and Dr. Tajie Harris during this training period. This project was only feasible with their collaborative support and their lab members. Dr. Ewald's expertise in *T. gondii's* immunobiology helped me shape many essential scientific foundations for the project. In addition to the ease of accessing her for scientific input, her support during the manuscript revision was vital for its success. I appreciate Dr. Harris's insights on brain immunology, especially regarding *T. gondii* infection, which helped me grow scientific rigor and precision.

I want to thank Dr. Xiao-Yu (Harry) Zhao from Dr. Ewald's lab for his instrumental help throughout the project. In addition to coordinating with me throughout the many rounds of *T. gondii* infection, Harry was a natural scientist who helped me see where an experimental design could be improved. Scientific exchange with him eased my learning about *T. gondii's* immunobiology. I also thank Lydia Sibley, Dr. Piotr Przanowski, and Dr. Michael Kovacs for their project support. Lydia demonstrated the indispensable value of having a peer network to lean on. In addition to the experimental support, her input on the early draft of the manuscript and thesis allowed me to refine my scientific writing. I appreciate Piotr for his ingenious skills in bioinformatic analysis. Without his help, we would not have been able to dissect the transcriptomic data for the project concretely. Thank you, Mike, for teaching me about flow cytometry and explaining the different brain meningeal layers. I also appreciate other collaborators who contributed to the project, including Dr. Shengyuan Wang, Dr. Wenjie Liu, and Dr. Andrea R. Merchak. Thank you for providing the scientific expertise from your field to address related questions in the project.

I also want to thank my friends who supported me along this journey. Thank you, Dr. Tiffany T. Terry and Dr. Jianhao Zeng, for your peer support and advice during the first few years in the lab. Your scientific spirit and determination spoke through your actions and helped set examples of how to advance in graduate training. Thank you, Dr. Ying Jiang, for the animal training and colony support during the COVID period. Without your help, this animal research work would have taken much longer. Thank you, Dr. Xian Zhou, for the immunology input on my flow cytometry data and other professional advice. You showed me the importance of community in research. To Alexys and Elaina, meeting and getting to know you during this journey has been a great pleasure. Not only being wonderful lab mates, but your positive outlook and receptivity to constructive feedback demonstrate the core value of constant refinement in a research scientist.

Lastly, I thank my parents, Mr. Liem T. Nguyen and Dr. My T. Lam. Your unwavering support for me and my older brother has been the backbone of our success. The sacrifice you made during our teenage years allowed us to cultivate personal and professional growth and enabled us to adapt to a new culture and country. You showed us how contributing to society and others is the best way to receive and develop ourselves.

Chapter 1: Introduction

1.1 Overview of treatment approaches for primary brain tumors.

1.1.1. Current standard of care for malignant primary brain tumors exampled by glioblastoma and medulloblastoma.

Primary brain tumors affect approximately 1,323,121 individuals in the United States [1], with the annual incidence for malignant brain tumors being ~7 per 100,000 individuals [2] and ~25,000 new cases expected in 2024 [3]. Of these tumors, glioblastoma is the most prevalent intracranial brain tumor in adults, while medulloblastoma is most common among pediatric patients. Approximately 49% of diagnosed malignant brain tumors in adults are glioblastoma [3], while medulloblastoma accounts for up to 20% of all childhood brain tumors [4].

Current standard treatment against malignant primary brain tumors is usually multimodal and involves three major approaches, including surgical resection, radiotherapy, and chemotherapy. Of the treatments, surgical resection is the preferred first-line intervention to reduce the tumor burden. Advances in MRI and fluorescence technologies, such as 5-aminolevulinic acid (5-ALA), an FDA-approved fluorescent probe that helps delineate tumor regions from normal brain regions, have helped neurosurgeons optimize the maximal safe area of resection [6]. However, challenges remain for the surgical approach. For instance, lower-grade glioma [5] and diffusive glioma [6] uptake 5-ALA less. Reactive astrocytes in nearby non-tumor areas could also uptake 5-ALA nonspecifically [6, 7]. Additionally, maximal resection of all tumor areas may not be possible. Tumors in eloquent areas, such as the language center, or deeper brain regions, such as the brain stem [12], or highly diffusive tumors are generally surgically inaccessible [13]. In all, while surgery in conjunction with 5-ALA offers immediate relief of tumor mass, it may not be applicable for all brain tumors and cannot guarantee complete removal of all cancer cells.

Along with surgical resection, radiotherapy, and chemotherapy are part of the standard treatment regimen for glioblastoma and medulloblastoma. A session of radio-chemotherapy followed by a course of chemotherapy alone as adjuvant therapies are integrated to enhance clinical efficacy [7-9]. With newly diagnosed glioblastoma, the standard regimen is six weeks of focal irradiation (2 Gy per day, 60 Gy in total) in concurrence with daily temozolomide, followed by six cycles of temozolomide alone. Temozolomide is the gold-standard chemotherapeutic agent for glioblastoma because it effectively crosses the blood-brain barrier and reaches the tumor cells [10]. For medulloblastoma, standard chemo-adjuvants include Vincristine, Cisplatin, and Cyclophosphamide [8-10].

Despite the combination of multiple treatments, malignant primary brain tumors remain among the most challenging tumors, being one of the leading causes of cancer mortality with a risk of treatment sequelae. The estimated 5-year survival rate for primary brain cancers is approximately 30%, compared to >90% for melanoma and 77% for kidney and renal cancers [4]. Tumor recurrence is inevitable for glioblastoma patients, and the current 5-year survival rate post-standard treatment is 9.8% [3]. For medulloblastoma, the 5-year survival rate post-standard treatment is between 50-90% [4]. However, significant sequelae, such as cognitive and neurologic decline or growth hormone deficiency, are present in up to 80% of survivors [9, 11, 12]. Additionally, early exposure to radiotherapy to treat pediatric brain tumors has been shown to increase the risk for secondary radiation-induced gliomas [13, 14].

1.1.2. Targeted therapies for primary brain tumors.

Beyond the traditional approaches of surgery, radiotherapy, and chemotherapy, an improved understanding of the tumor genomic landscape has brought new clinical efforts against primary brain tumors.

These approaches involve small inhibitors to target the molecular pathways tumor cells demonstrate dependence for their proliferation.

According to the 2021 World Health Organization (WHO) classification, the genomic landscape of glioblastoma is noted with unaltered Isocitrate dehydrogenase 1 (IDH1), unlike astrocytoma and oligodendroglioma [15]. Frequently mutated genes include mutations in *TERT* promoter and *TP53*, *PTEN* loss, alterations in *PIK3CA* and *PIK3R1*, and amplifications in *Epidermal Growth Factor Receptor* (*EGFR*) and *Platelet-Derived Growth Factor Receptor-alpha* (*PDGFRA)* [16]. The events underline glioblastoma's oncogenic dependence on the signaling pathways. Correspondingly, notable targeted therapies include EGFR inhibitors, PDGFR inhibitors, and mTORC1 inhibitors [17].

Considering 40% of patients exhibited *EGFR* amplification [18] and ~11% of patients have *PDGFRA* amplification [19], targeting these receptor tyrosine kinases (RTK) has garnered significant interest. Against EGFR, including *EGFRvIII* mutation that causes the receptor to stay constitutively active, inhibitors including Gefitinib, erlotinib, and dacomitinib have been tested in at least two-phase II trials [17, 20]. Gefitinib has been approved for non-small cell lung cancer and presumedly can cross the BBB with higher doses through passive permeability [21]. Erlotinib has been shown to accumulate in the cerebrospinal fluid (CSF) [22] and has shown a curative effect on patients with non-small-cell lung cancer with EGFR-activating mutations and brain metastases [23]. Dacomitinib is a recently developed EGFR inhibitor and has shown tumor-controlling effect over EGFR-amplified ± EGFRvIII glioblastoma multiforme (GBM) cells [20, 24]. Results from the clinical trials, however, were dismal. The overall survival range from the single-arm studies was 9.1 months, 6-8.6 months, and 7.8-10 months for gefitinib, erlotinib, and dacomitinib, respectively.

For PDGFR-amplified glioblastoma, inhibitors such as imatinib, sorafenib, and sunitinib have undergone phase II clinical trials [17]. Imatinib (Gleevec) was a paradigm for targeted therapy with the complete hematological response in chronic myeloid leukemia (CML) in a phase III trial [25]. Since imatinib inhibits ABL, BCR-ABL, PDGFRA, and c-KIT [26], it could apply to PDGFRA+ glioblastoma. Similarly, sorafenib and sunitinib can block several RTKs, including PDGFRA [17], and were shown to exert anti-glioma [27, 28] or pro-apoptotic effect over glioma cells [29] in preclinical studies. Similar to EGFR inhibitors, though, trials with PDGFR inhibitors also yielded similar results, with overall survival between 5-10 months [17, 20]. Via radiographic monitoring, complete and partial response was less than 10% for these inhibitors [17, 20].

Since PDGFR and EGFR lead to the activation of the mechanistic target of rapamycin (mTOR) [30], while *PIK3CA*/*PIK3R1* alterations and *PTEN* loss occur in >25% -40% patients [16, 31], aberrant activation of mTOR signaling is heavily implicated. Between the two working forms of mTOR (mTORC1 and mTORC2), mTORC1 has been shown to regulate glioblastoma growth and proliferation [32, 33], making it a primary therapeutic interest. However, against preclinical expectations, clinical trials for rapamycin and rapamycin derivatives (rapalogs), such as temsirolimus, sirolimus, and everolimus, yielded no clinical benefit [17]. Like RTK inhibitors, the overall survival for patients who received mTOR inhibitors was <10 months [17]. While second-generation mTOR inhibitors (dual PI3K/mTOR inhibitors and mTORC1/mTORC2 inhibitors) have gone through several phase I and two phase II clinical trials, no result has yet been reported [32]. The third-generation mTOR inhibitors (bivalent molecules RapaLink-1 and RapaLink-2 [34]) bring new promise with preclinical studies detecting glioblastoma control effect [35, 36].

For medulloblastoma, since current standard therapies are associated with long-term neurological sequelae and other long-term debilitating complications, targeted therapies could offer alternative treatments [37]. Based on the 2021 WHO classification, medulloblastoma can be molecularly defined into four major subtypes: WNT-activated, SHH-activated and TP53-wildtype, SHH-activated and TP53-mutant, non-WNT/non-SHH (comprised of the formerly known group 3 and group 4 tumors) [15]. Since WNT signaling is involved in

essential physiological activities such as bone formation [38] and maintenance of intestinal stem cells [39], targeting this pathway is undesirable. On the other hand, non-WNT/non-SHH medulloblastoma have a low rate of somatic mutations, making them disadvantaged for targeted therapy [37].

Targeted therapy efforts have been attempted on SHH-activated tumors [40]. Briefly, in these tumors, various genetic mutations support the overt activity of Hedgehog signaling through activating the Smoothened (SMO) receptor and expressing its downstream effector, such as oncogenic transcriptional factor GLI1. Given the tumor's dependence on SMO and GLI, inhibiting these molecules should dampen tumor growth. However, despite SMO inhibitors (vismodegib and sonidegib) gaining approval for other cancer types and producing encouraging efficacy in preclinical studies, clinical application in Shh-medulloblastoma patients has been uncertain. While vismodegib induced tumor regression in a patient, the response was transient [41]. In a phase II study, the Overall Response Rate (ORR) for Sonidegib was 18.8% in adult patients and 0% in children [37]. Furthermore, vismodegib and sonidegib were found to have a limiting toxicity effect of inducing premature growth plate fusion in children [42]. On the other hand, while GLI antagonist (GANT)-61, discovered from a cell-based screen, demonstrated specific inhibitory activity against GLI proteins in many cancer cell lines, their instability at physiological conditions makes them not applicable as a translational therapy [37].

1.1.3. Challenges with targeted therapies.

A major significant hurdle to targeted therapies in primary brain tumors is tumor heterogeneity. In glioblastoma, an earlier attempt to classify the tumor into molecular subtypes (Proneural, Neural, Classical, and Mesenchymal) [43] was found to be impractical as multiple subtypes could be detected in the same tumor biopsy, implying the presence of intratumoral heterogeneity [44]. Another study that performed single-cell RNA-sequencing of 28 tumors and re-evaluated TCGA specimens further found that tumor cells could exist in four cellular states with plasticity and be influenced by the tumor microenvironment [44]. Most recently, ATAC-seq and Hi-C analyses, which interrogate chromatin landscape, found evidence suggesting that intratumoral heterogeneity exists at an epigenetic level and chromothripsis could be one of the earliest drivers for tumor evolution [45].

For medulloblastoma, even though these tumors can be categorized into molecular subgroups, tumor heterogeneity also exists. Re-evaluation of genome-wide DNA methylation and gene expression data with a more advanced approach, such as similarity network fusion (SNF), has found that each sub-group could be further delineated into more specific categories [46]. For example, using this method, Shh-activated medulloblastoma could be placed into four subtypes: Shh-α, Shh-β, Shh-γ, Shh-δ, Shh-ε [46]. In another study, using single-cell RNA (sc-RNA) sequencing and lineage tracing, Oscasio et al. detected a spectrum of progenitor-differentiation and stem cell markers expressed in tumors from a SHH-driven medulloblastoma mouse model [47]. Such cellular diversity could underlie the persistence of proliferative tumor cells in the mice post-treatment with targeted therapy Vismodegib [47].

The existence of tumor heterogeneity would support clonal escape from targeted drugs in multiple ways. First, tumor clones that do not strongly express the targeted molecules would be exempted from the drug's inhibitory effect. In a preclinical study with glioblastoma patient-derived tumors with EGFR inhibitor, while cells with high EGFRvIII expression were eliminated, EGFRvIII ^{low} cells survived the drug treatment [48]. Second, different cancer clones could evolve to have divergent oncogenic dependence. For example, some glioblastoma tumors have been found to express genetic alterations in both EGFR and PDGFRa [44]. EGFR, PDGFRa, and MET coactivation at protein levels were also detected in various glioblastoma cell lines and clinical samples [49]. The observations suggest that targeting individual RTK may not completely abrogate the downstream proliferative machinery of clones that are not dependent on these RTK. As expected, evaluating clinical samples from patients who developed resistance against EGFR-inhibitor found that although EGFR was

dephosphorylated and PDGFR was not highly activated, the phosphorylated Akt and mTOR levels stayed high [50].

Overall, considering the limited options of standard treatment and the poor response rate to targeted therapy attempts due to tumor heterogeneity and drug resistance, there is an urgent need for alternative therapies for malignant primary brain tumors.

1.2. Immunotherapy – the new frontier against cancers.

1.2.1. Discovery of cancer immune surveillance.

In the early twentieth century, when modern oncology was still nascent [51], Dr. William Bradley Coley conducted the first review of cases of spontaneous tumor remission that coincided with acute infections [52]. Then, using a mixture of heat-killed *Streptococcus pyogenes* (*S. pyogenes*) and *Serratia marcescens*, he showed that intratumoral injection of this cocktail into inoperable tumors could induce tumor remission in up to 50% of patients [53, 54]. Notably, transient febrile response was associated with a longer five-year survival rate [54, 55]. In some cases, tumors distant from the local injection also resolved, suggesting a systemic effect [56, 57]. However, given the lack of tools and the growing interest in radiotherapy at the time [58], "Coley's toxins" were not further pursued.

The cancer immune-surveillance concept starts to gain recognition with the discovery of T cells [59] and the development of immunodeficient mouse models such as athymic "Nude" mice [60], recombination activating gene 2 (RAG2) knockout mice [61], and others. Briefly, mice that either received a thymectomy as neonates [59] or failed to develop a thymus [60, 62] were found to have immunological defects and would readily support engraftment of human tumor cells [63]. Later murine experiments showed that, after exposure to MCA carcinogen, mature lymphocyte-deficient mice formed tumors at a much higher frequency and severity [64]. The findings corroborate the clinical observation that immunodeficient or immunosuppressed individuals have greater incidences of cancer [65].

Further studies showed that tumor immune surveillance depends on a tumor-specific T-cell response. In addition to RAG2-/- mice, other MCA carcinogenic studies using Perforin-/- [66] and IFNγ-/- mice found that Perforin-/- [67] recapitulate similar results as RAG2-/-. Although Perforin and IFNγ are effector molecules produced primarily by T cells and Natural Killer (NK) cells, *RAG2* genes are explicitly needed to generate antigen receptor diversity in T and B cells, and the NK cell compartment is intact in these mice [68]. Hence, increased tumor incidence in MCA-treated mice is due to the lack of T cells and the mouse's inability to mount tumor-specific T cell response [61].

Evaluating tumor cells has since discovered the expression of tumor-specific antigens, which could be induced by oncoviral genes (among cancers with oncogenic virus etiology) or hypermutations [69]. These tumor-specific antigens would enable the host immune system to distinguish tumor cells from normal cells, similar to self/non-self-recognition during pathogenic infection [70]. Since T cells can build immunological memory, successful establishment of anti-tumor T cell response would confer long-term tumor surveillance.

In addition to T cells, it should be noted that other immune cell types, such as NK cells, macrophages, and neutrophils, have been observed to contribute to immune surveillance. For example, NK cells have also been shown to contribute to reducing the tumor incidence in mice exposed to sarcoma-inducing carcinogens [71]. Similarly, neutrophils, the most abundant innate leukocytes, could be activated by TNF-α to express nitric oxide (NO) and control tumor growth [72]. Finally, treatment with antineoplastic agent Paclitaxel in ovarian cancer has been shown to activate macrophages in the tumor into a pro-inflammatory state via Toll-like-receptor 4

(TLR4) [73]. Mice with loss of TLR4, specifically in macrophages, lose the enhanced tumor control effect, suggesting that these macrophages are necessary for anti-tumoral response [73].

1.2.2. Major Immunotherapy approaches.

a) Non-cellular therapies.

Considering the role of T cells in cancer immune surveillance, boosting the overall T cell activities would bring immune surveillance to an optimal level. Following this principle, two primary systemic therapies have been pursued: immune checkpoint inhibitors (ICIs) to restrain the activity of inhibitory receptors on T cells and cytokine-based therapies to supplement T cells with essential signals.

ICIs are antibody-based therapies that bind the receptors on T cells or the inhibitory ligand on the target or stromal cells. T cells detected in many solid tumors have been observed to enter an exhausted or dysfunctional state, in which they lost the ability to produce effector cytokines, reduced expansion, and sustained upregulation of co-inhibitory receptors such as PD-1 and CTLA-4 [74]. Anti-PD1 blocks PD-1-expressing T cells from engaging with PD-L1 expressed on tumor cells or local antigen-presenting cells (APCs). On the other hand, anti-PD-L1 prevents PD-L1 from being recognized by T cells to abrogate the activation of PD-1 signaling in T cells. Other checkpoint receptors have been identified in T cells, including LAG-3, TIM-3, and TIGIT [75]. Clinically, anti-CTLA-4 and anti-PD1 have been approved in 18 different types of cancer [76]. Pembrolizumab and Nivolizumab (the earliest anti-PD-1 [77]) have received FDA approval for multiple other solid tumors, including melanoma, non-small cell lung cancer, urothelial cancer, and others [77]. A potential challenge for ICIs is the possibility of patients developing anti-drug antibodies due to the immunogenicity of the administered antibodies [78].

Cytokines are soluble mediators, and in the context of immunology, they work to modulate the immune response. So far, only interleukin-2 (IL-2) and interferon-α (IFNα) have received FDA approval as cancer immunotherapies [79]. IL-2 directly affects T-cell activity by helping rescue T cells from anergy, which occurs when they can neither produce IL-2 by themselves nor undergo secondary expansion after a re-encounter with antigen [80]. Anergy may occur when T cells are primed without the presence of both specific antigen (signal 1) and co-stimulation (signal 2) [80]. Anergy could also happen when immune-suppressive factors in the environment activate transcriptional factor NFAT in T cells, which would upregulate the expression of anergy-inducing genes [81]. IFNα was initially employed for its direct anti-proliferative activity over tumor cells at high doses [79]. However, its immunostimulatory property as an essential signal for dendritic cell (DC) maturation is increasingly recognized [79].

b) Cellular therapies.

Another immunotherapy approach is to deliver tumor-specific T cells in a controlled manner. To this goal, prominent approaches include Chimeric antigen receptor (CAR)-T cells, engineered T cell receptor (TCR) T cells, and tumor-lysate pulsed DC vaccines.

CAR-T are T cells that have been engineered to express chimeric antigen receptors. While physiological activation of TCR involves phosphorylation of the intracellular CD3z subunit and building an immunological synapse to recruit co-stimulatory receptors such as CD28 [82], CAR's structure contains the units that would make T cell activation more efficient. Since its intracellular domain contains the CD3z unit and co-stimulatory receptor, it can bypass a full immunological synapse [83, 84]. In addition, CAR's antigen binding domain acts like a monomer antibody that can bind surface antigens independent of the presence of major histocompatibility complex class I (MHC-I) [84]. Currently, there are six FDA-approved CAR-Ts. Tisagenlecleucel

(anti-CD19 CAR-T) produced 80% complete response (CR) with evidence of immunological memory in relapsed or refractory B-cell acute lymphoblastic leukemia (ALL) patients [85].

Another approach with direct T cell therapy is TCR-engineered T cells. These cells express a transgenic T cell receptor that would recognize selected epitopes [86]. Although the cells rely on regular T cell activation pathways, they offer other windows of advantage. As the MHC presentation pathway can sample antigens derived from intracellular proteins and cell membranes, the repertoire of targetable antigens for TCR-T is broader than CAR-T [86]. Additionally, the epitope density needed to induce TCR-T activation is lower than CAR-T [86]. Taking advantage of TSAs such as melanoma antigen recognized by T cells 1 (MART-1) and mesothelin, several clinical trials have been done with partial response and a few complete response cases observed in MART-1 [87]. However, addressing the side toxicity due to a poor differential expression of some tumor-specific antigens would be necessary.

Lastly, in addition to tumor-specific antigens, other classes of antigens have been identified that have reasonable differential expression between tumor and non-tumor tissues. These include cancer-associated antigens and cancer-germline antigens [88]. While tumor-specific antigens are not encoded in the normal host genome, cancer-associated antigens are encoded in the normal genome but either over-expressed in tumor cells or have undergone posttranslational modifications [88]. Cancer-germline antigens are encoded in the host genome, but their expression in normal adult tissues is limited [88]. As the antigens could help differentiate tumors from normal tissues, they could be used to instruct tumor immunological recognition. Autologous DC vaccine was developed following this principle. Briefly, patient-derived DCs were harvested and then incubated with either recombinant tumor antigens or patient-derived tumor lysate in conjunction with stimulatory factors such as GM-CSF ex vivo [89]. As DCs are the canonical professional antigen presentation cells, successful development of APC presenting tumor antigen would support T cell priming and initiate the immune surveillance against the cancer cells. This approach has gained momentum with Sipuleucel-T, a DC-based vaccine against prostate cancer cells, getting FDA approval in 2010 for Hormone-Refractory Prostate Cancer [90].

c) Microbial therapies

In addition to non-cellular and cellular immunotherapies, delivery of microbial agents into the tumors has been shown as an effective means to enact cancer immune surveillance. The transient fever observed in the patients treated with Coley's Toxins was due to an innate immune response against the pathogen-associated molecular patterns (PAMPs) or danger-associated molecular pattern signals (DAMPs). The local inflammatory response was a necessary step to trigger the activation of adaptive immunity against the pathogens and, along the way, the tumor cells.

Bacillus Calmette–Guérin (BCG).

Intravesical delivery of BCG for non-invasive bladder cancer was the first FDA-approved immunotherapy in cancer in 1990 [91]. In initial controlled experiments, mice with BCG pre-infection survived longer than uninfected mice post-tumor engraftment [92]. Later clinical trials in bladder cancer patients confirmed BCG's tumor-controlling effect [93, 94]. Mechanistically, local immune activation and tumor-specific CD4+ T cell response development have been identified as the main driver for the tumor-control effect. As BCG presents PAMPs to the local environment, levels of pro-inflammatory cytokines such as Interferon gamma (IFNγ) and Tumor-necrosis factor (TNF) at the tumor sites were much higher in tumor sites of treated mice [95, 96] coinciding with infiltration of APC's and lymphocytes [97]. Innate immunity helps orchestrate the actual adaptive immune effectors. BCG benefits would be abolished in athymic mice [98] and T-cell-depleted mice [99]. Earlier studies found BCG-specific T-cell expansion in the bladder-draining lymph node [100]. However, mice re-challenged with tumor cells could control them, suggesting that tumor-specific T cells were mounted

[101]. Antonelli et al. further showed that CD4+ T cells contributed more to the improved survival via IFNγ, as deleting IFNγ-R expression on tumor cells would undo the BCG effects [101].

Oncolytic virus.

Like BCG, oncolytic viruses work by triggering the innate immune response in the tumor. However, they have the additional benefit of directly killing tumor cells, given how they were engineered to have higher tropism toward tumor cells. T-VEC (Imlygic) has received FDA approval for melanoma [102]. Via preclinical studies, tumor cells co-cultured with selected viruses were found to be infected and lysed [103, 104]. Taking advantage of identified functions of essential genes in herpes simplex virus (HSV) type 1, T-VEC was engineered to harbor the selected traits. For example, *ICP34.5* deletion promotes tumor cell selectivity [105], while disabling *ICP47* enhances antigen presentation in the infected cells [106]. On the other hand, transgenic expression of human granulocyte-macrophage colony-stimulating factor (*GM-CSF*), an important growth factor for myeloid cell maturation and activation [102, 107], would support the activation of tumor-associated macrophages. Clinical evaluation of melanoma patients who received T-VEC found increased T cells and fewer Treg in the tumor lesions [108]. Mechanistically, the oncolytic virus could induce both antiviral and anti-tumoral immune responses in addition to the tumor cell-killing selectivity. Viral PAMPs would trigger the release of type 1 IFNs and DAMPs, initiating Th1 response against intracellular pathogens [109]. The immunogenic cell death of tumor cells would activate local APC while releasing viral particles and tumor-associated antigens, enhancing tumor cell recognition by the host's immune system [110].

1.2.3. Results from notable clinical trials that test the efficacy of immunotherapies for primary brain cancers.

Among the mentioned immunotherapies, only two approaches have reached phase III clinical trials in brain tumors, all were for glioblastoma adult patients. For pediatric brain cancer patients, most of the immunotherapy trials are ongoing phase I/II trials. Only on phase II with immune checkpoint inhibitor (NCT03130959) and one phase II trial with DCs vaccine (NCT00014573) have been completed [111, 112]. The phase III clinical trials for glioblastoma include checkpoint inhibitors (Checkmate 143 [113], CheckMate 498 [114], and Checkmate 548 [115]) and autologous DCs vaccine (DCVax-L [116]).

The Checkmate trials evaluated nivolumab (anti-PD-1 monoclonal antibodies) in patients with either recurrent glioblastoma (Checkmate 143), newly diagnosed glioblastoma with unmethylated MGMT promoter (Checkmate 498), or newly diagnosed glioblastoma with methylated MGMT promoter (Checkmate 548) [117]. In terms of treatment comparison per study, Checkmate 143 evaluated nivolumab's effect compared to bevacizumab, the most recently approved drug for recurrent glioblastoma. Checkmate 498 compared [radiotherapy + nivolumab] to [radiotherapy + temozolomide]. Lastly, Checkmate 548 assessed [temozolomide + radiotherapy + nivolumab] versus standard of care [temozolomide + radiotherapy] [113-115]. Temozolomide was omitted in Checkmate 498 to avoid the possibility of lymphopenia and immunosuppression. The results were dismal, with comparable median OS between treatment and control groups observed in Checkmate 143 and Checkmate 548. Median OS was slightly worse in the treatment group in Checkmate 498 [117].

Among the explanations proposed for the failure of the nivolumab trials include poor T cell presence in the brain tumor at baseline, alternative inhibitory signaling for T cells in brain tumors, and timing of administration [117]. If T cells were absent in brain tumors, then systematic activation of the T cells still cannot ensure their trafficking to the tumors [117]. As explained in section 1.3.2., assorted lines of clinical evidence have converged to the conclusion that gliomas are generally void of T cells in the untreated state. Then, there have been contradictory findings on the effects of anti-PD-1 and anti-CTLA-4 combinatorial therapy between an earlier phase I trial [118] and a preclinical study with GL261 [119]. Whereas the combo therapy worked in a preclinical model, it failed in the phase I trial, underlying a gap between the murine glioma and patient glioma. Potentially immunosuppressive signaling other than PD-1 and CTLA-4 may be present in patients.

In the DCVax-L phase III trial, newly diagnosed glioblastoma or recurrent glioblastoma patients were given either DCVax-L or standard of care post-surgical procedure. Tumor tissues from the surgical resection and the patient-derived dendritic cells were used to produce the personalized DC vaccine. Patients received a total of ten doses of DCVax-L (at 0, 10, and 20 days, followed by 2, 4, 8, 12, 18, 24, and 30 months). Among the newly diagnosed glioblastoma patients, the median overall survival from randomization was 19.3 months for the DCVax-L group compared to 16.5 months for the control group. For recurrent glioblastoma, the median overall survival from relapse was 13.2 months for the DCVax-L group versus 7.8 months for the control group [116]. Two points should be noted about clinical trial design and analysis [117]. First, the study has an exclusionary criterion, removing patients who demonstrated evidence of early progression or pseudo-progression via MRI after standard chemoradiation, making the overall survival time more extended than other clinical studies [117]. Second, in the earlier report of the study, the trial had a crossover design, with ~90% of the enrolled patients receiving DCVax-L at some point [120]. The authors supplemented an external control population for the final report to increase statistical power due to the small number of control groups [116]. It is unclear whether all confounding factors were considered for the matched-external control populations [117].

In addition, similar to the Checkmate trials, DCVax-L trial lacked a longitudinal collection of biomarkers, which would have provided essential information on the therapy's activity and result interpretation. Knowing whether tumor-specific T cells developed post-vaccination and recruited to the tumor would have shown the vaccine's effectiveness. The modest success and high rate of tumor relapse, despite nine booster doses, imply that other variables exist. Having biomarkers such as T cell expansion and activation after a vaccine would have informed if mechanisms such as cancer immunoediting emerged in the later treatment rounds.

For pediatric patients with malignant primary brain cancer, phase II trial with DCs vaccine (NCT00014573) in combination with chemotherapy was completed but published data available to date [121]. In phase II NCT03130959 trial (CheckMate 908), high-grade relapsed medulloblastoma (n=30 patients), along with other malignant brain tumors, were treated with either Nivolumab alone or in combination with Ipilimumab (anti-CTLA-4) [122]. While combination therapy demonstrated a survival trend (40% of patients not showing tumor progression after four-months), the result lacks statistical power due to the small sample size. However, both treatment regimens did not bring major clinical benefits for the other aggressive pediatric brain cancers, including diffuse intrinsic pontine glioma and high-grade glioma.

1.3. Hurdles for T cell therapies in primary brain cancers.

1.3.1. The immune landscape in the brain at physiological state.

a) The blood-brain-barrier (BBB)

The BBB is an active and selective barrier for the brain parenchyma and has been shown to restrict the paracellular movement of T cells and other peripheral immune cells, keeping them away from the brain parenchyma [123]. The BBB is primarily comprised of a layer of endothelium, a basement membrane, and the glia limitans. Unlike vasculature at the peripheral organs, the BBB endothelial cells at the capillary and postcapillary venule lack fenestrations and are connected via tight and adherens junctions. Tight junctions are formed by membrane complexes (such as occludin and claudin-1/-3/-5/-12) and Junctional adhesion molecules (JAMs) [124]. Adherens junctions are made up of cadherins and vinculin [123]. The tight junctions make the BBB endothelium a continuous membrane that blocks the paracellular movements of most macromolecules [125]. At the physiological state, while blood-dissolved gases such as O_2 can diffuse through the BBB endothelium, polar nutrients such as glucose and amino acids need solute carriers to cross the BBB [125].

Other life-sustaining macromolecules like Leptin and Insulin rely on receptor-mediated transcytosis to enter the brain parenchyma [125].

On the abluminal (brain-facing) side of the BBB epithelium, there is another cellular covering called the glia limitans, made of the astrocytic end-feet [125]. The glia limitans and the BBB epithelium produce a layer of extracellular matrix (ECM) forming the basement membrane (also called the basal lamina), which is constituted of collagen IV, laminin, nidogen, and perlecan [126]. Along with the glia limitans, the basement membrane provides additional physical structures to the BBB [126]. The BBB endothelium is maintained via multi-cellular communications. For example, exposing endothelial cells to an astrocyte-conditioned medium or co-culture of the two cell types will induce tight junction formation in endothelial cells in vitro [127, 128]. BBB of pericyte deficient mice have been shown to have increased permeability to water and a range of tracers of low- and high-molecular weights [129]. Since neurons cannot regenerate, the restricted exchange at the BBB and the multi-cellular mechanisms to reinforce its integrity reflect BBB's priority in protecting the brain parenchyma from unwanted neurotoxic materials.

b) Microglia.

As the brain resident-macrophages, microglia have three major features. First, they are the primary immune cells that occupy the brain parenchyma at the steady state. Specifically, using congenic mouse models to prevent host-versus-graft rejection and sublethal irradiation to deplete circulating hematopoietic myeloid cells, Ginhoux et al. evaluated where the transplanted myeloid cells would engraft [130]. The authors found that three months post-transplant, while donor cells make up 30% of circulating leukocytes, spleen, and dermal macrophages, they were undetectable in the brains of recipient mice [130].

Second, microglia have been found to be ontogenically distinct from the tissue-resident macrophages in peripheral organs. Through a time-course imaging of *Cx3cr1*$^{gfp/+}$ knock-in mice, which labels both myeloid progenitors and microglia, the authors found that GFP+ microglia cell-of-origin derived from the yolk sac hematopoietic progenitor cells [130]. The disappearance of adult microglia through colony-stimulating factor 1 receptor deletion (*Csf-1r*$^{-/-}$), which prevents the development of yolk sac primitive myeloid progenitors, confirmed microglia origin [130].

Thirdly, microglia's cellular state is influenced by both their ontogeny and the brain environment. In a study, Bennett et al. engrafted other myeloid lineages into the microglia deficient *Csf-1r*$^{-/-}$ mice [131]. The authors found that while bone-marrow-derived monocytes readily occupied microglia space in the brain, the two cell types are still transcriptionally distinct [131]. This cell-specific difference between microglia and infiltrating myeloid cells extends into the brain inflammatory context. In the brain with chronic infection with *Toxoplasma gondii*, infiltrating myeloid cells, not microglia, express inducible nitric oxide synthase (iNOS) [132]. From the experimental autoimmune encephalomyelitis (EAE) mouse model, while microglia would elevate expression of molecules such as MHC-II, CD80, CD86, or CD40 to contribute to the inflammatory response, the expression level of MHC-II per cell was indicated to be lower than infiltrating monocytes [133]. These observations demonstrated that despite being considered a type of macrophage, microglia do not behave similarly to monocytes in the periphery.

c) The brain meningeal borders.

While the brain parenchyma is void of leukocytes and infiltrating lymphatic vasculature, the brain borders have been discovered to be potential sites for neuroimmune interactions [134]. First, the meningeal blood vasculature is continuous to those that permeate the brain parenchyma. While the endothelium of blood vessels in the arachnoid and pia maters is connected by tight junctions, blood vessels running through the dura mater resemble those in the peripheral tissues [123]. In particular, the dural sinuses, which receive the venous

drainage from the brain vasculature, were found to express higher levels of adhesion molecules Vcam1, Icam1, and P-selectin at physiological state, facilitating interaction between homeostatic T cells and local MHC-II+ APCs [135]. Following intraventricular injection of OVA antigen, expansion of OVA-specific T cells was detected at the dural sinuses, suggesting that the sinuses-associated APCs can uptake and present cerebrospinal fluid (CSF) antigens to the patrolling T cells [135].

Second, alongside the dural sinuses is the dural lymphatic vasculature. Labeled beads and fluorescent T cells delivered intraventricularly could be detected in these lymphatic vessels and the deep cervical lymph node (dCLN) [136]. This traffic was abrogated if the dural lymphatic vasculature had been ablated or the draining lymph vessel into the dCLN had been surgically ligated. The observations highlight the connection between the dural lymphatic vasculature and dCLN. In the context of experimental autoimmune encephalomyelitis (EAE) model, removing either of the two structures delayed the disease onset and ameliorated the disease severity, implying that antigen drainage from dural lymphatic vasculature helps induce or maintain the autoreactive T cells [136]. As the exchange between CSF and the brain interstitial fluid (ISF) can happen at the choroid plexus [123] and the paravascular spaces [137], CSF drainage from the dural lymphatic vasculature could support brain immune surveillance.

Lastly, the leptomeninges is a brain location where APCs other than microglia are located, including the brain-border-associated macrophages (BAMs) and dendritic cells (DCs) [138, 139]. BAMs share the same yolk-sac ontogeny as microglia and do not get replaced by infiltrating monocytes; however, unlike microglia, BAMs are not detected in the brain parenchyma at baseline [140]. Under homeostasis, BAMs-produced matrix metalloproteinases remodel the extracellular matrix of the leptomeningeal space and help regulate the CSF flow [141]. During EAE-induced neuroinflammation, leptomeninges have been observed to be an essential site for facilitating T cell trafficking into the brain. In a T cell tracking experiment, T cells were found to transgress the vascular walls of leptomeningeal blood vessels into the subarachnoid space [142]. Engagement with leptomeningeal BAMs helped brain-reactive T cells floating in CSF slow down, reattach, and enter the brain parenchyma [142]. Conventional DCs were later found to be the primary APCs that provide antigen-specific binding and license these T cells' entry into the brain [143]. Compared to dural meninges during EAE, leptomeninges have been found to accumulate more APCs, uptake more antigens, and activate the brain-reactive T cells more readily [144].

Considering the proximity of the brain borders – the dura mater and the leptomeninges – to the brain parenchyma, it would be necessary to elucidate further their roles in other brain immunological contexts, such as pathogenic infections and tumor immunotherapy.

