REVIEW ARTICLE

Tumor immune landscape of paediatric high-grade gliomas

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Abstract

Over the last decade, remarkable progress has been made towards elucidating the origin and genomic landscape of childhood high-grade brain tumors. It has become evident that pediatric high-grade gliomas (pHGGs) differ from adult HGGs with respect to multiple defining aspects including: DNA copy number, gene expression profiles, tumor locations within the central nervous system, and genetic alterations such as somatic histone mutations. Despite these advances, clinical trials for children with glioma have historically been based on ineffective adult regimens that fail to take into consideration the fundamental biological differences between the two. Additionally, although our knowledge of the intrinsic cellular mechanisms driving tumor progression has considerably expanded, little is known concerning the dynamic tumor immune microenvironment (TIME) in pHGGs. In this review, we explore the genetic and epigenetic landscape of pHGGs and how this drives the creation of specific tumor sub-groups with meaningful survival outcomes. Further, we provide a comprehensive analysis of the pHGG TIME and discuss emerging therapeutic efforts aimed at exploiting the immune functions of these tumors.

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Abbreviations: AA = anaplastic astrocytoma; aGBM = adult glioblastoma; aHGG = adult high-grade glioma; ATCL = allogeneic tumor cell lines; CAR = chimeric antigen receptor; CMMRD = constitutional mismatch repair deficiency; DAMP = damage associated molecular patterns; DC = dendritic cell; DIPG = diffuse intrinsic pontine glioma; DMG: diffuse midline glioma; FFPE = formalin fixed paraffin embedded; FLT3L = fms-like tyrosine kinase 3 ligand; GBM = glioblastoma; GCV = ganciclovir; GEMM = genetically engineered mouse model; HGG = high-grade glioma; IHC = immunohistochemistry; LGG = low grade glioma; MB = medulloblastoma; MHC = major histocompatibility complex; MMR = mismatch repair; NPC = neural progenitor cell; NSG = nod scid gamma; OPC = oligodendrocyte progenitor cell; OS = overall survival; PA = pilocytic astrocytoma; PFS = progression free survival; pHGG = pediatric high-grade glioma; pLGG = pediatric low-grade glioma; Poly-ICLC = polyinosinic-polycytidylic acid (poly[I:C]) stabilized by lysine and carboxymethylcellulose; PRC2 = polycomb repressive complex 2; PVSRIPO = polio-rhinovirus recombinant; RT = radiotherapy; SCID = severe combined immunodeficiency; TAM = tumor associated macrophage; TCR = t-cell receptor; TIL = tumor infiltrating lymphocytes; TK = thymidine kinase; TIME = tumor immune microenvironment; TME = tumor microenvironment; TMZ = temozolomide; WHO = world health organization; WT = wild type

Introduction

Central nervous system (CNS) neoplasms are the most common solid tumors in the pediatric population.[1, 2] Approximately 53% of all pediatric solid tumors are comprised of low (pLGG) and high-grade (pHGG) gliomas. pLGGs account for approximately two-thirds, and pHGGs one-third, of all pediatric CNS tumors.[3] High-grade gliomas include several disease entities such as; anaplastic astrocytoma, glioblastoma (GBM), diffuse midline glioma (DMG) with H3K27M mutation (WHO Grade IV), and diffuse intrinsic pontine glioma (DIPG). Less frequently encountered high-grade gliomas include anaplastic oligodendroglioma, oligoastrocytoma, and gliosarcoma. Together, these gliomas occur in 1 child per 100,000 children each year, yet despite their relative rarity they are responsible for the most cancer related deaths in children under the age of 19 years old.[1, 2, 4] To date, there have been very few predisposing conditions causally linked to the development of pHGG but conditions such as Li-Fraumeni syndrome, constitutional mismatch repair deficiency (CMMRD), neurofibromatosis type 1, and Turcot syndrome have been implicated.[5-7]

pHGGs within the brain are found in two general locations including the cortical hemispheres (~50%) or the midline/brainstem (~50%)(collectively referred to as DMG)(**Figure 1a**). Hemispheric pHGGs (supratentorial) are typically less aggressive than those found in the midline or brainstem (infratentorial). Further, hemispheric pHGGs usually have a median age at diagnosis of 13 years old and median survival of 18 months, as opposed to 7-9 years old and 11 months respectively in DMGs.[8, 9] Much is known about the intrinsic cellular mechanisms driving tumor progression in pHGGs, yet the tumor immune microenvironment (TIME) has been less comprehensively characterized. In this review we discuss the historical data describing the TIME of pHGGs and explore the implications that the TIME has on tumor dynamics and therapeutic efforts.

Current Standard of Care

As with adult HGGs (aHGG), the most effective treatment strategy for pHGGs is maximal safe tumor resection.[10-13] However, the diffusely infiltrative nature of HGGs precludes complete tumor resection in nearly all cases, owing to inevitable tumor recurrence. In one study (CCG-945), patients who underwent gross total resection had a 5 year progression free survival (PFS) of 35%, and those who received sub-total resection had a 5 year PFS of 17%.[12] In cases where over 90% of the tumor is resected, 5 year PFS is significantly greater than those with less successful resection.[11] Due to the location of DMGs in delicate regions of the brain such as the brainstem and thalamus, these tumors are typically rendered inoperable, contributing to their dismal outlook. Treatment of DMGs thus relies heavily on radiotherapy with the use of corticosteroids for symptom management and cerebral edema.[14, 15] pHGGs are treated with a range of radiation doses with most patients receiving between 50-60 Gy of fractionated radiation spread over approximately 6 weeks of 30 fractions.[1, 6, 14] Unlike aHGGs, pHGGs typically do not respond to chemotherapies and the addition of chemotherapy with radiotherapy adds little benefit.[16] Further, unlike aLGGs and aHGGs which frequently harbor the prognostically favorable *MGMT* promoter methylation, the frequency of this genetic alteration is lower in pHGGs, and is unclear whether this has prognostic value in the pediatric setting.[16-20] The limited number of long term pHGG survivors are often left with detrimental side effects including endocrine morbidity, psychiatric and neurocognitive impairments, developmental disorders, and a high incidence of secondary

tumors.[4-6] These deleterious outcomes further highlight the necessity for novel treatment modalities that extend prognostic outcome while minimizing toxicities in children with pHGG.

Historically, therapeutic strategies for pHGG have been adapted from their adult counterparts and have therefore ignored the innate biological differences between the two. Immune checkpoint blockade strategies targeting adult systemic cancers have largely failed to produce benefits in pHGG, with the exception of patients harboring CMMRD.[21, 22] Further, there have been few attempts to target immunological properties of these tumors, most likely because the role of the TIME in pHGGs has overwhelmingly been ignored despite the abundance of non-neoplastic cell types found within them. Henceforth, we must put more emphasis on delineating the role of the TIME in pHGGs including the cell types involved, their biological functions within the tumor, and their potential for therapeutic targeting.

Genetic and Epigenetic Landscape of pHGGs

The genetic differences between pHGGs and aHGGs are striking, highlighting the need to develop tailored therapeutic strategies specific for pHGG. Although pHGGs share the same defining histologic feature of aHGGs including diffusely infiltrating tumor margins, vigorous mitotic activity, microvascular hyperplasia, and pseudopalisading necrosis, the similarities diverge from here (**Figure 1a**).[23, 24] Both pHGGs and aHGGs possess a high frequency of *PDGFRA* amplification and mutation, however these alterations are more frequent in pHGGs (up to 40%) and constitute the most common genetic alteration in these tumors (**Figure 1b**).[9, 25, 26] Despite demonstrated activation of the PI3K pathway in pHGGs, alterations in aHGG subtype-defining genes *EGFR* and *PTEN* are less frequent in pHGGs.[26] *TERT* promoter mutations which are frequent in aHGGs are present in only 2% of DMGs and 3% of hemispheric pHGGs.[27] Further, chromosomal alterations are much more common in aHGG compared to pHGGs.[9, 26]

A prominently unique feature of pHGGs is the presence of mutations in histone encoding genes. Up to 50% of all cases possess mutations in *H3F3A* or *HIST1H3B*, including 60-80% of DIPGs, 60% of midline HGGs, and 15% of hemispheric pHGGs.[9, 28] These mutations were first identified through whole genome sequencing in 2012 by two separate groups, Schwartzentruber et al.[29] and Wu et al.,[30] where they discovered DIPGs exclusively possessed heterozygous somatic mutations encoding lysine to methionine alterations at lysine 27 (K27M) in the *H3F3A* and

HIST1H3B genes.[27] These mutations were not found in hemispheric pHGGs; however, somatic mutations encoding glycine to arginine and less frequently glycine to valine at glycine 34 (G34R/V) of the *H3F3A* gene are found in up to 15-20% of these tumors.[29, 31] K27M mutations are primarily found in brainstem/midline pHGGs and G34R/V mutations are exclusively found in hemispheric pHGGs, yet the exact mechanism for these location-based differences have yet to be revealed.

The presence of histone mutations appears to be the driving factor of subtype-specific groupings of pHGG. A recent meta-analysis of over 1,000 pHGGs by Mackay et al.[9] demonstrated unique survival statistics for each histone mutant subgroup. H3.1 and H3.3K27M mutations confer the worst survival and occur in younger patients, while H3.3G34R/V mutations confer slightly extended survival over K27M and histone wild type (WT) tumors (**Figure 1b**).[9, 29] Activating mutations in *ACVR1* are frequently found in H3.1K27M tumors and less frequently in H3.3K27M tumors.[27, 32] Almost all G34R/V tumors possess *TP53* mutations and ~50% also have *ATRX-DAXX* mutations.[29, 31] H3K27M tumors arising outside of the pons also possess *ATRX* loss, but at lower frequencies than G34R/V tumors.[29, 33, 34] Despite possessing similar co-mutations with IDH1 mutant tumors, histone-mutant pHGGs do not harbor *IDH1* mutations, and only ~6% of all pHGGs do.[31, 34] Further, histone-mutant pHGGs rarely have *EGFR* amplifications, *BRAF* V600E or *NF1* mutations, and they typically do not possess receptor tyrosine kinase fusions (*MET, FGFR2, NTRK2/3*) that are otherwise found in hemispheric histone-wild type tumors.[9, 34, 35] Heterozygous loss of chromosome arm 10q is also found in all subgroups of pHGG, including histone-mutant subgroups.[9, 34, 35]

Despite having a more indolent course of disease, DMGs harboring H3.1K27M and H3.3K27M mutations have similar overall survival and event-free survival compared to pHGGs and are therefore now classified as WHO Grade IV tumors even in the absence of high-grade histology.[36, 37] Tumors possessing K27M mutations are thought to arise from neural precursor cells during CNS development.[38] These pHGGs usually possess *TP53* overexpression or loss of function mutations as well as amplifications of *PDGFRA*.[27-29] Histone H3.1 is produced during the S-phase of the cell cycle and is utilized for the packaging of newly synthesized DNA. H3.3 is synthesized during interphase and is deposited on actively transcribed genes by the ATRX/DAXX complex independent of the cell cycle.[39-41] Although histone H3 mutant proteins make up only 3-17% of the total H3 pool in DIPGs, almost complete loss of H3K27me3 has been observed.[42] Even though

there is global loss of H3K27M methylation, it has been shown that patient-derived DMG cells harboring the H3.3K27M mutation retain high methylation at a small number of domains such as the *HOXD* locus. This is in contrast to H3.1K27M-bearing cells which do not retain methylation in these domains.[41] The mutated histone proteins inhibit the enzymatic activity of the Polycomb Repressive Complex 2 (PRC2) subunit EZH2, which leads to global loss of H3K27me3/me2 methylation and increased H3K27 acetylation (**Figure 2**).[42, 43] PRC2 target genes are involved in developmental processes related to stem cell regulation, cellular proliferation, and cellular differentiation.[44, 45] Through single-cell RNA sequencing of human and murine DIPG K27M mutant tumors, groups have shown that few genes are down-regulated in comparison to other tumor types, and PRC2 target genes are upregulated as a result of hypomethylation.[46, 47] It has also been demonstrated that the majority of cells within DIPGs are most similar to self-renewing oligodendrocyte progenitor cells (OPCs), indicating the H3K27M mutation likely prohibits OPC differentiation into oligodendrocytes.[46]

Much is known regarding histone wild-type and K27M mutant gliomas. Less is known regarding pHGGs harboring H3G34R/V mutations, which arise predominately in the hemispheres of the brain. H3G34R/V mutations occur in up to 15% of hemispheric pHGGs, and in a similar manner to K27M tumors, they possess H3K36me3 hypomethylation; however, this is observed only on affected histone H3 mutant proteins.[40, 42] The loss in methylation is due to the close proximity of the H3G34 position to the K36 location, leading to a diminishment of SETD2 enzymatic activity and subsequent loss of H3K36me3.[42, 48] Interestingly, much like aGBM, H3G34R/V mutant tumors have been shown to possess a methylated *MGMT* promoter, however this finding does not confer a survival advantage in patients treated with temozolomide.[9, 35, 49] Unlike H3G34R/V mutant pHGGs, H3K27M pHGGs do not harbor *MGMT* promoter methylation.[50] It has also been found that H3G34R/V tumors have a greater mutational burden than other pHGG subgroups, likely due to deficiencies in the DNA chaperone proteins ATRX and DAXX, which also aid in DNA repair.[51] Although currently unexplored, these characteristics may be useful in the development of subtype-specific targeted immunotherapies.

In the absence of mutations in histone encoding genes, there are three main subgroups that arise from consensus clustering of 450k methylation data, and these groups have been termed "WT-A", "WT-B", and "WT-C".[49] A large majority of WT-A samples were found to be hemispheric, resemble LGG subgroups, and were driven by *BRAF V600E*, *NF1,* and *TP53*

mutations, as well as receptor tyrosine kinase fusions such as *MET*, *FGFR2*, and *NTRK2/3*.[49] Interestingly, these tumors had upregulation of cytokine signaling and had the best overall survival of the three histone wild-type subgroups - reflective of historical findings suggesting pLGGs have increased cytokine and chemokine signaling compared to their pHGG counterparts.[52] DNA methylation analysis by Grabovska, et al. further demonstrated WT-A tumors possessed greater monocyte infiltration and greater tumor infiltrating lymphocyte (TIL) infiltration compared to WT-B and WT-C tumors.[53] It was also found that lower B-cell and CD8 T-cell infiltration in this group correlated with worse survival. Other mutations commonly found in histone wild type tumors that don't necessarily fall into specific subtypes include *CDKN2A/B* deletions, *RB1* mutations, receptor tyrosine kinase alterations (*MET, IFG1R, NTRK2*), PI3K pathway alterations (*PTEN, PIK3CA, TSC2, PIK3R1*), and MAPK pathway mutations (*BRAF* and *NF1*).[9, 49] Although not specifically discussed in this review, infant HGGs are also largely driven by receptor tyrosine kinase alterations (*ALK, ROS, NTRK, MET*) and possess their own subgroupings.[54]

Stratification between WT-B and WT-C tumors is less robust, yet differences do exist between them. WT-B tumors were found more evenly dispersed among the brain's anatomic locations, had strong upregulation of MYC target genes, and had the worst overall survival of the three histone WT subgroups.[9] Further, they demonstrate the lowest level of CD4 T-cell infiltration between the three subgroups.[53] WT-C tumors were mostly hemispheric in nature yet their expression signature reflected that of midline tumors. These tumors harbored *PDGFRA* and *MET* amplifications, and were associated with the proneural gene signature of adult GBM, which is characterized by a relatively inert immunologic landscape.[9, 55]

It is well-established that pHGGs are highly heterogenous in terms of region-specific differences in: age at diagnosis, molecular alterations, and survival. To date, in depth studies investigating how the tumor location (midline vs cerebral hemispheres) or presence of onco-histones (H3K27M or H3G34R/V) impact the TIME are lacking. We believe a focus on these areas will propel therapeutic discovery, therefore we will now thoroughly discuss our current understanding of the pHGG TIME.

Immune Landscape of pHGGs

Studies specifically designed to enhance our understanding of the TIME of pHGGs are sparse yet informative. Several studies have broadly compared pLGGs to pHGGs; however, as the importance of the TIME in aHGG subtypes with respect to survival and response to therapy has dramatically increased, the pHGG TIME is becoming more heavily studied. A study done by Griesinger et al.,[56] demonstrated through flow cytometry that the number of infiltrating CD45+ and CD11b+ myeloid cells is significantly higher in pediatric pilocytic astrocytoma (PA) and ependymoma compared to GBM and medulloblastoma (MB) samples. The GBM and MB samples also displayed an immunosuppressed phenotype with a high proportion of CD163 and CD206 expression, as well as a smaller frequency of CD4+ and CD8+ T-cells compared to PA and ependymoma samples. It must be noted that this study did not provide sample sizes, however it was one of the first cases to analyze the immune infiltrate of pHGGs.

Much of our knowledge of the pHGG TIME has been obtained through immunohistochemical analyses. Lieberman et al.,[52] used a set of pLGG (19 PA, 4 GG), pHGG (1 AA, 16 GBM), and DIPG (n=9, autopsy) samples for tissue microarray analysis and compared findings to normal adjacent brain tissue (n=12). There were minimal differences in the amount of CD68+CD163+ tumor associated macrophages (TAMs) between tumor types, however pLGG and pHGG samples had significantly more TAMs compared to control tissue while DIPG samples did not. The same trend held true for CD3+ and CD8+ T-cells, highlighting the scarcity of lymphocytes in DIPGs. NanoString analysis on CCL2-4 demonstrated higher expression in pLGGs compared to pHGGs and DIPGs, while pHGGs and DIPGs had higher expression in CXCL-8 over pLGG and non-tumor tissue. Interestingly, pHGG and DIPG samples had the highest expression of PDL1 again suggesting the TIME of pHGGs is immunosuppressive in nature. Lastly, VEGFA expression was highest in DIPGs and pHGGs, likely indicative of the highly vascularized nature of these tumors.[52] Robinson et al.,[57] performed multiplex immunohistochemical analysis of 18 pLGG (5 pleomorphic xanthoastrocytoma, 7 ganglioglioma, and 6 PA) and 8 pHGG (4 histone WT, 3 H3G34R, 1 H3K27M) tissue samples. This analysis revealed pLGGs have significantly higher amounts of CD3+ T-cell infiltration, however CD3 staining was highly variable among all samples. The same group also performed single-cell mass cytometry (CyTOF) on eight pLGGs and one pHGG (fresh tumors), and they found the predominant infiltrating T-cell subtype was CD69+CD45RO+ tissue resident memory cells. Further, it was observed TCF1+ T-cells localized in perivascular areas while CD103+ T-cells were further away from CD31+ blood vessels.[57] The

number of high-grade samples in this study were low, yet they corroborate other findings of low T-cell infiltration in pHGGs.

A more integrated approach comparing pLGGs and pHGGs was taken by Plant et al.,[58] where they performed immunohistochemistry (IHC) on formalin fixed-paraffin embedded (FFPE) tumor samples and supplemented this data with flow cytometry on fresh tumor samples. IHC on 27 pediatric GBMs and 32 pLGGs demonstrated significantly greater infiltration of CD45+ myeloid cells, CD8+ T-cells, and PD1+ cells in pLGGs. The majority of all samples were negative for PDL1 expression, and there were no correlations between IHC markers and overall survival in pHGGs. Additionally, OncoPanel™ results were available for a subset of patients which showed there were 7 or fewer mutations per tumor type, indicating the mutational load of pLGGs and pHGGs is small. Flow cytometry on 10 pLGGs and 3 pHGGs further demonstrated a higher level of lymphocytic infiltration in pLGGs compared to pHGGs, however statistical significance was not met. Interestingly, pHGG samples had significantly higher levels of CD45+CD19+ B-cells and CD38+IgD+ activated B cells compared to pLGGs, yet the significance of these findings was not determined. In the same study, frozen blood samples from 4 pLGG and 4 pHGG patients was used for T-cell receptor (TCR) sequencing. This analysis found there is little sharing of T-cell clones between tumor and corresponding blood samples, likely due to the limited T-cell infiltrate in these tumors.[58] Overall, this study once again demonstrated the paucity of an immunogenic microenvironment in pHGGs, especially with respect to infiltrating T-cells. These results suggest other immune cell types such as TAMs should take precedent when studying the TIME of pHGGs.