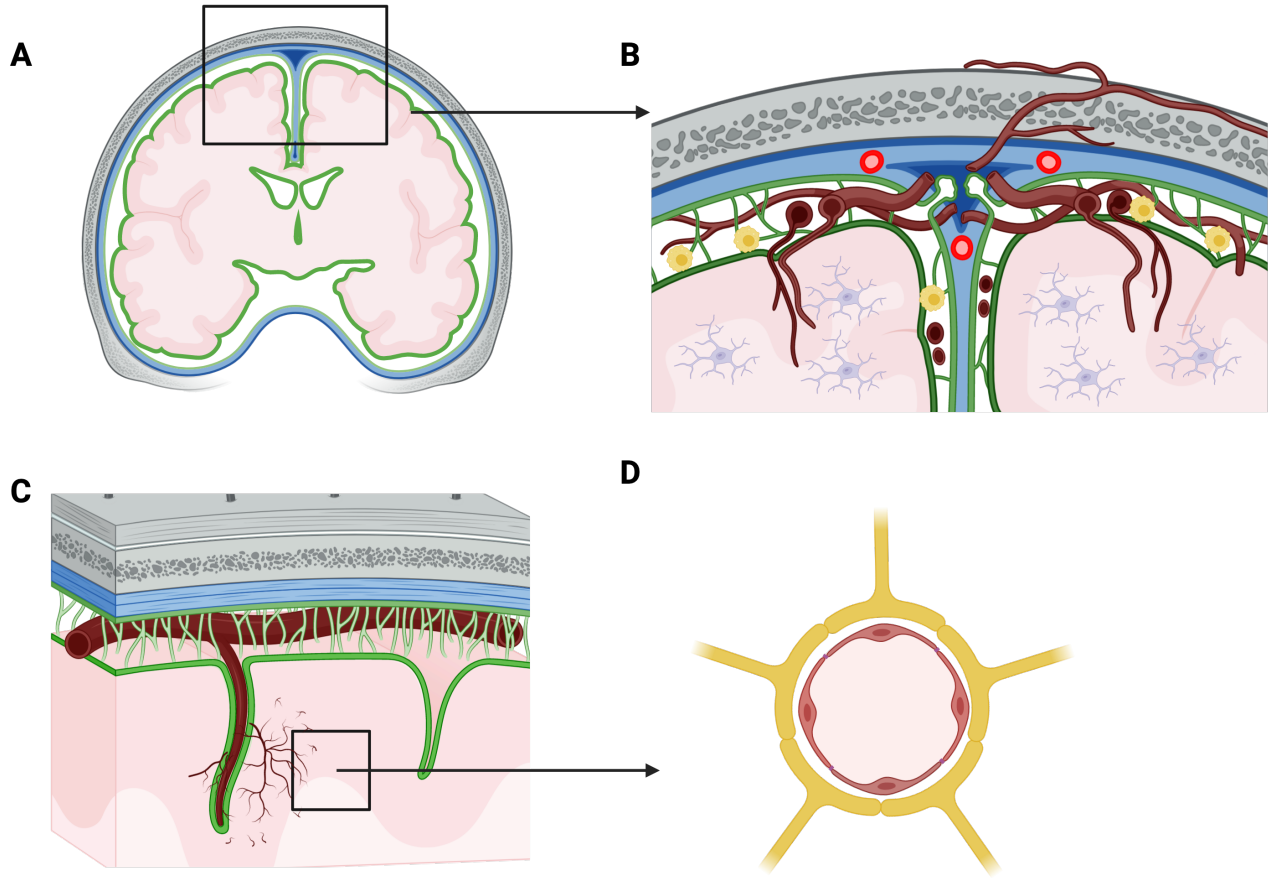


Figure 1.1: **Gross brain anatomy at baseline.**
A) Cross-section view of the brain in the coronal plane without any blood vasculature to demonstrate the location of the brain meningeal borders. The brain (pink) is enveloped by the leptomeninges (green) and suspended in CSF (white). Underneath the skull (gray) are the dura meninges (blue) and subdural space (light blue). Dural lymphatic vessels are located at the roof of the skull (boxed region).
B) Zoomed-in cross-sectional view of the superior sagittal sinus, a part of the dural venous sinuses. The dura meninges (blue) contain both blood vessels (maroon) and the dural lymphatic vessels (red). In the CSF (white), border-associated myeloid cells (yellows) are located. Microglia (light purple) are the sole immune cells within the brain parenchyma at homeostasis.
C) A representative image of the meninges is shown in the cross-section view. The brain parenchyma (pink) is covered with leptomeninges (green). Larger blood vessels (maroon) run between the pia and arachnoid mater, making up the leptomeninges (green). Blood vessels at the capillary vasculature (boxed region) do not interact with leptomeninges.
D) A representative image of the BBB at the capillary level. Endothelial cells (maroon) are covered with astrocytic end-feet (yellow). In between the two cellular layers is the basement membrane (white).

1.3.2. The immune landscape of primary brain tumors.

a) The BBB of brain tumors

Compared to the BBB in normal brains, BBB in low-grade glioma and majority of medulloblastoma subtypes is largely intact, which would deter T cell infiltration at the physiological state. Among the higher-grade glioma and Wnt-medulloblastoma subtype [145], BBB in tumor noted for heterogeneity that spans from the physical integrity of BBB to the expression profile of transmembrane receptors on the BBB endothelium. These factors contribute to brain tumor progression and present obstacles to effective delivery of drug or immune adjuvant into the tumor parenchyma.

Regarding BBB integrity, 'leakier' BBB has been observed to be more pronounced in high-grade tumors wherein Magnetic resonance imaging (MRI) detects increased levels of contrast agents in the tumors [146]. At the physiological state, contrast agents such as Gadolinium would not readily cross BBB [147]. Therefore, the accumulation of contrast agents would indicate a disrupted BBB. Clinically, contrast enhancement is rarely observed in lower-grade gliomas such as Grade I-II tumors [148, 149]. Notably, BBB leakage has been observed to varying degrees among clinical gliomas histologically diagnosed as grade II-III. For example, in a study that reviewed patients with MRI data within one month of the histological diagnosis (n=927), Pallud et al. observed that while contrast enhancement was absent in ~85% of the tumors, 10% patients showed enhancement with a patchy and faint pattern, and ~5% had nodular-like pattern enhancement [150]. In another study, Wang et al. evaluated patients with grade III anaplastic glioma (n=216) [151]. The authors found evidence that BBB disruption in this progressive glioma class was significantly higher in tumors without IDH1 mutation, a critical molecular feature for WHO classification of high-grade-glioma [151]. Notably, the enhancement pattern of tumor cases with BBB leakage ranged from ring-like to patchy and nodular. The heterogeneous pattern of contrast enhancement implies that BBB disruption does not occur at a similar rate across the tumor. Considering that the BBB is intact in lower-grade gliomas and only progressively becomes disrupted in aggressive tumors, transcellular migration by immune cells is unlikely to be supported at baseline.

Even when tumors display features disrupted BBB, evidence indicates this does not readily translate into improved drug delivery to the tumors [152]. In addition to the heterogeneity of disruption in the tumor BBB, brain tumors have been observed to overexpress ATP-binding cassette transporters, a class of efflux pump [153]. In this study, ABCA13 overexpression was also associated with decreased progression-free survival in glioblastoma patients (n=51) receiving radiation with temozolomide [153]. Via animal preclinical models, efflux pumps Abcb1 and Abcg2 [154, 155], Pgp, and Mrp1 [156] have been shown to reduce anticancer agent delivery to cancer cells.

b) At baseline, primary brain tumors are T cell-quiescent and potentially lack active myeloid cell infiltration.

The number of tumor-infiltration lymphocytes is a biomarker for patient responsiveness toward ICIs [157]. Tumeh et al. found that clinical response to anti-PD-1 treatment had significantly higher CD8-, PD-1- and PD-L1-expressing cells within the tumor or tumor margin pretreatment [158]. The findings suggest that pretreatment tumor microenvironment classification into "T-cell infiltrating tumor," "T-cell excluded tumor," or "T-cell absent tumor" would improve treatment prognosis

From bioinformatic analysis to direct techniques such as fluorescence-activated cell sorting (FACS), clinical assessments have indicated that brain tumors usually fall in the T-cell absent category. Employing MCPcounter, a gene-expression signature algorithm to quantify immune cell abundance in transcriptional data, Bockmayr et al. found that brain tumors (n=1422) and medulloblastoma (n=763) had minimal expression of lymphocytes (T cells, B cells, and NK cells) [159]. For medulloblastoma, similar result was found in a later study that evaluated the DNA methylation profile of the clinical samples [160]. Correspondingly, over IHC, Thakur et al. confirmed that medulloblastoma was essentially T cell absent in comparison to solid tumors in the periphery (Ewing Sarcoma, Osteosarcoma, and Rhabdomyosarcoma) (n=20 for each group) [161]. More robustly, in the recent phase II trial with immune checkpoint inhibitor for 166 pediatric brain cancer patients (including 30 patients with medulloblastoma), low to absent CD8+ tumor-infiltrating T cells were observed across all clinical samples [122]. For glioblastoma, via flow cytometry, Klemm et al. found that T cells were negligible in glioblastoma (n=14), as were the non-tumor brain controls (n=3) [162]. Likewise, in another study using FACS, the average % of T cells was found to be 0.79% in glioblastoma (n=5), 0.40% in medulloblastoma (n=6) relative to 0.02% in non-tumor controls [163], indicating the minimal presence of T cell in brain tumors. More recently, employing

the single-cell mass cytometry (CyTOF) approach, Friebel et al. also observed T cell absence, and the immune composition of glioma (n=22) consisted primarily of myeloid cells [164].

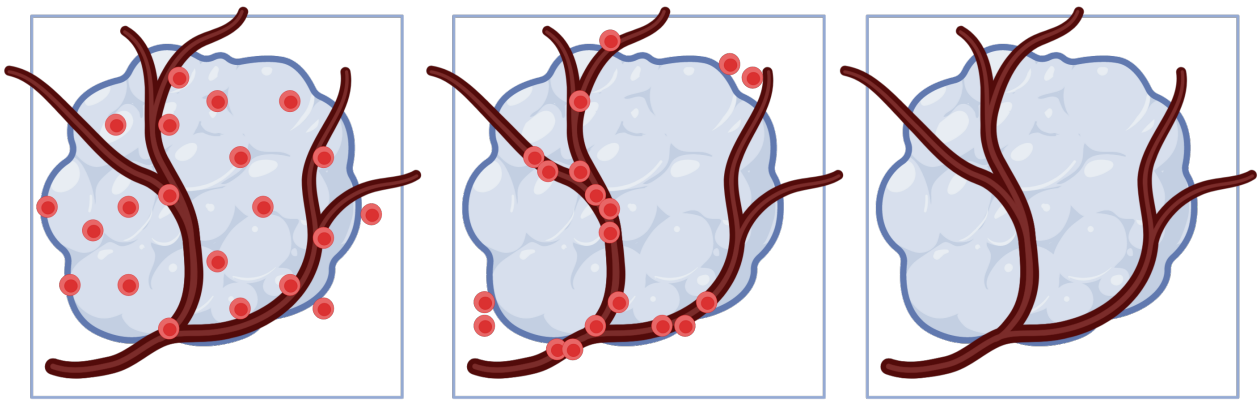


Figure 1.2: **Classification of tumor microenvironment**
A) "T-cell infiltrating tumor" is a tumor with T cells (red) present throughout the tumor parenchyma (blue) as well as at the tumor edge and tumor vasculature (maroon).
B) "T-cell excluded tumor" is one where T cells are detectable within tumor proximity but limited to the border of the tumor and not present in the tumor core.
C) "T-cell absent tumor" lacks T cell presence both in the tumor core as well as in the neighboring non-tumor regions.

In evaluating the myeloid composition in the brain, differential expression of specific cell surface markers such as CD45, P2RY12, and CD49d have often been used to distinguish microglia from peripheral myeloid cells [162]. However, such an approach may not be very reliable. For example, while microglia (CD45low) were thought to be distinguishable from monocyte-derived macrophages (CD45high), a hypoxic environment could upregulate CD45 expression in myeloid cells [165]. Hypoxic brain tumors could upregulate CD45 expression in microglia. In the study by Klemm et al. that evaluated the immune landscape of glioblastoma, purinergic receptor P2RY12, which was thought to be expressed primarily in microglia [131, 166], was also detected on monocyte-derived macrophages [162]. CD49d (integrin alpha 4) was proposed to help distinguish microglia ($CD49d^-$) from peripheral monocytes ($CD49d^+$) [162]. However, in another study, CD49d was not among the makers that stayed elevated in monocytes upon migrating into the brain parenchyma [131]. Likewise, the P2ry12 level may reduce when microglia are in an "activated" state [133]. To sum up, the expression level of surface markers may fluctuate depending on the different environmental contexts. Therefore, to distinguish the identity of myeloid cells in the brain, lineage tracing methods would be the more robust options.

Using a lineage tracing approach, our lab's previous characterization of a GEMM for medulloblastoma demonstrated that no monocytes could be detected throughout tumor development [167]. Specifically, mice with GFP-labeled medulloblastoma background (Ptch1KO/WT; p53KO/KO; Math1-GFP) were crossed with mice that can distinguish microglia from monocyte-derived macrophages (Cx3cr1-CreERI; Rosa26-LSL-tdTomato) [167]. A short Tamoxifen treatment would label microglia and circulating monocytes by inducing tdTomato expression. Since circulating monocytes have a faster turnover rate, Tdtomato+ monocytes would be cleared from the body after 28 days, while microglia retain the tdTomato expression. To avoid the possibility of Tdtomato+ monocytes infiltrating tumors, Yao et al. performed tamoxifen treatment at postnatal day 7 (P7), well in advance of the tumor initiation period (P40). The authors found that only tdTomato+ myeloid cells were detected in medulloblastoma, while the host circulating monocytes are tdTomato- [167]. The findings suggest that monocytes do not actively infiltrate brain tumors at baseline.

Overall, clinical findings suggest that, at baseline, primary brain tumors are essentially a T-cell desert. In terms of peripheral myeloid cell status, while there is no definite evidence from clinical data, lineage tracing experiment in a GEMM medulloblastoma suggests that peripheral myeloid cells do not actively infiltrate the tumor, throughout most of the tumor development period.

1.3.3. The immunosuppressive barriers present in primary brain cancers.

a) Brain tumors lack support for T cell recruitment and entry.

One of the major challenges for T cell therapies in solid tumors is poor T cell trafficking [168]. Activated T cells rely on a homing receptor to receive the recruiting cues to migrate to the target site [169]. Th1 effector T cells primarily depend on CXCR3, which recognizes CXCL11, CXCL10, and CXCL9 to be attracted to the site of inflammation [169]. The chemoattractants are often interferons-inducible and could be expressed by various cells in the local organ [170]. Then, the interaction between adhesion molecules on T cells and adhesion ligands on endothelial cells helps slow the T cells before mediating the tethering and extravasation process [170]. Although various adhesion molecules are identified, ICAM-1 and VCAM-1 have been found to elevate and contribute to T cell response during multiple brain inflammatory contexts. For example, ICAM-1 and VCAM-1 were upregulated in the BBB endothelial cells during brain infection with *Toxoplasma gondii (T. gondii)* [171] and lymphocytic choriomeningitis virus [172] as well as experimental autoimmune encephalomyelitis (EAE) [173].

Among the available clinical evidence on the baseline level of T cell-homing signals, antigen presentation activity, and general metabolic milieu, most studies were performed in the context of glioblastoma. This is likely because there has been a higher clinical urgency for improved therapy with this aggressive brain cancer. While limited, the clinical data and the absence of T cells in medulloblastoma (as discussed in section 1.3.2.) suggest that medulloblastoma likely shares a similar problem with T cell recruitment [174, 175].

For glioblastoma, the clinical consensus indicates a lack of T cell chemoattractant and adhesion molecules. In a study, via immunohistochemistry, Sharma et al. found that > 70% of astrocytoma and >50% of advanced glioblastoma samples lack CXCL10 or do not have a strong expression [176]. Via q/RT-PCR over clinical samples, Weenink et al. also observed that CXCL9, CXCL10, and ICAM-1 expression levels were undetectable in low-grade glioma [177]. Regarding adhesion molecules, via immunohistochemistry, Mäenpää et al. found weak to no VCAM1 signals in the majority of tumor vessels of brain tumors (13 glioblastoma grade IV, 7 astrocytoma grade I-III, and 7 Oligodendroglioma grade II) [178]. For ICAM-1, the expression level in tumor vessels was equivalent to the background normal brain vessels [178]. For medulloblastoma, using NanoString for spatial transcriptomic analysis, most medulloblastoma samples were found to lack T-cell chemoattractant [174]. The only exception was WNT-subtype medulloblastoma, which can induce fenestrated vasculature and accumulate higher levels of chemotherapy [145].

In addition to chemokines and adhesion molecules, antigen-specific engagement at the brain borders has been implicated in controlling T cell entry in several contexts. In an adoptive transfer EAE model, where activated Myelin oligodendrocyte glycoprotein (MOG)+ T cells were transferred into a naïve recipient, loss of MHC-II in cDCs led to a significant reduction in EAE severity [143]. At the steady state, these cDCs are located in the leptomeninges at low numbers [143]. An earlier study observed T-cell diapedesis and scanning activities in the same area prior to invading the CNS tissue [179]. These observations support the idea that cDCs have a licensing role in T cell entry into the brain parenchyma. Aside from DC engagement, antigen-specific interaction with the brain endothelium has also been shown to regulate T cell entry [180]. Using experimental cerebral malaria [181], Fain et al. found that disabling endothelial expression of MHC-I significantly reduced

CD8 T-cell presence in the brain and malaria encephalitis [181]. Following these findings, if the number of tumor-specific cDCs at the leptomeninges could be increased or if expression of tumor-antigen could be specifically induced in tumor vasculature, T cell entry into brain tumors would be significantly improved.

The lack of homing signals and APCs in local brain borders would potentially contribute to the poor dissemination of CAR-T into brain tumors. In two orthotopic brain tumor studies, CAR-T tumor control activity was found to depend on the mode of delivery, with intravenous (IV) delivery of CAR-T failing to improve animal survival while intra-ventricular (ICV) injection enacted CAR-T benefits [182, 183]. Histological evaluation of the IV-received mice found that CAR-T cells were absent in the tumors, implying BBB's poor T cell recruiting effect in brain tumors [182]. Although CAR-T delivered via ICV demonstrates a controlling effect in the studies, this would require tumor cells to be bathed in the CSF directly. Such conditions do not commonly occur in human brain tumors, but those metastasized to the leptomeninges [183].

b) Brain tumors lack support for T cell activity.

After entry, a T-cell supportive environment in solid tumors is necessary to retain and keep them activated for effective anti-tumoral immune response. From CAR-T studies, major T cell barriers in solid tumors include the metabolically hostile environment (hypoxia and low nutrient), minimal expression of antigen presentation and co-stimulatory molecules, and the development of suppressive factors (such as tolerogenic myeloid cells and CD4+ regulatory T cells) [168]. As with other solid tumors, multiple of these immunosuppressive activities have been detected in brain tumors.

In two studies updating phase I results of CAR-T therapies for recurrent glioblastoma patients, investigators found that while the therapies are generally tolerable, clinical response was either transient or did not meet the objective radiographic response. In one study, three patients received an intraventricular infusion of CARv3-TEAM-E T cells, which targets EGFRvIII while secreting T-cell–engaging antibody molecules to enable them also to recognize glioblastoma cells expressing wild-type EGFR [184]. In another study, bivalent CAR T cells, which target EGFR and IL13Rα2 expressed cells, were given intrathecally to six patients [185]. In both studies, radiographic response was observed within a few days. However, the response was transient, and tumors remained detectable for one month. It should be noted that in these advanced patients, CAR-T cells were detected in the peripheral blood, indicating that the BBB was disrupted in the advanced tumors. However, the T cell number was minimal and returned to baseline at the study endpoint. Effector cytokines (IFNy and TNFa) and CAR-T cell numbers in the CSF sharply declined 7 days post-infusion [184, 185]. Considering that CAR-T expansion is a predictive marker for therapeutic response in hematologic cancers [186], these observations imply that the brain tumor environment is actively dampening CAR-T activity and viability.

For effector T cells to exert their function, a milieu that supports their metabolic profile is essential. Unlike T cells in the naïve or memory compartments, effector T cells rely on aerobic glycolysis and oxidative phosphorylation [187]. In turn, they have a higher demand for oxygen and metabolites such as glucose in the environment. Limitations in oxygen and low glucose availability would render T cells dysfunctional [188]. However, elevation of Hypoxia-inducible factor 1 (HIF-1) has been observed in brain tumors [189]. HIF-1 is a central regulator of cellular oxygen homeostasis, and its expression is only induced by hypoxic conditions [190]. Using RNA-seq and hallmark gene sets, Kim et al. observed that hypoxia signature was more common in high-grade glioma cores than periphery regions [191]. Similarly, via qPCR over fresh-frozen medulloblastoma clinical samples, the HIF1A mRNA level was much higher in the tumors than in non-tumor match samples [175]. The evidence suggests hypoxia varies by the tumor region and could negatively impact T cell metabolic fitness.

Aside from a metabolically supportive environment, antigen-specific interaction is necessary for effector T cell's function. MHC-I engagement is required for effector CD8+ T cell's cytotoxic activities, while MHC-II is needed for CD4+ effector T cells to exert their T helper function. However, the quantity of MHC-I and MHC-II in brain tumors at baseline has been found to be poor or heterogeneous. Via immunohistochemistry, expression of beta-2 microglobulin (B2m), an invariant subunit of MHC-I complexes, has been observed to be predominantly weak (34%) or moderate (38.8%) among glioma samples (57 IDH1 wild-type gliomas; 46 other primary brain tumors). Notably, its protein and transcript levels are less in low-grade glioma samples, with strong B2m detection only observed in ~15% of these samples [192]. Similarly, Kilian et al. found that MHCII expression was heterogeneous among glioblastoma patients via in situ RNA hybridization of clinical samples and evaluation of the sc-RNAseq glioma datasets. Although primarily detected in myeloid cells, MHCII expression was spatially heterogeneous, and not all myeloid cells express similar levels [193]. Also, in these studies, lower MHC-I and MHC-II detection was associated with lower T cell presence or response to checkpoint inhibitors. Tang et al. found high B2m expression correlated with immune cell infiltration in glioma samples [192]. From patient samples that underwent neoadjuvant αPD-1 trial, Kilian et al. found that $MHCII^{high}$ tumors had a more favorable clinical course than $MHCII^{low}$ counterparts. Using the GL261 murine glioma model, the authors showed that MHC-II expression on blood-born tumor-infiltrating myeloid cells is indispensable for immune checkpoint blockade [193].

In addition to antigen presentation, T-cell activity depends on the availability of co-stimulatory molecules CD80 and CD86 in the local environment. Recognized by CD28 on T cells, activation of CD80/CD86-CD28 signaling enhances T-cell survival and lowers T cell activation threshold via mediating the lipid raft recruitment for immunological synapse [194]. However, via q/RT-PCR and IHC staining using glioblastoma samples (n=47), expression of CD80 and CD86 was found to be predominantly on the lower end of the spectrum at both transcript and protein levels [195]. In another study, CD86 was observed to be minimally expressed in grade II low-grade glioma samples via qRT-PCR and western blot [196]. Likewise, via flow cytometry, the mean fluorescence intensity (MFI) of CD80 was minimally elevated in glioblastoma samples compared to the matched patient blood control samples [197].

Other notable T cell-suppressive activities in brain tumors include the presence of Treg, lack of T cell survival cytokines, and the presence of tolerogenic microglia. In limited cases like high-grade-glioma, the tumor's BBB is dysfunctional, and T cells could be detected within the tumor [198]. Accordingly, regulatory T cell (Treg) numbers have been detected in the tumors [199]. Several preclinical studies found enhanced survival in glioma-bearing mice treated with Treg depletion [200-202], suggesting Treg is potentially an immune-suppressive factor in glioblastoma. For medulloblastoma, previous characterization from our lab's GEMM model found the level of IL-2 and IL-15, essential cytokines for T cell survival [203], to be much less in the tumors compared to non-tumor cerebella [167]. We also found that the tumor is enriched with IL-4, which programs the local microglia into a pro-tumoral state. While these microglia have the appearance of activated microglia, they do not produce pro-inflammatory cytokines. Instead, they produce IGF1, a potent growth factor for tumor cells [167].

c) The brain is sensitive to inflammatory changes

Building an effective but inoffensive inflammatory response in the brain is not an easy goal. As the organ that houses billions of neurons and responsible for complex functions, from physiological to emotional regulation and cognitive execution, the brain is sensitive to environmental changes, including pro-inflammatory response.

Acute overt inflammation has been found to be intolerable for the brain. For example, acute encephalitis is associated with poor outcomes, either a high fatality rate or permanent neurological sequelae [204, 205].

While this could be due to direct cellular injuries or cell death by the presence of the etiological pathogens, the degree of neuroinflammation also plays a role. Specifically, preclinical studies have found that when brain endothelial cells cross-present pathogenic antigens, non-specific activation of CD8 T-cells in the brain [181, 206, 207] could lead to excessive cerebral edema [208] and responsible for the high fatality rate of cerebral malaria.

Similarly, studies on the pathophysiology of immune effector cell-associated neurotoxicity syndrome (ICANS) associated with cancer immunotherapies have highlighted the importance of monitoring endothelial cell activation and the degree of BBB disruption. ICANS risk has been observed in the systemic infusion of IFNs, IL-2 [209, 210], and CAR-T [211]. In a study with 133 patients treated with anti-CD19 CAR-T, the activity of the angiopoietin (Ang)–Tie2 axis, which regulates endothelial cell activation, could inform the risk for ICANS [212]. At homeostasis, Ang1 is constitutively produced and binds the endothelial Tie2 receptor. Exposure to inflammatory cytokines would release Ang2, which is stored in endothelial Weibel-Palade bodies. Ang2 engagement with Tie2 would increase endothelial activation and microvascular permeability. The level of Ang2 and ratios Ang2:Ang1 in the serum were significantly higher in patients experiencing severe ICANS compared with those with less severe neurotoxicity. In the patients, the degree of endothelial cell activation was also associated with a higher risk for overt cerebral edema [212]. However, it should be noted that fatal cerebral edema has also been observed in the absence of activated CAR-T cells within the brain [213] and in non-immunological assaults such as stroke [214]. In both contexts, acute tissue swelling and uncontrolled fluid imbalance in the brain have been implicated as the primary drivers of the cytotoxic edema effect.

On the other hand, prolonged exposure to low levels of inflammation, such as multiple sclerosis and Alzheimer's disease, would also be undesirable. In a prospective cohort study, an increased presence of peripheral leukocytes in the brain was associated with aging-related patterns such as reduction of total brain and gray matter volumes [215]. Furthermore, infection in the setting of a pre-existing chronic neuro-inflammatory disease could exacerbate the inflammatory response in the brain and worsen clinical outcomes [216]. For example, lipopolysaccharide (LPS) injection into mice with Alzheimer's or murine prion disease induced a much higher expression of pro-inflammatory cytokines such as IL-1β and TNF-α in the brain and aggravated acute sickness [217, 218].

Considering the brain's sensitivity to inflammatory changes, identifying the appropriate immune-regulatory signaling that could support anti-tumor T cell activity without harming the non-tumor neighboring cells would be crucial.

1.4. *Toxoplasma gondii* as a potential biological agent to remodel brain tumor environment for T cell therapies.

1.4.1. Overview of *T. gondii* immune biology in intermediate mammalian hosts

a) *T. gondii* life cycle in intermediate hosts.

Toxoplasma gondii (T. gondii) are protozoan parasites with two sets of hosts: definite hosts, which include all animals in the Felidae family, and the intermediate hosts, which encompass over 350 warm-blooded vertebrates, including rodents and humans [219, 220].

Within the intermediate host, *T. gondii* has two life stages: tachyzoites and bradyzoites [221, 222]. During the acute infection, *T. gondii* exists primarily in the tachyzoite form, which rapidly replicates in the parasitophorous vacuole. Since the membrane of the vacuole is generated from the host plasma membrane, this avoids fusion with endo/lysosomal compartments [221, 223] and facilitates *T. gondii* replication while evading cell-automonmouse immune detection. Over the first three weeks of infection, parasites are cleared

from in most infected cells. Chronic infection is maintained in non-dividing cells mainly neurons, cardiac muscle, skeletal muscle, and retinal cells. In these tissues, parasites enter the slowly replicating bradyzoite form and form cyst structures that persist in the host life-long [221].

b) Type II *T. gondii*, the most prevalent strain in humans, induces mild to asymptomatic acute infection.

T. gondii can be distinguished into three clonal lineages: type I, II, and III [224, 225]. The clonal divergence occurred approximately 10,000 years ago [226]. The timeline of the clonal expansion coincided with human agriculture development and cat domestication [226]. Correspondingly, it is estimated that 30% of the human population is chronically infected with *T. gondii* [219]. While *T. gondii* prevalence has lowered with improved food hygiene standards and practices, recent epidemiological evaluations still detect a significant number of people seropositive for *T. gondii*: from 10% in the United States [227] to 50% in France [228].

Among the three clonal lineages, Type II strains have been the most prevalent within the human population. Type II makes up 60-80% of the clinical samples collected from immune-compromised patients who developed toxoplasmosis in North America and Europe [229, 230]. Interestingly, Type I is known to be the most virulent strain with an LD_{100} of under ten parasites in mice, compared to LD_{50} of 100–1000 for Type II and LD_{50} of ~100,000 to 1 million for Type III [231]. Type I hypervirulence is attributed to the strain-specific secreted effectors called rhoptry proteins 5 (ROP5), ROP17, and ROP18, which would inactivate IRGa6 and IRGb6 function [232, 233]. Whether a similar mechanism occurs in the human host is still unclear. However, considering the pervasiveness of Type II strains among humans, an evolutionary trade-off between moderate virulence in exchange and success in establishing long-term infection in the host is implied.

c) *T. gondii* infection is controlled by the Th1 immune response.

As an obligate intracellular pathogen, *T. gondii* infection induces a Th1 immune response [234]. During *T. gondii* acute and early chronic infection, when *T. gondii* exist predominantly in the form of tachyzoites, Th1 immunity could be readily detected via elevated levels of IFNγ in the sera and infected tissues [235]. IFNγ is an effector molecule secreted by activated lymphocytes, including CD4+ helper T cells, cytotoxic CD8+ T cells, and NK cells. IFNγ is crucial to control *T. gondii* at both acute and chronic infection, as shown in the extensive antibody depletion experiments. During acute infection, CD8+ T cells and NK cells appear to be the major cell producers for IFNγ and have been shown to account for mice survival [236, 237]. During chronic infection, CD4+ T cell and CD8+ T cell depletion would result in *T. gondii* uncontrolled reactivation in the brain and fatality to a similar degree as mice treated with anti-IFNγ antibodies [238], suggesting the essential role of the two T cell populations at this stage.

In addition to cell-mediated killing (where *T. gondii* infected cells would be directly lysed by CD8+ effector T cells through the release of granulysin and other cytotoxic products [239]), IFNγ mediated cell-autonomous immunity is a primary mechanism to keep *T. gondii* burden in check. IFNγ-induced expression of IFN-responsive genes such as immunity-related GTPases (IRGs, mouse) and guanylate-binding proteins (GBPs, human and mouse) target the parasite vacuole and prevent tachyzoite accumulation. For example, in vitro studies with human macrophages have found that IFNγ induces expression of GBP1, which attacks the parasite vacuole [240]. Unlike humans, mice relied on IFNγ-induced expression of IRGs such as IRGM1 and IRGM3 to detect the parasitic vacuole. Additionally, IFNγ also upregulates the expression of inducible nitric oxide synthase (iNOS) [241, 242], elevating reactive nitrogen species in the cell that would be detrimental to *T. gondii* [243, 244].

Not limited to innate immune cells in the periphery, IFNγ-regulated cell-autonomous immunity is essential to control parasites at distant infection sites such as the brain. Microglia-specific deletion of STAT1, the intracellular responder for IFNγ, led to a significantly higher parasite burden in the brain and fatal encephalitis

despite no impairment in T cell number and activation [132]. Similarly, mice with STAT1 deficient astrocytes were found to carry higher cyst burden and higher mortality due to toxoplasmosis [245]. This corroborates with an earlier study using cultured human astrocytes that found IFNγ, coupled with IL-1b, controlled intracellular multiplication of tachyzoites [241]. Most recently, using primary neuron cultures (humans and mice), IFNγ stimulation promoted parasitic vacuole destruction and *T. gondii* clearance [246]. Corroborating with these observations, evidence suggests a co-evolution between *T. gondii* virulent proteins and the IRG genes in the murine, a central intermediate host choice for *T. gondii* [221, 247].

Considered the essential role of IFNγ in *T. gondii* control, the immunological intersection between innate immunity, which provides the early immunological signals about *T. gondii* infection, and the adaptive immunity is an important study area. From studies on myeloid cell, Toll-like-receptors (TLRs) and inflammasome have been shown to play important role in providing the initial inflammatory cues. For example, *T. gondii* derived profilin (from phagocytosed or dysfunctional parasites) binds and activates TLR-11 on the endosome of Dendritic cells, inducing the production of IL-12 from the cells [248]. IL-12 is among the key inducers of IFNγ production and Th1 response [249]. On the other hand, an inflammasome is an immunological module that is assembled from activated intracellular sensors for danger/metabolic stress, adaptors and inflammatory caspases. The main outcomes from inflammasome activation include the release of IL-1 family cytokines and inflammatory cell death process such as pyroptosis. Sensors NLRP1 (in mice), NLRP3 (in mice and humans), and AIM2 (in humans) have been found to participate in inflammasome activation through detection *T. gondii* components (such as parasitic DNA) [221]. Unlike canonical inflammasome response, pyroptotic cell death has not been observed in mouse or human cells [221]. Instead, alternative cellular apoptosis was activated (in the human system) [240, 250].

It should be noted that *T. gondii* has been observed to evolve strategies to manipulate the host's immune response through the release of various effectors into the host cells. The effectors are delivered via secretory organelles known as the rhopies (ROP) and the dense granules (GRA) [221]. For example, while GRA15 activation of NF-kB and GRA24 activation of p38 MAPK promotes expression of IL-12 and IL-18 [251, 252], ROP16 dampens IL-12 production via phosphorylation of STAT6 [253]. *T. gondii* inhibitor of STAT1-dependent transcription (TgIST) can bind STAT1 and alter their interaction with other cis-regulatory molecules in the nucleus, thereby suppressing IFNγ-STAT1 induced transcriptional activity [254]. Overall, these activities imply the ability of *T. gondii* to fine-tune the host's immunity to support their survival and transmission.

1.4.2. *T. gondii* are genetically tractable.

T. gondii's biology and pathophysiology have been study subjects for over a hundred years. *T. gondii's* genomic landscape is well-characterized, and the genetic engineering toolbox for the microorganism is growing. Among the Apicomplexa, which include the malaria-causing Plasmodium, *T. gondii* is considered the most genetically tractable microorganism [231]. Conditional knockout studies have provided clinically crucial information on *T. gondii*. For example, the microbe's metabolism helps identify anti-folate medications to target the parasites [255]. Other studies shed light on the tachyzoite-bradyzoite differentiation and the host-pathogen interaction [256]. Importantly, exogenous expression of epitope tags, reporter constructs, and heterologous proteins using mammalian cell biology has been easily achieved, and the transfection efficiency is regularly over 50% [231].

Importantly, engineered *T. gondii* has shown promising immune modulation that has helped mount an anti-tumoral response in several preclinical studies. Based on the discovery that carbamoyl phosphate synthetase (CSP) II is essential for *T. gondii* de novo pyrimidine synthesis, Bzik et al. developed a uracil auxotrophic strain of *T. gondii*, which requires uracil supplement in the culture media and if injected in vivo these *T. gondii* would be cleared shortly [257]. Intratumoral injection of these CSP-deficient *T. gondii* into grafted solid tumors such

as B16F10 melanoma, Lewis lung carcinoma, and UpK10 tumors generated reliable tumor-controlling effects and tumor-specific T cell response [258]. In another study using ovarian carcinoma models, intraperitoneal injection of *T. gondii* enacted tumor immunosurveillance and immune recall capabilities [259]. This was found to be dependent on the reprogramming of $CD11c^+$ antigen-presenting cells at the tumor-grafted site upon being invaded by the parasites [259]. More recently, with the advance of CRISPR-Cas9, another attenuated *T. gondii* strain has been developed. Via deleting the gene encoding for Dense granule protein 17, an essential factor for parasitophorous vacuole [260], these parasites' virulence is significantly dampened in vitro and in vivo. Intratumoral injection of ΔGRA17 parasites in combination with PD-L1 inhibitors also achieves anti-tumoral immunity and memory [261].

1.4.3. *T. gondii* infection in the brain is controlled by T cell response.

a) *T. gondii* naturally disseminates to the brain.

As transmission among the intermediate host is a major dissemination route for *T. gondii*, cyst formation is essential for the *T. gondii* to pass to the next host. Compared to cells that turn over regularly, non-dividing cells such as muscle cells and neurons would provide stability for *T. gondii* cyst to establish chronic infection [262, 263]. Through a series of experiments (from in vitro culture to histological assessment of infected brain slices and in vivo parasite tracking), neurons have been found to be the primary cyst-bearing cell type during chronic infection in the brain [264-266]. Accordingly, in the brain, *T. gondii* were detected only in the brain parenchyma and not in the brain meninges [267]. Regarding cyst distribution within the brain, murine studies found that although *T. gondii* cysts could be detected anywhere in the brain, the cerebral cortices are where up to 70% of cysts are located [268, 269].

How *T. gondii* traverse into the brain parenchyma is an actively investigated topic, and three models for their entry route have been proposed. Evidence indicates that the process may rely on more than one route. Since *T. gondii* could transmigrate via a paracellular process across the intestinal epithelium [270], which shares some features with the BBB [271], one model proposed that *T. gondii* could cross in-between BBB epithelium. However, no direct in vivo evidence has been found [272]. Additionally, because *T. gondii* are obligate intracellular pathogens, they are unlikely to survive the travel in the circulation to reach the BBB. The second model for *T. gondii* brain dissemination is translocation across the BBB endothelium via the paracellular process. During early acute infection (~3-5 days post-infection), green-fluorescent protein (GFP) labeled tachyzoites have been detected in the brain, with the highest numbers in the intravascular areas[273]. Tachyzoite egress from the BBB endothelium is required for *T. gondii* detection in the brain parenchyma, supporting that endothelial cell is a replicative niche for *T. gondii* [274]. In addition, *T. gondii* can hitchhike the infected myeloid cells. As *T. gondii*-infected dendritic cells have been found to be a means for *T. gondii* to migrate to other peripheral organs such as the spleen [275], CD11b+ monocytes carrying tachyzoites may our similar activities. In the brains of infected mice at the later acute infection phase (15 days post-infection), locations of CD11b+ monocytes have been correlated with clusters of tachyzoites [276]. These findings suggest infiltrating CD11b+ monocytes may also transport *T. gondii* into the brain parenchyma. Considering the different time windows for endothelial cells and monocyte studies, *T. gondii* could employ both endothelial cells and myeloid cells to establish their presence in the brain.

b) In the brain, while *T. gondii* cysts are immune-quiescent, T cell recruitment is observed during early chronic infection.