Studies done by our lab have further supported historical findings of scarce T-cell infiltration in pHGGs.[59] Through IHC analysis of human FFPE pHGG tissue samples, including 12 DIPG (7/12 K27M) and 11 pGBM (3/11 G34R), we found very few CD3+ and CD8+ T-cells present, and no differences between subtype or tumor location. On a larger cohort of 33 samples, including 17 DIPG (10/17 K27M) and 16 pGBM (4/16 G34R), we did however find considerable infiltration of IBA1+ TAMs among all tumor types, with high sample-to-sample variability. Again, no differences were observed between tumor subtype, molecular subgroup, or tumor location but this does not preclude the existence of these differences. To make conclusive observations concerning subtype-specific differences, larger studies must be done. Regression analysis revealed significant correlations between CD3 and IBA1 positivity in matched samples, as well as CD3 and

CD31, CD3 and PDGFRb, IBA1 and CD31, PDGFB, and PDGFRb, suggesting the stromal compartment of the tumor may play a pivotal role in influencing immune cell infiltration.

To supplement our findings using IHC, we also performed NanoString on 22 human pHGG samples, including 10 DIPG (8/10 K27M) and 12 pHGG (3/12 G34R). Interestingly, there were considerable differences between the inflammatory signaling pathways between the two HGG subtypes. Unsupervised hierarchical clustering of signaling pathway scores indicated the majority of DIPG samples cluster together while their hemispheric counterparts cluster together, with DIPGs possessing greater expression of genes involved in almost all inflammatory pathways. Further, cell type analysis indicated DIPGs had greater representation of genes associated with T-cells, dendritic cells (DCs), Th1 cells, natural killer cells, neutrophils, and macrophages. When examining differentially expressed genes, DIPGs had significant expression of genes involved in the CXCR2 signaling pathway including *CXCL1, CXCL2, CXCL5, CXCL6,* and *IL8*. These results were further supported by NanoString on 11 murine pHGGs including 5 H3.3K27M DIPGs and 6 H3.3WT hemispheric GBMs. This analysis again demonstrated DIPGs, tumors found in the brainstem, were more inflammatory than hemispheric pHGGs. Lastly, when we performed unsupervised clustering of human and murine pHGGs, we found that hemispheric pHGGs clustered together and DIPGs clustered together, regardless of species, indicating that the tumor location, molecular subtype, or both influence the inflammatory microenvironment of pHGGs. Our results suggest despite the lack of infiltrating T-cells, pHGGs still possess an inflammatory TIME characterized by considerable TAM infiltration (**Figure 3**), again highlighting the emerging role of TAMs in pHGG. We are currently investigating subtype-specific differences in pHGGs to determine how the molecular signature or tumor location dictates the TIME.

A study by Mackay et al.[49] on the HERBY phase II randomized trial in pHGGs further supported several key findings on our understanding of the immune response in pHGGs. The HERBY trial was designed to study the efficacy of adding an anti-angiogenic agent, bevacizumab, to the current standard of care which included radiotherapy (RT) and-or temozolomide (TMZ) in 121 patients with newly diagnosed non-brainstem pHGG. As previously demonstrated in the field, they found histone mutant tumors (both K27M and G34R) were considerably immune cold based on the absence of immunohistochemical staining of CD8+ T-cells. PXA-like HGGs however were significantly enriched for CD8+ T-cells, as were four hypermutator cases that were found to have mutations in *POLE, POLD1,* and *MLH1*. Patients with tumors possessing greater CD8+ T-cell

infiltration had a significantly better overall survival, and were more likely to respond to TMZ/RT with the addition of bevacizumab. It is important to note however, that 17/18 cases with a high presence of CD8+ T-cells were hemispheric tumors, further supporting the notion of a relatively barren TIME of midline and brainstem pHGGs. Additionally, the impact of tumor resection on survival is difficult to control for, and ascribing positive results to high CD8+ T-cell infiltration must be done cautiously. These results are in contrast to findings from Bailey et al.[60] who demonstrated through CIBERSORT that the presence of CD8+ T cells in histone WT hemispheric tumors negatively correlated with survival. A case report by Bouffet et al.[21] of two siblings aged 6 and 3.5 years old both harboring *PMS2* and *POLE* germline mutations, demonstrated that mismatch repair (MMR) deficiency induces a significantly higher mutational load in pGBM. Because of their relatively high mutational burden (both over 20,000 mutations per exome), the children were selected for nivolumab (anti-PD1) treatment. After 9 and 5 months of therapy, both patients' tumors were significantly reduced and a reduction in peritumoral edema and nodular lesions was also observed. The patients were declared clinically stable after treatment, highlighting the importance of neoantigens and their influence on the response to immunotherapy. Although MMR deficient pHGGs are infrequent, evidence suggests a significant prognostic value exists in determining the presence of MMR deficiencies in patients with pHGGs.

Another histological study by Jha et al.,[61] compared PDL1 expression in 126 adult and pediatric DMGs, and found PDL1 expression to be highest in H3K27M/IDH1 wild type DMGs in both age groups. PDL1 expression significantly correlated with TIL infiltration in both age groups and patients whose tumors were PDL1 negative had a longer overall survival compared to PDL1 tumor positive patients. Pediatric DMGs did not differ from adult DMGs in terms of CD3+ T-cells, however adult DMGs had significantly greater CD8+ T-cells present, suggesting immunotherapeutic approaches must be tailored not only to specific tumor types, but also to specific age groups as well.

Other important findings from the Mackay et al.[49] analysis of the HERBY trial showed that pHGGs with MAPK alterations had a greater T-cell signature and M2 macrophage response in RNA-seq data compared to those that did not have MAPK alterations. Further, immunohistochemical staining for CD68 in pHGGs found heterogenous infiltration of TAMs that were found to cluster around areas of necrosis and perivascular lymphocytes. Another study found in xenograft models of DIPG into NOD scid gamma (NSG) mice that there were CD68+ and

CD163+ cells resembling macrophages and microglia in the tumor parenchyma, with limited CD3+ T-cell infiltration.[62] Due to the limited infiltration of T-cells with pHGGs, analyses focused on TAMs may prove to be more beneficial to our understanding of the TIME. Lin et al.,[63] performed immunofluorescence on DIPG tissue obtained from biopsy and found DIPGs and adult GBMs had high IBA1+ staining compared to normal cortical tissue. They also performed flow cytometry on early post-mortem DIPG tissue samples and found DIPGs had a smaller CD45+CD11b- lymphoid population compared to adult GBMs. DIPGs however had significantly greater infiltration of CD45+CD11b+ TAMs compared to adult GBMs. TAMs from six DIPGs, four adult GBMs, and three pediatric cortical control samples were flow sorted and RNA sequencing was performed. They did not find an enrichment of M1 or M2 associated genes in DIPG TAMs, indicating they may not necessarily fall discretely into classically activated or alternatively activated phenotypes. When compared to control samples, DIPG TAMs have upregulated genes associated with interferon gamma signaling, type 1 interferon signaling, and antigen processing and presentation. Yet when differentially expressed genes between aGBMs and DIPGs were compared, inflammatory genes were upregulated in aGBM samples, including *IL6, CCL4, IL1A, IL1B,* and *CCL3*. To determine if the DIPG tumor cells were producing inflammatory signals, they analyzed the cytokine and chemokine secretome using an ELISA array on DIPG patient-derived cell cultures. Their findings again indicated that DIPG tumor cells produce significantly fewer cytokines and chemokines than aGBMs.[63] RNA sequencing data further supported these findings, and in general point towards the conclusion that DIPGs are non-inflammatory when compared to aGBM, however they did not directly compare DIPG to non-brainstem pHGGs. These results highlight two key consistent findings; DIPGs possess sparse T-cell infiltration yet are characterized by high TAM infiltration, and the inflammatory profile of DIPG associated TAMs are quite different from aGBM TAMs.

Immunotherapeutic Studies

Due to the limited efficacy of chemotherapy and limited longitudinal response to radiotherapy, there has been increasing efforts to harness the immune system for anti-tumor treatment in pHGGs. Ideally, targeted immunotherapies would be designed to eliminate tumor cells while sparing permanent neurological dysfunction. Targeting tumor specific neoepitopes

would achieve this and limit autoimmune reactions. Several therapeutic strategies are emerging as likely candidates for pHGG including CAR-T cell therapy, tumor vaccines, and viral therapies. All of these approaches are designed to induce tumor-specific T-cell killing while limiting pathological insults to adjacent normal brain tissue and vasculature, including blood brain barrier integrity.

Checkpoint Blockade:

Checkpoint blockade in adult GBM has historically shown no benefit to overall survival (OS) either alone or in combination with bevacizumab (anti-VEGF). The underlying mechanisms driving resistance to immunotherapy are yet to be determined, however work with immunocompetent genetically engineered mouse models (GEMMs) of aGBM has shown that resistance is conserved across genetic subtypes and is not improved with adjuvant radiotherapy.[64] Checkpoint blockade in the pediatric population has also shown little promise, with the exception of patients harboring hypermutator phenotypes.[21] The limited amount of TILs in pHGGs may contribute to the lack of efficacy of checkpoint blockade therapy, however the pHGG TIME should be further examined to determine the exact underlying mechanisms responsible for resistance. There are several ongoing phase I and II clinical trials investigating the use of anti-PD1 and anti-CTLA4 therapies in children with newly diagnosed or refractory HGGs (**Table 1**).[65-67] After the conclusion of these trials, it will be imperative to report both successful and unsuccessful cases. Combinatorial therapeutic strategies will likely be most efficacious, and may be required, for the treatment of pHGGs and require further investigation. Currently, other immune-modulatory strategies show promise and will therefore be further discussed in this review.