During early chronic infection, when tachyzoites can be readily detected in the brains of infected mice, T-cell infiltration has been robustly detected in the brain of infected mice [276-278]. Studies indicated that tachyzoite's presence reprograms the brain milieu to support the entry and activity of T cells from this Th1 response. During chronic infection in healthy hosts, once *T. gondii* have developed cyst structures, *T. gondii* reactivation and cyst rupture is rare. At this stage, the brain immunological landscape returns to the

physiological state, evident through the minimal detection of T cells and microglial activation close to the *T. gondii* cyst in an incidental clinical case report [279]. Likewise, mouse strains that are more resistant to *T. gondii* infection have been found to be absent of brain inflammation despite the presence of cyst in low numbers [278, 280]. More definitely, two-photon time-lapse imaging of infected brain tissue has observed that CD8+ T cells recruited to the brain ignored intact cyst-bearing neurons [281]. Several factors have been proposed to explain the lack of immune response against bradyzoite and cysts. One possibility is the difference in antigenic epitope expressed by the tachyzoites and the bradyzoites [282].

How T cell response develops in the brain during the early chronic infection is an actively studied area. In terms of immune cell profile in the brain at this period, myeloid cells (including microglia, monocyte-derived myeloid cells, and DCs) and T cells have been observed to be two major populations [276, 283]. Other lymphocytes (such as B cells and NK cells), and innate cells (such as neutrophils) have been minimally detected. The inflammatory response from myeloid cells and nearby non-classical immune cells, including astrocytes and endothelial cells, plays an essential role to recruit and retain activated T cells in the brain parenchyma. Activated T cells then release effector cytokines such as IFNγ to build the local Th1 immune response. This feedforward immune response resolves by latent chronic infection, when *T. gondii* enter the cyst structure.

To recruit T cells in a *T. gondii* infected brain, inflammatory cues such as chemokines and pro-inflammatory cytokines have been found to elevate. Among the known Th1 T cell chemoattractant(s), CXCL9, CXCL10 and CCL5 have been to be upregulated, at both transcriptional and protein levels, in the brain of infected BALB/c mice [284]. Considered that BALB/c are more resistant against *T. gondii* infection, T cell response in the brain of the mice would be closer representative to human infection. The chemokines were found to be mediated by IFNγ as IFNγ knockout mice were not able to elevate the cytokines. CXCL9 and CCL5 have been shown to be primarily produced by microglia, whereas CXCL10 was mostly expressed in astrocytes [285]. CXCL9 and CXCL10 is recognized by CX1CR3 receptor on T cells and is well known to aid with Th1 T cell migration [286]. CXCL9 was found to be necessary for T cell accumulation to areas of tachyzoite proliferation and CXCL9-depletion via antibody led to greater number of inflammatory foci with tachyzoite presence [287]. CXCL10 also helped with T cell migration, improving their velocity to identify the target cells [288].

Upon recruited to the inflammatory sites, interactions with the endothelial cells are necessary to facility T cell entry into the brain parenchyma. In the *T. gondii* infected brain, elevated expression of adhesion molecules ICAM-1 and VCAM-1 on BBB endothelial cells have been found to develop very early during acute infection [289] implying this happened before T cell arrival [290]. VCAM-1 could be recognized by VLA-4 (or Integrin α4β1) while ICAM-1 would be recognized by LFA-1 on activated T cells. Blocking VLA-4 activity via antibody dampened the amount of T cell recruitment and led to a higher parasitic burden in the brain [291, 292]. Although ICAM-1 has been shown to support T cell recruitment during infection with Sinbis and Theiler's viruses [292], it is unclear whether it contributes to T cell recruitment during *T. gondii* infection. LFA, however, has been implicated to be involved in DC recruitment to *T. gondii* infected brain [293].

Although there is yet direct evidence on the specific factors that induce the expression of ICAM-1 and VCAM-1 on BBB endothelial cells, they are known to be induced by a variety of signals including pro-inflammatory cytokines such as TNF-α and IL-1β [294, 295]. VCAM-1 could also be induced by presence of reactive oxygen species and microbial stimulation of the endothelial cell TLR [295]. Considered that BBB endothelial cells have been shown to be a replicative niche for *T. gondii* and infected circulating myeloid cells could be detected early during acute infection, it is possible that the presence of *T. gondii* and the inflammatory response by the infected myeloid cells activate the BBB endothelial cells prior to T cell

presence. Other studies have identified additional danger signals such as IL-33 from oligodendrocytes and astrocytes [296] and IL-1a from microglia [297], which may also contribute to endothelial cell activation.

Another to-be-explored area of research for T cell response in the *T. gondii* infected brain is whether T cells 'licensing' (the regulation of T cell entry into the brain parenchyma) occurs and which cell type and immune-signaling participate in this activity. In an adoptive transfer experimental autoimmune encephalitis mouse model, MHC-II deficient conventional DCs led to reduced T cell detection in the brain and disease severity [143]. Likewise, in an experimental cerebral malaria model, the activity of discrete MHC-I molecules on endothelial cells, which cross-present malarial antigens, has been shown to modulate the number of anti-malaria T cells detected in the brain [181]. Considering that *T. gondii* can infect endothelial cells and recruit monocytes throughout the brain, it is possible that T cell 'licensing' may occur.

Upon entry into the brain parenchyma, antigen-specific engagement is necessary for T cell retention in the brain [298]. In an *T. gondii* infection experiment, mice received ex vivo activated OT-1 cells (which are OVA-specific CD8+ T cells) [281]. Although OT-1 cells were detected in the brain, they could not expand, and their population quickly declined. Transplanting OT-1 cells into mice infected with OVA-expressing *T. gondii* supported the accumulation and expansion [291]. The affinity of TCR to MHC presented epitope has also been shown to contribute to T cell retention in the brain and ability to develop into resident-like memory populations [299]. The identity of the antigen-presenting cells that engage with recruited CD8+ T cells during *T. gondii* infection is an ongoing investigation. Via imaging, transient contacts with CD11b+ cells and granuloma-like structures containing parasites has been observed [281]. Interaction with CD11c+ was also observed [293]. Since neurons has been shown to not be able to express MHC-I even during transgenic expression of IFNg by oligodendrocyte [300] and no interaction has been observed between CD8+ T cells and intact cyst, it is unclear whether neurons can effectively present to these cells.

It is important to note that *T. gondii*-induced Th1 immunity in the brain comprises not only inflammatory mediators but also immune-regulatory signaling to prevent fatal immunopathology. Examples include IL-10 and ICOS-L. Mice unable to produce IL-10 (IL-10 $^{-/-}$), an immunosuppressive cytokine, would experience lethal immunopathological response [301]. Even when they could survive chronic infection, they were found to eventually succumb to overt inflammatory response characterized by increased numbers of CD4+ T cells and macrophages along with elevated inflammatory cytokines in the brain [302]. Independently, ICOS are co-stimulatory receptors on T cells that hold both actional and regulatory functions over T cell response [303]. In the context of *T. gondii* infection, disabling ICOS signaling using antibodies to block ICOS-Ligand contributed to an expanded T cell number in the brain [303].

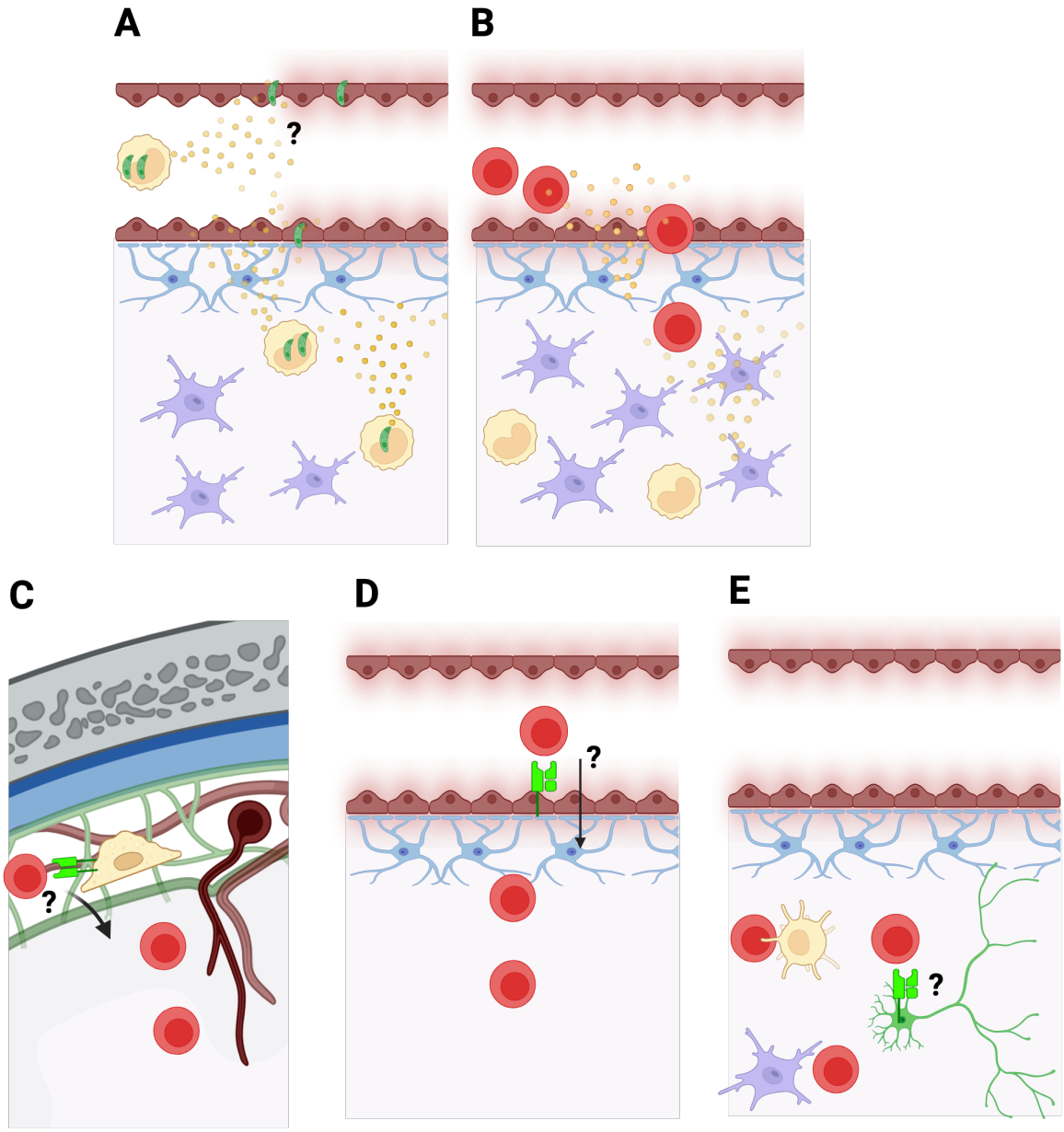


Figure 1.3: **T cell recruitment process to the brain during *T. gondii* infection.**
A) During early infection, BBB endothelial cells (maroon) become activated (glowing) by expressing T cell homing ligands VCAM-1 and ICAM-1. Endothelial cell expression of VCAM-1 and ICAM-1 could happen via inflammasome activation due to *T. gondii* presence in the limited infected cells, or under the influence of pro-inflammatory cytokines (yellow particles), such as TNF-a and IL-1b, released by infected myeloid cells (yellow cells with green *T. gondii*).
B) T cells (red) are recruited to the brain through a multistep process - first guided by chemokine signals (yellow particles) such as CXCL9, CCL5 from microglia (purple) and CXCL10 from astrocytes (blue). Then, interaction with VCAM-1 and ICAM-1 ligands on the endothelial cells help slow them down and
C - D) T cell licensing may regulate T cell entry during *T. gondii* infection. Activity of MHC-II (bright green) on DC (yellow) in the leptomeninges (**C**) and MHC-I (bright green) on endothelial cells (**D**) have been implicated for T cell entry in other brain inflammatory contexts.
E) Antigen-specific interaction with APCs has been shown to be necessary to support T cell retention and expansion in the brain post *T. gondii* infection. However, the specific cell type involved in this process is unclear.

c) Concerns about *T. gondii* in brain cancer context.

It is essential to acknowledge that as an opportunistic parasite, *T. gondii* presents a significant public health concern among immune-compromised or immune-incompetent individuals such as AIDS patients and newborns [304]. In healthy immune-competent individuals, cyst rupture is rare and quickly controlled [305, 306]. In immune-compromised or deficient individuals, though, *T. gondii* recrudescence could happen in an uncontrolled manner and may lead to lethal encephalitis. Prophylactic and therapeutic interventions against toxoplasma reactivation involve primarily sulfadiazine and pyrimethamine [255, 307]. While the drugs cannot target bradyzoites in cyst structures, they target tachyzoite effectively by inhibiting a key enzyme in the folic acid synthesis pathway of the parasites named Dihydropteroate Synthase [255, 308]. It is worth noting that cancer patients usually require chemotherapy, which is an immunosuppressive agent. However, there has been only one case report where intervention with anti-folate medication was required [309]. The patient had been receiving prednisone and cyclophosphamide for ten months, however. This suggests that prolonged exposure to high-level immunosuppressive drugs is more likely to support *T. gondii* reactivation.

Some epidemiological studies have suggested an association between *T. gondii* infection and increased risk for brain tumors [310, 311]. Most studies rely on *T. gondii* seropositivity (presence of anti-*T. gondii* antibodies) as the readout for infection status and glioma prevalence to assess the association. Since seropositivity only informs the activity of B cell response, it cannot provide the timing of infection or severity. In a recent prospective study, researchers evaluated glioma incidence among seropositive patients three to fifteen years prior [310], providing a more definite window between infection and brain cancer detection. Since acute infection in humans only spans days to weeks, the tumor must have developed during the chronic infection phase. As high-grade glioma is known to have disrupted BBB (section 1.3.2.) and brain injuries can release brain-derived soluble immune-suppressive mediators [312], *T. gondii* may reactivate after advanced glioma breaches the BBB and cause systemic immune-suppression.

Overview of this dissertation

In this dissertation, I pursued a proof-of-principle study to evaluate *T. gondii's* ability to induce effective Th1 immunity in the brain in the context of brain tumors using the GEM medulloblastoma model. The overarching hypothesis is that *T. gondii* infection, commonly occurring in nature with murine and human hosts, can remodel the brain tumor microenvironment's T-cell desert nature without major adverse effects.

In this chapter, I provided an overview of the current clinical approaches for primary brain cancers, the challenges contributing to the T-cell desert character of brain tumors, and the prospects of *T. gondii* as a microbial agent to remodel the brain tumor environment. Briefly, given the dim prospects of primary brain cancers, alternative therapies are needed to improve patient outcomes. Immunotherapy is an attractive avenue, with the DC vaccine demonstrating some clinical benefit. However, the clinical consensus on brain tumor environment is that T cells are generally absent in the tumors at baseline. Brain tumors also demonstrate additional immunosuppressive mechanisms, including the immune-tolerogenic profile of tumor-associated microglia at homeostasis. The brain sensitivity for pro-inflammatory changes and the presence of BBB are significant hurdles for regular treatment modalities such as cytokine and CAR-T infusions. Considering the long coevolution between *T. gondii* and the mammalian intermediate hosts, including humans and murine, the Th1 immunity in the brain to control *T. gondii's* presence is essential for the parasite's success in nature. It is unclear whether *T. gondii*-induced Th1 immunity could be developed in brain tumors efficiently and tolerably.

In Chapter 2, I and other co-authors set out to address the three research goals. First, does brain tumor presence significantly impact the hosts' ability to control *T. gondii* infection? Second, can *T. gondii* induce T cell response in tumors, which has been known to be immune-suppressive? Lastly, to probe the underlying mechanism that supports T cell recruitment, I evaluated the presence of IFNγ (the central modulator for Th1 immunity) and its effect on the myeloid cells in the tumors.

We found that the presence of brain tumors did not significantly affect the host's ability to control *T. gondii* and their overall well-being. We also found that, despite the immunosuppressive nature of medulloblastoma, they posed a minimal barrier to *T. gondii* induced T cell response. T cells were recruited to a similar degree with normal cerebella for infected wild-type mice and could be activated ex vivo. Lastly, we found that *T. gondii* infection significantly elevated IFNg in the tumor environment. Accordingly, transcriptional evaluation of the enriched myeloid cells from the tumors demonstrated multiple IFNγ-induced responses that would support T cell recruitment and activity.

In Chapter 3, I integrated the current findings with previous observations to discuss several potential future directions. Given the implications on myeloid cell status, I proposed combinatorial therapy that may further potentiate T cell response in the tumor environment. Regarding *T. gondii*, I proposed the next optimal engineering goal: recapitulate the T cell recruitment effect without conferring long-term persistence of *T. gondii* in the hosts. Another major direction is toward building immune surveillance against brain tumors. Albeit not addressed in this thesis, an additional future direction should be considered is using *T. gondii* as a study model to elucidate the underlying molecular and cellular activities that support Th1 immunity and Th1 recall response in the brain of *T. gondii* infected host. The knowledge and engineering efforts could provide the path toward building a tunable and effective anti-tumoral T-cell response against brain tumors.

Chapter 2: *Toxoplasma gondii* infection supports the infiltration of T cells into brain tumors.

2.1. Introduction

Primary brain tumors are among the deadliest cancers, with a ~30% 5-year survival rate [313], and ~10% for glioblastoma [2]. Although T cell-based immunotherapies have shown clinical benefits in many types of solid tumors [314-316], recent clinical trials using the immune checkpoint inhibitor anti-PD-1 for glioblastoma patients - CheckMate 143, CheckMate 498, and Checkmate 548 - did not significantly improve patient survival [113, 317, 318]. The most likely explanation is the lack of functional T cells in brain tumors. Except for tumors that develop outside of the blood-brain-barrier (BBB) [319, 320] and advanced glioma with a dysfunctional BBB [198, 321], many reports showed that primary brain tumors have very limited T cell infiltration [113, 159, 162]. These findings are consistent with the notion that a T-cell-accessible tumor microenvironment (TME) is a critical prerequisite for immunotherapy to be effective [322, 323].

The BBB limits the accessibility of peripheral immune cells into the brain, protecting neural cells from inflammatory damage, but also shields brain tumors from immune surveillance [324]. Additionally, our lab and others have found that the brain TME adopts an immune suppressive state, which would limit the activation of T cells even if they gain access to the tumor mass [167, 325]. Early attempts to overcome these problems faced significant challenges. For example, systemic infusion of pro-inflammatory cytokines, including IL-2 and interferons, led to minimal benefit while exposing patients to the risk of cytokine release syndrome [326, 327]. Delivering adjuvants or CAR-T cells through direct placement in the ventricle or the tumor site, showed limited toxicity with elevated IFNγ in cerebrospinal fluid (CSF) and increased presence of $CD8^+$ T cells [328, 329]. However, such implantation requires invasive surgical procedures that are not feasible for all brain tumor locations and would not be efficacious for highly invasive tumors [330].

Microbial-based therapy is a cornerstone of cancer immunotherapy. The use of Bacillus Calmette-Guerin (BCG) to treat non-muscle invasive bladder cancer (NMIBC) was the first FDA-approved immunotherapy [331-333] and has been shown to promote effective tumor immunosurveillance with limited side effects [334-336]. *Toxoplasma gondii* is a unicellular protozoan parasite with a long evolutionary relationship with mammalian hosts [337]. The type II strain of *T. gondii* is associated with asymptomatic infection in humans [225, 338] It naturally traffics to the central nervous system and induces a sustained IFNγ /Th1 immune response [225, 339]. Of interest, preclinical studies have shown that direct injection of attenuated strains of *T. gondii* into peripheral solid tumors promoted anti-tumoral immune responses. Specifically, the uracil auxotroph strain *cps*, which can be grown in tissue culture but is non-replicative in vivo, has been shown to remodel the TME and enact tumor-reactive T cells against highly aggressive ovarian cancer and melanoma models [258, 259]. RH*Δgra17*, which lacks the essential virulence effector dense granule protein 17 (GRA17), was shown to extend survival of mice grafted with B16-F10 melanoma, MC38 colon adenocarcinoma, or murine Lewis lung carcinoma when combined with checkpoint inhibitors [261]. In the case of brain tumors, *T. gondii* is particularly enticing due to its natural dissemination to the brain and its effective recruitment of peripheral T cells into the brain [340].

The attenuated strains used in previous studies are cleared before trafficking to the central nervous system, thus, they would not be applicable in the setting of brain tumors for the current project. Therefore, to test *T. gondii*'s capacity to elicit a Th1 immune response in brain tumors, in this study we chose a *T. gondii* strain with normal dissemination kinetics and immune modulation in the brain. For the tumor model, we chose a recently developed genetically engineered mouse model (GEMM) for Shh-subtype medulloblastoma [167]. Compared to other medulloblastoma GEMMs, this model has a relatively consistent penetrance and tumor latency, where most mice develop large tumors around 90 days of age, which accommodates the kinetics of *T. gondii* infection [167, 341]. We chose to avoid tumor-grafting models because

graft-associated immunosuppression [312] could alter immunity to *T. gondii*, and thus confound the interpretation of results. In addition to these practical considerations, previous characterization of this model showed an absence of T cells along with the presence of tolerogenic/immunosuppressive myeloid cells in the TME, closely mimicking the findings in medulloblastoma patients [167]. Moreover, medulloblastoma develops in the cerebellum, a region of the brain where *T. gondii* is not highly prevalent in mice [268, 269]. Thus, if *T. gondii* can shift the immune profile of Shh medulloblastoma GEMM, then addressing similar challenges in brain tumors in other brain regions would be possible.

As a proof-of-principle study to explore the potential use of *T. gondii* as a microbial-based therapy for brain tumors, here we focused on three fundamental questions. First, is *T. gondii* infection well-tolerated in brain tumor-bearing hosts? Second, is *T. gondii* infection sufficient to facilitate the infiltration of functional T cells into the brain tumor microenvironment? Third, can *T. gondii* infection sufficiently reprogram the TME from an immune suppressive to a T cell-supportive state?

Given the prevalence and tolerability of *T. gondii* infection among immune-competent individuals, we hypothesized that *T. gondii* infection would be well-tolerated in medulloblastoma-developing mice that have yet to reach the latent stage while capable of efficiently recruiting T cells and remodeling the TME into a T-cell supportive state. By addressing the above three questions, the project aims to evaluate a potential but yet-to-be-characterized capacity of *T. gondii*'s "bug-as-drug" – its immune-modulatory capacity in the context of brain tumors. If *T. gondii* can transform brain TME into a T-cell hospitable state without causing significant harm to the tumor-bearing-hosts, the results could open new avenues for immunotherapy to target brain cancers – through direct *T. gondii* engineering and through a better understanding of how to build tolerable Th1 immunity in brain tumors.

2.2. Results

2.2.1. *T. gondii* infection was well-controlled in Medulloblastoma-bearing mice.

a) *T. gondii* burden among the infected hosts.

Although *T. gondii* infection does not cause significant health concerns for intermediate hosts, including humans and mice, this tolerability depends on a functional immune system. However, literature reports have indicated that brain tumors or brain injury can exert systemic immune suppression to a certain degree [312, 342]. In the glioblastoma study, it is unclear the tumor grade of the evaluated cases [342]. As BBB of high-grade glioma brain tumors are more dysfunctional, immune-suppressive mediators could be released from the tumors in a similar manner as in the study with brain injuries [312]. We hypothesize that tumors during the developing stage (before reaching latency) would not impact the host immunity and impair its ability to control *T. gondii* infection. If *T. gondii* infection in the context of developing medulloblastoma leads to significant fatality, this project would have to be aborted. To test the above hypothesis, we focus on the *T. gondii* burden via their genomic DNA content in representative organs of the infected hosts because an immune-compromised host would not be able to control *T. gondii*.

T. gondii infection was given at postnatal day 50, when medulloblastoma just becomes detectable [167]. To evaluate the effectiveness of immune control against *T. gondii* in medulloblastoma-bearing mice relative to wildtype (WT) littermates, *T. gondii* burden was measured in genomic DNA by qPCR at two time points: at 12 days post-infection (DPI), when *T. gondii* resides in peripheral organs, and at 27 DPI, when *T. gondii* starts establishing infection in the brain [268, 269, 343, 344] (Fig. 2.1.A) [267, 345, 346].

At 12 DPI, *T. gondii* burden in the heart (Fig. 2.1.B) and lung (Fig. 2.1.C), selected representative organs [132, 343, 344], showed no significant difference between tumor-bearing mice and WT littermates. At 27 DPI,

T. gondii burden was first measured in the forebrain, a region distal to the tumor site. To account for stochastic differences in regional brain colonization by the parasite, the entire forebrain (excluding the cerebellum and midbrain) was compared [268, 269, 344]. Infected medulloblastoma-bearing mice had statistically similar *T. gondii* burdens as infected-WT mice (Fig. 1D), indicating that any immune modulatory effects of the tumor did not negatively impact the immune control of *T. gondii* in brain regions distant from the tumor.

To evaluate parasite burden in the cerebellum where its clearance could be more directly impaired by medulloblastoma-associated immune suppression, we separated tumors from the nearby, tumor-free cerebellar regions guided by the tdTomato expression in the tumors. There was no difference between the *T. gondii* burden in the tumor areas and in paired, tumor-free cerebellar regions (Fig. 2.1.E), indicating that medulloblastoma posed no negative impact on *T. gondii* control in the tumor mass.

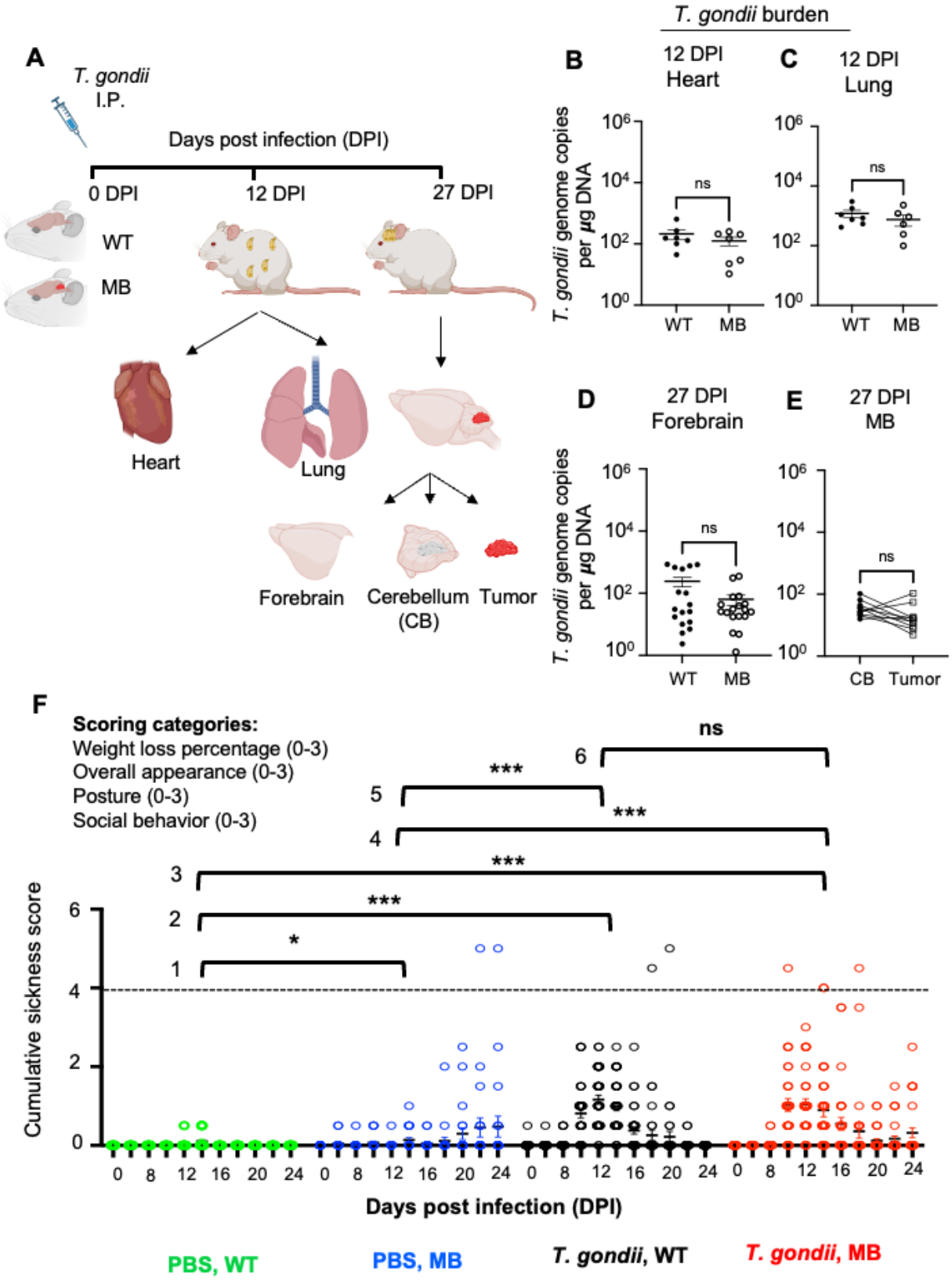

A
T. gondii
I.P.
Days post infection (DPI)
0 DPI
12 DPI
27 DPI
WT
MB
Heart
Lung
Forebrain
Cerebellum (CB)
Tumor
T. gondii burden
B
12 DPI
Heart
C
12 DPI
Lung
T. gondii genome copies per µg DNA
ns
WT
MB
D
27 DPI
Forebrain
E
27 DPI
MB
CB
Tumor
F
Scoring categories:
Weight loss percentage (0-3)
Overall appearance (0-3)
Posture (0-3)
Social behavior (0-3)
Cumulative sickness score
Days post infection (DPI)
PBS, WT
PBS, MB
T. gondii, WT
T. gondii, MB

Figure 2.1: Medulloblastoma does not impact *T. gondii* burden and infection outcome in mice.

A) Schematic of *T. gondii* infection and tissue harvesting procedures to evaluate parasite burden in medulloblastoma-bearing mice (MB) and wild-type (WT) littermates. Briefly, mice were given an intraperitoneal injection (I.P.) of 100 Me49 tachyzoites and harvested at either 12 days post-infection (DPI) or 27 DPI. At 12 DPI, when T. gondii are primarily in the periphery organs, the heart and lung were collected. At 27 DPI, when T. gondii has disseminated to the brain, the forebrain was collected. Additionally, to assess parasite burden in medulloblastoma, the tumor mass and nearby tumor-free cerebellar regions (CB) were collected, respectively.

B - C) Parasite burden in the periphery was equivalent between WT and MB mice. At 12 DPI, hearts (B) and lungs (C) from infected WT (n=12) and infected MB mice (n=12) were collected. Genomic DNA was extracted and subjected to qPCR. Signals from *T. gondii* specific 529-bp repeat element were normalized to a standard curve of 3 to 300,000 *T. gondii* genome copies to calculate the actual *T. gondii* genomic copies in the samples. Unpaired two-tailed T-test.

D) Parasite burdens in the forebrains were equivalent between WT and MB mice. At 27 DPI, forebrain samples from infected WT (n=18) and infected MB mice (n=18) were collected. Extracted genomic DNA was subjected to qPCR evaluation as described above. Unpaired two-tailed T-test.

E) Parasite burdens were equivalent between tumor and tumor-free cerebellar regions in MB mice. At 27 DPI, tumors and adjacent tumor-free cerebella from infected MB mice (n=10) were isolated and measured for parasite burden. Paired two-tailed T-test.

F) *T. gondii* infection did not exacerbate sickness in MB mice. Sickness was monitored in uninfected PBS-injected WT mice (green, n=15), uninfected MB mice (blue, n=22), infected WT mice (black, n=37), and infected MB mice (red, n=36). The dashed line denotes the euthanasia-reaching point. Each dot represents one mouse. The total sickness score was analyzed using a Poisson mixed-effects model explaining the sickness symptoms by treatment group. The main model was followed by Tukey pairwise differences.

*$p < 0.05$, **$p < 0.005$, *** $p < 0.001$, ns=not significant.
Images Created with BioRender.com

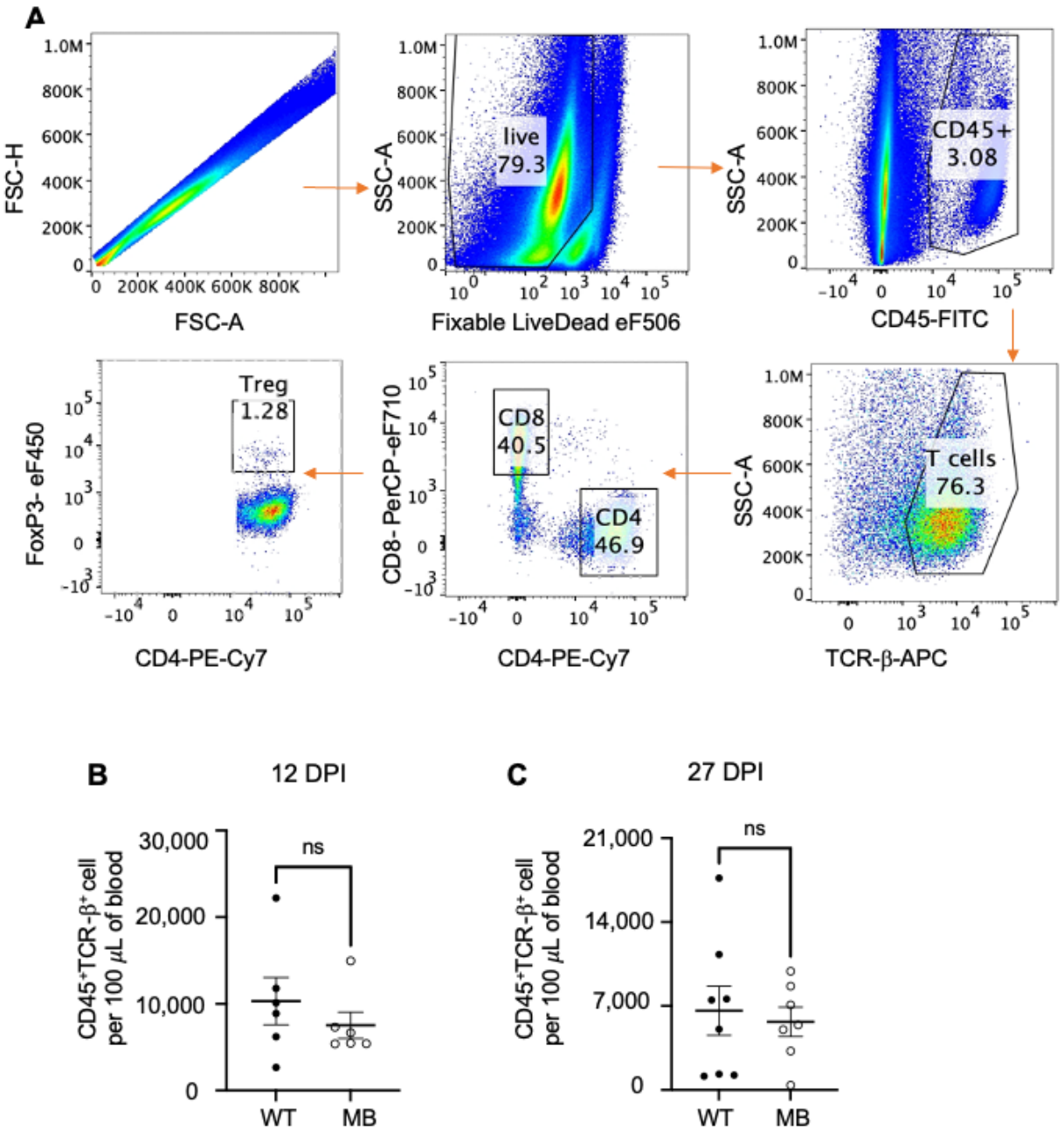
b) Assessment of other health parameters in the infected hosts.

To verify that medulloblastoma presence does not lead to significant systemic immune impairment upon *T. gondii* infection, we assessed the number of T cells, which is the principal cellular responder to control *T. gondii* [238, 347, 348]. Via flow cytometry, T cells in the peripheral blood was measured using gating strategy as shown in Supp Fig. 1A. We found that T cell numbers were similar between infected WT mice and infected medulloblastoma mice at 12 DPI (Supp Fig. 1B) and 27 DPI (Supp Fig. 1C). In addition, the amount of T cells recruited to the forebrain, as shown through percentage among live cells (Supp. Fig. 2A) and cell count (Supp. Fig. 2B), was not different between WT and medulloblastoma-bearing mice at 27 DPI. Taken together, these results suggest that the overall host immune response to *T. gondii* infection was not impaired by the presence of brain tumors.

T. gondii infection could impact the host's well-being in aspects such as transient weight loss [349] and behavioral changes [350], and the presence of brain tumor can aggravate these effects. To determine whether the presence of both *T. gondii* infection and medulloblastoma would exacerbate the adverse effects on the animal's well-being, we devised a scoring system over four categories (body weight, overall appearance, posture, and social behavior) to assess animal health throughout the experiment (Fig. 2.1.F). Each category was given a score on a scale of 0 (for no change) to 3, and mice that reached a combined score of 4 and higher were euthanized immediately as the humane endpoint (Materials and Methods section 2.3). As expected, tumor presence alone and infection alone led to significantly higher sickness scores than uninfected WT littermates (#2, #3 vs. #1, Fig. 2.1.F). Importantly, infected tumor-bearing mice had a similar sickness score to infected WT (#6, Fig. 2.1.F). Although the sickness score was higher in the infected tumor-bearing mice than in uninfected medulloblastoma mice (#4, Fig. 2.1.F), this is associated with infection rather than an exacerbation of late-stage tumor-associated health problems (#5, Fig. 2.1.F).

T. gondii infection has been found as an associated risk factor for brain tumors development [310, 311]. However, a seropositive test does not inform the specific infection time point [311] while the prospective study evaluated brain tumor frequency in the 13 years following blood collection, well beyond the acute and early acute infection stage. As the first controlled study with brain-tumor bearing mice, we asked whether early chronic infection with *T. gondii* would alter the tumor development. To answer this, we dissected out the tumor regions and measured the tumor weight. We found that tumor burden was equivalent between uninfected and infected tumor-bearing mice (Supp. Fig. 3). This suggests that early chronic *T. gondii* infection neither exacerbates nor suppresses tumor growth.

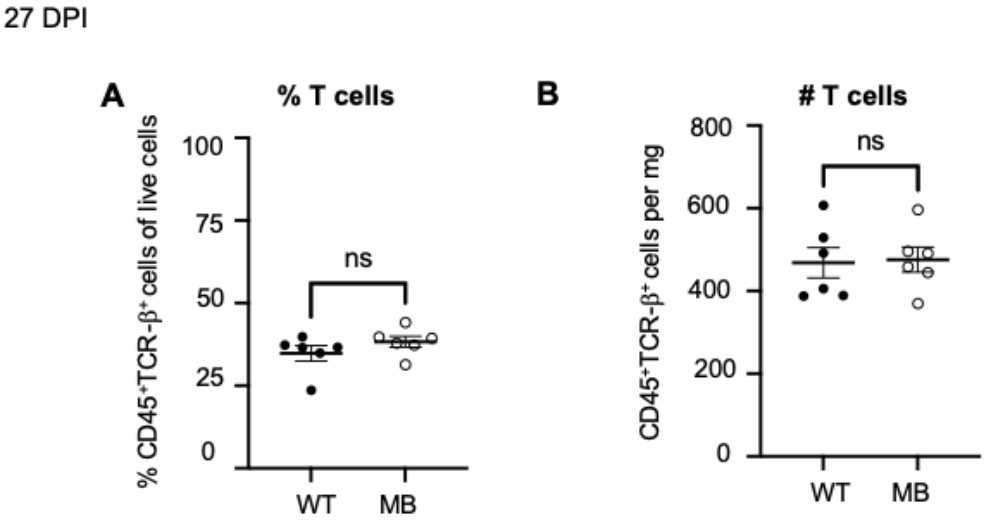
Taken together, our findings indicate that *T. gondii* infection was well-controlled in tumor-bearing mice, did not majorly impact the overall well-being of the animals and did not affect the tumor progression.



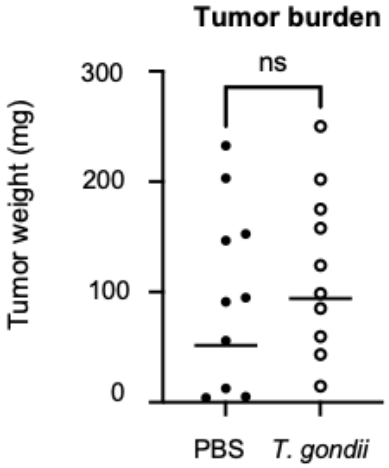
Supplemental Figure 1 Medulloblastoma did not significantly impact the number of T cells in the peripheral blood following *T.* gondii infection.

A) Gating strategy for T cell abundance and composition. Shown is a representative tumor sample from an infected MB mouse being gated for T cell number and composition: singlet -> Live -> CD45+ cells -> TCR-b+ cells -> CD8+ cells or CD4+ cells. Then, among the CD4+ population, Foxp3+ cells were gated. This strategy was used for Fig. 2B-C, Fig. 3A-C, Supp. Fig. 2, and Supp. Fig. 4.

B-C) Flow cytometric evaluation of the number of $CD45^{+}TCR\text{-}b^{+}$ T cells in peripheral blood at 12 DPI (**B**) and 27 DPI (**C**). Gating as described above. N=6-8 mice per group pooled from two experiments. Unpaired two-tailed t-test.



Supplemental Figure 2: *T. gondii*-elicited T cell infiltration into the forebrain was not altered by the presence of medulloblastoma at 27 DPI.
Forebrains were isolated from infected MB mice (n=6) and infected WT littermates (n=6). Flow cytometry evaluates the $CD45^{+}TCRb^{+}$ cell percentage among live cells (**A**) and cell count normalized to tissue weight (**B**). Gating strategy as shown in Supp. Fig. 1A (singlet -> Live -> CD45+ -> TCR-b+). Unpaired two-tailed t-test.



Supplemental Figure 3: Tumor burden was not different between infected and uninfected medulloblastoma-bearing (MB) mice at 27 DPI.
Tissue weights were measured from the dissected tdTomato+ tumor regions of infected MB mice (n=10) and uninfected MB mice (n=10). Unpaired two-tailed t-test.

2.2.2. *T. gondii* infection efficiently recruited functional T cells into Medulloblastoma.

Although *T. gondii* infection was sufficient to facilitate T cell trafficking into the forebrain of medulloblastoma-bearing mice (Supp. Fig. 2), as the cerebellum is known to harbor less *T. gondii* than other regions of the brain [268, 269], it is unclear whether T cell response could develop in the cerebella of infected mice. To answer this question, we performed a time-course study to track the kinetics of T cell abundance in the cerebella of WT mice. The abundance of CD45+ TCR-b+ T cells was assessed by flow cytometry at 14-, 27-, 45-, and 120 DPI, which indicated that T cell infiltration peaked in the cerebella of WT mice at 27 days (Supp. Fig. 4).

Since tumors are known to be an immune suppressive environment toward T cells [167], we then asked whether *T. gondii* infection could recruit T cells into the tumor mass medulloblastoma as efficiently as the cerebella of infected WT littermates at the early chronic infection (Fig. 2.2.A). To address this question, we performed flow cytometry analysis to quantify T cell abundance. We found that T cells were significantly increased in the tumors of infected mice compared to uninfected medulloblastoma, both in terms of the percentage of T cells relative to all live cells (Fig. 2.2.B) and the number of T cells adjusted to tissue weight. The latter value accounted for the cellularity difference between normal cerebella and the cell-dense tumor areas (Fig. 2.2.C). Importantly, the number of T cells adjusted to tissue weight was similar between the tumors of infected medulloblastoma mice and the cerebella of infected WT mice, suggesting that T cell entry was not significantly dampened by the immune suppressive TME (Fig. 2.2.C).

Solid tumors often exhibit immune cell exclusion by confining T cells to the tumor stromal border [351] via structural, signaling, or cell-contact-dependent barriers [352]. These barriers could impede the recruited T cells from infiltrating the tumor parenchyma. To determine whether T cells in the tumor mass could infiltrate into the tumor parenchyma, we performed immunofluorescent staining to assess the spatial distribution of CD3+ T cells (Fig. 2.2.D). Laminin, a glycoprotein component of the basement membrane of blood vessels and the leptomeninges (which include the arachnoid and pia mater) was chosen as the staining target to label these borders of the brain tumors. The distribution of T cells relative to Laminin+ vessels was then determined (Fig. 2.2.E). We found that in uninfected medulloblastoma mice, the number of T cells in tumors was near zero, similar to uninfected normal cerebella (Fig. 2.2.D, left four panels). In response to *T. gondii* infection, T cells were detected in both tumors and cerebella (Fig. 2D, right four panels). Laminin co-staining indicated that both vessel- (Fig. 2.2.F) and parenchyma-associated (Fig. 2.2.G) T cells were significantly elevated in infected tumors. The T cell distribution was similar to those observed in infected WT cerebella. Taken together, these results indicate that *T. gondii* infection is sufficient to recruit T cell infiltration beyond the stromal borders into the parenchyma of medulloblastoma.

In some of the limited reports that identified T cells within brain tumors, regulatory T cells (Tregs) could be enriched in the tumor and were found to exert inhibitory effects over nearby effector T cell activity [162, 353, 354]. Hence, it is important to evaluate the presence and abundance of these cells among the recruited T cells. To do so, we examined the T cell composition in the tumors of infected tumor-bearing mice and cerebella of infected WT mice. We found that the proportions of CD4+ T cells (Fig. 2.3.A), CD8+ T cells (Fig. 2.3.B) were similar between these two groups. Moreover, CD4+Foxp3+ Tregs (Fig. 2.3.C) were similar and accounted for less than five percent of total T cells, suggesting that TME did not skew T cell composition significantly.

Solid tumor environment is known to be a metabolically hostile environment toward effector T cells with effector T cells often become dysfunctional [187] Considered the proximity of the recruited T cells and the tumor cells, we asked if the recruited T cells were still functional. A common to assess T cell functionality is to measure their ability to be reactivated ex vivo [355, 356]. Dysfunctional T cells would have reduced effector cytokines production after ex vivo restimulation. To answer this question, tumor and adjacent tumor-free

cerebella from the infected medulloblastoma-bearing mice were separated, dissociated, then restimulated ex vivo with PMA and Ionomycin for 6 hours and stained for IFNγ for flow cytometry (Supp. Fig. 5). A similar proportion of IFNγ +CD4+ cells (Fig. 2.3.D) and IFNγ +CD8+ cells (Fig. 2.3.E) were detected between tumor-free cerebella and tumors. Likewise, there was no difference in median fluorescence intensity of IFNγ in CD4+ T cells (Fig. 2.3.F) and CD8+ T cells (Fig. 2.3.G) between the two groups. Taken together, our data indicate that the tumor microenvironment did not inhibit *T. gondii*-induced T cell infiltration and their capacity for cytokine production ex vivo.

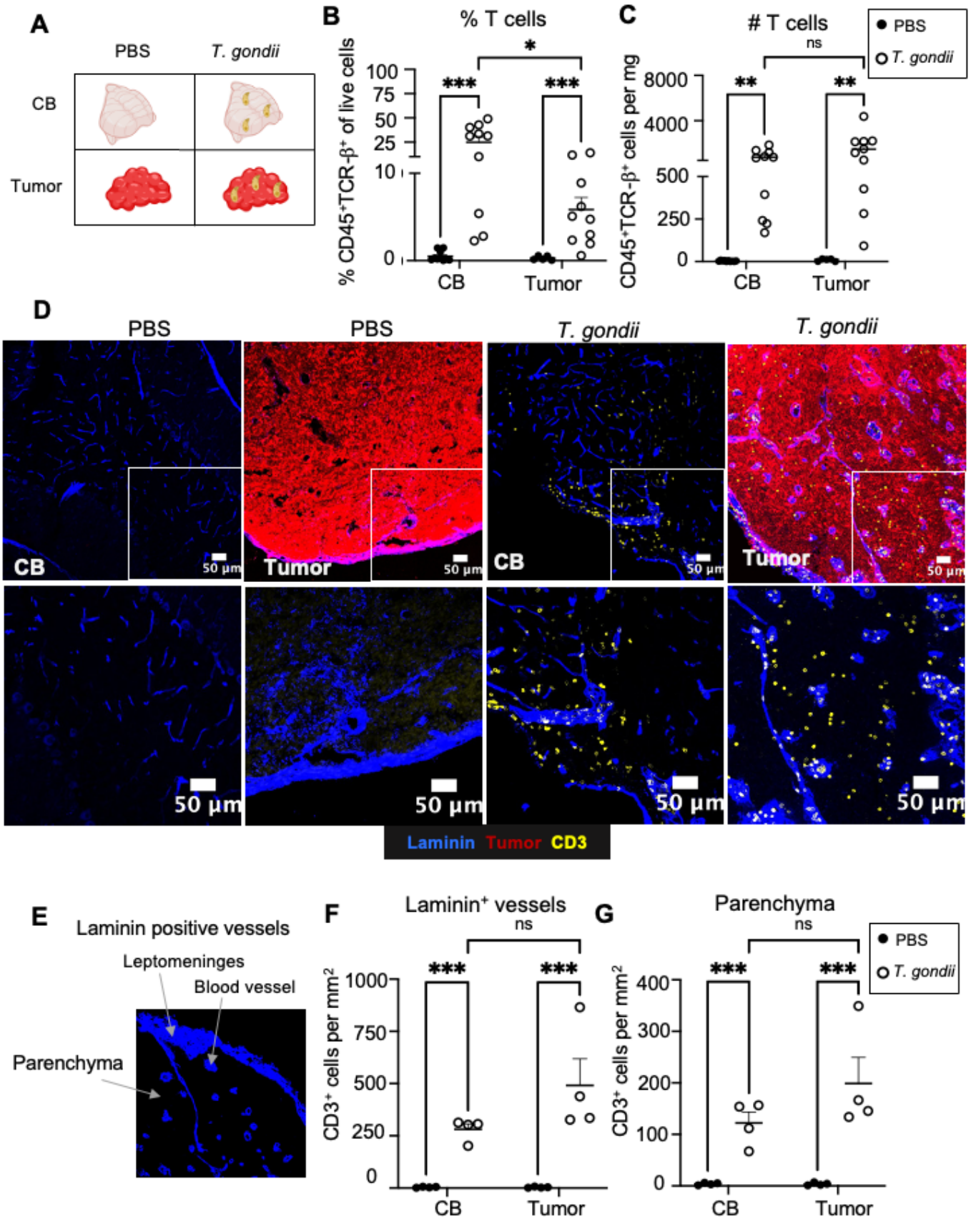

A
PBS
T. gondii
CB
Tumor
B
% T cells
% CD45+TCR-β+ of live cells
100
75
50
25
10
0
***
***
*
CB
Tumor
C
T cells
ns
PBS
T. gondii
CD45+TCR-β+ cells per mg
8000
4000
500
250
0
**
**
CB
Tumor
D
PBS
PBS
T. gondii
T. gondii
CB
Tumor
CB
Tumor
50 µm
Laminin Tumor CD3
E
Laminin positive vessels
Leptomeninges
Blood vessel
Parenchyma
F
Laminin+ vessels
ns
CD3+ cells per mm2
1000
750
500
250
0
***
***
CB
Tumor
G
Parenchyma
ns
PBS
T. gondii
CD3+ cells per mm2
400
300
200
100
0
***
***
CB
Tumor

Figure 2.2: T cells were recruited with similar efficiency into the tumor mass and the tumor-free cerebellum of infected mice.

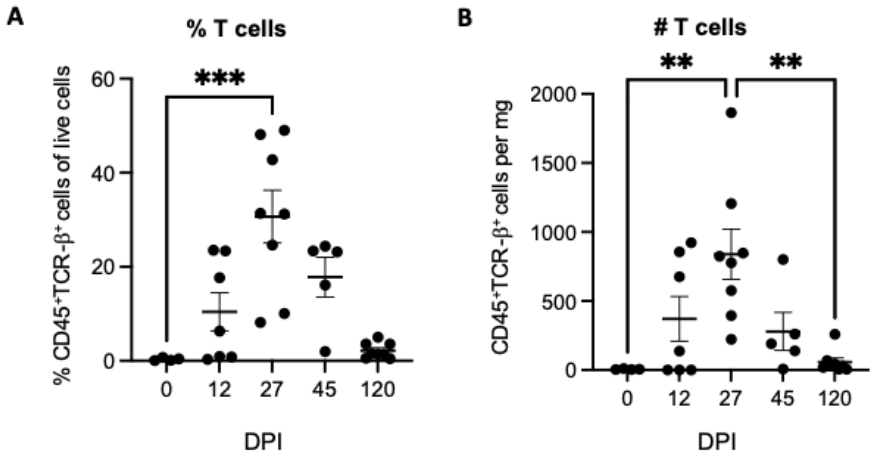
A) Diagram of samples collected to evaluate T cell abundance and distribution. At 27 DPI, cerebella (CB) from WT mice and tumors from MB mice were collected for downstream analysis via flow and immunofluorescence staining.

B – C) At 27 DPI, similar amounts of T cells were detected in the cerebella of infected WT mice as in tumors of infected MB mice. The percentage of CD45+TCRb+ T cells among live cells (**B**) and T cell number relative to tissue weight (**C**) in the cerebella of uninfected WT mice (n=10), the cerebella of infected WT mice (n=10), tumors of uninfected MB mice (n=5), and tumors of infected MB mice (n=10) were evaluated via flow cytometry as described in Supp. Fig 1A.

D-G) At 27 DPI, T cells were found in equivalent amounts at the borders (Laminin+ vessels) and cerebellar parenchyma of infected WT mice (n=4) and tumors of infected MB mice (n=4). Representative confocal images of the spatial distribution of T cells (CD3+, yellow) relative to Laminin positive vessels (blue) within WT cerebella and tumor regions (tdTomato+, red) of uninfected and infected mice **(D).** The lower panels are zoom-ins of the boxed areas of the upper panels. Scale bar = 50 µm. Laminin-positive vessels demarcate leptomeninges and blood vessels (**E**). CD3+ T cell colocalization with laminin (**F)** or the brain parenchyma (**G**) was quantified. Data represents three images from each of 4 mice per group.

Statistical significance was determined by ordinary 2-way ANOVA with main effects only, followed by Tukey pairwise comparisons (**B, C, F,** and **G**).

* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.
Images Created with BioRender.com



Supplemental Figure 4: Kinetic analysis of *T. gondii*-induced T cell infiltration into the cerebellum.
Cerebella from uninfected mice or day 0 (n= 4) and *T. gondii* infected mice at 12 DPI (n=7), 27 DPI (n=8), 45 DPI (n= 5), and 120 DPI (n=8) were assessed via flow cytometry for CD45+TCR-b+ cell presence through percentage of these cells among live cells (**A**) and cell number normalized to tissue weight (**B**).

Gating strategy as shown in Supp. Fig. 1A (singlet -> Live -> CD45+ -> TCR-b+). One-way ANOVA followed by Tukey post-hoc test.

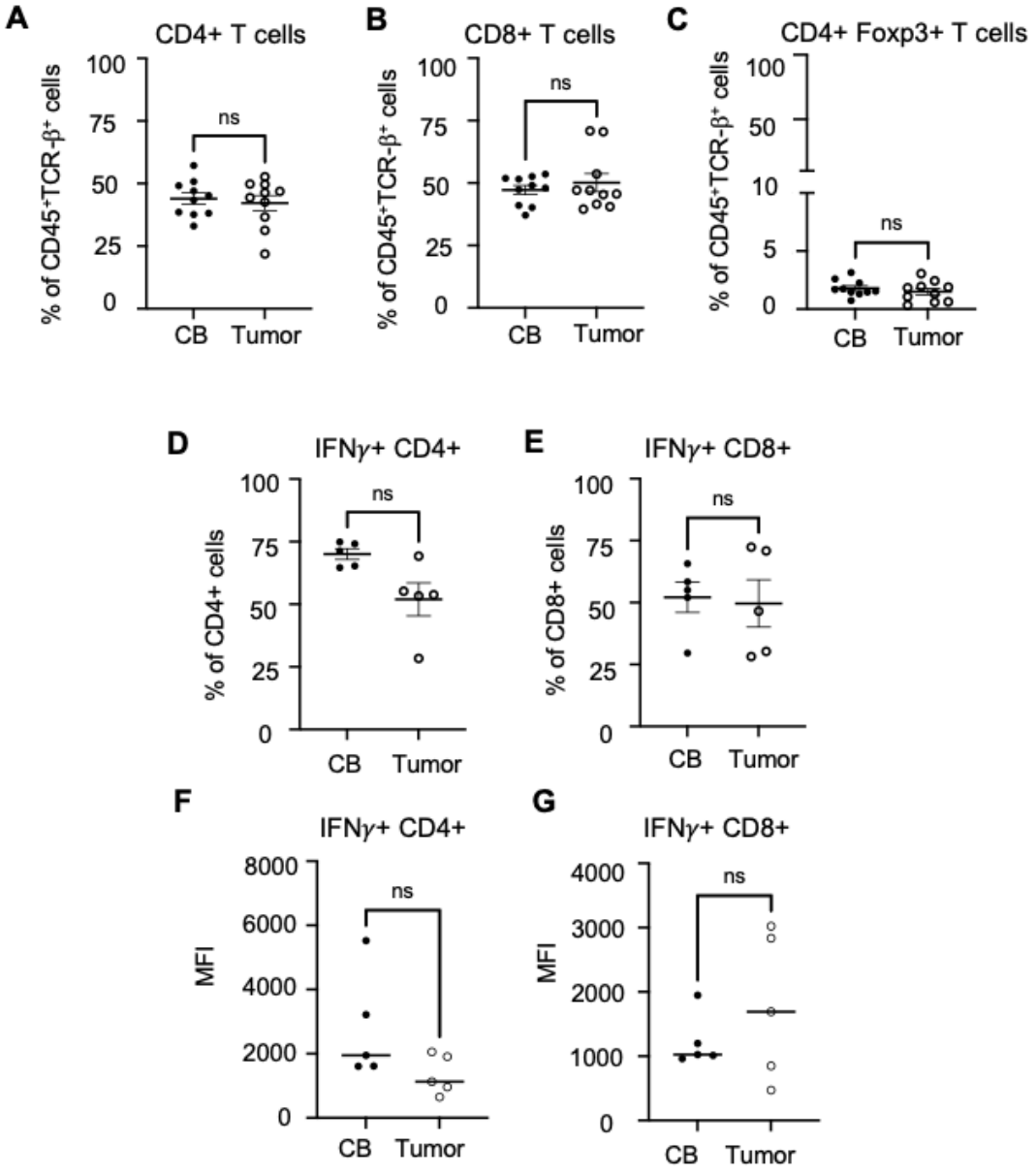
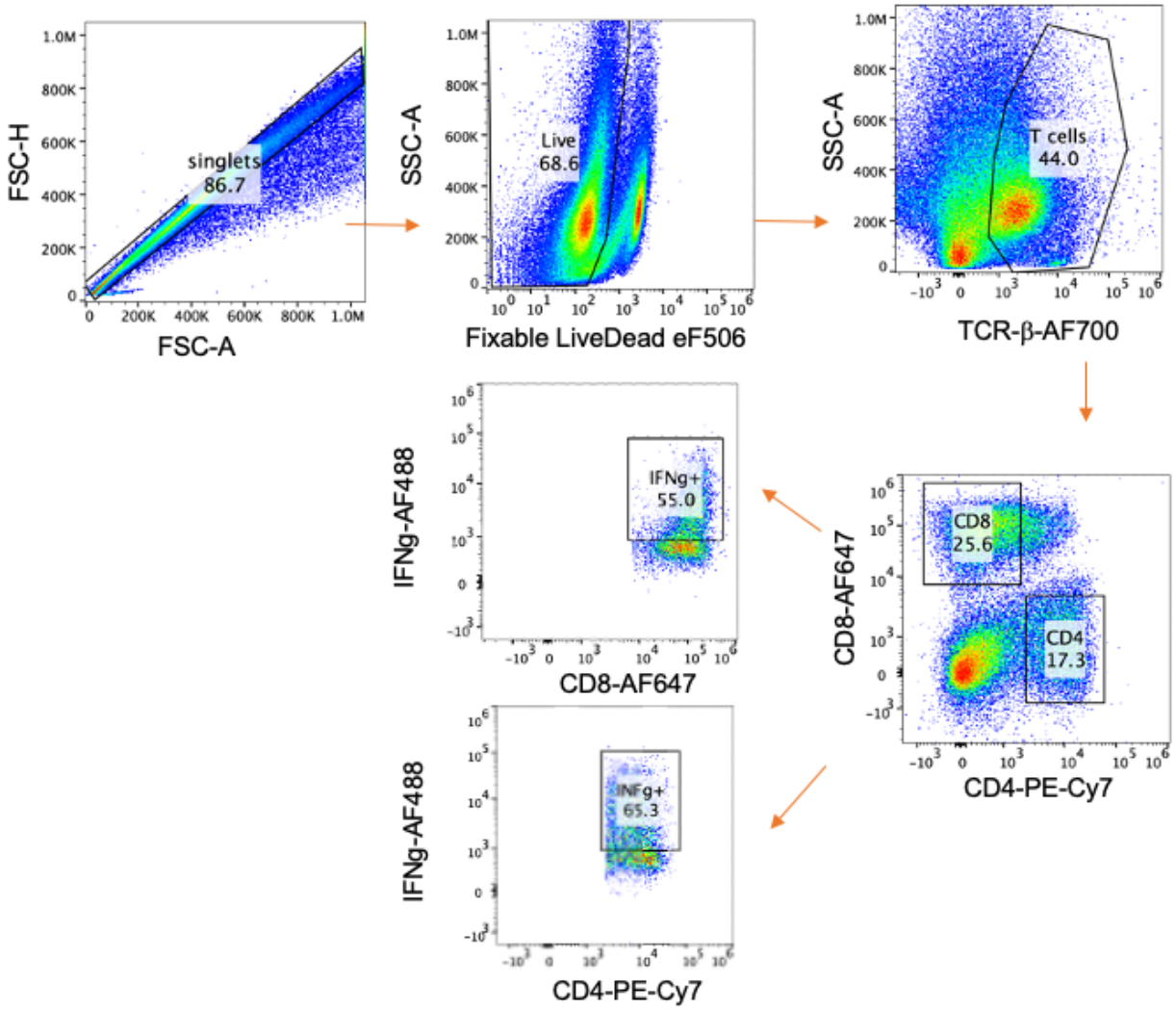


Figure 2.3: T cell composition and activation signatures induced by *T. gondii* infection were similar between tumor regions of MB mice and cerebella of WT mice.

A – C) At 27 DPI, T cells recruited to the tumors of infected MB mice shared a similar composition with those in the cerebella of infected WT mice. Flow cytometric quantification of the frequency of CD4+ T cells (**A**), CD8+ T cells (**B**), and CD4+Foxp3+ regulatory T cells (**C**) among CD45+TCRb+ T cells from the cerebella of infected WT mice (n=10) and tumors of infected MB mice (n=10)**.** The gating strategy is described in Supp. Fig. 1A. Unpaired two-tailed t-test.

D – G) T cells from tumors and adjacent non-tumor cerebella of infected MB mice (n=5) were evaluated for their ability to produce effector cytokines ex-vivo. After tissue dissociation, samples underwent 6 hours of PMA/Ionomycin restimulation in the presence of Brefeldin A.
Flow cytometric evaluation detected no significant differences in the frequency of IFNγ+CD4+ (**D**) and IFNγ+CD8+ cells (**E**) among total CD4+ and CD8+ T cells, between the cerebella and tumor regions. The gating strategy is described in Supp. Fig. 5. Paired two-tailed t-test.

Flow cytometric quantification for Median Fluorescent Intensity (MFI) of IFNγ signals among CD4+ T cells (**F**) and CD8+ cells (**G**). Paired two-tailed t-test. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, ns = not significant.



Supplemental Figure 5: Flow cytometry gating strategy for T cell activation.
Representative non-tumor cerebellar region from an infected MB mouse being gated for T cell activation following PMA-Ionomycin and incubation with brefeldin A. Cells were gated as follows: singlet -> Live -> TCR-b+ -> CD8+ or CD4+ -> IFNg+. The gating strategy was used in Figure 3D-G.

2.2.3. *T. gondii* infection's locally elevated IFNγ remodeled Medulloblastoma into a T-cell accessible and supportive state.

The presence of T cells in the tumor mass indicated that *T. gondii* infection can overcome the immune-suppressive nature of TME. As the principal modulator of Th1 immune response, IFNγ is critical to control *T. gondii* infection [132, 357, 358]. Moreover, IFNγ receptor signaling activation in stromal cells attracts and retains effector T cells [359, 360], and is considered a major signature of immunotherapy responsiveness in solid tumors [361-363]. Thus, we asked if *T. gondii* infection was sufficient to upregulate IFNγ in the TME, which was previously characterized by a Th2 cytokine profile [167]. We found that both IFNγ transcripts (Fig. 2.4.A) and protein (Fig. 2.4.B) were significantly elevated in tumors of infected medulloblastoma mice compared to uninfected tumors.

Next, we hypothesized that IFNγ would likely change the myeloid cell populations and their activation state in the TME. Via flow cytometry, CD11b+ myeloid cells represented the other major immune population in the TME after T cells, in comparison to uninfected medulloblastoma (Supp. Fig. 6). In contrast, B cells, NK cells, and neutrophils were far less abundant. Previous characterization of this GEMM of medulloblastoma reported the presence of microglia, not peripheral myeloid cells [167]. However, *T. gondii* has been shown to recruit peripheral myeloid cells into the brains of infected mice [276, 283]. Although microglia and infiltrating bone marrow-derived myeloid cells are ontogenically and functionally distinct [130], both of these CD11b+ populations express IFNγ receptor and positively regulate Th1 immunity in the brain to control *T. gondii* infection [132, 283]. Acknowledging that the relative abundance of microglia-derived and infiltrating monocyte-derived CD11b+ cells likely differ between uninfected and infected medulloblastoma samples we sought to evaluate the transcriptional profile of CD11b+ cells within the tumor environment after *T*. gondii infection.

To enrich for myeloid cells, we made single cell suspensions from the tumor regions. Since some activated T cells have been shown to express CD11b [364, 365], we depleted T cells with CD3+ microbeads prior to enriching the myeloid cells with CD11b+ microbeads (Supp. Fig. 7A). Using q/RT-PCR, the purity of myeloid cells was confirmed by quantifying the enrichment of CX3CR1, a myeloid cell-specific chemokine receptor [366] (Supp. Fig. 7B), and the depletion of Math1, a tumor cell-specific gene [367] (Supp. Fig. 7C).

Principal component analysis showed that myeloid cells from in *T. gondii*-infected medulloblastoma segregated from myeloid cells populations isolated from uninfected medulloblastoma (Supp. Fig. 8A). A total of 1,259 genes were significantly differentially enriched when these two samples were compared (Supp. Fig. 8B). Gene Set Enrichment Analysis (GSEA) identified "IFNγ response" as the top of the hallmark gene set elevated in infected myeloid cells (Fig. 2.4.C), in support of our hypothesis that IFNγ led to changes in myeloid cell transcript expression and/or recruitment of distinct myeloid cell types from the blood. The transcripts associated with the "IFNγ response" gene family included increased enrichment of *Stat1* and *Irf1*, the transcription factors downstream of IFNγ-receptor signaling (Fig. 2.4.D). In addition, IFNγ-inducible genes that support T-cell functions were detected, including *Cxcl9*, a major T cell chemoattractant [368], and *Cd86,* a co-stimulatory ligand for T cell activation [369]. Antigen presentation is generally suppressed in brain tumors [193, 370, 371], however, IFNγ is known to promote antigen processing and presentation in myeloid cells [372, 373]. Correspondingly, multiple genes related to major histocompatibility complex Class II pathways were enriched in myeloid cells isolated from the tumors of infected mice, including *Cd74*, *Ciita*, *H2-Aa*, and *H2-DMa*.

To verify the upregulation of antigen presentation gene products, immunostaining for MHC-II and Iba-1, a myeloid marker, was performed. There was a significant increase in the percentage of Iba1+ cells that co-expressed MHC-II from the tumors of *T. gondii*-infected mice in comparison to uninfected mice (Fig. 2.4. E-F).

These results collectively indicate that myeloid cells in the TME after *T. gondii* infection are immunologically activated, likely driven by the presence of IFNγ in the local tissue.

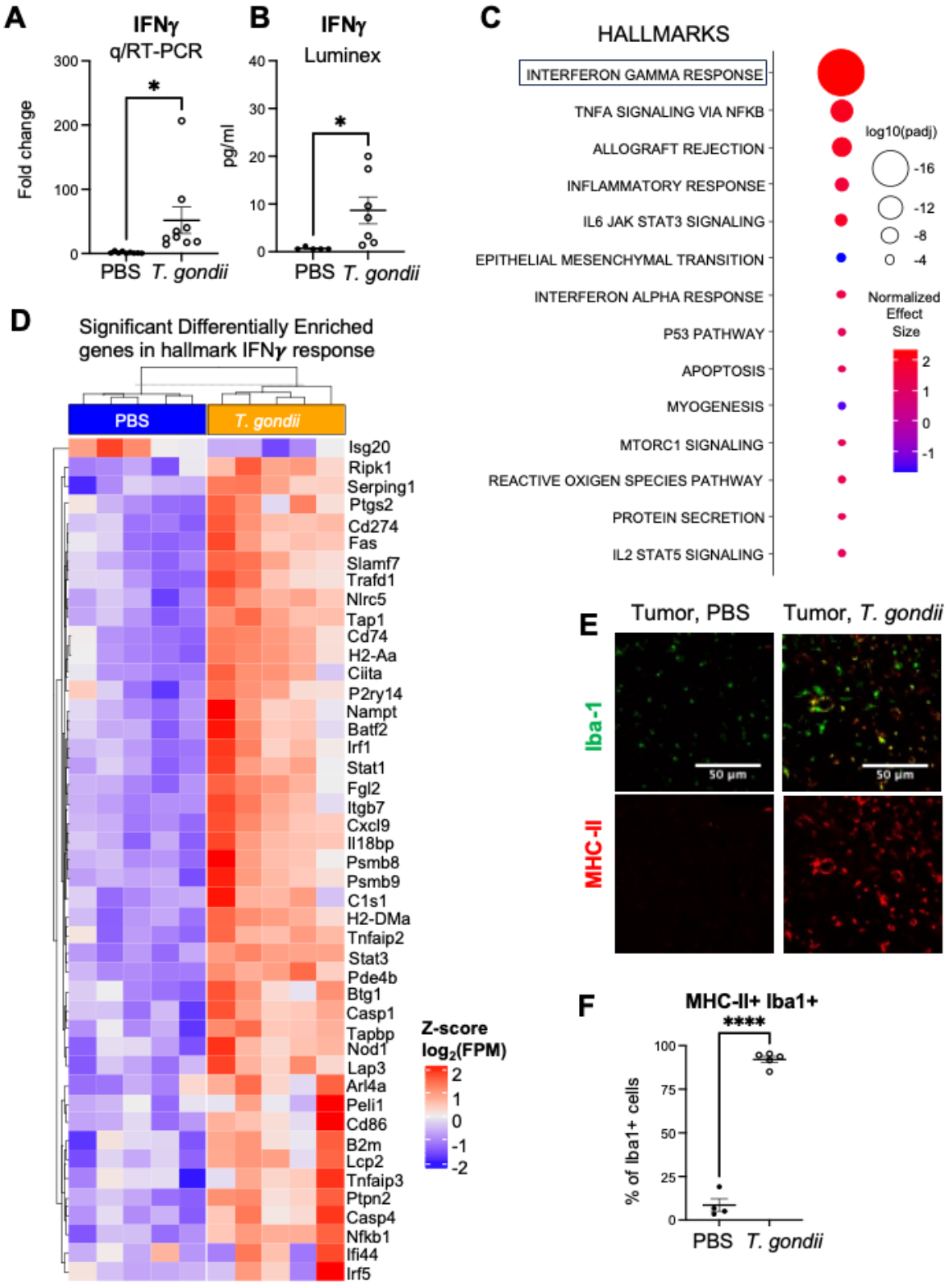


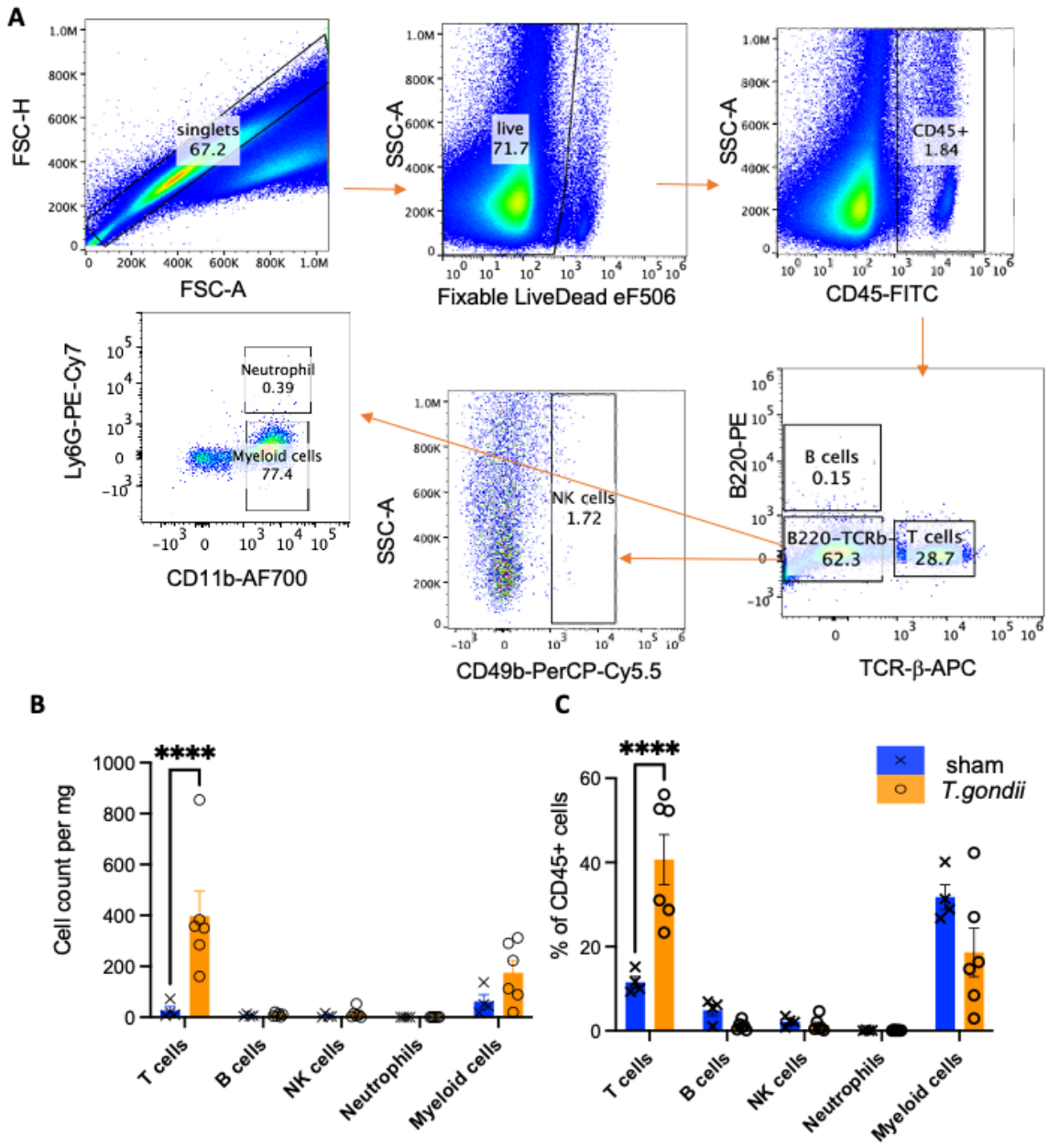
Figure 2.4: T. gondii infection leads to a pro-inflammatory myeloid cell population in medulloblastoma at 27 DPI.