CAR T-Cell Therapies and Tumor Peptides

The list of tumor-specific antigens for pHGGs is low, yet due to the distinct expression of mutated histone proteins in H3K27M and H3G34R pHGGs, there have been increasing efforts to develop chimeric antigen receptor (CAR) T-cell and peptide therapies specifically designed to target these proteins. Mutant onco-histone proteins are ideal targets because normal brain tissue does not express these tumor specific antigens, therefore risks of off-target toxicity is low. Further,

due to the low mutational burden of these tumors, epitope spreading and antigen escape may be limited, allowing for a more durable response to immunotherapy. Because of the relatively impermeable BBB, monoclonal antibodies do not easily infiltrate into the brain parenchyma, which may explain their lack of success in pHGG. On the other hand, activated immune cells, including T cells and DCs, can cross the BBB.[68] CAR T-cells are produced by collecting a patient's lymphocytes via leukapheresis, engineering a single-chain variable fragment of a particular antibody with the T-cell receptor signaling domain CD3, and often including co-stimulatory domains in the engineered cells.[69] The CAR T-cells are then re-introduced into the patient where they can recognize tumor-specific antigens without necessarily being stimulated by antigen presenting cells, thus allowing for a robust and effective cytolytic immune response to take place (**Figure 4**). To date, there have been very few studies investigating the efficacy of CAR T-cell therapies in pHGG. There are currently multiple ongoing phase I clinical trials involving the use of IL13Ra2, HER2, and EGFR modified CAR T-cells for adult and pediatric HGG, however in order to achieve maximum therapeutic responses, more tailored approaches might be necessary to account for the biologic characteristics of pHGG.[67, 70]

Majzner et al.[71] assessed the efficacy of a B7-H3 CAR T-cell for pediatric solid tumors. B7-H3 is believed to be a checkpoint molecule that is aberrantly expressed in a variety of cancers while being minimally expressed in normal tissue.[72] The authors showed B7-H3 is highly expressed in pediatric solid tumors, including HGGs and DIPG.[71] Staining for B7-H3 demonstrated it was ubiquitously expressed in all tumor cells, a desirable trait for CAR-T cell therapy. Interestingly, B7-H3 staining intensity was less in DIPG samples compared to HGGs, indicating that this therapy may have efficacy in both tumor types, although this was not tested. When co-cultured with patient derived DIPG cultures, the B7-H3 CAR T cells produced high levels of IFNγ, TNFa, and IL2 indicating the CAR T-cells possess high cytotoxic potential. The authors did not test the engineered B7-H3 CAR T-cells in mouse models of pHGG or DIPG, however they did observe significant reductions in tumor growth and a subsequent extension of survival in NSG medulloblastoma xenografts. A phase I clinical trial involving the use of B7-H3 CAR T cells is currently active for their use in DIPG or refractory pHGG (NCT04185038).

Another study by Mount et al.[73] sought out to identify potential targets for CAR T-cell therapy in DIPG. Using patient derived cell cultures, they found the disialoganglioside GD2 was highly expressed on the surface of all H3K27M mutant DIPG cultures examined while expression

was much lower on H3-WT DIPG samples. Unlike other gangliosides, GD2 is minimally expressed in normal tissue and has extensive expression in multiple tumor types.[74] Mount et al.[73] generated GD2 specific CAR T-cells and demonstrated *in vitro* cytotoxic specificity for H3K27M DIPG tumor cells, but not H3WT DIPG tumor cells, through the production of IFNγ and IL2. They further demonstrated specificity by CRISPR-Cas9 mediated deletion of GD2 synthase in patient derived cell cultures and found the cytotoxic effects of GD2 CAR T-cells were ablated. Using postmortem DIPG patient tissue, they created mouse xenografts into NSG mice to test the therapeutic efficacy of their GD2 CAR T-cells. Significant tumor clearance was observed in GD2 CAR intravenously treated animals resulting in prolonged survival; however, a small portion of H3K27M+ tumor cells were found to remain that did not express GD2. It is possible these GD2 negative tumor cells could survive through selective pressure and eventually re-populate the eradicated tumor. Combination therapies may reduce the likelihood of such events. Further histological analysis revealed extensive neuroinflammation within the brainstem and ventricles, including infiltration of CAR T cells and IBA1+ TAMs, especially around apoptosing cells. Unfortunately, ventriculomegaly and hydrocephalus was observed in a subset of animals with fourth ventricular compression. The authors hypothesize this treatment-induced cerebral edema was due to on-target tumor-specific cytotoxic activity and not due to cytotoxicity on non-tumor cells.

Further analysis of GD2 CAR T-cells and other CAR constructs in immunocompetent mouse models should be pursued to delineate the mechanisms underlying treatment induced edema. This is especially important for tumors located in the brainstem such as DIPGs, however it would also be important for hemispheric pHGGs as well. Other investigational studies are currently being conducted on the use of HER2 and EGFR-specific CAR T-cells in clinical trials; however, due to the limited frequency of EGFR amplifications in pHGG, the outcomes of these trials may not be as promising as pHGG-specific therapies.[67, 75] A phase I clinical trial for the use of GD2 CAR T-cells modified with a constitutively signaling cytokine receptor, C7R, is currently active for children with DIPG or HGG (NCT04099797). The addition of the cytokine receptor allows for IL-7 signaling to occur within the engineered T-cells without effecting bystander lymphocytes, thus causing the CAR T-cells to survive longer and provide a more durable response.[76]

Approaches utilizing tumor specific peptides arising from oncohistone proteins have also been pursued to induce the cytotoxic T cell response (**Figure 5**). Ochs et al.[77] recently described the identification of a H3K27M 27mer peptide (H3K27M p14-40) that binds to HLA-A*0201. Humanized mice expressing HLA-A*0201, HLA-DRA*0101, and HLA-DRB*0101, while lacking major histocompatibility complex (MHC) class I and II (A2.DR1 mice), were used to study vaccination with the identified peptide. Vaccination in a non-tumor setting induced mutation-specific CD4 and CD8+ T cell responses including proliferation and the production of IFNγ. Utilizing a subcutaneous sarcoma model in A2.DR1 mice, H3K27M peptide vaccination resulted in reduced tumor size and increased infiltration of IFNγ producing CD4 and CD8+ T cells compared to vaccination with the wild type H3 peptide. Ideally, vaccination into an MHC-humanized glioma model would be preferred, however the authors state that such a model does not exist. The authors also demonstrated the ability to detect H3K27M-specific cytotoxic T cells in the peripheral blood of three patients harboring H3K27M mutant gliomas. Similarly, Chheda et al.[78] discovered a 10mer peptide (H3K27M p26-35) that binds with high affinity to HLA-A*0201. Using human peripheral blood samples from HLA-A*0201^{+} DIPG patients and healthy donors, they found the peptide stimulated antigen-specific increases in IFNγ production. To demonstrate H3K27M cytotoxic specificity, they cloned a TCR specific for HLA-A*0201^{+}H3K27M^{+} glioma cells and found that it significantly induced *in vitro* IL2 and IFNγ production in J76CD8$^{+/-}$ Jurkat T cells and primary human CD8^{+} T cells, respectively. They further demonstrated the cytotoxic capability of TCR transduced T cells in response to HLA-A*0201^{+}H3K27M^{+} glioma cells *in vitro*. Most importantly, *in vivo* activity of their TCR-transduced T cells was tested in NSG mice bearing H3.3K27M mutant U87 glioblastoma cells. They found that mice receiving the TCR-transduced T cells had a significant reduction in tumor size compared to mock transduced T cells. The majority of the infiltrating CD4 and CD8+ T cells within the tumors were positive for the HLA-A*0201-H3K27M dextramer, and there were even dextramer positive T cells found in the peripheral blood. Unfortunately, survival could not be assessed, as the animals receiving the mock-transduced and TCR-transduced T cells succumbed to graft-versus-host disease. The same group has recently implemented a pilot study to evaluate an H3.3K27M-26-35 peptide vaccine in children with H3.3K27M+ DIPG or HGG (NCT02960230) and their results are encouraging.[79]

There are multiple studies reporting the immune stimulatory effects of polyinosinic-polycytidylic acid (poly[I:C]) stabilized by lysine and carboxymethylcellulose (poly-ICLC)

treatment alone against aHGG and pLGG, making it a potentially effective immune adjuvant for pHGG as well.[80-84] A study by Pollack et al.[85] investigated the combination of three glioma associated antigen peptides combined into a vaccine administered to DIPG or non-brainstem HGG patients. They identified HLA-A2 restricted epitopes from IL13Rα2, EphA2, and survivin peptides and administered them in combination with poly-ICLC. No instances of high-grade reactions or autoimmunity occurred in the patient cohort. Vaccine induced immune responses as denoted by IFNγ production was observed in 13 out of 21 patients, with no discrimination between patients who received prior radiotherapy alone or in combination with chemotherapy. Interestingly the IL13Rα2 epitope invoked the highest magnitude of responses, and in one patient the immune response diminished after receiving high-dose dexamethasone and returned after dexamethasone tapering occurred. This highlights the importance of determining optimal timing and therapeutic strategies combining the use of immunotherapies with dexamethasone as a remedy for treatment-associated inflammation. The median survival of the overall patient cohort of this study was 13.3 months from diagnosis, 12.7 months for brainstem tumors, and 25.1 months for non-brainstem HGGs. More importantly, two patients had disease-free status; these patients had undergone prior gross total resection, again emphasizing the extent of tumor resection directly affects disease outcome in pHGGs. Although survival was negligibly extended, this study effectively showed the safety and immunogenic capability of combinatorial peptide vaccines for pHGG.

Dendritic Cell Therapy

Dendritic cells (DCs) are the professional antigen processing cells in the body and are responsible for priming cytotoxic lymphocytes during the adaptive immune response.[86] Once DCs have encountered antigen, they migrate to draining lymph nodes via CCR5 and CCR7 upregulation, where they then activate the T-cell response.[87] This process can be triggered or amplified through the isolation and "training" of DCs to specific antigens in a process known as DC vaccination (**Figure 6**). There have been few studies investigating the use of DC vaccines in pHGG. Initial studies by Caruso et al.[88] demonstrated the safety and feasibility of RNA based DC vaccines in patients with recurrent high-grade brain tumors. While most patients in the study had eventual disease progression, one patient with AA had stable disease after 5 vaccines and 21 months of follow up. Unfortunately, the authors were unable to detect significant *in vitro*

production of IFNγ or T cell proliferation with treated patient samples. In a larger cohort of patients with relapsed HGG, De Vleeschouwer et al.[89] demonstrated the efficacy of autologous DC adjuvant therapy after tumor resection in adults and children. No analysis of the immune response was performed, however survival analysis indicated younger patients under the age of 35 years old had a better OS compared to older patients. Additionally, patients who had a greater extent of tumor resection had better progression free survival (PFS) and OS compared to those who had sub-optimal resection. A similar study was performed by Ardon et al.[90] in a cohort of 33 recurrent pHGGs using autologous DC vaccination. At the time of last follow up, there were 7/33 patients still alive with multiple patients surviving 35-85 months. Five of the long-term survivors also received chemotherapy during or after immunotherapy, suggesting combination therapy is more effective. Another study by Lasky et al.[91] further supported these findings where 2/3 patients receiving autologous tumor lysate DC vaccination in combination with chemotherapy survived 40 and 51 months respectively. For a disease with such dismal prognosis these initial results were encouraging.

A recent study by Benitez-Ribas et al.[92] investigated the feasibility and safety of administering autologous DCs pulsed with tumor cell line lysate to nine newly diagnosed children with DIPG. Patient DCs were collected by leukapheresis after the discontinuation of dexamethasone and were loaded with lysate obtained from eight allogeneic DIPG tumor cell lines (ATCL) *ex vivo*. Mature, ATCL pulsed DCs were then administered intradermally three to six weeks after the completion of radiotherapy, followed by once every other week during the induction phase, and then a subsequent booster three months later in the maintenance phase. Before administration, DCs were confirmed to have increased expression of CD80 (co-stimulatory molecule), CD83 (activation), CCR7 (migration), and MHC-II (antigen presentation) indicating a mature and activated population of DCs following ATCL loading. No adverse reactions were noted during the course of the study, demonstrating the safety of DC administration to patients. Analysis of peripheral blood and CSF indicated ATCL-specific proliferative responses and the production of IFNγ by patient T-cells. Unfortunately, at the time of being published, there were no clinical outcome data available for this study: however, it is the first study to demonstrate the feasibility of ATCL pulsed DC therapy for DIPG. Although this method was adapted for the use of tumor cell line lysate, it could be applied to individual patient tumors as well. This would likely be costly and resource intensive, however it may be a more effective and personalized approach as compared

to using a generalized cocktail of tumor cell lines. Since this approach would require a surgical biopsy or tumor sample after resection, further considerations would be required for tumors located in precarious regions such as the brainstem and midline regions (thalamus/hypothalamus) where surgery poses potential irreversible harm. The efficacy and feasibility of actively personalized vaccines has recently been demonstrated in adult GBM and will likely serve as templates for their use in pHGG.[93, 94] One challenge to consider for DC vaccination therapies is there must be adequate time for vaccine production, which may allow for subsequent growth of remaining tumor to cause morbidity and mortality. There also must be enough time between the weaning of steroids and other chemotherapeutic agents before vaccination.

Viral Therapies

To date, clinical trials investigating the use of oncolytic viruses for the treatment of pHGGs and DIPG are in early stages and have not progressed to phase 2 studies. Most studies have been performed *in vitro,* with minimal supporting *in vivo* studies. Tejada et al.[95, 96] previously examined the safety and efficacy of DNX-2401 for the treatment of newly diagnosed DIPG. DNX-2401 (also known as Delta-24-RGD) is an oncolytic adenovirus that targets cells with a defective Rb pathway. The virus demonstrated therapeutic efficacy in recurrent human adult HGG, murine models of pHGG, and murine models of DIPG and therefore was adapted for use in human pHGG.[97-100] In an immunocompetent murine model of aHGG utilizing the GL261 glioma cell line injected into C57BL/6 mice, Jiang et al.[99] performed intratumoral injections of Delta-24-RGD. They observed an increase in NK cell, CD3+, CD4+, and CD8+ lymphocyte infiltration as well as activated APC populations after injections. Further, they noted an increase in IFNγ production as well as a significant survival advantage in treated mice, demonstrating the effectiveness of the virus to elicit an anti-tumor immune response. Utilizing both immunodeficient and immunocompetent mouse models of pHGG and DIPG, Martina-Velez et al.[98] further supported the immune stimulatory abilities of Delta-24-RGD. Intratumoral injection resulted in a significant increase in survival accompanied by an increase in CD3+, CD4+, and CD8+ lymphocyte infiltration. They also analyzed human tumor samples *in silico* and determined the majority of pHGGs and DIPGs are susceptible to Delta-24-RGD infection based on the aberrant expression of Rb pathway genes such as *CCND1, CDKN2A,* and *E2F1.* These findings resulted in a study by the same authors where

they combined Delta-24-RGD with radiotherapy to study the synergistic effects in pHGG and DIPGs.[98] They found combination therapy significantly extended survival in both pHGG and DIPG bearing mice as compared to single treatment experimental arms. Combination therapy resulted in the greatest infiltration of cytotoxic lymphocyte populations and increased expression of granzyme B and IFNγ mRNA compared to viral monotherapy or radiotherapy alone. However, it is important to note that both monotherapies also induced an immune response as well. A case report on a patient with a H3.3K27M mutated DIPG treated with DNX-2401 validated the safety of administering the oncolytic virus to pediatric patients.[96] The virus was injected immediately after tumor biopsy into the resection cavity using the biopsy tract and no virus-related toxicities were noted 4 weeks after injection. Unfortunately, the authors did not provide any immunological studies or survival statistics on the patient.

Multiple labs have studied the use of oncolytic herpes simplex virus *in vitro.* Cockle et al.[101] studied the efficacy of HSV1716 to inhibit cell migration and invasion of pHGG and DIPG cell lines *in vitro.* They found that HSV1716 significantly reduced both aspects of cell motility. *In vitro* time-lapse imaging demonstrated that HSV1716 treatment induced a loss of cell polarity and reduction in cell velocity compared to controls. Injection of an HSV1716 treated DIPG cell line into the fourth ventricle of NSG mice resulted in reduced infiltration of tumor cells into surrounding brain structures. No immune analyses were performed however. Friedman et al.[102] studied the oncolytic HSV G207 and HSV M002, due to their clinical significance for use in early phase I clinical trials (NCT02457845 and NCT02062827 respectively).[102, 103] They found that pediatric patient derived tumor cells were more sensitive to both viruses than adult GBM tumor cells *in vitro*. This sensitivity was accounted for by increased expression of the HSV entry molecule CD111 on the surface of the pediatric tumor cells compared to the adult tumor cells. Treatment of tumor bearing mice resulted in an increase in survival with M002, however these were nude mice lacking an intact immune system.[102] These results suggest CD111 expression may be a useful biomarker for predicting the efficacy of HSV therapy in the pediatric population.

The promising effects of a recombinant polio-rhinovirus chimera (PVSRIPO) vaccine in recurrent aGBM - in which 8 patients survived to 24 months, and 5 survived to 36 months - has prompted ongoing investigational use of PVSRIPO in recurrent malignant glioma in children (NCT03043391).[104] Other viruses studied for oncolytic potential in pHGG include parvovirus and picornavirus. Due to the success of H1-parvovirus (H-1PV) in aHGG and rat glioma models,

Josupeit et al. studied H-1PV in adult and pediatric HGG glioma stem cell neurosphere cultures to determine the ability of H-1PV to eradicate glioma stem cells *in vitro* and *in vivo*.[105, 106] Using four pHGG cell lines, including two DIPG and two pGBM lines, it was shown H-1PV induces cell death and a reduction in metabolic activity in a viral-dose dependent manner.[107] Engraftment of virally infected aGBM stem cells into NSG mice was reduced along with tumor volume compared to mock infected mice, however, again this analysis was performed in immunocompromised mice and no pediatric cell lines were investigated. Using the seneca valley virus 001 (SSV-001), a member of the picornavirus family which has shown tumoricidal effects in MB, Liu et al.[108] demonstrated SSV-001 also has anti-tumor activity in pHGG.[108, 109] Freshly isolated human pHGG tumor cells from xenograft mouse models were treated with the virus *in vitro* (MOI=0.5), resulting in significant cell death in 4/6 models tested, with 2/6 resistant to SSV-001 induced cell death. Infection of glioma stem-like neurospheres was also demonstrated, indicating SSV-001 can also target historically treatment-resistant populations. A single injection of SSV-001 into Rag2-SCID mice with orthotopically xenografted tumors significantly extended survival compared to placebo treated mice. Analysis of sialic acids on permissive and resistant tumor cells indicated SSV-001 infection was higher in cells with more α2,6 and α2,3-linked sialic acids, indicating SSV-001 viral therapy may have differential activity in humans and should therefore be investigated further to determine which patients will best respond to treatment. Future studies involving H-1PV and SSV-001 oncolytic tumor viruses need to be performed in immunocompetent mouse models to determine their oncolytic capacity when the innate and adaptive immune systems are intact.

An impressive study by Mendez et al.[68] demonstrated the efficacy of adenoviral delivery of thymidine kinase (TK) and fms-like tyrosine kinase 3 ligand (Flt3L) gene therapy in an immunocompetent mouse model of ACVR1 mutant DIPG. This approach utilizes TK to convert the prodrug ganciclovir (GCV) into GCV-triphosphate which induces DNA damage and cell death (**Figure 7**).[110] Further administration of Flt3L recruits DCs into the TME, allowing them to process antigens from dying tumor cells and induce cytolytic T-cell activity.[111] Using the sleeping beauty transposase system, which allows the integration of transposon DNA into the host genome through a cut-and-paste method, ACVR1 mutant or ACVR1 wild type tumors were induced with co-mutations short hairpin p53 and mutant NRAS in C57BL/6 mice. Primary neurospheres obtained from generated tumors were first treated *in vitro* with TK and ganciclovir to demonstrate TK gene therapy induces the generation of damage associated molecular patterns (DAMPs). In response to

TK and ganciclovir treatment, the neurospheres released significant levels of ATP, calreticulin, and HMGB1. To demonstrate the efficacy of TK gene therapy *in vivo*, neurospheres were then injected into the pons of recipient C57BL/6 mice, and after tumor formation mice were intratumorally injected with TK and Flt3L alone or in combination with radiotherapy. TK/Flt3L therapy significantly increased the median survival of mice from 18 days to 36 days compared to saline treated animals. The addition of radiotherapy did not add an additional survival benefit to TK/Flt3L therapy, however it also did not reduce the efficacy of the viral therapy. Additionally, viral therapy induced a significant increase in intratumoral CD8 T cell infiltration while sparing deleterious neuroinflammation in normal brain tissue. The CD8 T cells had significantly greater IFNγ production, proliferation, and cytolytic potential compared to CD8 T cells isolated from saline treated animals. This study established a working immunocompetent mouse model for testing adenoviral gene therapy in DIPG. The same model could also be used for preclinical studies in other pHGG tumor types harboring different genetic driver mutations. Moving forward, careful considerations must be made and diligent preclinical analysis done to ensure minimal neuroinflammation results after the administration of oncolytic viruses in pHGG and DIPG. Because of the unresectable nature of many DIPGs and pHGGs, therapeutic mechanisms stimulating the patient's immune system may provide benefit to current treatment strategies.