A - B) IFNγ was elevated in the tumor mass of MB mice infected with *T. gondii*. Via q/RT-PCR of bulk-tissue RNA, an elevated transcript level of IFNγ was detected in the tumors of infected MB mice (N=9 per group) (**A**). Correspondingly, via Luminex, IFNγ protein level was significantly higher in the protein lysate of tumors from infected MB mice (n=7) relative to those from uninfected MB mice (n=5) (**B**). Unpaired two-tailed T-test.

C - D) IFNγ-induced transcripts were detected in the CD11b+ myeloid cells isolated from tumors of infected mice. CD11b+ myeloid cells were enriched from tumor samples of uninfected MB mice (n=5) and infected MB mice (n=5) using CD11b+ microbeads. Sample purity was validated as shown in Supp. Fig. 7. Gene Set Enrichment Analysis of RNA-seq data identified 'Interferon gamma response' as the top hallmark of significantly changed genes in enriched myeloid cells from T. gondii infected mice (**C**). Heatmap shows the z-score value for differentially enriched genes (padj<0.05) belonging to the 'Interferon gamma response' hallmark (**D**). The Z-score was calculated using log2(FPM).

E - F) Immunostaining validates the increased expression level of MHC-II protein in myeloid cells within TME. Representative confocal image from tumor regions that were immunostained with Iba1 (green) to label myeloid cells and MHC-II (red) (**E**). The number of MHC-II+ Iba1+ cells in tumors from uninfected (n=4) or *T. gondii*-infected mice (n=5) was quantified (**F**). Unpaired two-tailed T-test.

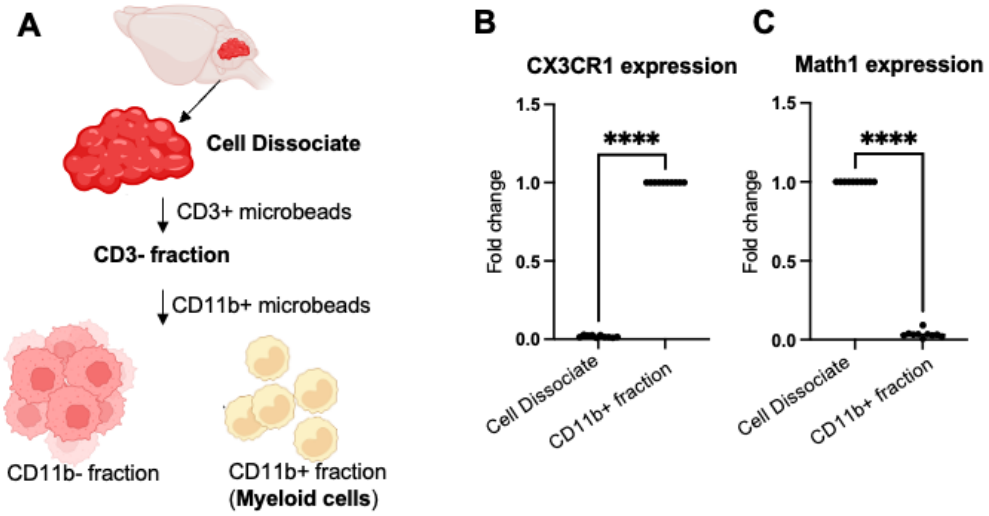
* $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$.



Supplemental Figure 6: T cells and myeloid cells are the major CD45+ cell populations in medulloblastoma at 27 DPI.

A) Representative flow gating strategy to evaluate CD45+ immune cell profile from an infected tumor sample: singlet -> live -> CD45+ -> B220+ for B cells, TCR-b+ for T cells. Then, from the B220-TCR-b- population, CD49b+ cells were gated as NK cells, CD11b+Ly6G+ as neutrophils, and CD11b+Ly6G- were gated as myeloid cells. FMO and full-panel stained splenocytes were used as controls.

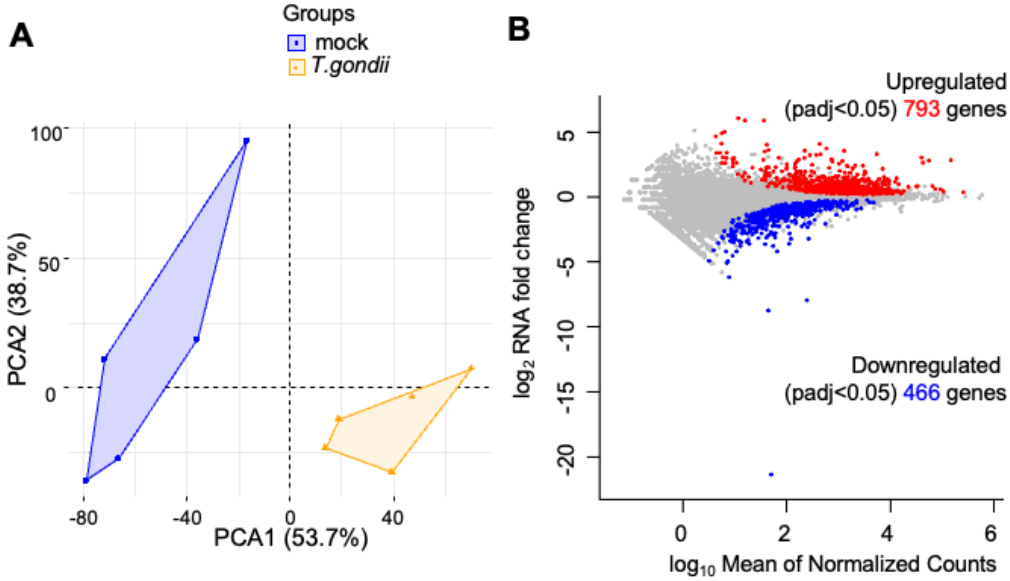
B - C) Flow cytometric evaluation for major immune cell presence in tumors from uninfected MB mice (n=4) and infected MB mice (n=6). Cell count per mg (**B**) and frequency of CD45+ cells (**C**) were assessed for T cells ($CD45^+TCR\text{-}b^+$), B cells ($CD45^+B220^+$), NK cells ($CD45^+B220^-TCR\text{-}b^-CD49b^+$), Neutrophils ($CD45^+B220^-TCR\text{-}b^-CD11b^+Ly6G^+$), and Myeloid cells ($CD45^+B220^-TCR\text{-}b^-CD11b^+Ly6G^-$). Two-way ANOVA.



Supplemental Figure 7: CD11b microbeads efficiently enrich myeloid cells from the tumors.
A) Schematic of CD11b+ myeloid cell collection. After dissecting out tdTomato+ tumor regions, samples were dissociated into a single-cell suspension. Using CD3+ microbeads, T cells were depleted from the samples. Then, using CD11b microbeads, the CD11b+ fraction was collected for further assessment.

B) Via q/RT-PCR, CD11b+ samples were found to be significantly enriched with the myeloid marker CX3CR1 compared to the cell dissociates. N=5 per group. Unpaired two-tailed T-test.

C) Via q/RT-PCR, tumor granule neuron precursor-specific (TuGNP) Math1 expression was significantly less in CD11b^{+} samples than in the cell dissociates. N=5 per group. Unpaired two-tailed T-test.



Supplemental Figure 8: Transcriptional changes in CD11b+ myeloid population were significantly different between tumors from uninfected and *T. gondii* infected mice.

A) Unbiased principal component analysis of all the mapped genes demonstrates that biological replicates cluster together by their experiment condition. N= 5 per group.
B) MA-plot compares the expression of all genes (red denotes activated genes, blue for repressed genes, and light-gray for all other genes) in myeloid samples from the tumors of infected MB mice relative to uninfected samples. N= 5 per group.

2.3. Discussion

In summary, employing the GEM model that endogenously developed medulloblastoma, we found that the presence of brain tumors did not significantly impact the host's ability to control *T. gondii* infection. Importantly, we observed that by the early chronic infection phase (when *T. gondii* has disseminated to the brain), *T. gondii* infection induced effective T cell arrival into the tumor masses. The presence of tumor cells posed minimal barriers to T cell recruitment, infiltration, composition, and activatable status. To understand how *T. gondii* infection remodeled the tumor environment toward a T-cell amenable state, we assessed and found IFNg, the principal modulator of Th1 immunity, to be elevated in the tumor masses. Correspondingly, transcriptional evaluation of the myeloid cells, another major tumor immune cell group, detected the IFNγ response. IFNγ-induced expression for antigen processing and presentation molecules, T cell chemoattractant *Cxcl9*, and co-stimulatory molecule *CD86* likely contributed to the T cell supportive changes in brain tumors.

Brain cancers, especially primary tumors, are often referred to as a 'T cell desert' [159, 162, 374]. However, promoting an overly pro-inflammatory T cell response risks damaging the brain. Clinical efforts to prime anti-tumoral T cells against brain tumors have spanned from systemic infusion of IL-2 and IFNγ [326, 375] to regional delivery of cytokines and CAR-T cells [328, 329]. However, systemic cytokine infusion has been shown to carry major neurotoxicity and other adverse side effects, such as cytokine storm, without significant benefits [326, 327]. Accountable factors for this observation include the short half-life of cytokines and the difficulty in reaching effective levels in the brain tumor mass. Intrathecal delivery, which may reduce the likelihood of systemic toxicity, still does not guarantee access into the brain parenchyma [376, 377] and may only function effectively for tumors that have access to the CSF [183]. In contrast, *T. gondii* has evolved a strategy to efficiently enter the brain while promoting an effective T cell response throughout the brain to control the parasite burden, ensuring host survival for *T. gondii* transmission. Harnessing a multi-faceted mechanism honed by natural selection is, therefore, an appealing strategy to boost T cell entry into brain tumors. As the first step in evaluating *T. gondii*'s potential as a 'bug-as-drug' therapy, this study addresses three foundational questions: can *T. gondii* be well controlled by a brain tumor bearing host? Can *T. gondii* effectively recruit T cells into the immune-suppressive TME? And if yes, are the recruited T cells in an activatable state? Moreover, *T. gondii* infection can be leveraged as a model system to understand the changes in the TME that are capable of breaking the tolerogenic environment imparted by the brain tumor for wider applications.

As for any 'bug-as-drug' strategy, even as a long-term goal, the prerequisite is to mitigate the risk of parasites killing the patients. In this case, we first asked whether systemic immune suppression imparted by brain tumors [312, 342] could impair control of *T. gondii* infection and, in turn, lead to animal death. Our results of similar *T. gondii* burden and overall sickness degree between infected medulloblastoma mice and infected WT mice suggest that, even though brain tumors may compromise systemic immunity to a certain level, the medulloblastoma mice were still able to mount an effective immune response to control *T. gondii* infection and ensure animal viability. However, as discussed below, parasite engineering approaches will be required to develop future therapeutic applications.

The observation that *T. gondii* is sufficient to elicit T-cell infiltration into the brain tumors is groundbreaking. Many studies have established that the brain environment poses a major barrier to peripheral immune surveillance. Primary brain tumors have a negligible number of peripheral immune cells, including T cells, compared to non-tumor regions of the brain [159, 162, 164] and brain metastases attract fewer immune cells compared to their primary tumors [378, 379]. Therefore, the large number of T cells found in the tumor parenchyma upon *T. gondii* infection demonstrates the promise of this strategy to fundamentally improve immunotherapy for brain tumors in the future.

Finally, our data indicated that *T. gondii* infection is sufficient to elicit Th1 immunity in the brain. In conjunction to the elevated level of IFNγ, changes in myeloid cell transcript enrichment that are consistent with augmented IFNγ receptor signaling and T cell-supportive activities were detected. This included the increased transcription of chemoattractant *Cxcl9* for T cells, MHC-II for antigen presentation, and co-stimulatory ligands such as *Cd86*. *T. gondii* infection has been shown to alter the myeloid compartment by not only reprogramming brain-resident microglia toward a disease-associated-microglia (DAM)-like state but also recruiting bone-marrow derived monocytes into the brain [132, 283]. While our RNAseq analysis could not distinguish between altered gene expression in microglia and unique gene expression due to recruited monocytes, an overall shift of the myeloid compartment toward a T cell-supportive state demonstrates the TME-reprogramming capacity of *T. gondii*. The observations align with previous work, in which recruiting activated T cells into the brain depends on an inflammatory local environment[380].

While there is understandable caution associated with the application of infectious agents as cancer therapeutics, the success of BCG for high-grade, noninvasive bladder cancer exemplifies the power of such an approach. Originally developed as a vaccine for tuberculosis, epidemiological data [381] indicated that vaccinated individuals had a lower incidence of bladder cancer. This observation catalyzed studies to directly test the efficacy of local inoculation of BCG in the bladder to mount an immune response to the tumor [382, 383]. Compared to BCG, which was highly attenuated in the lab [384], at least two prevalent strains of *T. gondii* are associated with asymptomatic infection in immunocompetent adults (Type II, used here, and Type III) [224, 229, 338]. These strains can still be problematic for immune-compromised patients, particularly patients with HIV/AIDs [385], transplant recipients [386], and some chemotherapy patients [387, 388]. While this can be managed with medications [255, 389, 390], an ideal therapeutic strain will be sterilizable when no longer needed. To this end, a handful of short-lived, temperature-sensitive [391] and auxotrophic *T. gondii* strains [257] have been developed. Intra-tumoral injection of the uracil-auxotroph *cps* strain or the *gra17* mutant in combination with checkpoint inhibitors has produced long-term anti-tumoral immune responses in preclinical studies for peripheral tumors [258, 259, 261]. However, these engineered strains get cleared by the peripheral immune system before they can disseminate to the brain, and thus are not applicable for brain tumor therapies. Therefore, for brain tumor treatment, an engineered *T. gondii* strain that can withstand the peripheral immune responses to enter the brain and can then be eliminated from the patient once an anti-tumor immune response is successfully mounted would be ideal.

It should be noted that this study cannot directly assess whether TME-infiltrating T cells induced by *T. gondii* infection target tumor-specific antigens. While medulloblastoma may express tumor antigens, without knowing what the specific antigens are, it is not feasible to track them in vivo with this model. In the future, tumor cells that express a model antigen can be used to determine if *T. gondii* can prime anti-tumor T cell responses. Moreover, *T. gondii* has been engineered to express foreign proteins, such as the model antigen Ovalbumin [392], beta-lactamase [393], and cre-recombinase [394] in host cells, opening the door to its use as a tumor antigen delivery tool.

Overall, this proof-of-principle investigation demonstrated effective immune modulatory activities of *T. gondii* in the context of brain tumors, which should motivate both basic and translational studies in the future. For basic research, *T. gondii* can be used to deepen our understandings of essential signaling molecules and cell types needed to support an effective local Th1 immune response in brain cancers. For translational studies, it is highly attractive to engineer *T. gondii* strains that can first achieve optimal immune modulation in brain tumors and then self-terminate, to serve as microbial agents to facilitate immunotherapies for brain cancers.

2.4. Methods

2.4.1. Mice

Medulloblastoma mice (TG11ML/GT11ML, *p53KO*; Math1-Cre/WT; *Ptc-KO*/WT) and WT littermate mice (TG11ML/GT11ML, *p53KO*; *Ptc-KO*/WT). Stock mice were originated from TG11ML, GT11ML JAX# 030578 [395], Ptch1-KO JAX# 003081 [396], p53-KO JAX# 002101 [397], Math1-Cre JAX# 011104 [398]. Balanced numbers of female and male mice were used for each experimental condition. Power analysis was used to predetermine sample size of experimental mice. All mice were healthy, immune-competent, and not involved in any procedures prior to *T. gondii* infection. Animals were housed and cared for according to animal care guidelines approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Virginia.

2.4.2. Parasite strains and infection

The *T. gondii* Me49 strain, which stably expresses GFP and luciferase (Me49Gluc) [399], was used for all experiments. Me49GLuc was maintained on primary human foreskin fibroblasts (HFFs) in DMEM (Thermo) with 10% FBS until passages 3-5, when they were used for infection. Briefly, HFFs were scraped and passed through a 22g blunt end needle to liberate *T. gondii* (Instech Laboratories, catalog LS22/6S). Parasite suspension was then counted on the hemocytometer and diluted in PBS to a final dose of 100 parasites per 200 µL, which was given to each mouse via intraperitoneal injection.

2.4.3. Sickness score monitoring

After infection, mice were weighed every other day through the time of euthanasia. Mice were scored in four categories. Mice reaching a total score of 4 and higher were immediately euthanized. Weight loss percentage: 0 (no change or increase in body weight), 1 (5-10%), 2 (>10-20%), 3 (≥ 25%). Overall appearance: 0 (well-groomed, normal head shape), 0.5 (mild ruffled haircoat or mild domed head), 1 (ruffled haircoat, normal head shape), 2 (ruffled haircoat with mild domed head), 3 (ruffled haircoat with enlarged, domed head). Posture: 0 (no hunching), 0.5 (mildly hunched), 1 (hunched), 2 (unbalanced/swaying, head tilt), 3 (lying prone, ataxia, or hindlimb paralysis). Social Behavior: 0 (actively interacting with other mice), 1 (interactive but slower or less frequent compared to uninfected WT peers), 2 (reduced interaction but responsive when given environmental enrichment), 3 (lethargy or not responsive even if stimulated with environmental enrichment).

2.4.4. Assessment of parasite burden via real-time PCR

Genomic DNA (gDNA) was isolated from heart, lung, and forebrains. Tissues were subjected to mechanical homogenization in PBS using Omni TH tissue homogenizer (Omni International), followed by gDNA isolation using the Isolate II Genomic DNA Kit (Bioline, BIO-52067) per the manufacturer's instruction guide. For each tissue sample, 500 ng of DNA was loaded into the PCR reaction assay. *T. gondii* genomic presence was detected via PCR amplification of the 529 bp repeat element as described previously [345, 346], using the *Taq* polymerase-based SensiFAST Probe No-ROX Kit (Bioline, BIO-86005) and CFX384 Real-Time System (Bio-Rad). A standard curve of 3 to 300,000 genome copies was generated using 10-fold serial dilutions of pure *T. gondii* gDNA, which was then used to determine the total number of *T. gondii* genome copies per µg of gDNA.

2.4.5. Forebrain, Tumor, and Cerebellum processing

Using a previously developed method [167], mice were anaesthetized and perfused with PBS to remove circulating red-blood cells and immune cells. Cortices, tdTomato+ tumors, or normal cerebella were dissected, weighed, then digested with Papain (Worthington Biochemical) for 45 minutes at 37 ^{o}C before being triturated and passed through a 70 µm cell strainer. To remove the myelin portion, cell dissociates were applied to a percoll gradient (35%/65%) followed by centrifugation at 1300g for 30 minutes. After aspirating the myelin top layer, cell pellets were washed and resuspended in complete RPMI media (cRPMI; 10% heat inactivated FBS [Gibco], 1% penicillin/streptomycin [Gibco], 1% sodium pyruvate [Gibco], 1% non-essential amino acids [Gibco], and 0.001% 2-Mercaptoethanol [Life Technologies]).

2.4.6. Evaluation of T cells in peripheral blood

After ketamine anesthesia, 200 µL of blood per mouse was collected by cardiac puncture. Blood sample was placed in a 1.5 mL microtube pre-filled with 50 µL of 0.5M EDTA [400] and gently flicked to distribute the anticoagulant evenly throughout the sample. The samples were briefly spun at 300g, treated with ACK (Ammonium-Chloride-Potassium) Lysing Buffer for 2 min to remove red blood cells, washed with complete RPMI media and used for flow cytometric evaluation.

2.4.7. Ex vivo T cell restimulation assays

Cells isolated from tumors or cerebella were restimulated for 6 hours at 37 °C with PMA (0.1 µg/mL) and Ionomycin (0.5 µg/mL) in the presence of brefeldin A (10 µg/mL, Selleck Chemicals) to inhibit cytokine release. Cells were washed with complete RPMI media and stained for surface markers, followed by intracellular cytokine staining.

2.4.8. Flow cytometry

After tissue dissociation, single-cell suspensions were plated into a 96-well plate for flow immunostaining. First, samples were stained with fixable Livedead dye eFluor 506 (1/1000 dilution in 1X PBS) for 15 min at room temperature. After a wash with FACS buffer (2 µg/mL BSA, 0.2% EDTA in 1X PBS), samples were resuspended in 50 µL of Fc block (1 µg/mL CD16/CD32 Bioscience 553141, 0.1% rat γ-globulin Invitrogen PI31885) for 10 minutes at 4°C before primary antibodies were added in FACS buffer to a final 1/200 dilution. Samples were then incubated for 30 minutes at 4°C. After a wash, samples were fixed with Fixation/ Permeabilization Diulent and Concentrate (Invitrogen, 00-5223-56 and Invitrogen, 00-5123-43) overnight at 4°C. For intracellular target staining, samples would be washed and further stained with antibodies diluted in 1X Permeabilization Buffer (Invitrogen, 00-8333-56) for 30 minutes at 4°C, washed and resuspended in FACS buffer. Data acquisition was done on an Attune NxT at the UVA Flow Cytometry Core and analyzed using FlowJo.

Antibodies used in experiments included: CD45-FITC (eBioscience 11-0451-82), TCR-b-APC (eBioscience 17-5961-82), TCR-b-AF700 (Biolegend 109224), CD8a-AF647 (Biolegend 100724), CD4-PE-Cy7 (eBioscience 25-0041-82), CD8a-PerCP-Cy5.5 (eBioscience 45-0081-82), Foxp3-eF450 (eBioscience 48-5773-82), IFNγ-AF488 (eBioscience 53-7311-82), B220-PE (eBioscience 12-0452-82), CD49b-PerCP-Cy5.5 (Biolegend 103519), CD11b-AF700 (Biolegend 101222), Ly6G-PE-Cy7 (Biolegend 127617).

2.4.9. Immunofluorescence

Post PBS perfusion and brain collection, cerebellar regions with tumors were dissected out in sagittal plane under the microscope. The dissected tissue was fixed in 4% PFA (24 hours at 4°C), dehydrated in 30% sucrose (24 hours at 4°C), then embedded in optimal cutting temperature (O.C.T.) medium. Using a cryostat, histological samples were cut into 20 µm thick sections. For immunostaining, sample slides were dried at room temperature for at least 1 hour before being blocked with 10% serum in PBST (PBS + 0.3-0.5% Triton X) for at

least 30 minutes at room temperature. Samples were then placed in primary antibodies (diluted in 1% serum PBST) overnight at 4°C. After 3-5 washes with PBST, samples were stained with fluorophore-conjugated secondary antibodies (1:250) overnight at 4°C. After further washes to remove excess secondary antibodies, sample slices were mounted in 70% glycerol with coverslips. Image acquisition was performed on Zeiss LSM700, Zeiss LSM900, and Leica DMi8 Thunder Imager with TIRF at 20X magnification.

Quantification of T cells in TME

For each experimental mouse, three representative sections were immunostained with anti-Laminin (Sigma L9393, 1:75 dilution) and anti-CD3 (clone 17A2, Biolegend 100201, 1:100 dilution) to label T cells. For each tissue section, we took a tiled image at 20X magnification. tdTomato was used to identify the tumor region. Using ImageJ, the number of parenchyma-infiltrating T cells was determined by counting CD3+ cells not associated with Laminin+ stromal borders per unit area. Likewise, the number of T cells in the blood vessels or leptomeninges was calculated by counting T cells colocalized with Laminin and normalized to the tissue area (μm), which was calculated by ImageJ.

Quantification of Myeloid cells in TME

Representative sections were immunostained for Iba1 (wako 019-19741l, dilution 1:200) to visualize myeloid cells and MHC-II (clone M5/114.15.2, eBioscience 13-5321-82, dilution 1:200) to assess antigen presentation activity. Colocalized MHC-II+ Iba1+ cells were counted using ImageJ then divided against the total Iba1+ cells to calculate the percentage of myeloid cells expressing MHC-II.

2.4.10. Luminex

Fresh-frozen tumors were homogenized in Tissue Extraction Reagent I (Life Technologies) supplemented with protease inhibitors and phosphatase inhibitors. Samples were mechanically homogenized and left on a rocking platform at 4 ^{o}C for 30 minutes. After a centrifuge at 14,000 rpm for 15 minutes, lysates were collected, and protein concentrations were measured by Pierce™ BCA protein Assay Kit (Thermo Scientific). Samples were then adjusted to 1 mg/mL in Tissue Extraction Reagent I and submitted to UVA Flow Cytometry Core Facility to run a Multiplex Mouse Th1/Th2 Cytokine Panel, which detected IFNγ, IL-2, IL-3, IL-4, IL-5, IL-6, IL-10, IL-13, and IL-15.

2.4.11. Enrichment of CD11b+ myeloid cells

After tumor harvest, Papain digestion and myelin removal using Percoll as described above, the tumor cell suspensions were treated with anti-CD3e+ microbeads (Miltenyi 130-094-973) and run through the autoMACS® Pro Separator to deplete CD3+ T cells from the samples. Then, the negative fractions were treated with anti-CD11b magnetic beads (Meltinyi Biotec, 130-093-634) and run through the autoMACS Pro Separator using the “Double-positive selection” program, from which CD11b+ fractions were collected. Myeloid purity and identity were confirmed via q/RT-PCR.

2.4.12. RNA extraction, cDNA amplification, library construction

Total RNAs of CD11b+ myeloid cells were extracted using the RNeasy micro kit (QIAGEN) according to the manufacturer’s instruction. RNA quality was assessed by Bioanalyzer (Agilent Technologies) with RNA integrity number (RIN) of ≥ 6.5 were selected for library construction. Illumina TruSeq Stranded Total RNA Library prep kit (#20020596, Illumina) was used to prepare the libraries per Illumina protocol specifications. Ribozero beads, included in the kit, were used to deplete the rRNA. Concentrations of the libraries were determined using the Qubit Flex (#Q33327, Fisher) and the Qubit 1X dsDNA HS Assay kit (#Q33230, fisher). The libraries were pooled together at a 4nM concentration and loaded on a Nextseq 550 using a NextSeq 500/550 High Output Kit v2.5 (150 Cycles) (#20024907, Illumina).

2.4.13. RNAseq analysis

All sequence reads files (in fastq format) successfully passed 'Basic Statistic' check by FastQC (v0.11.5) [401]. The reads were aligned to the mouse genome assembly (mm10) using STAR (v2.7.9a) [402] and quantified with RSEM (v1.3.0) [403]. Each sample demonstrated a count of mapped reads greater than 12 million, or a mapping rate above 84%.

Unbiased principal component analysis was performed to ensure that replicates group together and that variation is observed between uninfected control and *T. gondii* infection (Supp. Fig. 9A). Differential gene expression analysis was conducted using the standard DESeq2 (v1.36.0) pipeline [404] to identify differentially expressed genes with a false discovery rate less than 0.05 (Supp. Fig. 9B). To correct for possible differences between females and males, sex was included in DESeq2 as a covariance. Gene Set Enrichment Analysis (GSEA) [405] was performed in R with the fgsea package [406] with hallmark gene sets, using $\log_2$(Fold Change) to rank all the genes (Fig. 4C).

Heatmaps were generated with ComplexHeatmap R package [407] on significantly changed genes (padj<0.05) in the hallmark of "Interferon Gamma response" (Fig. 4D).

2.4.14. Quantitative RT-PCR

Total RNA was isolated from tissues using RNeasy plus kit (QIAGEN), from which an equivalent amount of RNA from each sample was reverse transcribed to make cDNA with iScript cDNA synthesis kit (Bio-Rad). Real-time qPCR was performed using SyBR green/Rox PCR Master Mix (Kapa Biosystems) and ABI StepOnePlus real-time PCR system, following manufacturer's instruction. Relative levels of cDNA for the interest genes were normalized with the level of Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH). Primer details: GAPDH (forward-CAGGTTGTCTCCTGCGACTT, reverse-ATGTAGGCATGAGGTCCAC), IFNγ (forward-ACTGGCAAAAGGATGGTGAC, reverse-GACCTGTGGGTTGTTGACCT), CX3CR1 (forward-TCTTCACGTTCGGTCTGGTG, reverse-GAAGAAGGCAGTCGTGAGCT), Math1 (forward- AACGGCGCAGGATGCA, reverse-TTGAAGGACGGGATAACGTTG).

2.4.15. Assessment of tumor weight

After euthanasia and perfusion with PBS, tdTomato+ tumor regions were carefully dissected out from the brain under Olympus Macro Zoom Microscope MVX10 and weighed with Mettler Toledo AB104-S analytical balance.

2.4.16. Statistical analysis

GraphPad PRISM 10 (La Jolla, CA) was used for most statistical analyses and figure generation. For two group comparisons, data were assessed for normality. Then based on whether samples were paired, paired or unpaired T test was used. For multiple comparisons, ANOVA and related tests would be applied. Sickness scores were multiplied by two to accommodate assessment via Poisson mixed-effects model in R (version 4.3.3) and Tukey post-hoc contrasts were corrected for multiple testing across a family of 5 comparisons using the emmeans package. Specific tests are indicated in the figure legends. Data are represented as individual data points with mean ± standard error of the mean (SEM). Statistical significance is noted by $*p<0.05$; $**p<0.01$; $***p<0.001$; ns, not significant.

Chapter 3: Future Directions

3.1. Immune activities of myeloid cells in the tumor could be further augmented to support T cell response in brain tumors during the *T. gondii* infection model.

3.1.1 Hypothesis, rationales, and preliminary data.

a) Hypothesis: *T. gondii* induced Th1 immunity is orchestrated by IFNγ. Although IFNγ was elevated in medulloblastoma after *T. gondii* infection, the transcriptional profile of enriched CD11b+ myeloid cells from infected tumors shows a lack in upregulation of IFNγ-inducible CD40, a major T-cell activating molecule, and inducible nitric oxide synthase (iNOS), a proinflammatory marker for anti-tumoral macrophages. Considered that IL4 is elevated in medulloblastoma at baseline and is known to antagonize IFNγ, IL4 may suppress IFNγ induced expression of CD40 and iNOS in tumor's CD11b+ myeloid cells. Given the location and abundance of microglia in tumor at baseline, deleting IL4Ra expression in microglia and infiltrating monocytes could potentiate IFNγ response and improve T cell recruitment and activity in tumors.

b) Rationale and preliminary data:

Myeloid cells are known to help orchestrate T cell immunity in anti-tumoral response and Th1 infection. In the context of tumors, effective anti-tumoral T cell response is accompanied by CD40 and iNOS expressed myeloid cells including infiltrating monocytes. CD40 is receptor for CD40L, which is expressed on activated CD4+ T cells. In multiple preclinical solid caners, CD40 activation help facilitating anti-tumoral control from supporting intratumoral APCs maturation [408], reinvigorating T cell response [409], and promoting the inflammatory response of the overall tumor milieu [410]. Antibody blocking CD40:CD40L engagement abrogated the survival benefit [411] while activation using agonist antibodies improved T cell and tumor-control [408, 409].

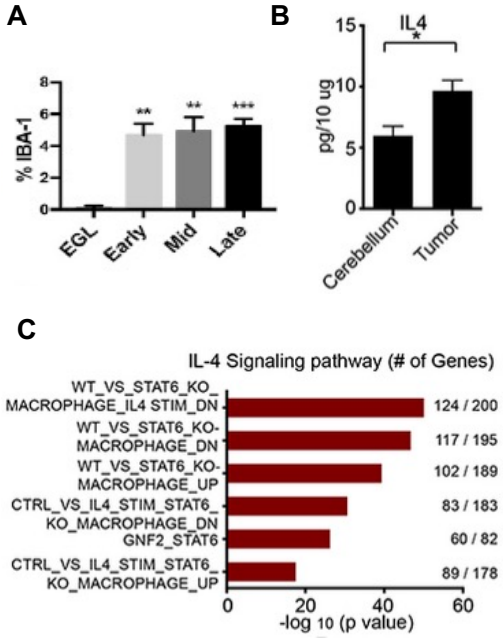


Figure 3.1.: At baseline, medulloblastoma has higher number of microglia and IL4 than non-tumor areas. **A)** Microglia density was sparse at pre-malignancy (external germinal layer, EGL) but significantly increases during tumor development. **B)** Level of IL4 in the tumor, detected via Luminex. **C)** Pathway analysis on the differentially expressed genes between tumor-associated microglia and normal microglia from WT-brain.

iNOS expression in tumor associated macrophages has also been shown to support anti-tumoral T cell response [411, 412]. In murine tumor models, chemical inhibiting iNOS activity or genetic deletion of *Nos2* gene undermined T cell response against the cancer cells [409, 411]. In a spontaneous melanoma model, mice that developed tumor-specific immunity had an accumulation of CCR2+ Ly6C^{high} inflammatory monocytes in the skin [413]. Depleting this cell population or their reactive oxygen species products increased tumor formation rate.

During *T. gondii* infection in the brain, monocytes are recruited to the brain [276, 283], and CD40 and iNOS have been shown to support *T. gondii* control. Expression of iNOS elevates the local level of nitric oxide and has direct *T. gondii* killing effect [414]. In vitro culture with microglia infected with tachyzoites and treated with either anti-CD40 antibody or control antibody found that CD40 induced autophagy marker LC3 in microglia [415].

At baseline, microglia were the only immune cells detected in our lab GEM medulloblastoma model. There were many more microglia in tumors than in the nearby non-tumor regions and the higher density of the Iba1+ microglia developed early on and persisted throughout tumor development (Fig. 3.1.A). In conjunction, a higher level of IL4 was detected in the tumors (Fig. 3.1.B). Since IL4 is a potent mediator for Th2 cell-mediated immune response, we tested whether it could affect the tumor-associated microglia (TAMg). Indeed, evaluating the differentially expressed genes between TAMg and microglia detected an enrichment of genes in IL4-signaling pathway (Fig. 3.1.C).

During *T. gondii* infection, along with the elevation of IFNγ (Fig. 2.4.A-B), assessment the transcriptional profile of CD11b+ myeloid cells detected an enrichment of genes in the IFNγ-signaling (Fig. 2.4.C-D). The observations suggest a change in both the cellular state and the composition of myeloid cells in the tumors. Surprisingly, antigen presentation activities were detected in the myeloid cells post-infection, we failed to detect transcriptional upregulation in other IFNγ-inducible genes. Expression of *CD40* (Fig. 3.2.A) and *Nos2* (Fig. 3.2.B) was not significantly upregulated between the baseline TAMg and the enriched myeloid cells post-infection.

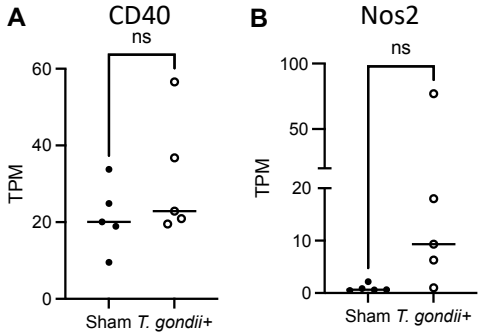


Figure 3.2.: Relative to tumor-associated microglia, multiple IFNγ inducible pro-inflammatory genes stayed unelevated in the enriched CD11b+ myeloid cells post *T. gondii* infection. **A)** CD40 and **B)** Nos2. Transcripts Per Million (tpm)

IL4 is potentially responsible for the lack in upregulation of *CD40,* and *Nos2*. IFNγ and IL4 are known to trigger opposing activation programs in myeloid cells. Reduced expression of *CD40* [416] and *Nos2* [417]were observed in macrophage and microglia cultures that were treated with both cytokines. For *CD40*, IL4-activated STAT6 was found to occupy *CD40* promoter and inhibit IFNγ induction. Additionally, IFNγ-inducible genes that requires auxiliary transcription factors (such as JUNB and C/EBPβ) are more sensitive to IL4 inhibitory effects [416]. For *Nos2*, a coactivator was found to be taken up by STAT6 in a competitive manner, suppressing its expression [417].

In summary, while an enrichment for IFNγ-signaling genes was detected in the myeloid cells of tumors post infection, IFNγ inducible *CD40* and *Nos2* genes were not upregulated at transcript level. Considering that IL4, an IFNγ antagonist, is present in the tumor, blocking IL4 signaling in myeloid cells can improve the expression of CD40 and iNOS, molecules with anti-tumoral T-cell function, and augment T cell response in the tumors.

3.1.2 Proposed experiments

a) Inactivation of IL4 signaling will increase CD40 and iNOS expression during *T. gondii* challenge.

Targeting IL4-receptor via Cre-recombinase has been established via deleting an essential region of IL4R-a gene [418]. IL4-receptor is a heterodimer composed of IL4Ra with either a common gamma-chain or IL13Ra1 [419]. To disable IL4Ra expression in myeloid cells specifically, CX3CR1 promoter driven Cre expression would be used. CX3CR1 is a fractalkine receptor that is strongly expressed among myeloid cells, making its promoter activity and recombinase expression reliable [420]. Since Cre expression is now expressed under CX3CR1 promoter activity, I will have to incorporate $Cx3CR1^{Cre}$; $IL4Ra^{fl/fl}$ the system into a Math1-GFP medulloblastoma model ($Ptch1^{KO/wildtype}$; $p53^{KO/KO}$). A potential caveat with this experimental system is that IL4Ra-disabled myeloid cells may exacerbated the inflammatory response during *T. gondii* infection. IL4 has been shown to be part of the immune-regulatory mechanism to prevent overt inflammation and immunopathology during *T. gondii* infection [421, 422].

First, I will verify the IL4ra expression level the myeloid cells of (*Cx3CR1^{Cre}; IL4Ra$^{fl/fl}$; Ptch1 $^{KO/WT}$; p53$^{KO/KO}$*; also called "IL4Ra-MoMB") in comparison to littermate (*Cx3CR1^{Cre}; IL4Ra$^{WT/WT}$; Ptch1 $^{KO/WT}$; p53$^{KO/KO}$*; also called "IL4Ra+MoMB") at physiological state. I will collect brain dissociate and blood samples from both IL4Ra-MB mice and IL4Ra+MB mice. Via flow cytometry, I will check IL4Ra expression level among the CD45+CD11b+. I expect that IL4Ra expression will be much lower for myeloid cells from IL4Ra-MoMB mice than those coming from IL4Ra+MoMB mice.