Concluding Remarks

Remarkable progress has been made in the last decade elucidating the origin and genomic landscape of childhood brain tumors. Much is known regarding the intrinsic cellular mechanisms driving tumor progression, yet the role of the TIME is still poorly understood. It is apparent the TIME is a key driver of immunotherapy resistance through the orchestration of multiple complementary mechanisms blocking effector immune cell infiltration and activation. Therefore, the success of immunotherapy-based clinical trials and conventional cancer cell-targeted therapies hinges on a more mature understanding of the pHGG TIME. The utilization of pHGG GEMMs will help address these needs. Multiple groups have utilized these models to characterize the TME of pHGGs, demonstrating they faithfully recapitulate the human disease with regards to histologic hallmarks, tumor genetics, and immune infiltrate.[47, 59, 68, 112, 113] Because the majority of studies are done using patient derived xenograft (PDX) models alone that exhibit an immunocompromised

status and species incompatibilities between immune cells and signaling molecules, there is a need for more studies investigating the use of immunotherapies in immunocompetent mouse models of pHGG.[64]

Despite the growing popularity of studying the TIME of pHGGs, we have yet to determine why pHGGs have fewer infiltrating immune cells compared to pLGGs and other tumor types. This review has demonstrated pHGGs have low amounts of infiltrating T-cells, yet they still recruit macrophages, including brain resident microglia and bone marrow derived monocytes. Although efforts designed to efficiently target tumor cells have been studied, it is imperative to determine if microglia and infiltrating monocytes have differential functions in pHGG and how amenable they are for therapeutic use. These distinctions between microglia and BMDMs are of paramount importance in light of recent advances illuminating substantial differences between the two populations in terms of their ontogeny, proliferative capacity, locations, and biological functions.[114-118] Determining whether the TAM lineage has functional consequences in pHGG is an important topic that needs addressed. Further, although TAMs are genetically stable, their expression profiles are responsive to signals derived from tumor cells, therefore cancer cell heterogeneity inevitably leads to heterogeneity within the TIME, impacting the effectiveness of therapeutic strategies. This leads to another important question; how do TAMs respond to chemotherapy, radiotherapy, and immunotherapy? Do they help accelerate cytotoxic clearing of tumor cells or do they contribute to therapeutic resistance? Answering these questions will then enable us to design more effective therapeutic strategies that can be easily tested in immunocompetent mouse models and PDX models of pHGG, and eventually converted into human clinical trials.

Additional considerations should incorporate the possibility of cell-type specific, location-based differences within the brain, including microglia, myeloid cells, and other cell types important for immune regulation. As previously discussed, tumor mutations can be specific to the location of the tumor within the brain, such as K27M mutations found in brainstem gliomas. It is now known brain microenvironments are conducive to cultivating particular transcriptional and phenotypic profiles of the constituent cells present. For example, neuronal diversity has been documented through single cell RNA sequencing analysis, demonstrating different subsets of neurons can be found in specific anatomic locations of the brain.[119] Microglia have been demonstrated to be found in different densities, display distinct morphologic features, and possess

unique transcriptomic profiles depending on the brain region sampled.[120-123] Myeloid cell diversity among brain compartments has also been described.[124] This suggests a tumor found in the brainstem may possess a unique cellular milieu compared to a hemispheric tumor for more reasons than tumor genetics alone. We have demonstrated one example of this in our GEMMs of hemispheric pHGG and brainstem DIPG, where we found that BBB permeability is influenced not by H3.3K27M status but rather by tumor location.[125] Although these concepts are not highly novel, they have been largely ignored in brain tumor studies. Understanding how individual microenvironments are conducive for the phenotypic profiles of constituent cells will facilitate our understanding of the overall immune profile of pHGGs, therefore enhancing therapeutic discovery efforts and allowing us to better predict outcomes of immunotherapeutic strategies in clinical trials.

One example where our efforts utilizing GEMMs will lead to deeper understandings of therapeutic resistance in pHGG is by studying how, and why, patients with CMMRD respond better to immunotherapy. Establishing a causal link between biallelic MMR mutations and checkpoint inhibitor response in pHGG will allow us to mechanistically dissect the underlying reasons for checkpoint inhibitor resistance in the broader pHGG population, and will therefore enable us to design therapies bypassing these mechanisms. Moving forward, preclinical studies involving mouse models of pHGG will be critical for the advancement of immunotherapies as they will allow us to determine effective therapeutic combinations as well as delineate the mechanisms driving treatment resistance. Further, because standard treatment protocols involve the use of immune-modulating steroids for the management of cerebral edema, GEMMs will enable us to determine optimal timing and treatment strategies for pHGG.[126, 127] In conclusion, a deeper understanding of the pHGG TIME is essential for the development and progression of more efficacious therapeutic strategies for this dismal disease.

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Competing interests

The authors report no competing interests.

Figure 1. Clinical features of pediatric high-grade gliomas. (A) T2 flair magnetic resonance imaging of a DIPG in the coronal plane and a post-contrast T1 MRI H3/IDH WT hemispheric pHGG in the transverse plane. Corresponding H&Es displaying major histologic characteristics including microvascular proliferation (yellow arrow), pseudopalisading necrosis (red arrow), and brisk mitotic activity (black arrow). (B) 40-60% of pHGGs have PDGFRA amplification. Half of pHGGs have histone mutations; including H3.1/H3.3K27M mutations which arise in the brainstem and midline, and H3.3G34R/V mutations which are hemispheric tumors. The other half of pHGGs are histone wildtype, but commonly possess CDKN2A and TP53 mutations, and typically arise in the hemispheres of the brain.

Figure 2. Histone mutations in pediatric high-grade glioma. (A) In histone wild-type pHGGs, there is maintenance of global H3K27me3 across the genome. (B) However, in *H3.1* or *H3.3K27M* mutant pHGGs, the methyltransferase activity of the EZH2 enzymatic unit of the PRC2 complex is inhibited, resulting in global hypomethylation and increased acetylation. (C) In *H3.3G34R/V* mutant pHGGs, there is loss of H3K27me3 but only on affected histone proteins due to the proximity of the G34 position to K36.

Figure 3. Pediatric high-grade glioma tumor microenvironment. The pHGG tumor microenvironment is composed of a vascularized tumor bulk with invasive tumor margins. Infiltrating bone marrow derived tumor associated macrophages are found in perivascular areas while brain resident microglia are found at the periphery of the tumor. T-cells are typically sparse, yet are found in perivascular areas.

Figure 4. Chimeric antigen receptor T-cell therapy in pediatric high-grade glioma. CAR T-cell therapies that have been investigated for pre-clinical use in pediatric high-grade gliomas include anti-GD2, anti-IL13Rα2, anti-B7H3, and K27M-peptide specific CARs. CAR T-cell therapy stimulates the adaptive immune response, allowing for direct tumor cell killing and priming of antigen presenting cells to further amplify the immune response.

Figure 5. Tumor peptide vaccination in pediatric high-grade glioma. Patient-derived tumor cell lines from a tumor bank or tumor cells from surgical resection are cultured to produce tumor antigens. Tumor cells are lysed to release the tumor antigens, which are then administered to the patient to stimulate antigen presenting cells and cytotoxic T-cells to kill tumor cells.

Figure 6. Dendritic cell vaccination in pediatric high-grade glioma. Dendritic cells are collected through leukapheresis, cultured in vitro, and stimulated with tumor cell lysate or tumor RNA. Mature antigen-loaded dendritic cells are then re-administered back into the patient to stimulate the adaptive immune response and cause cytotoxic killing of tumor cells.

Figure 7. Viral therapy in pediatric high-grade glioma. A tumor-cell specific virus is injected into the tumor bulk or resection cavity. A pro-drug such as ganciclovir is then administered and

subsequently converted by the virus to a cytotoxic agent, which kills tumor cells and induces and amplifies an active immune response.

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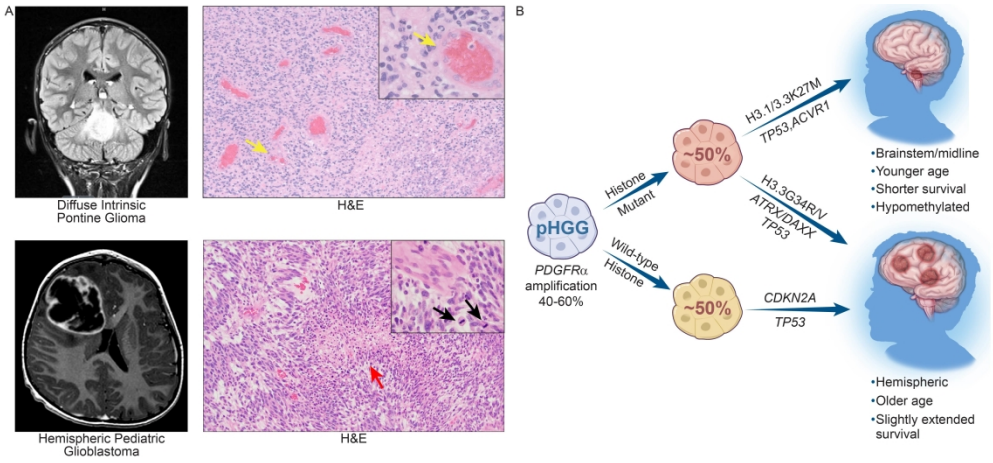

Figure 1. Clinical features of pediatric high-grade gliomas. (A) T2 flair magnetic resonance imaging of a DIPG in the coronal plane and a post-contrast T1 MRI H3/IDH WT hemispheric pHGG in the transverse plane. Corresponding H&Es displaying major histologic characteristics including microvascular proliferation (yellow arrow), pseudopalisading necrosis (red arrow), and brisk mitotic activity (black arrow). (B) 40-60% of pHGGs have *PDGFRA* amplification. Half of pHGGs have histone mutations; including H3.1/H3.3K27M mutations which arise in the brainstem and midline, and H3.3G34R/V mutations which are hemispheric tumors. The other half of pHGGs are histone wildtype, but commonly possess *CDKN2A* and *TP53* mutations, and typically arise in the hemispheres of the brain.