Then, via q/RT-PCR and flow cytometry, I will evaluate CD40 and iNOS expression in CD11b+ myeloid cells from tumors of IL4Ra-MoMB, in comparison to myeloid cells from the tumors of IL4Ra+MoMB at 27 DPI. Assuming that IL4Ra deletion disables STAT6 activity, the transcriptional expression of CD40 and iNOS would be upregulated in the presence elevated IFNγ. Both CD40 and iNOS transcript level would be higher in IL4Ra-MoMB myeloid cells than IL4Ra+MoMB myeloid cells. Via flow cytometry, the percentage of myeloid cells that express CD40 (CD45+ CD11b+CD40+) among total myeloid samples, would be higher in myeloid cells from IL4Ra-MoMB tumors than IL4Ra+MoMB tumors. The mean fluorescence intensity (MFI) for CD40 would also be higher in IL4Ra-deficient myeloid cells than in IL4Ra-intact myeloid cells.

b) inactivation of IL4 signaling in TAM will enhance T cell response in tumors *T. gondii* infection.

If CD40 and iNOS protein expression could be elevated in myeloid cells post infection, the higher density of microglia in the tumor baseline could turn the tumor environment into an immune-"hotspot". Since T cells from tumors and non-tumor cerebellar regions have been previously shown to be equivalently reactivatable with PMA and Ionomycin (Fig. 2.3), infiltrating T cells are not negatively impacted by tumor cells and IL4 presence. Since both MHC-I and MHC-II transcription expression was upregulated in the myeloid cells post infection, I hypothesize that there would not a be preferential recruitment for either CD4+ or CD8+ T cell.

To assess the abundance of T cells in tumors, I will use a dual approach with flow cytometry and immunofluorescence. Via flow cytometry, I expect that the cell counts of CD45+ TCRb+ cells, normalized to tissue weight, and their frequency among live cells would be higher in tumors from IL4Ra-MoMB mice than tumors from IL4Ra+MoMB mice. Although the percentage of CD4+ and CD8+ T cell subsets among total T cells is likely to stay similar between the two groups, the cell count of both T cell subsets will be higher in tumors with IL4Ra-disabled TAMg. Via immunofluorescence for CD3 and Laminin, I expect the percentage of CD3+ T cells detected in the parenchyma will be higher in tumors with IL4Ra-disabled TAMg than in normal tumors.

In several tumor contexts, higher expression of iNOS and CD40 on myeloid cells is associated with higher T cell functionality [411, 423]. Hence, T cell activity is likely to be higher in tumors with IL4Ra-disabled TAMg. To assess the recruited T cell functionality, I will evaluate the frequency of proliferative T cells among total T cells via flow cytometry. In conjunction, via Luminex with fresh frozen tumor samples and in situ hybridization, I measure the IFNγ and Granzyme B levels and their spatial distribution in the tumor. I anticipate that IFNγ and Granzyme B levels would be higher in tumors with IL4Ra-disabled TAMg than in normal tumors. Histologically, the IFNγ and Granzyme B signals will be detected higher in areas with CD40+ and iNOS+ expressing myeloid cells.

3.2. Evaluate whether a self-terminating *T. gondii* upon bradyzoite differentiation could induce a similar degree of T cell entry in brain tumors.

3.2.1 Hypothesis, rationales, and preliminary data.

a) Hypothesis:

Compared to wild-type *T. gondii*, a self-terminating *T. gondii* strain during the cyst biogenesis stage can recapitulate the T cell recruitment effect into the brain of WT mice and the tumors of MADM medulloblastoma mice without conferring chronic infection.

b) Rationale and preliminary data:

Although *T. gondii* infection enables T cell recruitment and activation in brain tumors, the risk of chronic infection with wild-type *T. gondii* is undesirable. An engineered *T. gondii* strain that could replicate the T cell infiltration phenotype in the brain without persisting in the tumor-bearing host would be a more applicable microbial agent. The detection of *T. gondii* (Fig. 2.1.E) and IFNγ elevation in medulloblastomas (Fig. 2.4.A-B) during wild-type *T. gondii* infection suggest that the two variables are essential for T cell response in the tumors. However, like other IFNs, clinical trials for IFNγ systematic infusion in recurrent glioblastoma and pediatric high-grade glioma have poor tolerability and yielded minimal clinical benefit [424, 425]. Even when IFNγ could be delivered to the tumor, given the molecule's short half-life (30 minutes via intravenous injection and 4.5 hours after intramuscular injection) [426], repeated injection would be necessary to sustain its bioavailability.

Regarding the second biological variable (local presence of *T. gondii* in the brain), *T. gondii* likely have entered the differentiation process at 27 DPI [427]. *T. gondii* tachyzoite form provides access to the parasite components that could activate the host innate immunity or antigenic secreted molecules (e.g., GRA6, ROP5 [298]). These materials would be more sequestered from the host cells when *T. gondii* enter the mature cyst form. However, the differentiation process from tachyzoite to mature bradyzoite is known to be a continuum. Through in-vitro work, intermediate stages where tachyzoite and bradyzoite proteins could be co-expressed have been observed [428].

Given the above characteristics of tachyzoite, local delivery of replication-deficient tachyzoites to the brain may reproduce the T cell response effect. However, studies with *CPS* (an uracil-auxotrophic *T. gondii* strain that can only replicate in vitro when uracil supplemented) [257], underline two major hurdles with replication-deficient *T. gondii*. First, because *CPS* cannot replicate in the host and die within a cell-cycle division, they cannot effectively prime, build the adaptive T cell response, and recruit them to the local sites in the brain in time. In a preclinical melanoma study, anti-tumoral immune response requires multiple rounds of *CPS* intratumoral injection to induce IL-12 and adaptive response in the local tumors [258]. Second, *CPS* cannot effectively cross the BBB. Intravenous transfer of monocytes infected with *CPS* supported only a transient presence of *T. gondii* in the brain [274]. *CPS* presence was limited to the BBB endothelial cells compartment and could not be detected in the brain parenchyma.

Instead of an avirulent form, a conditionally self-terminating form of *T. gondii* would make a stronger fit for its ability to produce a systemic Th1 immune response and disseminate to the brain naturally. To achieve this goal, we would need a transgenic *T. gondii* that expresses the recombinase enzyme after *T. gondii* has arrived at the to-be chronically infected organs. Then, loss of an essential gene will be mediated by loxP sites to eliminate *T. gondii*. Regarding the promoter choice to temporally control the recombinase expression, a gene that regulates the cyst wall's biogenesis would confer two advantages. First, the development of the cyst wall occurs in a more defined time window than the differentiation tachyzoite to bradyzoite period [428]. The more definite expression window would minimize 'leakiness' in the system, as we would not want to impact the

viability of the tachyzoites. Second, the transcriptional network regulating the differentiation into bradyzoite involves multiple molecules such as BFD1 and several ApiAP2 members [427]. Such a condition could undermine the 'efficiency' of the promoter activity as the promoter may not be strongly expressed. Among proteins contributing to cyst wall formation, glycoprotein CST1 is essential for cyst wall integrity and functionally contributes to *T. gondii* persistence during chronic infection [429]. Loss of CST1 led to fewer cyst numbers and significantly impaired the structural integrity of the remaining cysts, which became thinner with disrupted underlying region. These findings underline *T. gondii's* dependence on CST1 for cyst building and their survival in chronic infection.

Concerning the essential gene to be targeted, I propose calcium-dependent protein kinase 1 (CDPK1). Previously developed avirulent CPS target pathway that would be more dependent by *T. gondii* during the active infection phase. To effectively target all *T. gondii*, the essential gene must be necessary for *T. gondii* regardless of their form. CDPK1 is a member of the CDPKs, the serine/threonine kinases found only in plants and apicomplexan parasites [430]. Functionally, the cytosolic protein participates in diverse biological activities *T. gondii,* including attachment [431] and micronemes exocytosis from infected cells [432]. Genetic deletion of *CDPK1* suppresses the parasite's motility, host-cell invasion, and egress from infected fibroblast cell culture [432, 433]. As a primary target in a developing therapeutic class against *T. gondii*, studies on the CDPK1 selective inhibitors also demonstrate CDPK1's importance for *T. gondii* fitness in vivo. Murine models with dampened CDPK1 activity led to a significantly less *T. gondii* burden in peritoneal fluid, spleen, and brain in the infected mice during acute toxoplasmosis [434, 435].

To build the self-terminating *T. gondii*, we will employ CRISPR/Cas9 technology to introduce the Cre gene into the CST1 promoter region and flanking the *CDPK1* gene with loxP sites. CRISPR/Cas9 has shown efficiency in targeted gene editing and helped generate multiple transgenic *T. gondii* strains [433]. As *Me49-GFP-eLuc* was the *T. gondii* strain evaluated in the MADM Medulloblastoma mice, all genetic manipulations will be incorporated on this strain background to ensure a proper comparison for the self-terminating strain. We will generate a *Me49-GFP-eLuc* strain with $CST1^{Cre}$; $CDPK1^{f/f}$ incorporated into the genomic background (*CC-Me49*). The control strain only expresses $CST1^{Cre}$ (*Co-Me49*).

3.1.2 Proposed experiments

a) Evaluate cyst number and the overall parasite burden of *CC-Me49* using in vitro and in vivo platforms.

It is important to verify that the engineered strain maintains a similar infectivity (as parental Type II *T. gondii*) during acute infection. To test this, I will harvest the peritoneal fluid, the heart, and the lung at 12 DPI when acute infection peaks. Via q/PCR to measure *T. gondii* DNA content in the host tissue, I anticipate that *T. gondii* burden from *CC-Me49* infected mice will be similar to *Co-Me49* infected mice.

To test whether the *CC-Me49* will clear themselves from the infected host by chronic infection as they are designed for, I will perform qPCR to detect parasitic DNA presence in the brain at 27 and 45 DPI. At the early chronic infection (27DPI), when *T. gondii* still undergo differentiation from tachyzoite into bradyzoite, I expected that the parasitic burden would be similar between *CC-Me49* infected mice and *Co-Me49* infected mice. However, at latent chronic infection (45 DPI), there would be significantly less *T. gondii* DNA content in *CC-Me49* infected mice when.

To check that *Co-Me49* infected mice do not form cysts, I will collect the brains and perform a cyst count on the brain homogenate. As *Co-Me49* and *CC-Me49* are GFP positive, GFP+ cyst quantity could also be assessed via flow cytometry or confocal microscope. Importantly, to verify that *CC-Me49* infected mice are completely *T. gondii* free, I would collect the infected *CC-Me49* brain homogenate and administer it orally into

immunocompromised mice NU/J (which lack T cells) and IFNγ-knockout mice. A lack of T cells and IFNγR activity would lead to fatal infection [238, 358]. *CC-Me49* are absent in the brain homogenate, NU/J and IFNγR-knockout mice would not succumb to toxoplasmosis.

b) Evaluate Th1 T response between *CC-Me49* and *Co-Me49* infections in WT mice.

Having known that *CC-Me49* can self-terminate during *T. gondii* encystation but does not cause higher or lower *T. gondii* burden in the infected host, I will test whether *CC-Me49* can induce efficient systemic T cell response (during acute infection) and local T cell response in the brain (during early chronic infection).

To assess the host's systemic Th1 immune response and magnitude, I will measure the level of IFNγ and TNFa in the sera using Luminex or ELISA. I expect that the overall Th1 immune response would be similar between *CC-Me49* infected mice and *Co-Me49* infected mice. To ensure that *Co-Me49* infected mice mount a similar T cell response as *Co-Me49* infected mice, I will perform antigen restimulation assay on splenocytes of the mice using *Soluble T. gondii antigen*. Via flow cytometry, I expect that the number of IFNγ+ CD4+ TCRb+ cells and their frequency among CD45+ would be similar between *CC-Me49* infected mice and *Co-Me49* infected mice. A similar observation would occur for IFNγ+ CD8+ TCRb+ cells.

Then, to assess whether *CC-Me49* is capable of building T cell response in the brain of a similar degree as *Co-Me49* infection, I will harvest brain samples at 0 DPI, 20 DPI, 30 DPI, 45 DPI, and 80 DPI. Via flow cytometry, I will measure T cell abundance via frequency among live cells and T cell number normalized to tissue weight. At 20 DPI and 30 DPI, when *T. gondii* just begins the differentiation process into bradyzoite, I expect the number of T cells recruited to the brain of *CC-Me49* infected mice would be similar to *Co-Me49* infected ones. However, at 45 DPI, when encystation would have started in most *T. gondii*, the number of T cells in the brain of *CC-Me49* infected mice would be less than *Co-Me49* infected mice. At 80 DPI, when mature immune-quiescent cysts have developed in the *Co-Me49* infected mice, I expect negligible T cells to be detected in both *CC-Me49* and *Co-Me49* infected mice.

To assess the functionality of the T cell recruited to the brain, I will collect immune dissociates from the infected brain at 27 DPI and perform a restimulation assay with PMA, ionomycin, and Brefeldin A (a Golgi inhibitor). Through flow cytometric evaluation, I anticipate that T cells from the brains of *CC-Me49* infected mice and *Co-Me49* infected mice will have a similar number of IFNγ-expressing T cells (both CD4+ and CD8+ compartments) post-restimulation. Additional parameters for T cell activation status include proliferative activity (via Ki67 expression) and expression of other effector molecules (such as TNFa and Granzyme b).

c) Evaluate *CC-Me49*'s T cell recruitment effect into the tumors of MADM medulloblastoma mice.

Since the tumor microenvironment is known to be immune-suppressive, it is important to understand whether the T cell response due to the limited presence of *CC-Me49* in the brain would be impacted by tumor presence.

First, I will test whether *CC-Me49* can induce effective T cell response in the medulloblastoma as *Co-Me49* at 27 DPI, when T cell response peaks in the brain. For this question, I will harvest tumors from medulloblastoma mice that have been infected with either *CC-Me49* or *Co-Me49*. Via flow cytometry, I will evaluate the recruited T cell number. I expect the CD45+ TCRb+ cell count (normalized to tissue weight) and their percentage among live cells would be similar between *CC-Me49* infected and *Co-Me49* infected groups. The number of CD4+ T cells, CD8+ T cells, and Treg are expected to also stay identical between the two groups. Via immunohistochemistry, I will assess whether *CC-Me49* recruited T cells can infiltrate the tumor parenchyma as effectively as *Co-Me49* recruited T cells. Using Laminin and CD3 antibodies, I expect that the number of CD3+ T cells detected beyond the Laminin+ blood vessels and leptomeninges would be equivalent

between *CC-Me49* and *Co-Me49* infected mice. To ascertain that *CC-Me49* recruited T cells are as functional in the tumors as *Co-Me49*, I will perform restimulation assay with PMA, ionomycin, and Brefeldin A. I anticipate that the number of T cells expressing effector cytokine IFNγ (IFNγ+ CD4+ T cells and IFNγ+ CD8+ T cells) will also be similar between *CC-Me49* infection and *Co-Me49* infection.

Second, I will evaluate whether the tumor's immunosuppressive environment could shorten *CC-Me49* induced T cell response. For this question, I will compare T cell quantity in the tumors from *CC-Me49* infected medulloblastoma mice and the cerebella from *CC-Me49* infected WT mice at 20, 27, and 35 DPI. I hypothesize that the T cell response kinetics in the tumor would depend more on the overall *T. gondii* burden in the brain than the local tumor environment. *T. gondii-induced* T cell response has been shown to occur throughout the brain [276, 348] correlating with *T. gondii* spatial distribution in the brains infected mice [269, 436]. As *T. gondii's* burden in the forebrain is similar between tumor-bearing mice and WT mice, the same is assumed to apply to *CC-Me49* infection. Via flow cytometry, I anticipate that T cell kinetics in the tumors of *CC-Me49* infected medulloblastoma mice would be indistinguishable from that in the cerebella of *CC-Me49* infected WT mice. At 20 and 27 DPI, when *CC-Me49* has yet to enter encystation, T cells would be readily detected in the tumors of infected medulloblastoma mice and cerebella of infected WT mice. At 35 DPI, when more cyst formation occurs and less *CC-Me49* is present in the brain, T cell quantity will drop in both groups.

3.3. Additional future directions for consideration.

a) *T. gondii* infection may induce immune-surveillance of OVA-expressing brain tumor.

To test for cancer immune-surveillance, tumor that express antigenic epitope such as Ovalbumin (OVA) would be the ideal choice as OVA specific immune response could be assessed via different immunological tools. However, the currently available engrafted brain tumor models (with or without OVA expression) grow too aggressively, killing mice within a couple of weeks, and are often detected with T cells at baseline [437-439]. In the future, the development of an OVA-expressing, slow-growing brain tumor models that recapitulates the T-cell desert TME at baseline would be essential for the brain tumor immunology field.

Assuming an OVA-expressing brain tumor model is developed, *T. gondii* infection may promote immune surveillance of brain tumor cells. In addition to the ability to recruit T cells to the brain, *T. gondii* infection has been shown to support the maturation of conventional dendritic cells (cDC) and expansion of *T. gondii*-specific T cells in the deep cervical lymph node (DCLN) [267]. As the dural lymphatic vasculature could receive brain- and CSF-derived molecules and DCLN are the primary sites to receive drainage from the lymphatic vessels [136, 440]; brain tumor-antigens priming could occur at the DCLN during *T. gondii* infection. Although Kovacs et al. found that disrupting the meningeal lymphatic drainage via ligation surgery did not alter the T cell response in the brain, this does not necessarily subvert DCLN's role in sampling and priming for brain-derived materials. Since *T. gondii* is known to establish chronic presence in peripheral tissues such as skeletal muscles, *T. gondii*-specific T cell response can be mounted in the periphery and less dependent on DCLN's activity.

To test whether OVA-specific T cell response has been mounted in the host, one approach is to detect OVA-specific T cells among the immune cells recruited to the tumor. At 6-8 weeks post-infection (when the expansion of cDC in the meninges and T cell in the DCLN was detected), OVA-tetramer could be used to enriched for OVA-specific T cells. Alternatively, adoptive transfer of congenic OT-1 (OVA-specific CD8+ cells) and OT-2 T cells (OVA-specific CD4+ cells) and detection of their presence in the tumors could inform whether these T cells can home to the tumors. If OVA-specific has been mounted, they should home to the target site.

Although an ex vivo restimulation assay using OVA antigen or an ex vivo killing assay with tumor cells may provide more definite evidence for an anti-tumoral T cell response, the total immune cells (including APCs and

T cells) recruited to the tumors may not be as abundant as those in other immunological organs such as the spleen and lymph node. A tumor-rechallenge experiment in the same mice would be a more optimal alternative.

b) Combining *T. gondii* with other therapies to improve brain tumor immune surveillance.

Considered that most primary brain tumors locate behind the BBB and brain immune-surveillance depends primarily on the brain-derived-antigens availability in the CSF and their being sampled at the DCLN, one approach to boost brain tumor immune surveillance is to increase the tumor-antigen abundance in the CSF. To this goal, increase tumor cell killing via available therapies is an appealing option. Among the current therapies for brain cancer patients, systemic chemotherapy and radiotherapy may not be applicable as they are known to be immunosuppressive and could negatively impact the host ability to control *T. gondii*. However, focal radiation [441] and tumor-treating field [442] could provide several advantages. In addition to the direct tumor killing, the tumor dead cell fragments from these treatments would enhance tumor antigen release into the CSF. In turn, this would improve tumor-sampling rate and T cell priming at the DCLN.

Independently, improving the dural lymphatic drainage could increase the detection of tumor cell antigens at the DCLN. Although still under preclinical studies, VEGF-C (a lymphangiogenic factor) has been shown to promote dural lymphatic vasculature growth [443] and support anti-tumoral response in some implanted brain tumor models [444, 445]. However, in these preclinical models, T cells could be detected in the tumors at baseline, suggesting VEFG-C works by boosting the pre-existing cancer immunosurveillance, likely through the activity of DCLN. Although it is unknown whether VEGF-C alone could enact a similar effect in brain tumor without T cells at baseline, combining VEFG-C with *T. gondii*'s ability to increase antigen sampling and T cell priming in the DCLN could further boost tumor antigen sampling.

c) OVA-expressing *T. gondii* infection can build effective anti-tumoral response against OVA-expressing brain tumors.

An alternative method that would be sufficient to mount an anti-brain tumor T-cell response is to incorporate the tumor antigens into the microbes. As presented above, there are two major hurdles for brain tumor antigen priming: a) brain tumor antigens need to be abundantly present in the CSF, and b) dural lymphatic vasculature needs to work effectively to deliver the tumor antigens to the DCLN. These issues highlight the dependence on DCLN for brain tumor immune surveillance. However, this reliance can be circumvented entirely by using a *T. gondii* strain expressing tumor antigens. Preclinically, this hypothesis could be tested using an OVA-expressing brain tumor and OVA-expressing *T. gondii* such as Pru-OVA [392]). These OVA-expressing *T. gondii* strains have been shown to induce OVA-specific immune response as transferred OT-1 cells readily migrate to the brain of the infected mice. Utilizing OVA-expressing *T. gondii* strain in an OVA-expressing brain tumors setting is expected to mount anti-tumoral response.

d) Other avenues that could be combined with *T. gondii*.

As *T. gondii* remodels the brain tumor environment into a T cell-hospitable state, combining it with CAR-T or TCR-engineered T cells would allow CAR-T cells to be recruited and fully exert their tumor-killing effect. Preclinically, a representative experiment would be an adoptive transfer of OT-1 T cells into *T. gondii*-infected mice implanted with OVA-expressing brain tumors. OT-1 cells can be pre-labeled with cell-tracker dye such as CSFE to monitor and track their expansion. Successful migration to the tumor site and activation would lead to OT-1 cell expansion.

Likewise, immune adjuvants that could enhance T cell activity specifically in brain tumors could support tumor killing without majorly impacting nearby brain tumor regions. For example, as tumor cells and local APCs have been shown to express PD-L1 under the influence of IFNγ [446], anti-PDL1 could boost T cell activity in the local brain tumors.

Concluding Remarks

Malignant primary brain tumors, such as glioblastoma and medulloblastoma, are devastating diseases with poor response to the current standard of care and debilitating treatment sequelae. Immunotherapy presents a new frontier in cancer treatment with clinical benefits observed in multiple cancers, including hematological and solid tumors. However, attempts with immunotherapies in glioblastoma have yielded unsatisfactory results. From patient sample evaluation, the clinical consensus that brain tumors are a T-cell desert highlights the need to improve T cell infiltration into the tumors to enact their tumor-control response.

Although the brain is highly sensitive to inflammatory changes, *T. gondii* infection in the brain is controlled by Th1 immunity and does not cause adverse responses in immune-competent hosts. The tolerable immune response in the brain is likely a product of the long co-evolution relationship between the protozoans and the mammalian intermediate hosts, explaining the parasite's success among the Apicomplexans (with up to 30% human populations presumed to carry *T. gondii*).

Taking advantage of *T. gondii's* ability to induce the desirable Th1 immune response in the brain, this dissertation addresses three primary questions to evaluate the potential of *T. gondii* as a "bug-as-drug" candidate. First, considering that brain tumors could exert systemic immune suppression, we asked whether *T. gondii* could be controlled in the brain-tumor-bearing mice. Second, we asked whether T cell response induced by *T. gondii* infection could develop in the tumors, against their immune-suppressive nature. Lastly, we evaluated how *T. gondii* infection remodels the local tumor microenvironment, especially the myeloid cell compartment, to support T cell activity in the tumors.

Using mice that formed medulloblastoma endogenously and had intact immune system, we found that *T. gondii* infection was well-controlled among the infected tumor-bearing mice, indicating that the hosts' immune system was functional. Strikingly, we found that *T. gondii* infection could recruit T cells into brain tumors as efficiently as the brains of infected WT mice. The T cells exhibited no significant change in composition and functionality, indicating that the tumor environment poses a minimal barrier to the recruited T cells. Elevation of local IFNγ in the local tumor environment likely contributes to T cell arrival and activity through the upregulated expression of T cell chemoattractant and antigen processing/presentation molecules.

Overall, this dissertation demonstrated that *T. gondii* infection could overcome the immune-suppressive brain tumor environment and induce an efficient T-cell response. Future studies could further explore the immune-signaling mechanisms underlying the *T. gondii*-induced Th1 immune response in the brain. Independently, given the genetic tractability of *T. gondii* and promising results in several preclinical tumor models, developing a self-terminating *T. gondii* with added features such as tumor antigen would be a highly attractive avenue.

References

1. Ostrom, Q.T., et al., *CBTRUS Statistical Report: Primary Brain and Other Central Nervous System Tumors Diagnosed in the United States in 2016-2020.* Neuro Oncol, 2023. **25**(12 Suppl 2): p. iv1-iv99.
2. Schaff, L.R. and I.K. Mellinghoff, *Glioblastoma and Other Primary Brain Malignancies in Adults: A Review.* JAMA, 2023. **329**(7): p. 574-587.
3. Siegel, R.L., A.N. Giaquinto, and A. Jemal, *Cancer statistics, 2024.* CA: A Cancer Journal for Clinicians, 2024. **74**(1): p. 12-49.
4. Mahapatra, S. and M.J. Amsbaugh, *Medulloblastoma*, in *StatPearls*. 2024: Treasure Island (FL).
5. Valdes, P.A., et al., *Quantitative fluorescence using 5-aminolevulinic acid-induced protoporphyrin IX biomarker as a surgical adjunct in low-grade glioma surgery.* J Neurosurg, 2015. **123**(3): p. 771-80.
6. Stepp, H. and W. Stummer, *5-ALA in the management of malignant glioma.* Lasers Surg Med, 2018. **50**(5): p. 399-419.
7. Suero Molina, E., S. Schipmann, and W. Stummer, *Maximizing safe resections: the roles of 5-aminolevulinic acid and intraoperative MR imaging in glioma surgery-review of the literature.* Neurosurg Rev, 2019. **42**(2): p. 197-208.
8. De Braganca, K.C. and R.J. Packer, *Treatment Options for Medulloblastoma and CNS Primitive Neuroectodermal Tumor (PNET).* Curr Treat Options Neurol, 2013. **15**(5): p. 593-606.
9. Martin, A.M., et al., *Management of pediatric and adult patients with medulloblastoma.* Curr Treat Options Oncol, 2014. **15**(4): p. 581-94.
10. Othman, R.T., et al., *Overcoming multiple drug resistance mechanisms in medulloblastoma.* Acta Neuropathol Commun, 2014. **2**: p. 57.
11. Chevignard, M., et al., *Core deficits and quality of survival after childhood medulloblastoma: a review.* Neurooncol Pract, 2017. **4**(2): p. 82-97.
12. Dennis, M., et al., *Neuropsychological sequelae of the treatment of children with medulloblastoma.* J Neurooncol, 1996. **29**(1): p. 91-101.
13. Mesbahi, T., et al., *Glioblastoma Following Treated Medulloblastoma After 29 Years in the Posterior Fossa: Case Report and Review of Literature.* Front Oncol, 2022. **12**: p. 760011.
14. Nantavithya, C., et al., *Observed-to-expected incidence ratios of second malignant neoplasms after radiation therapy for medulloblastoma: A Surveillance, Epidemiology, and End Results analysis.* Cancer, 2021. **127**(13): p. 2368-2375.
15. Louis, D.N., et al., *The 2021 WHO Classification of Tumors of the Central Nervous System: a summary.* Neuro Oncol, 2021. **23**(8): p. 1231-1251.
16. Yan, Y., et al., *Landscape of Genomic Alterations in IDH Wild-Type Glioblastoma Identifies PI3K as a Favorable Prognostic Factor.* JCO Precis Oncol, 2020. **4**: p. 575-584.
17. Lau, D., S.T. Magill, and M.K. Aghi, *Molecularly targeted therapies for recurrent glioblastoma: current and future targets.* Neurosurg Focus, 2014. **37**(6): p. E15.
18. Felsberg, J., et al., *Epidermal Growth Factor Receptor Variant III (EGFRvIII) Positivity in EGFR-Amplified Glioblastomas: Prognostic Role and Comparison between Primary and Recurrent Tumors.* Clin Cancer Res, 2017. **23**(22): p. 6846-6855.
19. Ozawa, T., et al., *PDGFRA gene rearrangements are frequent genetic events in PDGFRA-amplified glioblastomas.* Genes Dev, 2010. **24**(19): p. 2205-18.
20. Lin, B., et al., *EGFR, the Lazarus target for precision oncology in glioblastoma.* Neuro Oncol, 2022. **24**(12): p. 2035-2062.
21. Chen, Y., et al., *Pharmacokinetic and pharmacodynamic study of Gefitinib in a mouse model of non-small-cell lung carcinoma with brain metastasis.* Lung Cancer, 2013. **82**(2): p. 313-8.

22. Deng, Y., et al., *The concentration of erlotinib in the cerebrospinal fluid of patients with brain metastasis from non-small-cell lung cancer*. Mol Clin Oncol, 2014. **2**(1): p. 116-120.
23. Bai, H. and B. Han, *The effectiveness of erlotinib against brain metastases in non-small cell lung cancer patients*. Am J Clin Oncol, 2013. **36**(2): p. 110-5.
24. Zahonero, C., et al., *Preclinical Test of Dacomitinib, an Irreversible EGFR Inhibitor, Confirms Its Effectiveness for Glioblastoma*. Mol Cancer Ther, 2015. **14**(7): p. 1548-58.
25. O'Brien, S.G., et al., *Imatinib compared with interferon and low-dose cytarabine for newly diagnosed chronic-phase chronic myeloid leukemia*. N Engl J Med, 2003. **348**(11): p. 994-1004.
26. Iqbal, N. and N. Iqbal, *Imatinib: a breakthrough of targeted therapy in cancer*. Chemother Res Pract, 2014. **2014**: p. 357027.
27. Siegelin, M.D., et al., *Sorafenib exerts anti-glioma activity in vitro and in vivo*. Neurosci Lett, 2010. **478**(3): p. 165-70.
28. de Bouard, S., et al., *Antiangiogenic and anti-invasive effects of sunitinib on experimental human glioblastoma*. Neuro Oncol, 2007. **9**(4): p. 412-23.
29. Joshi, A.D., et al., *Evaluation of tyrosine kinase inhibitor combinations for glioblastoma therapy*. PLoS One, 2012. **7**(10): p. e44372.
30. Martini, M., et al., *PI3K/AKT signaling pathway and cancer: an updated review*. Ann Med, 2014. **46**(6): p. 372-83.
31. Wang, S.I., et al., *Somatic mutations of PTEN in glioblastoma multiforme*. Cancer Res, 1997. **57**(19): p. 4183-6.
32. Papavassiliou, K.A. and A.G. Papavassiliou, *The Bumpy Road towards mTOR Inhibition in Glioblastoma: Quo Vadis?* Biomedicines, 2021. **9**(12).
33. Jhanwar-Uniyal, M., et al., *Distinct signaling mechanisms of mTORC1 and mTORC2 in glioblastoma multiforme: a tale of two complexes*. Adv Biol Regul, 2015. **57**: p. 64-74.
34. Rodrik-Outmezguine, V.S., et al., *Overcoming mTOR resistance mutations with a new-generation mTOR inhibitor*. Nature, 2016. **534**(7606): p. 272-6.
35. Zhang, Z., et al., *Brain-restricted mTOR inhibition with binary pharmacology*. Nature, 2022. **609**(7928): p. 822-828.
36. Fan, Q., et al., *A Kinase Inhibitor Targeted to mTORC1 Drives Regression in Glioblastoma*. Cancer Cell, 2017. **31**(3): p. 424-435.
37. Gatto, L., et al., *Molecular Targeted Therapies: Time for a Paradigm Shift in Medulloblastoma Treatment?* Cancers (Basel), 2022. **14**(2).
38. Houschyar, K.S., et al., *Wnt Pathway in Bone Repair and Regeneration - What Do We Know So Far*. Front Cell Dev Biol, 2018. **6**: p. 170.
39. Perochon, J., L.R. Carroll, and J.B. Cordero, *Wnt Signalling in Intestinal Stem Cells: Lessons from Mice and Flies*. Genes (Basel), 2018. **9**(3).
40. Ramaswamy, V., C. Nor, and M.D. Taylor, *Erratum: p53 and Medulloblastoma*. Cold Spring Harb Perspect Med, 2016. **6**(4): p. a029579.
41. Rudin, C.M., et al., *Treatment of medulloblastoma with hedgehog pathway inhibitor GDC-0449*. N Engl J Med, 2009. **361**(12): p. 1173-8.
42. Robinson, G.W., et al., *Irreversible growth plate fusions in children with medulloblastoma treated with a targeted hedgehog pathway inhibitor*. Oncotarget, 2017. **8**(41): p. 69295-69302.
43. Verhaak, R.G., et al., *Integrated genomic analysis identifies clinically relevant subtypes of glioblastoma characterized by abnormalities in PDGFRA, IDH1, EGFR, and NF1*. Cancer Cell, 2010. **17**(1): p. 98-110.
44. Sottoriva, A., et al., *Intratumor heterogeneity in human glioblastoma reflects cancer evolutionary dynamics*. Proc Natl Acad Sci U S A, 2013. **110**(10): p. 4009-14.

45. Mathur, R., et al., *Glioblastoma evolution and heterogeneity from a 3D whole-tumor perspective*. Cell, 2024. **187**(2): p. 446-463 e16.
46. Cavalli, F.M.G., et al., *Intertumoral Heterogeneity within Medulloblastoma Subgroups*. Cancer Cell, 2017. **31**(6): p. 737-754 e6.
47. Ocasio, J.K., et al., *scRNA-seq in medulloblastoma shows cellular heterogeneity and lineage expansion support resistance to SHH inhibitor therapy*. Nat Commun, 2019. **10**(1): p. 5829.
48. Nathanson, D.A., et al., *Targeted therapy resistance mediated by dynamic regulation of extrachromosomal mutant EGFR DNA*. Science, 2014. **343**(6166): p. 72-6.
49. Stommel, J.M., et al., *Coactivation of receptor tyrosine kinases affects the response of tumor cells to targeted therapies*. Science, 2007. **318**(5848): p. 287-90.
50. Hegi, M.E., et al., *Pathway analysis of glioblastoma tissue after preoperative treatment with the EGFR tyrosine kinase inhibitor gefitinib--a phase II trial*. Mol Cancer Ther, 2011. **10**(6): p. 1102-12.
51. Sakai, T. and Y. Morimoto, *The History of Infectious Diseases and Medicine*. Pathogens, 2022. **11**(10).
52. Dobosz, P. and T. Dzieciatkowski, *The Intriguing History of Cancer Immunotherapy*. Front Immunol, 2019. **10**: p. 2965.
53. Starnes, C.O., *Coley's toxins in perspective*. Nature, 1992. **357**(6373): p. 11-2.
54. Hoption Cann, S.A., J.P. van Netten, and C. van Netten, *Dr William Coley and tumour regression: a place in history or in the future*. Postgrad Med J, 2003. **79**(938): p. 672-80.
55. Nauts HC, F.G., *End results in lymphosarcoma treated by toxin therapy alone or combined with surgery and/or radiation or with concurrent bacterial infection* 1969, New York: Cancer Research Institute Inc. .
56. Mitsis, D., V. Francescutti, and J. Skitzki, *Current Immunotherapies for Sarcoma: Clinical Trials and Rationale*. Sarcoma, 2016. **2016**: p. 9757219.
57. Hellstrom, K.E. and I. Hellstrom, *From the Hellstrom paradox toward cancer cure*. Prog Mol Biol Transl Sci, 2019. **164**: p. 1-24.
58. Thariat, J., et al., *Past, present, and future of radiotherapy for the benefit of patients*. Nat Rev Clin Oncol, 2013. **10**(1): p. 52-60.
59. Miller, J.F., G.F. Mitchell, and N.S. Weiss, *Cellular basis of the immunological defects in thymectomized mice*. Nature, 1967. **214**(5092): p. 992-7.
60. Pantelouris, E.M., *Absence of thymus in a mouse mutant*. Nature, 1968. **217**(5126): p. 370-1.
61. Shinkai, Y., et al., *RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement*. Cell, 1992. **68**(5): p. 855-67.
62. Zhang, Z., et al., *Insights on FoxN1 biological significance and usages of the "nude" mouse in studies of T-lymphopoiesis*. Int J Biol Sci, 2012. **8**(8): p. 1156-67.
63. Fogh, J., J.M. Fogh, and T. Orfeo, *One hundred and twenty-seven cultured human tumor cell lines producing tumors in nude mice*. J Natl Cancer Inst, 1977. **59**(1): p. 221-6.
64. Shankaran, V., et al., *IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity*. Nature, 2001. **410**(6832): p. 1107-11.
65. Gatti, R.A. and R.A. Good, *Occurrence of malignancy in immunodeficiency diseases. A literature review*. Cancer, 1971. **28**(1): p. 89-98.
66. van den Broek, M.E., et al., *Decreased tumor surveillance in perforin-deficient mice*. J Exp Med, 1996. **184**(5): p. 1781-90.
67. Street, S.E., E. Cretney, and M.J. Smyth, *Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis*. Blood, 2001. **97**(1): p. 192-7.
68. Roth, D.B., *V(D)J Recombination: Mechanism, Errors, and Fidelity*. Microbiol Spectr, 2014. **2**(6).
69. Kufe DW, P.R., Weichselbaum RR, et al., *Categories of Tumor Antigens*. 6th ed., Holland-Frei Cancer Medicine: Hamilton (ON): BC Decker.

70. Houghton, A.N. and J.A. Guevara-Patino, *Immune recognition of self in immunity against cancer.* J Clin Invest, 2004. **114**(4): p. 468-71.
71. Wolf, N.K., D.U. Kissiov, and D.H. Raulet, *Roles of natural killer cells in immunity to cancer, and applications to immunotherapy.* Nat Rev Immunol, 2023. **23**(2): p. 90-105.
72. Finisguerra, V., et al., *MET is required for the recruitment of anti-tumoural neutrophils.* Nature, 2015. **522**(7556): p. 349-53.
73. Wanderley, C.W., et al., *Paclitaxel Reduces Tumor Growth by Reprogramming Tumor-Associated Macrophages to an M1 Profile in a TLR4-Dependent Manner.* Cancer Res, 2018. **78**(20): p. 5891-5900.
74. Saeidi, A., et al., *T-Cell Exhaustion in Chronic Infections: Reversing the State of Exhaustion and Reinvigorating Optimal Protective Immune Responses.* Front Immunol, 2018. **9**: p. 2569.
75. Cai, L., et al., *Targeting LAG-3, TIM-3, and TIGIT for cancer immunotherapy.* J Hematol Oncol, 2023. **16**(1): p. 101.
76. Lee, J.B., H.R. Kim, and S.J. Ha, *Immune Checkpoint Inhibitors in 10 Years: Contribution of Basic Research and Clinical Application in Cancer Immunotherapy.* Immune Netw, 2022. **22**(1): p. e2.
77. Gong, J., et al., *Development of PD-1 and PD-L1 inhibitors as a form of cancer immunotherapy: a comprehensive review of registration trials and future considerations.* J Immunother Cancer, 2018. **6**(1): p. 8.
78. Pinato, D.J.J., et al., *Anti-drug antibodies related to CTLA-4, PD-1, or PD-L1 inhibitors across tumour types: A systematic review.* Journal of Clinical Oncology, 2023. **41**(16_suppl): p. e14600-e14600.
79. Berraondo, P., et al., *Cytokines in clinical cancer immunotherapy.* Br J Cancer, 2019. **120**(1): p. 6-15.
80. Bachmann, M.F. and A. Oxenius, *Interleukin 2: from immunostimulation to immunoregulation and back again.* EMBO Rep, 2007. **8**(12): p. 1142-8.
81. Crespo, J., et al., *T cell anergy, exhaustion, senescence, and stemness in the tumor microenvironment.* Curr Opin Immunol, 2013. **25**(2): p. 214-21.
82. Yokosuka, T. and T. Saito, *The Immunological Synapse, TCR Microclusters, and T Cell Activation*, in *Immunological Synapse*, T. Saito and F.D. Batista, Editors. 2010, Springer Berlin Heidelberg: Berlin, Heidelberg. p. 81-107.
83. Rafiq, S., C.S. Hackett, and R.J. Brentjens, *Engineering strategies to overcome the current roadblocks in CAR T cell therapy.* Nat Rev Clin Oncol, 2020. **17**(3): p. 147-167.
84. Dotti, G., et al., *Design and development of therapies using chimeric antigen receptor-expressing T cells.* Immunol Rev, 2014. **257**(1): p. 107-26.
85. Maude, S.L., et al., *Tisagenlecleucel in Children and Young Adults with B-Cell Lymphoblastic Leukemia.* N Engl J Med, 2018. **378**(5): p. 439-448.
86. Baulu, E., et al., *TCR-engineered T cell therapy in solid tumors: State of the art and perspectives.* Sci Adv, 2023. **9**(7): p. eadf3700.
87. Chandran, S.S. and C.A. Klebanoff, *T cell receptor-based cancer immunotherapy: Emerging efficacy and pathways of resistance.* Immunol Rev, 2019. **290**(1): p. 127-147.
88. Gubin, M.M., et al., *Tumor neoantigens: building a framework for personalized cancer immunotherapy.* J Clin Invest, 2015. **125**(9): p. 3413-21.
89. Perez, C.R. and M. De Palma, *Engineering dendritic cell vaccines to improve cancer immunotherapy.* Nat Commun, 2019. **10**(1): p. 5408.
90. Anassi, E. and U.A. Ndefo, *Sipuleucel-T (provenge) injection: the first immunotherapy agent (vaccine) for hormone-refractory prostate cancer.* P T, 2011. **36**(4): p. 197-202.
91. Leary, W.E., *F.D.A. Backs A Treatment For Cancer Of Bladder*, in *The New York Times*. 1990.

92. Old, L.J., D.A. Clarke, and B. Benacerraf, *Effect of Bacillus Calmette-Guerin infection on transplanted tumours in the mouse.* Nature, 1959. **184(Suppl 5)**: p. 291-2.
93. Cookson, M.S., et al., *The treated natural history of high risk superficial bladder cancer: 15-year outcome.* J Urol, 1997. **158**(1): p. 62-7.
94. Han, R.F. and J.G. Pan, *Can intravesical bacillus Calmette-Guérin reduce recurrence in patients with superficial bladder cancer? A meta-analysis of randomized trials.* Urology, 2006. **67**(6): p. 1216-23.
95. Böhle, A., et al., *Detection of urinary TNF, IL 1, and IL 2 after local BCG immunotherapy for bladder carcinoma.* Cytokine, 1990. **2**(3): p. 175-81.
96. Pichler, R., et al., *Intratumoral Th2 predisposition combines with an increased Th1 functional phenotype in clinical response to intravesical BCG in bladder cancer.* Cancer Immunol Immunother, 2017. **66**(4): p. 427-440.
97. Böhle, A., et al., *Effects of local bacillus Calmette-Guerin therapy in patients with bladder carcinoma on immunocompetent cells of the bladder wall.* J Urol, 1990. **144**(1): p. 53-8.
98. Ratliff, T.L., D. Gillen, and W.J. Catalona, *Requirement of a thymus dependent immune response for BCG-mediated antitumor activity.* J Urol, 1987. **137**(1): p. 155-8.
99. Ratliff, T.L., et al., *T-cell subsets required for intravesical BCG immunotherapy for bladder cancer.* J Urol, 1993. **150**(3): p. 1018-23.
100. Biot, C., et al., *Preexisting BCG-specific T cells improve intravesical immunotherapy for bladder cancer.* Sci Transl Med, 2012. **4**(137): p. 137ra72.
101. Antonelli, A.C., et al., *Bacterial immunotherapy for cancer induces CD4-dependent tumor-specific immunity through tumor-intrinsic interferon-γ signaling.* Proc Natl Acad Sci U S A, 2020. **117**(31): p. 18627-18637.
102. Andtbacka, R.H., et al., *Talimogene Laherparepvec Improves Durable Response Rate in Patients With Advanced Melanoma.* J Clin Oncol, 2015. **33**(25): p. 2780-8.
103. Moore, A.E., *The destructive effect of the virus of Russian Far East encephalitis on the transplantable mouse sarcoma 180.* Cancer, 1949. **2**(3): p. 525-34.
104. Moore, A.E., *Effect of inoculation of the viruses of influenza A and herpes simplex on the growth of transplantable tumors in mice.* Cancer, 1949. **2**(3): p. 516-24.
105. Chou, J. and B. Roizman, *The gamma 1(34.5) gene of herpes simplex virus 1 precludes neuroblastoma cells from triggering total shutoff of protein synthesis characteristic of programed cell death in neuronal cells.* Proc Natl Acad Sci U S A, 1992. **89**(8): p. 3266-70.
106. Goldsmith, K., et al., *Infected cell protein (ICP)47 enhances herpes simplex virus neurovirulence by blocking the CD8+ T cell response.* J Exp Med, 1998. **187**(3): p. 341-8.
107. Liu, B.L., et al., *ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties.* Gene Ther, 2003. **10**(4): p. 292-303.
108. Kaufman, H.L., et al., *Local and distant immunity induced by intralesional vaccination with an oncolytic herpes virus encoding GM-CSF in patients with stage IIIc and IV melanoma.* Ann Surg Oncol, 2010. **17**(3): p. 718-30.
109. Annunziato, F. and S. Romagnani, *Th1 Cells*, in *Encyclopedia of Immunobiology*, M.J.H. Ratcliffe, Editor. 2016, Academic Press: Oxford. p. 287-293.
110. Kaufman, H.L., F.J. Kohlhapp, and A. Zloza, *Oncolytic viruses: a new class of immunotherapy drugs.* Nature Reviews Drug Discovery, 2015. **14**(9): p. 642-662.
111. Schakelaar, M.Y., et al., *Cellular immunotherapy for medulloblastoma.* Neuro Oncol, 2023. **25**(4): p. 617-627.
112. Rechberger, J.S., et al., *Exploring the Molecular Complexity of Medulloblastoma: Implications for Diagnosis and Treatment.* Diagnostics (Basel), 2023. **13**(14).

113. Reardon, D.A., et al., *Effect of Nivolumab vs Bevacizumab in Patients With Recurrent Glioblastoma: The CheckMate 143 Phase 3 Randomized Clinical Trial*. JAMA Oncol, 2020. **6**(7): p. 1003-1010.
114. Omuro, A., et al., *Radiotherapy combined with nivolumab or temozolomide for newly diagnosed glioblastoma with unmethylated MGMT promoter: An international randomized phase III trial*. Neuro Oncol, 2023. **25**(1): p. 123-134.
115. Lim, M., et al., *Phase III trial of chemoradiotherapy with temozolomide plus nivolumab or placebo for newly diagnosed glioblastoma with methylated MGMT promoter*. Neuro Oncol, 2022. **24**(11): p. 1935-1949.
116. Liau, L.M., et al., *Association of Autologous Tumor Lysate-Loaded Dendritic Cell Vaccination With Extension of Survival Among Patients With Newly Diagnosed and Recurrent Glioblastoma: A Phase 3 Prospective Externally Controlled Cohort Trial*. JAMA Oncol, 2023. **9**(1): p. 112-121.
117. Chen, E., et al., *Lessons learned from phase 3 trials of immunotherapy for glioblastoma: Time for longitudinal sampling?* Neuro Oncol, 2024. **26**(2): p. 211-225.
118. Omuro, A., et al., *Nivolumab with or without ipilimumab in patients with recurrent glioblastoma: results from exploratory phase I cohorts of CheckMate 143*. Neuro Oncol, 2018. **20**(5): p. 674-686.
119. Reardon, D.A., et al., *Glioblastoma Eradication Following Immune Checkpoint Blockade in an Orthotopic, Immunocompetent Model*. Cancer Immunol Res, 2016. **4**(2): p. 124-35.
120. Liau, L.M., et al., *First results on survival from a large Phase 3 clinical trial of an autologous dendritic cell vaccine in newly diagnosed glioblastoma*. J Transl Med, 2018. **16**(1): p. 142.
121. Zahraa, I.K., et al., *Clinical investigations of immunotherapy for human primary brain tumors*. Neuroimmunology and Neuroinflammation, 2021. **8**(3): p. 154-73.
122. Dunkel, I.J., et al., *Nivolumab with or without ipilimumab in pediatric patients with high-grade CNS malignancies: Safety, efficacy, biomarker, and pharmacokinetics-CheckMate 908*. Neuro Oncol, 2023. **25**(8): p. 1530-1545.
123. Mastorakos, P. and D. McGavern, *The anatomy and immunology of vasculature in the central nervous system*. Sci Immunol, 2019. **4**(37).
124. Kadry, H., B. Noorani, and L. Cucullo, *A blood-brain barrier overview on structure, function, impairment, and biomarkers of integrity*. Fluids Barriers CNS, 2020. **17**(1): p. 69.
125. Abbott, N.J., et al., *Structure and function of the blood-brain barrier*. Neurobiol Dis, 2010. **37**(1): p. 13-25.
126. Xu, L., A. Nirwane, and Y. Yao, *Basement membrane and blood-brain barrier*. Stroke Vasc Neurol, 2019. **4**(2): p. 78-82.
127. Neuhaus, J., W. Risau, and H. Wolburg, *Induction of blood-brain barrier characteristics in bovine brain endothelial cells by rat astroglial cells in transfilter coculture*. Ann N Y Acad Sci, 1991. **633**: p. 578-80.
128. Lee, S.W., et al., *SSeCKS regulates angiogenesis and tight junction formation in blood-brain barrier*. Nat Med, 2003. **9**(7): p. 900-6.
129. Armulik, A., et al., *Pericytes regulate the blood-brain barrier*. Nature, 2010. **468**(7323): p. 557-61.
130. Ginhoux, F., et al., *Fate mapping analysis reveals that adult microglia derive from primitive macrophages*. Science, 2010. **330**(6005): p. 841-5.
131. Bennett, F.C., et al., *A Combination of Ontogeny and CNS Environment Establishes Microglial Identity*. Neuron, 2018. **98**(6): p. 1170-1183 e8.
132. Cowan, M.N., et al., *Microglial STAT1-sufficiency is required for resistance to toxoplasmic encephalitis*. PLoS Pathog, 2022. **18**(9): p. e1010637.
133. Greter, M., I. Lelios, and A.L. Croxford, *Microglia Versus Myeloid Cell Nomenclature during Brain Inflammation*. Front Immunol, 2015. **6**: p. 249.

134. Rustenhoven, J. and J. Kipnis, *Brain borders at the central stage of neuroimmunology.* Nature, 2022. **612**(7940): p. 417-429.
135. Rustenhoven, J., et al., *Functional characterization of the dural sinuses as a neuroimmune interface.* Cell, 2021. **184**(4): p. 1000-1016 e27.
136. Louveau, A., et al., *CNS lymphatic drainage and neuroinflammation are regulated by meningeal lymphatic vasculature.* Nat Neurosci, 2018. **21**(10): p. 1380-1391.
137. Iliff, J.J., et al., *A paravascular pathway facilitates CSF flow through the brain parenchyma and the clearance of interstitial solutes, including amyloid beta.* Sci Transl Med, 2012. **4**(147): p. 147ra111.
138. Faraco, G., et al., *Brain perivascular macrophages: characterization and functional roles in health and disease.* J Mol Med (Berl), 2017. **95**(11): p. 1143-1152.
139. Van Hove, H., et al., *A single-cell atlas of mouse brain macrophages reveals unique transcriptional identities shaped by ontogeny and tissue environment.* Nat Neurosci, 2019. **22**(6): p. 1021-1035.
140. Goldmann, T., et al., *Origin, fate and dynamics of macrophages at central nervous system interfaces.* Nat Immunol, 2016. **17**(7): p. 797-805.
141. Drieu, A., et al., *Parenchymal border macrophages regulate the flow dynamics of the cerebrospinal fluid.* Nature, 2022. **611**(7936): p. 585-593.
142. Schlager, C., et al., *Effector T-cell trafficking between the leptomeninges and the cerebrospinal fluid.* Nature, 2016. **530**(7590): p. 349-53.
143. Mundt, S., et al., *Conventional DCs sample and present myelin antigens in the healthy CNS and allow parenchymal T cell entry to initiate neuroinflammation.* Sci Immunol, 2019. **4**(31).
144. Merlini, A., et al., *Distinct roles of the meningeal layers in CNS autoimmunity.* Nat Neurosci, 2022. **25**(7): p. 887-899.
145. Phoenix, T.N., et al., *Medulloblastoma Genotype Dictates Blood Brain Barrier Phenotype.* Cancer Cell, 2016. **29**(4): p. 508-522.
146. Arvanitis, C.D., G.B. Ferraro, and R.K. Jain, *The blood-brain barrier and blood-tumour barrier in brain tumours and metastases.* Nat Rev Cancer, 2020. **20**(1): p. 26-41.
147. Montagne, A., A.W. Toga, and B.V. Zlokovic, *Blood-Brain Barrier Permeability and Gadolinium: Benefits and Potential Pitfalls in Research.* JAMA Neurol, 2016. **73**(1): p. 13-4.
148. Aiman, W., D.P. Gasalberti, and A. Rayi, *Low-Grade Gliomas*, in *StatPearls*. 2024: Treasure Island (FL).
149. Smirniotopoulos, J.G. and H.R. Jager, *Differential Diagnosis of Intracranial Masses*, in *Diseases of the Brain, Head and Neck, Spine 2020-2023: Diagnostic Imaging*, J. Hodler, R.A. Kubik-Huch, and G.K. von Schulthess, Editors. 2020: Cham (CH). p. 93-104.
150. Pallud, J., et al., *Prognostic significance of imaging contrast enhancement for WHO grade II gliomas.* Neuro Oncol, 2009. **11**(2): p. 176-82.
151. Wang, Y.Y., et al., *Patterns of Tumor Contrast Enhancement Predict the Prognosis of Anaplastic Gliomas with IDH1 Mutation.* AJNR Am J Neuroradiol, 2015. **36**(11): p. 2023-9.
152. Sarkaria, J.N., et al., *Is the blood-brain barrier really disrupted in all glioblastomas? A critical assessment of existing clinical data.* Neuro Oncol, 2018. **20**(2): p. 184-191.
153. Drean, A., et al., *ATP binding cassette (ABC) transporters: expression and clinical value in glioblastoma.* J Neurooncol, 2018. **138**(3): p. 479-486.
154. de Gooijer, M.C., et al., *ATP-binding cassette transporters restrict drug delivery and efficacy against brain tumors even when blood-brain barrier integrity is lost.* Cell Rep Med, 2021. **2**(1): p. 100184.
155. Lin, F., et al., *ABCB1, ABCG2, and PTEN determine the response of glioblastoma to temozolomide and ABT-888 therapy.* Clin Cancer Res, 2014. **20**(10): p. 2703-13.
156. Wang, F., et al., *Influence of blood-brain barrier efflux pumps on the distribution of vincristine in brain and brain tumors.* Neuro Oncol, 2010. **12**(10): p. 1043-9.

157. Teng, M.W., et al., *Classifying Cancers Based on T-cell Infiltration and PD-L1*. Cancer Res, 2015. **75**(11): p. 2139-45.
158. Tumeh, P.C., et al., *PD-1 blockade induces responses by inhibiting adaptive immune resistance*. Nature, 2014. **515**(7528): p. 568-71.
159. Bockmayr, M., et al., *Subgroup-specific immune and stromal microenvironment in medulloblastoma*. Oncoimmunology, 2018. **7**(9): p. e1462430.
160. Safaei, S., et al., *DIMEimmune: Robust estimation of infiltrating lymphocytes in CNS tumors from DNA methylation profiles*. Oncoimmunology, 2021. **10**(1): p. 1932365.
161. Thakur, M.D., et al., *Immune contexture of paediatric cancers*. Eur J Cancer, 2022. **170**: p. 179-193.
162. Klemm, F., et al., *Interrogation of the Microenvironmental Landscape in Brain Tumors Reveals Disease-Specific Alterations of Immune Cells*. Cell, 2020. **181**(7): p. 1643-1660 e17.
163. Griesinger, A.M., et al., *Characterization of distinct immunophenotypes across pediatric brain tumor types*. J Immunol, 2013. **191**(9): p. 4880-8.
164. Friebel, E., et al., *Single-Cell Mapping of Human Brain Cancer Reveals Tumor-Specific Instruction of Tissue-Invading Leukocytes*. Cell, 2020. **181**(7): p. 1626-1642 e20.
165. Kumar, V., et al., *CD45 Phosphatase Inhibits STAT3 Transcription Factor Activity in Myeloid Cells and Promotes Tumor-Associated Macrophage Differentiation*. Immunity, 2016. **44**(2): p. 303-15.
166. Jurga, A.M., M. Paleczna, and K.Z. Kuter, *Overview of General and Discriminating Markers of Differential Microglia Phenotypes*. Front Cell Neurosci, 2020. **14**: p. 198.
167. Yao, M., et al., *Astrocytic trans-Differentiation Completes a Multicellular Paracrine Feedback Loop Required for Medulloblastoma Tumor Growth*. Cell, 2020. **180**(3): p. 502-520 e19.
168. Albelda, S.M., *CAR T cell therapy for patients with solid tumours: key lessons to learn and unlearn*. Nat Rev Clin Oncol, 2024. **21**(1): p. 47-66.
169. Brinkman, C.C., J.D. Peske, and V.H. Engelhard, *Peripheral tissue homing receptor control of naive, effector, and memory CD8 T cell localization in lymphoid and non-lymphoid tissues*. Front Immunol, 2013. **4**: p. 241.
170. Weaver, K.M.C., *Janeway's Immunobiology*. 9th ed.: Garland Science.
171. Deckert-Schluter, M., et al., *Interferon-gamma receptor-mediated but not tumor necrosis factor receptor type 1- or type 2-mediated signaling is crucial for the activation of cerebral blood vessel endothelial cells and microglia in murine Toxoplasma encephalitis*. Am J Pathol, 1999. **154**(5): p. 1549-61.
172. Aydin, S., et al., *Antigen recognition detains CD8(+) T cells at the blood-brain barrier and contributes to its breakdown*. Nat Commun, 2023. **14**(1): p. 3106.
173. Hofmann, N., et al., *Increased expression of ICAM-1, VCAM-1, MCP-1, and MIP-1 alpha by spinal perivascular macrophages during experimental allergic encephalomyelitis in rats*. BMC Immunol, 2002. **3**: p. 11.
174. Chai, X., et al., *[Clinicopathological characteristics of the CD8(+) T lymphocytes infiltration and its mechanism in distinct molecular subtype of medulloblastoma]*. Beijing Da Xue Xue Bao Yi Xue Ban, 2024. **56**(3): p. 512-518.
175. Cruzeiro, G.A.V., et al., *HIF1A is Overexpressed in Medulloblastoma and its Inhibition Reduces Proliferation and Increases EPAS1 and ATG16L1 Methylation*. Curr Cancer Drug Targets, 2018. **18**(3): p. 287-294.
176. Sharma, I., et al., *Immunohistochemical expression of chemokine receptor CXCR3 and its ligand CXCL10 in low-grade astrocytomas and glioblastoma multiforme: A tissue microarray-based comparison*. J Cancer Res Ther, 2016. **12**(2): p. 793-7.
177. Weenink, B., et al., *Low-grade glioma harbors few CD8 T cells, which is accompanied by decreased expression of chemo-attractants, not immunogenic antigens*. Sci Rep, 2019. **9**(1): p. 14643.

178. Mäenpää, A., et al., *Lymphocyte adhesion molecule ligands and extracellular matrix proteins in gliomas and normal brain: expression of VCAM-1 in gliomas*. Acta Neuropathologica, 1997. **94**(3): p. 216-225.
179. Bartholomaus, I., et al., *Effector T cell interactions with meningeal vascular structures in nascent autoimmune CNS lesions*. Nature, 2009. **462**(7269): p. 94-8.
180. Galea, I., et al., *An antigen-specific pathway for CD8 T cells across the blood-brain barrier*. J Exp Med, 2007. **204**(9): p. 2023-30.
181. Fain, C.E., et al., *Discrete class I molecules on brain endothelium differentially regulate neuropathology in experimental cerebral malaria*. Brain, 2024. **147**(2): p. 566-589.
182. Brown, C.E., et al., *Optimization of IL13Ralpha2-Targeted Chimeric Antigen Receptor T Cells for Improved Anti-tumor Efficacy against Glioblastoma*. Mol Ther, 2018. **26**(1): p. 31-44.
183. Donovan, L.K., et al., *Locoregional delivery of CAR T cells to the cerebrospinal fluid for treatment of metastatic medulloblastoma and ependymoma*. Nat Med, 2020. **26**(5): p. 720-731.
184. Choi, B.D., et al., *Intraventricular CARv3-TEAM-E T Cells in Recurrent Glioblastoma*. N Engl J Med, 2024. **390**(14): p. 1290-1298.
185. Bagley, S.J., et al., *Intrathecal bivalent CAR T cells targeting EGFR and IL13Ralpha2 in recurrent glioblastoma: phase 1 trial interim results*. Nat Med, 2024. **30**(5): p. 1320-1329.
186. Sterner, R.C. and R.M. Sterner, *CAR-T cell therapy: current limitations and potential strategies*. Blood Cancer J, 2021. **11**(4): p. 69.
187. Kishton, R.J., M. Sukumar, and N.P. Restifo, *Metabolic Regulation of T Cell Longevity and Function in Tumor Immunotherapy*. Cell Metab, 2017. **26**(1): p. 94-109.
188. Thommen, D.S. and T.N. Schumacher, *T Cell Dysfunction in Cancer*. Cancer Cell, 2018. **33**(4): p. 547-562.
189. Kaur, B., et al., *Hypoxia and the hypoxia-inducible-factor pathway in glioma growth and angiogenesis*. Neuro Oncol, 2005. **7**(2): p. 134-53.
190. Ziello, J.E., I.S. Jovin, and Y. Huang, *Hypoxia-Inducible Factor (HIF)-1 regulatory pathway and its potential for therapeutic intervention in malignancy and ischemia*. Yale J Biol Med, 2007. **80**(2): p. 51-60.
191. Kim, A.R., et al., *Spatial immune heterogeneity of hypoxia-induced exhausted features in high-grade glioma*. Oncoimmunology, 2022. **11**(1): p. 2026019.
192. Tang, F., et al., *Impact of beta-2 microglobulin expression on the survival of glioma patients via modulating the tumor immune microenvironment*. CNS Neurosci Ther, 2021. **27**(8): p. 951-962.
193. Kilian, M., et al., *MHC class II-restricted antigen presentation is required to prevent dysfunction of cytotoxic T cells by blood-borne myeloids in brain tumors*. Cancer Cell, 2023. **41**(2): p. 235-251 e9.
194. Fuse, S., et al., *CD80 and CD86 control antiviral CD8+ T-cell function and immune surveillance of murine gammaherpesvirus 68*. J Virol, 2006. **80**(18): p. 9159-70.
195. Ahmed, M.H., et al., *Expression and Prognostic Value of CD80 and CD86 in the Tumor Microenvironment of Newly Diagnosed Glioblastoma*. Can J Neurol Sci, 2023. **50**(2): p. 234-242.
196. Qiu, H., et al., *Integrated Analysis Reveals Prognostic Value and Immune Correlates of CD86 Expression in Lower Grade Glioma*. Front Oncol, 2021. **11**: p. 654350.
197. Ott, M., et al., *Profiling of patients with glioma reveals the dominant immunosuppressive axis is refractory to immune function restoration*. JCI Insight, 2020. **5**(17).
198. Lohr, J., et al., *Effector T-cell infiltration positively impacts survival of glioblastoma patients and is impaired by tumor-derived TGF-beta*. Clin Cancer Res, 2011. **17**(13): p. 4296-308.
199. El Andaloussi, A. and M.S. Lesniak, *CD4+ CD25+ FoxP3+ T-cell infiltration and heme oxygenase-1 expression correlate with tumor grade in human gliomas*. J Neurooncol, 2007. **83**(2): p. 145-52.
200. Banissi, C., et al., *Treg depletion with a low-dose metronomic temozolomide regimen in a rat glioma model*. Cancer Immunol Immunother, 2009. **58**(10): p. 1627-34.

201. Grauer, O.M., et al., *Elimination of regulatory T cells is essential for an effective vaccination with tumor lysate-pulsed dendritic cells in a murine glioma model.* Int J Cancer, 2008. **122**(8): p. 1794-802.
202. El Andaloussi, A., Y. Han, and M.S. Lesniak, *Prolongation of survival following depletion of CD4+CD25+ regulatory T cells in mice with experimental brain tumors.* J Neurosurg, 2006. **105**(3): p. 430-7.
203. Cornish, G.H., L.V. Sinclair, and D.A. Cantrell, *Differential regulation of T-cell growth by IL-2 and IL-15.* Blood, 2006. **108**(2): p. 600-8.
204. Granerod, J. and N.S. Crowcroft, *The epidemiology of acute encephalitis.* Neuropsychol Rehabil, 2007. **17**(4-5): p. 406-28.
205. Johnson, R.T., *Acute encephalitis.* Clin Infect Dis, 1996. **23**(2): p. 219-224; quiz 225-6.
206. Swanson, P.A., 2nd, et al., *CD8+ T Cells Induce Fatal Brainstem Pathology during Cerebral Malaria via Luminal Antigen-Specific Engagement of Brain Vasculature.* PLoS Pathog, 2016. **12**(12): p. e1006022.
207. Howland, S.W., et al., *Brain microvessel cross-presentation is a hallmark of experimental cerebral malaria.* EMBO Mol Med, 2013. **5**(7): p. 984-99.
208. Seydel, K.B., et al., *Brain swelling and death in children with cerebral malaria.* N Engl J Med, 2015. **372**(12): p. 1126-37.
209. Leonard, J.P., et al., *Effects of single-dose interleukin-12 exposure on interleukin-12-associated toxicity and interferon-gamma production.* Blood, 1997. **90**(7): p. 2541-8.
210. Jereb, B., et al., *Addition of IFN-alpha to treatment of malignant brain tumors.* Acta Oncol, 1994. **33**(6): p. 651-4.
211. Goff, S.L., et al., *Pilot Trial of Adoptive Transfer of Chimeric Antigen Receptor-transduced T Cells Targeting EGFRvIII in Patients With Glioblastoma.* J Immunother, 2019. **42**(4): p. 126-135.
212. Gust, J., et al., *Endothelial Activation and Blood-Brain Barrier Disruption in Neurotoxicity after Adoptive Immunotherapy with CD19 CAR-T Cells.* Cancer Discov, 2017. **7**(12): p. 1404-1419.
213. Morris, E.C., et al., *Cytokine release syndrome and associated neurotoxicity in cancer immunotherapy.* Nat Rev Immunol, 2022. **22**(2): p. 85-96.
214. Mestre, H., et al., *Cerebrospinal fluid influx drives acute ischemic tissue swelling.* Science, 2020. **367**(6483).
215. Janowitz, D., et al., *Inflammatory markers and imaging patterns of advanced brain aging in the general population.* Brain Imaging Behav, 2020. **14**(4): p. 1108-1117.
216. Perry, V.H., T.A. Newman, and C. Cunningham, *The impact of systemic infection on the progression of neurodegenerative disease.* Nat Rev Neurosci, 2003. **4**(2): p. 103-12.
217. Sly, L.M., et al., *Endogenous brain cytokine mRNA and inflammatory responses to lipopolysaccharide are elevated in the Tg2576 transgenic mouse model of Alzheimer's disease.* Brain Res Bull, 2001. **56**(6): p. 581-8.
218. Combrinck, M.I., V.H. Perry, and C. Cunningham, *Peripheral infection evokes exaggerated sickness behaviour in pre-clinical murine prion disease.* Neuroscience, 2002. **112**(1): p. 7-11.
219. Robert-Gangneux, F. and M.L. Dardé, *Epidemiology of and diagnostic strategies for toxoplasmosis.* Clin Microbiol Rev, 2012. **25**(2): p. 264-96.
220. Sibley, L.D., et al., *Genetic diversity of Toxoplasma gondii in animals and humans.* Philos Trans R Soc Lond B Biol Sci, 2009. **364**(1530): p. 2749-61.
221. Zhao, X.Y. and S.E. Ewald, *The molecular biology and immune control of chronic Toxoplasma gondii infection.* J Clin Invest, 2020. **130**(7): p. 3370-3380.
222. Black, M.W. and J.C. Boothroyd, *Lytic cycle of Toxoplasma gondii.* Microbiol Mol Biol Rev, 2000. **64**(3): p. 607-23.

223. Mordue, D.G., et al., *Toxoplasma gondii resides in a vacuole that avoids fusion with host cell endocytic and exocytic vesicular trafficking pathways*. Exp Parasitol, 1999. **92**(2): p. 87-99.
224. Howe, D.K. and L.D. Sibley, *Toxoplasma gondii comprises three clonal lineages: correlation of parasite genotype with human disease*. J Infect Dis, 1995. **172**(6): p. 1561-6.
225. Sibley, L.D. and J.C. Boothroyd, *Virulent strains of Toxoplasma gondii comprise a single clonal lineage*. Nature, 1992. **359**(6390): p. 82-5.
226. Su, C., et al., *Recent expansion of Toxoplasma through enhanced oral transmission*. Science, 2003. **299**(5605): p. 414-6.
227. Jones, J.L., et al., *Toxoplasma gondii seroprevalence in the United States 2009-2010 and comparison with the past two decades*. Am J Trop Med Hyg, 2014. **90**(6): p. 1135-9.
228. Guigue, N., et al., *Continuous Decline of Toxoplasma gondii Seroprevalence in Hospital: A 1997-2014 Longitudinal Study in Paris, France*. Front Microbiol, 2018. **9**: p. 2369.
229. Howe, D.K., et al., *Determination of genotypes of Toxoplasma gondii strains isolated from patients with toxoplasmosis*. J Clin Microbiol, 1997. **35**(6): p. 1411-4.
230. Hosseini, S.A., et al., *Human toxoplasmosis: a systematic review for genetic diversity of Toxoplasma gondii in clinical samples*. Epidemiol Infect, 2018. **147**: p. e36.
231. Kim, K. and L.M. Weiss, *Toxoplasma gondii: the model apicomplexan*. Int J Parasitol, 2004. **34**(3): p. 423-32.
232. Fleckenstein, M.C., et al., *A Toxoplasma gondii pseudokinase inhibits host IRG resistance proteins*. PLoS Biol, 2012. **10**(7): p. e1001358.
233. Etheridge, R.D., et al., *The Toxoplasma pseudokinase ROP5 forms complexes with ROP18 and ROP17 kinases that synergize to control acute virulence in mice*. Cell Host Microbe, 2014. **15**(5): p. 537-50.
234. Thakur, A., H. Mikkelsen, and G. Jungersen, *Intracellular Pathogens: Host Immunity and Microbial Persistence Strategies*. J Immunol Res, 2019. **2019**: p. 1356540.
235. Hwang, Y.S., et al., *Characteristics of Infection Immunity Regulated by Toxoplasma gondii to Maintain Chronic Infection in the Brain*. Front Immunol, 2018. **9**: p. 158.
236. Shirahata, T., et al., *CD8+ T lymphocytes are the major cell population involved in the early gamma interferon response and resistance to acute primary Toxoplasma gondii infection in mice*. Microbiol Immunol, 1994. **38**(10): p. 789-96.
237. Denkers, E.Y., et al., *Emergence of NK1.1+ cells as effectors of IFN-gamma dependent immunity to Toxoplasma gondii in MHC class I-deficient mice*. J Exp Med, 1993. **178**(5): p. 1465-72.
238. Gazzinelli, R., et al., *Simultaneous depletion of CD4+ and CD8+ T lymphocytes is required to reactivate chronic infection with Toxoplasma gondii*. J Immunol, 1992. **149**(1): p. 175-80.
239. Dotiwala, F., et al., *Killer lymphocytes use granulysin, perforin and granzymes to kill intracellular parasites*. Nat Med, 2016. **22**(2): p. 210-6.
240. Fisch, D., et al., *Human GBP1 Differentially Targets Salmonella and Toxoplasma to License Recognition of Microbial Ligands and Caspase-Mediated Death*. Cell Rep, 2020. **32**(6): p. 108008.
241. Peterson, P.K., et al., *Human astrocytes inhibit intracellular multiplication of Toxoplasma gondii by a nitric oxide-mediated mechanism*. J Infect Dis, 1995. **171**(2): p. 516-8.
242. Chao, C.C., et al., *Effects of cytokines on multiplication of Toxoplasma gondii in microglial cells*. J Immunol, 1993. **150**(8 Pt 1): p. 3404-10.
243. Olias, P., et al., *Toxoplasma Effector Recruits the Mi-2/NuRD Complex to Repress STAT1 Transcription and Block IFN-γ-Dependent Gene Expression*. Cell Host Microbe, 2016. **20**(1): p. 72-82.
244. Freund, Y.R., N.T. Zaveri, and H.S. Javitz, *In vitro investigation of host resistance to Toxoplasma gondii infection in microglia of BALB/c and CBA/Ca mice*. Infect Immun, 2001. **69**(2): p. 765-72.

245. Hidano, S., et al., *STAT1 Signaling in Astrocytes Is Essential for Control of Infection in the Central Nervous System*. mBio, 2016. **7**(6).
246. Chandrasekaran, S., et al., *IFN-γ stimulated murine and human neurons mount anti-parasitic defenses against the intracellular parasite Toxoplasma gondii*. Nat Commun, 2022. **13**(1): p. 4605.
247. Müller, U.B. and J.C. Howard, *The impact of Toxoplasma gondii on the mammalian genome*. Curr Opin Microbiol, 2016. **32**: p. 19-25.
248. Yarovinsky, F., et al., *TLR11 activation of dendritic cells by a protozoan profilin-like protein*. Science, 2005. **308**(5728): p. 1626-9.
249. Lima, T.S. and M.B. Lodoen, *Mechanisms of Human Innate Immune Evasion by Toxoplasma gondii*. Front Cell Infect Microbiol, 2019. **9**: p. 103.
250. Fisch, D., et al., *Human GBP1 is a microbe-specific gatekeeper of macrophage apoptosis and pyroptosis*. Embo j, 2019. **38**(13): p. e100926.
251. Braun, L., et al., *A Toxoplasma dense granule protein, GRA24, modulates the early immune response to infection by promoting a direct and sustained host p38 MAPK activation*. J Exp Med, 2013. **210**(10): p. 2071-86.
252. Rosowski, E.E., et al., *Strain-specific activation of the NF-kappaB pathway by GRA15, a novel Toxoplasma gondii dense granule protein*. J Exp Med, 2011. **208**(1): p. 195-212.
253. Ong, Y.C., M.L. Reese, and J.C. Boothroyd, *Toxoplasma rhoptry protein 16 (ROP16) subverts host function by direct tyrosine phosphorylation of STAT6*. J Biol Chem, 2010. **285**(37): p. 28731-40.
254. Gay, G., et al., *Toxoplasma gondii TgIST co-opts host chromatin repressors dampening STAT1-dependent gene regulation and IFN-gamma-mediated host defenses*. J Exp Med, 2016. **213**(9): p. 1779-98.
255. Dunay, I.R., et al., *Treatment of Toxoplasmosis: Historical Perspective, Animal Models, and Current Clinical Practice*. Clin Microbiol Rev, 2018. **31**(4).
256. Mayoral, J., et al., *Toxoplasma gondii: Bradyzoite Differentiation In Vitro and In Vivo*. Methods Mol Biol, 2020. **2071**: p. 269-282.
257. Fox, B.A. and D.J. Bzik, *De novo pyrimidine biosynthesis is required for virulence of Toxoplasma gondii*. Nature, 2002. **415**(6874): p. 926-9.
258. Baird, J.R., et al., *Immune-mediated regression of established B16F10 melanoma by intratumoral injection of attenuated Toxoplasma gondii protects against rechallenge*. J Immunol, 2013. **190**(1): p. 469-78.
259. Baird, J.R., et al., *Avirulent Toxoplasma gondii generates therapeutic antitumor immunity by reversing immunosuppression in the ovarian cancer microenvironment*. Cancer Res, 2013. **73**(13): p. 3842-51.
260. Gold, D.A., et al., *The Toxoplasma Dense Granule Proteins GRA17 and GRA23 Mediate the Movement of Small Molecules between the Host and the Parasitophorous Vacuole*. Cell Host Microbe, 2015. **17**(5): p. 642-52.
261. Zhu, Y.C., et al., *Synergy between Toxoplasma gondii type I DeltaGRA17 immunotherapy and PD-L1 checkpoint inhibition triggers the regression of targeted and distal tumors*. J Immunother Cancer, 2021. **9**(11).
262. Alvarado-Esquivel, C., et al., *Prevalence of Toxoplasma gondii infection in brain and heart by Immunohistochemistry in a hospital-based autopsy series in Durango, Mexico*. Eur J Microbiol Immunol (Bp), 2015. **5**(2): p. 143-9.
263. Dakroub, H., et al., *Molecular Survey of Toxoplasma gondii in Wild Mammals of Southern Italy*. Pathogens, 2023. **12**(3).
264. Fischer, H.G., et al., *Host cells of Toxoplasma gondii encystation in infected primary culture from mouse brain*. Parasitol Res, 1997. **83**(7): p. 637-41.