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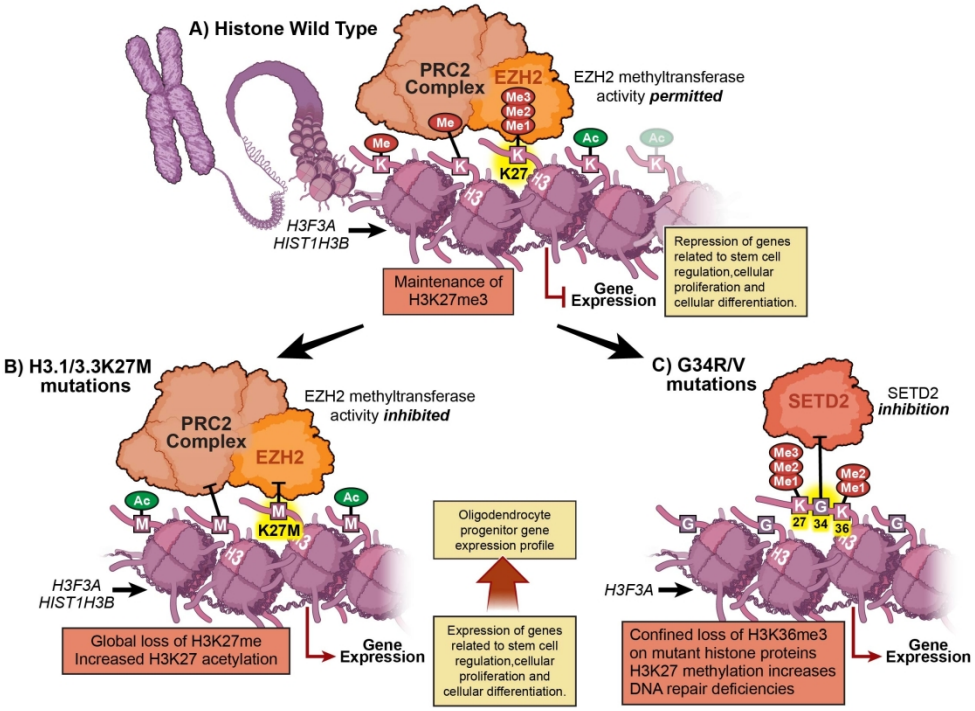

Figure 2. Histone mutations in pediatric high-grade glioma. (A) In histone wild-type pHGGs, there is maintenance of global H3K27me3 across the genome. (B) However, in H3.1 or H3.3K27M mutant pHGGs, the methyltransferase activity of the EZH2 enzymatic unit of the PRC2 complex is inhibited, resulting in global hypomethylation and increased acetylation. (C) In H3.3G34R/V mutant pHGGs, there is loss of H3K27me3 but only on affected histone proteins due to the proximity of the G34 position to K36.

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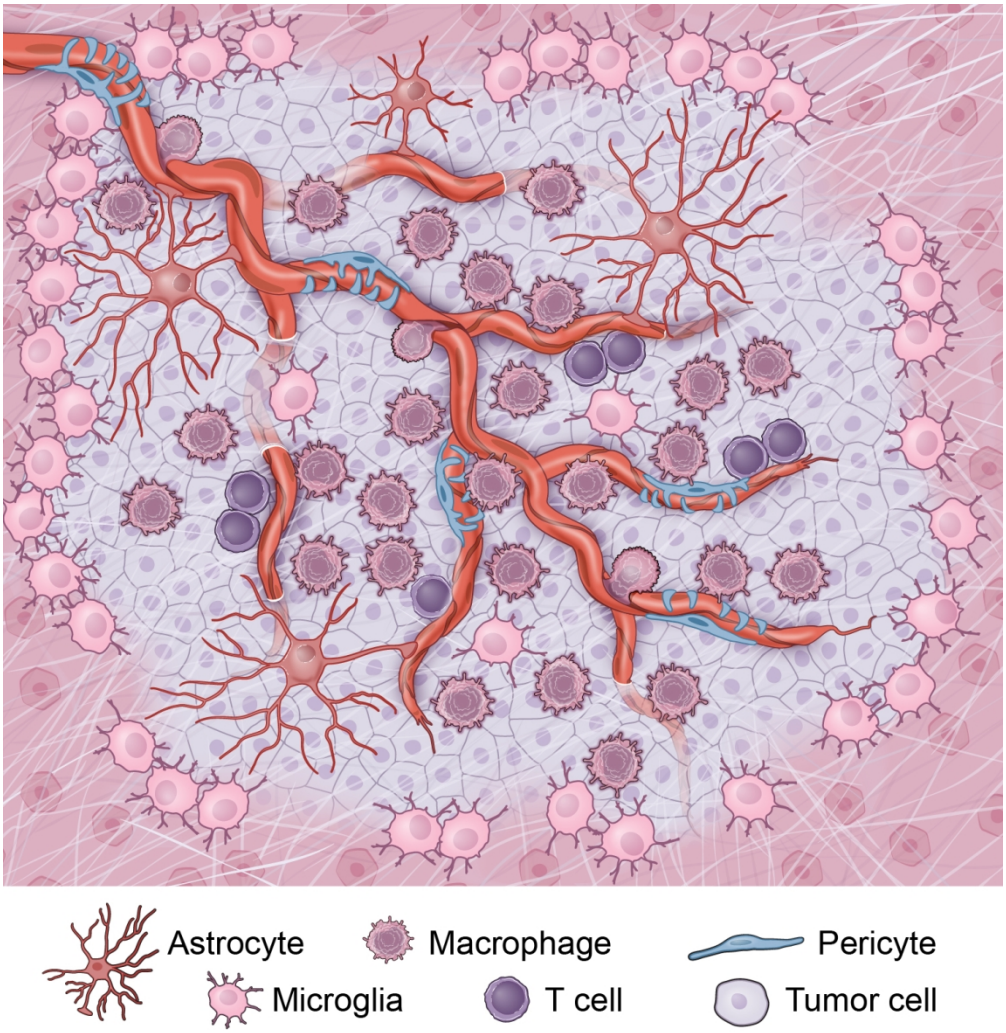

Figure 3. Pediatric high-grade glioma tumor microenvironment. The pHGG tumor microenvironment is composed of a vascularized tumor bulk with invasive tumor margins. Infiltrating bone marrow derived tumor associated macrophages are found in perivascular areas while brain resident microglia are found at the periphery of the tumor. T-cells are typically sparse, yet are found in perivascular areas.

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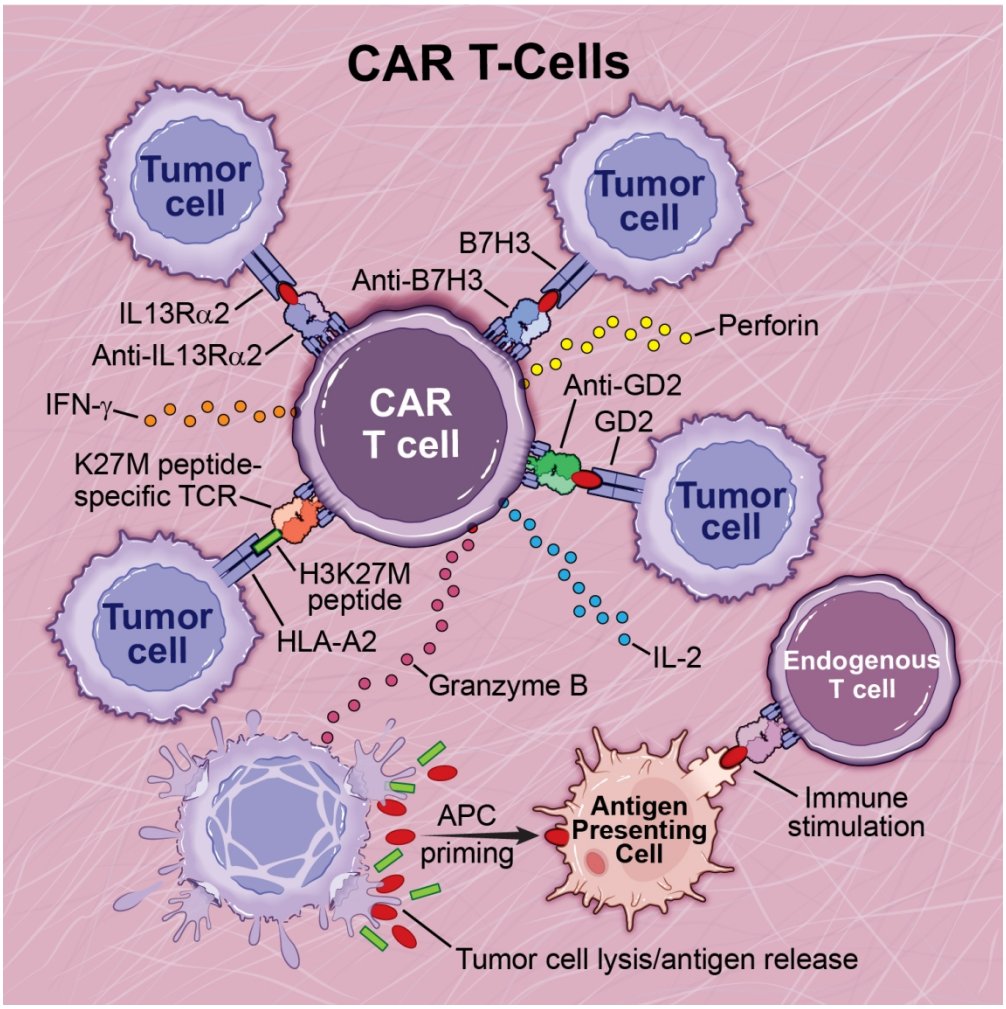

Figure 4. Chimeric antigen receptor T-cell therapy in pediatric high-grade glioma. CAR T-cell therapies that have been investigated for pre-clinical use in pediatric high-grade gliomas include anti-GD2, anti-IL13Rα2, anti-B7H3, and K27M-peptide specific CARs. CAR T-cell therapy stimulates the adaptive immune response, allowing for direct tumor cell killing and priming of antigen presenting cells to further amplify the immune response.