265. Melzer, T.C., et al., *Host Cell Preference of Toxoplasma gondii Cysts in Murine Brain: A Confocal Study.* J Neuroparasitology, 2010. **1**.
266. Cabral, C.M., et al., *Neurons are the Primary Target Cell for the Brain-Tropic Intracellular Parasite Toxoplasma gondii.* PLoS Pathog, 2016. **12**(2): p. e1005447.
267. Kovacs, M.A., et al., *Meningeal lymphatic drainage promotes T cell responses against Toxoplasma gondii but is dispensable for parasite control in the brain.* Elife, 2022. **11**.
268. Dellacasa-Lindberg, I., N. Hitziger, and A. Barragan, *Localized recrudescence of Toxoplasma infections in the central nervous system of immunocompromised mice assessed by in vivo bioluminescence imaging.* Microbes Infect, 2007. **9**(11): p. 1291-8.
269. Berenreiterova, M., et al., *The distribution of Toxoplasma gondii cysts in the brain of a mouse with latent toxoplasmosis: implications for the behavioral manipulation hypothesis.* PLoS One, 2011. **6**(12): p. e28925.
270. Barragan, A. and L.D. Sibley, *Transepithelial migration of Toxoplasma gondii is linked to parasite motility and virulence.* J Exp Med, 2002. **195**(12): p. 1625-33.
271. Daneman, R. and M. Rescigno, *The gut immune barrier and the blood-brain barrier: are they so different?* Immunity, 2009. **31**(5): p. 722-35.
272. Mendez, O.A. and A.A. Koshy, *Toxoplasma gondii: Entry, association, and physiological influence on the central nervous system.* PLoS Pathog, 2017. **13**(7): p. e1006351.
273. Olivera, G.C., et al., *Blood-brain barrier-restricted translocation of Toxoplasma gondii from cortical capillaries.* Elife, 2021. **10**.
274. Konradt, C., et al., *Endothelial cells are a replicative niche for entry of Toxoplasma gondii to the central nervous system.* Nat Microbiol, 2016. **1**: p. 16001.
275. Bierly, A.L., et al., *Dendritic cells expressing plasmacytoid marker PDCA-1 are Trojan horses during Toxoplasma gondii infection.* J Immunol, 2008. **181**(12): p. 8485-91.
276. Schneider, C.A., et al., *Imaging the dynamic recruitment of monocytes to the blood-brain barrier and specific brain regions during Toxoplasma gondii infection.* Proc Natl Acad Sci U S A, 2019. **116**(49): p. 24796-24807.
277. John, B., et al., *Dynamic Imaging of CD8(+) T cells and dendritic cells during infection with Toxoplasma gondii.* PLoS Pathog, 2009. **5**(7): p. e1000505.
278. Brown, C.R., et al., *Definitive identification of a gene that confers resistance against Toxoplasma cyst burden and encephalitis.* Immunology, 1995. **85**(3): p. 419-28.
279. Pusch, L., et al., *Persistent toxoplasma bradyzoite cysts in the brain: incidental finding in an immunocompetent patient without evidence of a toxoplasmosis.* Clin Neuropathol, 2009. **28**(3): p. 210-2.
280. Suzuki, Y., et al., *Susceptibility to chronic infection with Toxoplasma gondii does not correlate with susceptibility to acute infection in mice.* Infect Immun, 1993. **61**(6): p. 2284-8.
281. Schaeffer, M., et al., *Dynamic imaging of T cell-parasite interactions in the brains of mice chronically infected with Toxoplasma gondii.* J Immunol, 2009. **182**(10): p. 6379-93.
282. Wohlfert, E.A., I.J. Blader, and E.H. Wilson, *Brains and Brawn: Toxoplasma Infections of the Central Nervous System and Skeletal Muscle.* Trends Parasitol, 2017. **33**(7): p. 519-531.
283. Biswas, A., et al., *Ly6C(high) monocytes control cerebral toxoplasmosis.* J Immunol, 2015. **194**(7): p. 3223-35.
284. Wen, X., et al., *Predominant interferon-gamma-mediated expression of CXCL9, CXCL10, and CCL5 proteins in the brain during chronic infection with Toxoplasma gondii in BALB/c mice resistant to development of toxoplasmic encephalitis.* J Interferon Cytokine Res, 2010. **30**(9): p. 653-60.

285. Strack, A., et al., *Chemokines are differentially expressed by astrocytes, microglia and inflammatory leukocytes in Toxoplasma encephalitis and critically regulated by interferon-gamma*. Acta Neuropathol, 2002. **103**(5): p. 458-68.
286. Groom, J.R. and A.D. Luster, *CXCR3 in T cell function*. Exp Cell Res, 2011. **317**(5): p. 620-31.
287. Ochiai, E., et al., *CXCL9 is important for recruiting immune T cells into the brain and inducing an accumulation of the T cells to the areas of tachyzoite proliferation to prevent reactivation of chronic cerebral infection with Toxoplasma gondii*. Am J Pathol, 2015. **185**(2): p. 314-24.
288. Harris, T.H., et al., *Generalized Levy walks and the role of chemokines in migration of effector CD8+ T cells*. Nature, 2012. **486**(7404): p. 545-8.
289. Deckert-Schluter, M., et al., *Differential expression of ICAM-1, VCAM-1 and their ligands LFA-1, Mac-1, CD43, VLA-4, and MHC class II antigens in murine Toxoplasma encephalitis: a light microscopic and ultrastructural immunohistochemical study*. J Neuropathol Exp Neurol, 1994. **53**(5): p. 457-68.
290. Landrith, T.A., T.H. Harris, and E.H. Wilson, *Characteristics and critical function of CD8+ T cells in the Toxoplasma-infected brain*. Semin Immunopathol, 2015. **37**(3): p. 261-70.
291. Wilson, E.H., et al., *Behavior of parasite-specific effector CD8+ T cells in the brain and visualization of a kinesis-associated system of reticular fibers*. Immunity, 2009. **30**(2): p. 300-11.
292. Sa, Q., et al., *VCAM-1/alpha4beta1 integrin interaction is crucial for prompt recruitment of immune T cells into the brain during the early stage of reactivation of chronic infection with Toxoplasma gondii to prevent toxoplasmic encephalitis*. Infect Immun, 2014. **82**(7): p. 2826-39.
293. John, B., et al., *Analysis of behavior and trafficking of dendritic cells within the brain during toxoplasmic encephalitis*. PLoS Pathog, 2011. **7**(9): p. e1002246.
294. Bui, T.M., H.L. Wiesolek, and R. Sumagin, *ICAM-1: A master regulator of cellular responses in inflammation, injury resolution, and tumorigenesis*. J Leukoc Biol, 2020. **108**(3): p. 787-799.
295. Cook-Mills, J.M., M.E. Marchese, and H. Abdala-Valencia, *Vascular cell adhesion molecule-1 expression and signaling during disease: regulation by reactive oxygen species and antioxidants*. Antioxid Redox Signal, 2011. **15**(6): p. 1607-38.
296. Still, K.M., et al., *Astrocytes promote a protective immune response to brain Toxoplasma gondii infection via IL-33-ST2 signaling*. PLoS Pathog, 2020. **16**(10): p. e1009027.
297. Batista, S.J., et al., *Gasdermin-D-dependent IL-1alpha release from microglia promotes protective immunity during chronic Toxoplasma gondii infection*. Nat Commun, 2020. **11**(1): p. 3687.
298. Tsitsiklis, A., D.J. Bangs, and E.A. Robey, *CD8(+) T Cell Responses to Toxoplasma gondii: Lessons from a Successful Parasite*. Trends Parasitol, 2019. **35**(11): p. 887-898.
299. Sanecka, A., et al., *T Cell Receptor-Major Histocompatibility Complex Interaction Strength Defines Trafficking and CD103(+) Memory Status of CD8 T Cells in the Brain*. Front Immunol, 2018. **9**: p. 1290.
300. Horwitz, M.S., et al., *Primary demyelination in transgenic mice expressing interferon-gamma*. Nat Med, 1997. **3**(9): p. 1037-1041.
301. Gazzinelli, R.T., et al., *In the absence of endogenous IL-10, mice acutely infected with Toxoplasma gondii succumb to a lethal immune response dependent on CD4+ T cells and accompanied by overproduction of IL-12, IFN-gamma and TNF-alpha*. J Immunol, 1996. **157**(2): p. 798-805.
302. Wilson, E.H., et al., *A critical role for IL-10 in limiting inflammation during toxoplasmic encephalitis*. J Neuroimmunol, 2005. **165**(1-2): p. 63-74.
303. O'Brien, C.A., et al., *IL-10 and ICOS Differentially Regulate T Cell Responses in the Brain during Chronic Toxoplasma gondii Infection*. J Immunol, 2019. **202**(6): p. 1755-1766.
304. *1999 USPHS/IDSA guidelines for the prevention of opportunistic infections in persons infected with human immunodeficiency virus. U.S. Public Health Service (USPHS) and Infectious Diseases Society of America (IDSA)*. Infect Dis Obstet Gynecol, 2000. **8**(1): p. 5-74.

305. Sullivan, A.M., et al., *Evidence for finely-regulated asynchronous growth of Toxoplasma gondii cysts based on data-driven model selection*. PLoS Comput Biol, 2013. **9**(11): p. e1003283.
306. Ferguson, D.J., W.M. Hutchison, and E. Pettersen, *Tissue cyst rupture in mice chronically infected with Toxoplasma gondii. An immunocytochemical and ultrastructural study.* Parasitol Res, 1989. **75**(8): p. 599-603.
307. National Institutes of Health, C.f.D.C.a.P., HIV Medicine Association, and Infectious Diseases Society of America. . *Panel on Guidelines for the Prevention and Treatment of Opportunistic Infections in Adults and Adolescents with HIV. Guidelines for the Prevention and Treatment of Opportunistic Infections in Adults and Adolescents with HIV.* . Available from: https://clinicalinfo.hiv.gov/en/guidelines/adult-and-adolescent-opportunistic-infectio.
308. Fernandez-Villa, D., M.R. Aguilar, and L. Rojo, *Folic Acid Antagonists: Antimicrobial and Immunomodulating Mechanisms and Applications*. Int J Mol Sci, 2019. **20**(20).
309. Terao, I., et al., *[A case of miliary tuberculosis with cutaneous lesion].* Kekkaku, 1990. **65**(12): p. 821-5.
310. Hodge, J.M., et al., *Toxoplasma gondii infection and the risk of adult glioma in two prospective studies.* Int J Cancer, 2021. **148**(10): p. 2449-2456.
311. Abdollahi, A., et al., *Toxoplasma gondii infection/exposure and the risk of brain tumors: A systematic review and meta-analysis*. Cancer Epidemiol, 2022. **77**: p. 102119.
312. Ayasoufi, K., et al., *Brain cancer induces systemic immunosuppression through release of non-steroid soluble mediators.* Brain, 2020. **143**(12): p. 3629-3652.
313. Siegel, R.L., et al., *Cancer statistics, 2023.* CA Cancer J Clin, 2023. **73**(1): p. 17-48.
314. Robert, C., et al., *Nivolumab in previously untreated melanoma without BRAF mutation*. N Engl J Med, 2015. **372**(4): p. 320-30.
315. Westin, J.R., et al., *Survival with Axicabtagene Ciloleucel in Large B-Cell Lymphoma*. N Engl J Med, 2023. **389**(2): p. 148-157.
316. Rodriguez-Otero, P., et al., *Ide-cel or Standard Regimens in Relapsed and Refractory Multiple Myeloma*. N Engl J Med, 2023. **388**(11): p. 1002-1014.
317. Omuro, A., et al., *Radiotherapy combined with nivolumab or temozolomide for newly diagnosed glioblastoma with unmethylated MGMT promoter: An international randomized phase III trial*. Neuro Oncol, 2023. **25**(1): p. 123-134.
318. Lim, M., et al., *Phase III trial of chemoradiotherapy with temozolomide plus nivolumab or placebo for newly diagnosed glioblastoma with methylated MGMT promoter.* Neuro Oncol, 2022. **24**(11): p. 1935-1949.
319. Lyndon, D., et al., *Dural masses: meningiomas and their mimics*. Insights Imaging, 2019. **10**(1): p. 11.
320. Fang, L., et al., *The immune cell infiltrate populating meningiomas is composed of mature, antigen-experienced T and B cells*. Neuro Oncol, 2013. **15**(11): p. 1479-90.
321. Heimberger, A.B., et al., *Incidence and prognostic impact of FoxP3+ regulatory T cells in human gliomas.* Clin Cancer Res, 2008. **14**(16): p. 5166-72.
322. Binnewies, M., et al., *Understanding the tumor immune microenvironment (TIME) for effective therapy.* Nat Med, 2018. **24**(5): p. 541-550.
323. Gajewski, T.F., *The Next Hurdle in Cancer Immunotherapy: Overcoming the Non-T-Cell-Inflamed Tumor Microenvironment*. Semin Oncol, 2015. **42**(4): p. 663-71.
324. Engelhardt, B., P. Vajkoczy, and R.O. Weller, *The movers and shapers in immune privilege of the CNS*. Nat Immunol, 2017. **18**(2): p. 123-131.
325. Tang, F., et al., *Tumor-associated macrophage-related strategies for glioma immunotherapy.* NPJ Precis Oncol, 2023. **7**(1): p. 78.

326. Nagai, M. and T. Arai, *Clinical effect of interferon in malignant brain tumours.* Neurosurg Rev, 1984. **7**(1): p. 55-64.
327. Packer, R.J., et al., *Treatment of children with newly diagnosed brain stem gliomas with intravenous recombinant beta-interferon and hyperfractionated radiation therapy: a childrens cancer group phase I/II study.* Cancer, 1996. **77**(10): p. 2150-6.
328. Jiang, H., et al., *Combination of Immunotherapy and Radiotherapy for Recurrent Malignant Gliomas: Results From a Prospective Study.* Front Immunol, 2021. **12**: p. 632547.
329. Vitanza, N.A., et al., *Locoregional infusion of HER2-specific CAR T cells in children and young adults with recurrent or refractory CNS tumors: an interim analysis.* Nat Med, 2021. **27**(9): p. 1544-1552.
330. Nwagwu, C.D., et al., *Convection Enhanced Delivery in the Setting of High-Grade Gliomas.* Pharmaceutics, 2021. **13**(4).
331. Lamm, D.L., et al., *Maintenance bacillus Calmette-Guerin immunotherapy for recurrent TA, T1 and carcinoma in situ transitional cell carcinoma of the bladder: a randomized Southwest Oncology Group Study.* J Urol, 2000. **163**(4): p. 1124-9.
332. van der Meijden, A.P., et al., *Intravesical instillation of epirubicin, bacillus Calmette-Guerin and bacillus Calmette-Guerin plus isoniazid for intermediate and high risk Ta, T1 papillary carcinoma of the bladder: a European Organization for Research and Treatment of Cancer genito-urinary group randomized phase III trial.* J Urol, 2001. **166**(2): p. 476-81.
333. Ayed, M., et al., *[Results of BCG in the treatment of pTa and pT1 bladder tumors. Evaluation of a long protocol using 75 mg of Pasteur strain BCG].* Prog Urol, 1998. **8**(2): p. 206-10.
334. Kates, M., et al., *Intravesical BCG Induces CD4(+) T-Cell Expansion in an Immune Competent Model of Bladder Cancer.* Cancer Immunol Res, 2017. **5**(7): p. 594-603.
335. el-Demiry, M.I., et al., *Local immune responses after intravesical BCG treatment for carcinoma in situ.* Br J Urol, 1987. **60**(6): p. 543-8.
336. Pichler, R., et al., *Tumor-infiltrating immune cell subpopulations influence the oncologic outcome after intravesical Bacillus Calmette-Guerin therapy in bladder cancer.* Oncotarget, 2016. **7**(26): p. 39916-39930.
337. Robert-Gangneux, F. and M.L. Darde, *Epidemiology of and diagnostic strategies for toxoplasmosis.* Clin Microbiol Rev, 2012. **25**(2): p. 264-96.
338. Su, C., et al., *Identification of quantitative trait loci controlling acute virulence in Toxoplasma gondii.* Proc Natl Acad Sci U S A, 2002. **99**(16): p. 10753-8.
339. Matta, S.K., et al., *NADPH Oxidase and Guanylate Binding Protein 5 Restrict Survival of Avirulent Type III Strains of Toxoplasma gondii in Naive Macrophages.* mBio, 2018. **9**(4).
340. Weiss, L.M. and K. Kim, *Toxoplasma gondii: The Model Apicomplexan - Perspectives and Methods*. (Third Edition). 2020: Academic Press. 1222.
341. Huse, J.T. and E.C. Holland, *Genetically engineered mouse models of brain cancer and the promise of preclinical testing.* Brain Pathol, 2009. **19**(1): p. 132-43.
342. Chongsathidkiet, P., et al., *Sequestration of T cells in bone marrow in the setting of glioblastoma and other intracranial tumors.* Nat Med, 2018. **24**(9): p. 1459-1468.
343. Luo, W., et al., *Kinetics in parasite abundance in susceptible and resistant mice infected with an avirulent strain of Toxoplasma gondii by using quantitative competitive PCR.* J Parasitol, 1997. **83**(6): p. 1070-4.
344. Di Cristina, M., et al., *Temporal and spatial distribution of Toxoplasma gondii differentiation into Bradyzoites and tissue cyst formation in vivo.* Infect Immun, 2008. **76**(8): p. 3491-501.
345. Edvinsson, B., et al., *Real-time PCR targeting a 529-bp repeat element for diagnosis of toxoplasmosis.* Clin Microbiol Infect, 2006. **12**(2): p. 131-6.

346. Homan, W.L., et al., *Identification of a 200- to 300-fold repetitive 529 bp DNA fragment in Toxoplasma gondii, and its use for diagnostic and quantitative PCR*. Int J Parasitol, 2000. **30**(1): p. 69-75.
347. Suzuki, Y., F.K. Conley, and J.S. Remington, *Importance of endogenous IFN-gamma for prevention of toxoplasmic encephalitis in mice*. J Immunol, 1989. **143**(6): p. 2045-50.
348. O'Brien, C.A., et al., *CD11c-Expressing Cells Affect Regulatory T Cell Behavior in the Meninges during Central Nervous System Infection*. J Immunol, 2017. **198**(10): p. 4054-4061.
349. Melchor, S.J., et al., *IL-1R Regulates Disease Tolerance and Cachexia in Toxoplasma gondii Infection*. J Immunol, 2020. **204**(12): p. 3329-3338.
350. Gatkowska, J., et al., *Behavioral changes in mice caused by Toxoplasma gondii invasion of brain*. Parasitol Res, 2012. **111**(1): p. 53-8.
351. Chen, D.S. and I. Mellman, *Elements of cancer immunity and the cancer-immune set point*. Nature, 2017. **541**(7637): p. 321-330.
352. Pai, S.I., A. Cesano, and F.M. Marincola, *The Paradox of Cancer Immune Exclusion: Immune Oncology Next Frontier*. Cancer Treat Res, 2020. **180**: p. 173-195.
353. Karimi, E., et al., *Single-cell spatial immune landscapes of primary and metastatic brain tumours*. Nature, 2023. **614**(7948): p. 555-563.
354. El Andaloussi, A. and M.S. Lesniak, *An increase in CD4+CD25+FOXP3+ regulatory T cells in tumor-infiltrating lymphocytes of human glioblastoma multiforme*. Neuro Oncol, 2006. **8**(3): p. 234-43.
355. Severson, J.J., et al., *PD-1+Tim-3+ CD8+ T Lymphocytes Display Varied Degrees of Functional Exhaustion in Patients with Regionally Metastatic Differentiated Thyroid Cancer*. Cancer Immunol Res, 2015. **3**(6): p. 620-30.
356. Philipp, N., et al., *T-cell exhaustion induced by continuous bispecific molecule exposure is ameliorated by treatment-free intervals*. Blood, 2022. **140**(10): p. 1104-1118.
357. Collazo, C.M., et al., *The function of gamma interferon-inducible GTP-binding protein IGTP in host resistance to Toxoplasma gondii is Stat1 dependent and requires expression in both hematopoietic and nonhematopoietic cellular compartments*. Infect Immun, 2002. **70**(12): p. 6933-9.
358. Scharton-Kersten, T.M., et al., *In the absence of endogenous IFN-gamma, mice develop unimpaired IL-12 responses to Toxoplasma gondii while failing to control acute infection*. J Immunol, 1996. **157**(9): p. 4045-54.
359. Castro, F., et al., *Interferon-Gamma at the Crossroads of Tumor Immune Surveillance or Evasion*. Front Immunol, 2018. **9**: p. 847.
360. Thibaut, R., et al., *Bystander IFN-gamma activity promotes widespread and sustained cytokine signaling altering the tumor microenvironment*. Nat Cancer, 2020. **1**(3): p. 302-314.
361. Ayers, M., et al., *IFN-gamma-related mRNA profile predicts clinical response to PD-1 blockade*. J Clin Invest, 2017. **127**(8): p. 2930-2940.
362. Larson, R.C., et al., *CAR T cell killing requires the IFNgammaR pathway in solid but not liquid tumours*. Nature, 2022. **604**(7906): p. 563-570.
363. Boulch, M., et al., *A cross-talk between CAR T cell subsets and the tumor microenvironment is essential for sustained cytotoxic activity*. Sci Immunol, 2021. **6**(57).
364. Wagner, C., et al., *The complement receptor 3, CR3 (CD11b/CD18), on T lymphocytes: activation-dependent up-regulation and regulatory function*. Eur J Immunol, 2001. **31**(4): p. 1173-80.
365. Christensen, J.E., et al., *CD11b expression as a marker to distinguish between recently activated effector CD8(+) T cells and memory cells*. Int Immunol, 2001. **13**(4): p. 593-600.
366. Fogg, D.K., et al., *A clonogenic bone marrow progenitor specific for macrophages and dendritic cells*. Science, 2006. **311**(5757): p. 83-7.
367. Lee, Y., et al., *A molecular fingerprint for medulloblastoma*. Cancer Res, 2003. **63**(17): p. 5428-37.

368. Farber, J.M., *Mig and IP-10: CXC chemokines that target lymphocytes.* J Leukoc Biol, 1997. **61**(3): p. 246-57.
369. Schweitzer, A.N., et al., *Role of costimulators in T cell differentiation: studies using antigen-presenting cells lacking expression of CD80 or CD86.* J Immunol, 1997. **158**(6): p. 2713-22.
370. Badie, B., B. Bartley, and J. Schartner, *Differential expression of MHC class II and B7 costimulatory molecules by microglia in rodent gliomas.* J Neuroimmunol, 2002. **133**(1-2): p. 39-45.
371. Zhang, C., et al., *Comprehensive analysis of CTLA-4 in the tumor immune microenvironment of 33 cancer types.* Int Immunopharmacol, 2020. **85**: p. 106633.
372. Steimle, V., et al., *Regulation of MHC class II expression by interferon-gamma mediated by the transactivator gene CIITA*. Science, 1994. **265**(5168): p. 106-9.
373. Kenneth Murphy, C.W., *Janeway's immunobiolgy*. 9th ed. 2016: Garland Science.
374. Wang, M.M., et al., *Resistance to immune checkpoint therapies by tumour-induced T-cell desertification and exclusion: key mechanisms, prognostication and new therapeutic opportunities.* Br J Cancer, 2023. **129**(8): p. 1212-1224.
375. Sooreshjani, M., et al., *The Use of Targeted Cytokines as Cancer Therapeutics in Glioblastoma*. Cancers (Basel), 2023. **15**(14).
376. Johanson, C.E., et al., *Choroid Plexus Blood-CSF Barrier: Major Player in Brain Disease Modeling and Neuromedicine.* Journal of Neurology & Neuromedicine, 2018. **3(4)**: p. 39-58.
377. Fowler, M.J., et al., *Intrathecal drug delivery in the era of nanomedicine.* Adv Drug Deliv Rev, 2020. **165-166**: p. 77-95.
378. Ogiya, R., et al., *Comparison of immune microenvironments between primary tumors and brain metastases in patients with breast cancer.* Oncotarget, 2017. **8**(61): p. 103671-103681.
379. Taggart, D., et al., *Anti-PD-1/anti-CTLA-4 efficacy in melanoma brain metastases depends on extracranial disease and augmentation of CD8(+) T cell trafficking.* Proc Natl Acad Sci U S A, 2018. **115**(7): p. E1540-E1549.
380. Harris, M.G., et al., *Immune privilege of the CNS is not the consequence of limited antigen sampling.* Sci Rep, 2014. **4**: p. 4422.
381. PEARL, R., *CANCER AND TUBERCULOSIS**. American Journal of Epidemiology, 1929. **9**(1): p. 97-159.
382. Morales, A., D. Eidinger, and A.W. Bruce, *Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors.* J Urol, 1976. **116**(2): p. 180-3.
383. Bloomberg, S.D., et al., *The effects of BCG on the dog bladder.* Invest Urol, 1975. **12**(6): p. 423-7.
384. Herr, H.W. and A. Morales, *History of bacillus Calmette-Guerin and bladder cancer: an immunotherapy success story.* J Urol, 2008. **179**(1): p. 53-6.
385. Dian, S., A.R. Ganiem, and S. Ekawardhani, *Cerebral toxoplasmosis in HIV-infected patients: a review.* Pathog Glob Health, 2023. **117**(1): p. 14-23.
386. Martina, M.N., et al., *Toxoplasma gondii primary infection in renal transplant recipients. Two case reports and literature review.* Transpl Int, 2011. **24**(1): p. e6-12.
387. Frenkel, J.K., M. Amare, and W. Larsen, *Immune competence in a patient with Hodgkin's disease and relapsing toxoplasmosis.* Infection, 1978. **6**(2): p. 84-91.
388. Ali, M.I., et al., *Toxoplasma gondii in cancer patients receiving chemotherapy: seroprevalence and interferon gamma level.* J Parasit Dis, 2019. **43**(3): p. 464-471.
389. Eyles, D.E. and N. Coleman, *Synergistic effect of sulfadiazine and daraprim against experimental toxoplasmosis in the mouse.* Antibiot Chemother (Northfield), 1953. **3**(5): p. 483-90.
390. Leport, C., et al., *Treatment of central nervous system toxoplasmosis with pyrimethamine/sulfadiazine combination in 35 patients with the acquired immunodeficiency syndrome. Efficacy of long-term continuous therapy.* Am J Med, 1988. **84**(1): p. 94-100.

391. Lu, F., S. Huang, and L.H. Kasper, *The temperature-sensitive mutants of Toxoplasma gondii and ocular toxoplasmosis*. Vaccine, 2009. **27**(4): p. 573-80.
392. Pepper, M., et al., *Development of a system to study CD4+-T-cell responses to transgenic ovalbumin-expressing Toxoplasma gondii during toxoplasmosis*. Infect Immun, 2004. **72**(12): p. 7240-6.
393. Lodoen, M.B., C. Gerke, and J.C. Boothroyd, *A highly sensitive FRET-based approach reveals secretion of the actin-binding protein toxofilin during Toxoplasma gondii infection*. Cell Microbiol, 2010. **12**(1): p. 55-66.
394. Koshy, A.A., et al., *Toxoplasma co-opts host cells it does not invade*. PLoS Pathog, 2012. **8**(7): p. e1002825.
395. Henner, A., et al., *MADM-ML, a mouse genetic mosaic system with increased clonal efficiency*. PLoS One, 2013. **8**(10): p. e77672.
396. Goodrich, L.V., et al., *Altered neural cell fates and medulloblastoma in mouse patched mutants*. Science, 1997. **277**(5329): p. 1109-13.
397. Jacks, T., et al., *Tumor spectrum analysis in p53-mutant mice*. Curr Biol, 1994. **4**(1): p. 1-7.
398. Matei, V., et al., *Smaller inner ear sensory epithelia in Neurog 1 null mice are related to earlier hair cell cycle exit*. Dev Dyn, 2005. **234**(3): p. 633-50.
399. Boyle, J.P., J.P. Saeij, and J.C. Boothroyd, *Toxoplasma gondii: inconsistent dissemination patterns following oral infection in mice*. Exp Parasitol, 2007. **116**(3): p. 302-5.
400. Bhoopalan, V., E.E. Gardiner, and A. Kaur, *An Optimized Method of Collecting Murine Peripheral Blood and Dilution Correction for Accurate Blood Cell Enumeration*. Curr Protoc, 2023. **3**(5): p. e765.
401. *FastQC*. 2015.
402. Dobin, A., et al., *STAR: ultrafast universal RNA-seq aligner*. Bioinformatics, 2012. **29**(1): p. 15-21.
403. Li, B. and C.N. Dewey, *RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome*. BMC Bioinformatics, 2011. **12**(1): p. 323.
404. Love, M.I., W. Huber, and S. Anders, *Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2*. Genome Biology, 2014. **15**(12): p. 550.
405. Subramanian, A., et al., *Gene set enrichment analysis: a knowledge-based approach for interpreting genome-wide expression profiles*. Proc Natl Acad Sci U S A, 2005. **102**(43): p. 15545-50.
406. Korotkevich, G., et al., *Fast gene set enrichment analysis*. bioRxiv, 2021: p. 060012.
407. Gu, Z., *Complex heatmap visualization*. iMeta, 2022. **1**(3): p. e43.
408. Ho, P.C., et al., *Immune-based antitumor effects of BRAF inhibitors rely on signaling by CD40L and IFNgamma*. Cancer Res, 2014. **74**(12): p. 3205-17.
409. Hoves, S., et al., *Rapid activation of tumor-associated macrophages boosts preexisting tumor immunity*. J Exp Med, 2018. **215**(3): p. 859-876.
410. Kashyap, A.S., et al., *Optimized antiangiogenic reprogramming of the tumor microenvironment potentiates CD40 immunotherapy*. Proc Natl Acad Sci U S A, 2020. **117**(1): p. 541-551.
411. Marigo, I., et al., *T Cell Cancer Therapy Requires CD40-CD40L Activation of Tumor Necrosis Factor and Inducible Nitric-Oxide-Synthase-Producing Dendritic Cells*. Cancer Cell, 2016. **30**(3): p. 377-390.
412. Klug, F., et al., *Low-dose irradiation programs macrophage differentiation to an iNOS(+)/M1 phenotype that orchestrates effective T cell immunotherapy*. Cancer Cell, 2013. **24**(5): p. 589-602.
413. Pommier, A., et al., *Inflammatory monocytes are potent antitumor effectors controlled by regulatory CD4+ T cells*. Proc Natl Acad Sci U S A, 2013. **110**(32): p. 13085-90.

414. Schluter, D., et al., *Inhibition of inducible nitric oxide synthase exacerbates chronic cerebral toxoplasmosis in Toxoplasma gondii-susceptible C57BL/6 mice but does not reactivate the latent disease in T. gondii-resistant BALB/c mice.* J Immunol, 1999. **162**(6): p. 3512-8.
415. Portillo, J.A., et al., *The CD40-autophagy pathway is needed for host protection despite IFN-Gamma-dependent immunity and CD40 induces autophagy via control of P21 levels.* PLoS One, 2010. **5**(12): p. e14472.
416. Nguyen, V.T. and E.N. Benveniste, *IL-4-activated STAT-6 inhibits IFN-gamma-induced CD40 gene expression in macrophages/microglia.* J Immunol, 2000. **165**(11): p. 6235-43.
417. Hiroi, M., et al., *Anti-inflammatory cytokine interleukin-4 inhibits inducible nitric oxide synthase gene expression in the mouse macrophage cell line RAW264.7 through the repression of octamer-dependent transcription.* Mediators Inflamm, 2013. **2013**: p. 369693.
418. Herbert, D.R., et al., *Alternative macrophage activation is essential for survival during schistosomiasis and downmodulates T helper 1 responses and immunopathology.* Immunity, 2004. **20**(5): p. 623-35.
419. Andrews, A.L., et al., *IL-4 receptor alpha is an important modulator of IL-4 and IL-13 receptor binding: implications for the development of therapeutic targets.* J Immunol, 2006. **176**(12): p. 7456-61.
420. Bedolla, A., et al., *Finding the right tool: a comprehensive evaluation of microglial inducible cre mouse models.* bioRxiv, 2023: p. 2023.04.17.536878.
421. Roberts, C.W., et al., *Different roles for interleukin-4 during the course of Toxoplasma gondii infection.* Infect Immun, 1996. **64**(3): p. 897-904.
422. Suzuki, Y., et al., *IL-4 is protective against development of toxoplasmic encephalitis.* J Immunol, 1996. **157**(6): p. 2564-9.
423. Liu, P.S., et al., *CD40 signal rewires fatty acid and glutamine metabolism for stimulating macrophage anti-tumorigenic functions.* Nat Immunol, 2023. **24**(3): p. 452-462.
424. Lang, F.F., et al., *Phase 1b open-label randomized study of the oncolytic adenovirus DNX-2401 administered with or without interferon gamma for recurrent glioblastoma.* Journal of Clinical Oncology, 2017. **35**(15_suppl): p. 2002-2002.
425. Wolff, J.E., et al., *Maintenance treatment with interferon-gamma and low-dose cyclophosphamide for pediatric high-grade glioma.* J Neurooncol, 2006. **79**(3): p. 315-21.
426. Foon, K.A., et al., *A phase I trial of recombinant gamma interferon in patients with cancer.* Cancer Immunol Immunother, 1985. **20**(3): p. 193-7.
427. Cerutti, A., N. Blanchard, and S. Besteiro, *The Bradyzoite: A Key Developmental Stage for the Persistence and Pathogenesis of Toxoplasmosis.* Pathogens, 2020. **9**(3).
428. Dzierszinski, F., et al., *Dynamics of Toxoplasma gondii differentiation.* Eukaryot Cell, 2004. **3**(4): p. 992-1003.
429. Tomita, T., et al., *The Toxoplasma gondii cyst wall protein CST1 is critical for cyst wall integrity and promotes bradyzoite persistence.* PLoS Pathog, 2013. **9**(12): p. e1003823.
430. Cardew, E.M., C. Verlinde, and E. Pohl, *The calcium-dependent protein kinase 1 from Toxoplasma gondii as target for structure-based drug design.* Parasitology, 2018. **145**(2): p. 210-218.
431. Kieschnick, H., et al., *Toxoplasma gondii attachment to host cells is regulated by a calmodulin-like domain protein kinase.* J Biol Chem, 2001. **276**(15): p. 12369-77.
432. Lourido, S., et al., *Calcium-dependent protein kinase 1 is an essential regulator of exocytosis in Toxoplasma.* Nature, 2010. **465**(7296): p. 359-62.
433. Long, S., Q. Wang, and L.D. Sibley, *Analysis of Noncanonical Calcium-Dependent Protein Kinases in Toxoplasma gondii by Targeted Gene Deletion Using CRISPR/Cas9.* Infect Immun, 2016. **84**(5): p. 1262-1273.

434. Huang, W., et al., *SAR Studies of 5-Aminopyrazole-4-carboxamide Analogues as Potent and Selective Inhibitors of Toxoplasma gondii CDPK1*. ACS Med Chem Lett, 2015. **6**(12): p. 1184-1189.
435. Vidadala, R.S., et al., *Development of an Orally Available and Central Nervous System (CNS) Penetrant Toxoplasma gondii Calcium-Dependent Protein Kinase 1 (TgCDPK1) Inhibitor with Minimal Human Ether-a-go-go-Related Gene (hERG) Activity for the Treatment of Toxoplasmosis*. J Med Chem, 2016. **59**(13): p. 6531-46.
436. Castano Barrios, L., et al., *Behavioral alterations in long-term Toxoplasma gondii infection of C57BL/6 mice are associated with neuroinflammation and disruption of the blood brain barrier*. PLoS One, 2021. **16**(10): p. e0258199.
437. Khalsa, J.K., et al., *Immune phenotyping of diverse syngeneic murine brain tumors identifies immunologically distinct types*. Nat Commun, 2020. **11**(1): p. 3912.
438. van Hooren, L., et al., *CD103(+) regulatory T cells underlie resistance to radio-immunotherapy and impair CD8(+) T cell activation in glioblastoma*. Nat Cancer, 2023. **4**(5): p. 665-681.
439. Noffsinger, B., et al., *Technical choices significantly alter the adaptive immune response against immunocompetent murine gliomas in a model-dependent manner*. J Neurooncol, 2021. **154**(2): p. 145-157.
440. Ahn, J.H., et al., *Meningeal lymphatic vessels at the skull base drain cerebrospinal fluid*. Nature, 2019. **572**(7767): p. 62-66.
441. Shah, J.L., et al., *Stereotactic Radiosurgery and Hypofractionated Radiotherapy for Glioblastoma*. Neurosurgery, 2018. **82**(1): p. 24-34.
442. Mun, E.J., et al., *Tumor-Treating Fields: A Fourth Modality in Cancer Treatment*. Clin Cancer Res, 2018. **24**(2): p. 266-275.
443. Antila, S., et al., *Development and plasticity of meningeal lymphatic vessels*. J Exp Med, 2017. **214**(12): p. 3645-3667.
444. Song, E., et al., *VEGF-C-driven lymphatic drainage enables immunosurveillance of brain tumours*. Nature, 2020. **577**(7792): p. 689-694.
445. Hu, X., et al., *Meningeal lymphatic vessels regulate brain tumor drainage and immunity*. Cell Res, 2020. **30**(3): p. 229-243.
446. Abiko, K., et al., *IFN-gamma from lymphocytes induces PD-L1 expression and promotes progression of ovarian cancer*. Br J Cancer, 2015. **112**(9): p. 1501-9.