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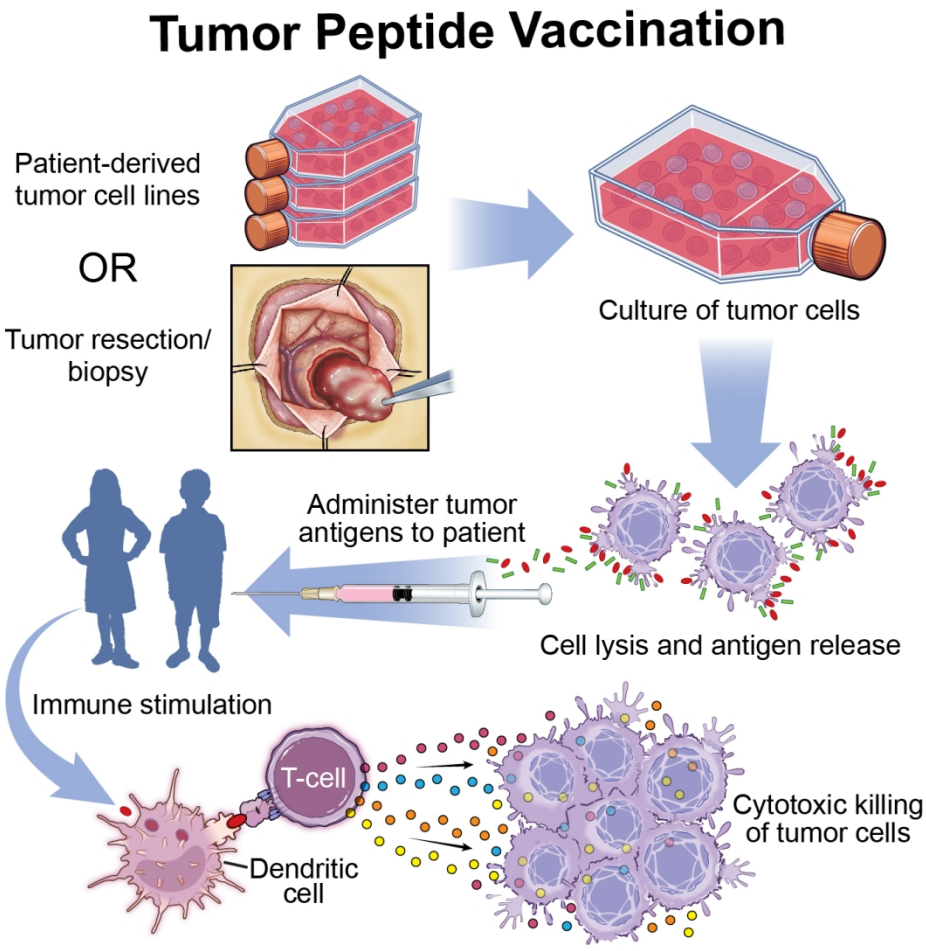

Figure 5. Tumor peptide vaccination in pediatric high-grade glioma. Patient-derived tumor cell lines from a tumor bank or tumor cells from surgical resection are cultured to produce tumor antigens. Tumor cells are lysed to release the tumor antigens, which are then administered to the patient to stimulate antigen presenting cells and cytotoxic T-cells to kill tumor cells.

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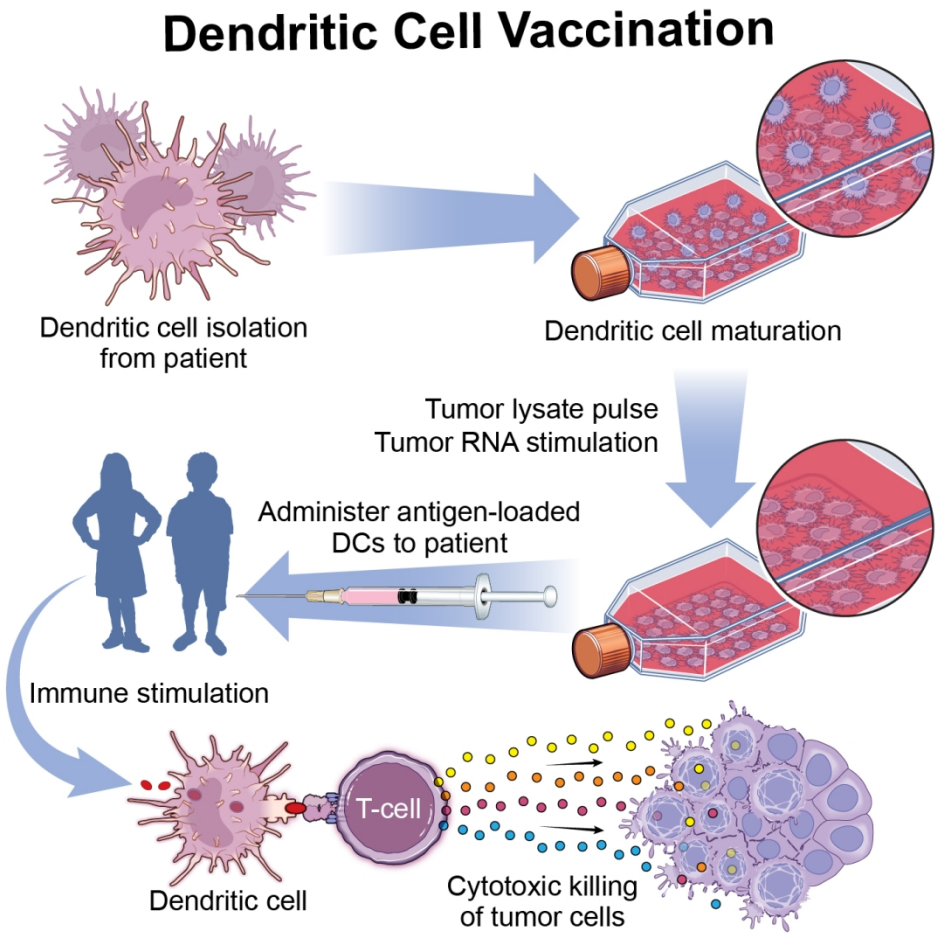

Figure 6. Dendritic cell vaccination in pediatric high-grade glioma. Dendritic cells are collected through leukapheresis, cultured in vitro, and stimulated with tumor cell lysate or tumor RNA. Mature antigen-loaded dendritic cells are then re-administered back into the patient to stimulate the adaptive immune response and cause cytotoxic killing of tumor cells.

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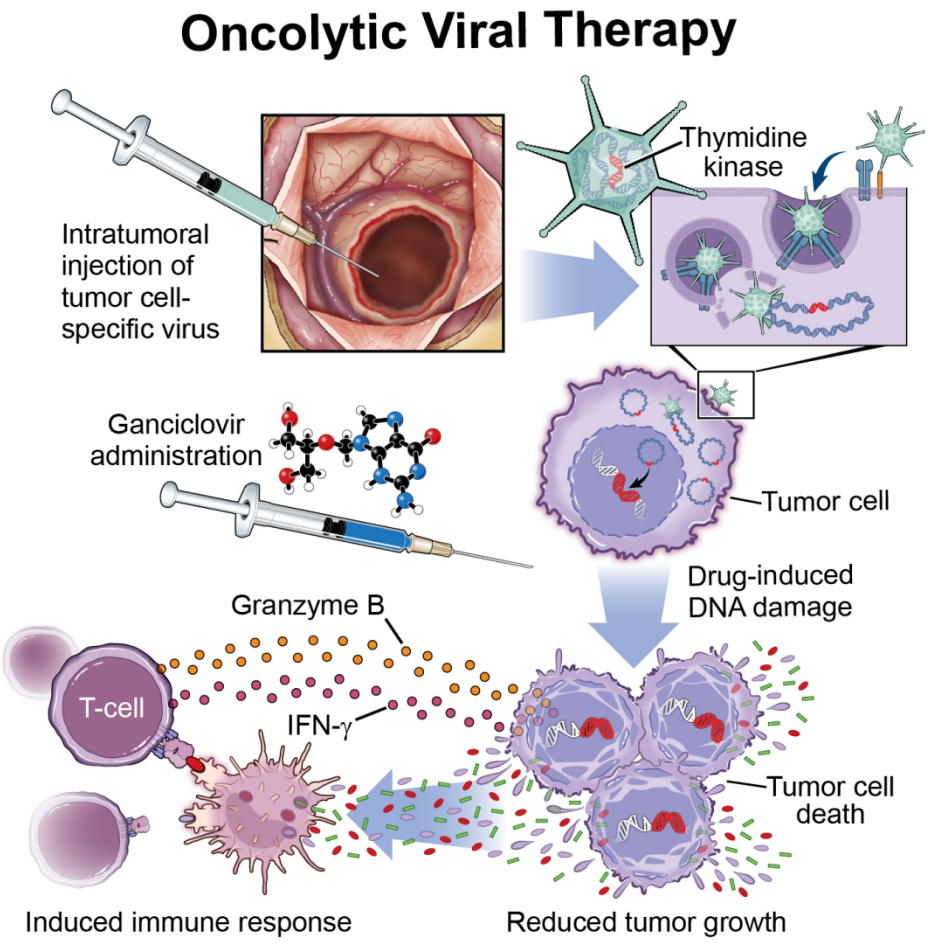

Figure 7. Viral therapy in pediatric high-grade glioma. A tumor-cell specific virus is injected into the tumor bulk or resection cavity. A pro-drug such as ganciclovir is then administered and subsequently converted by the virus to a cytotoxic agent, which kills tumor cells and induces and amplifies an active immune response.

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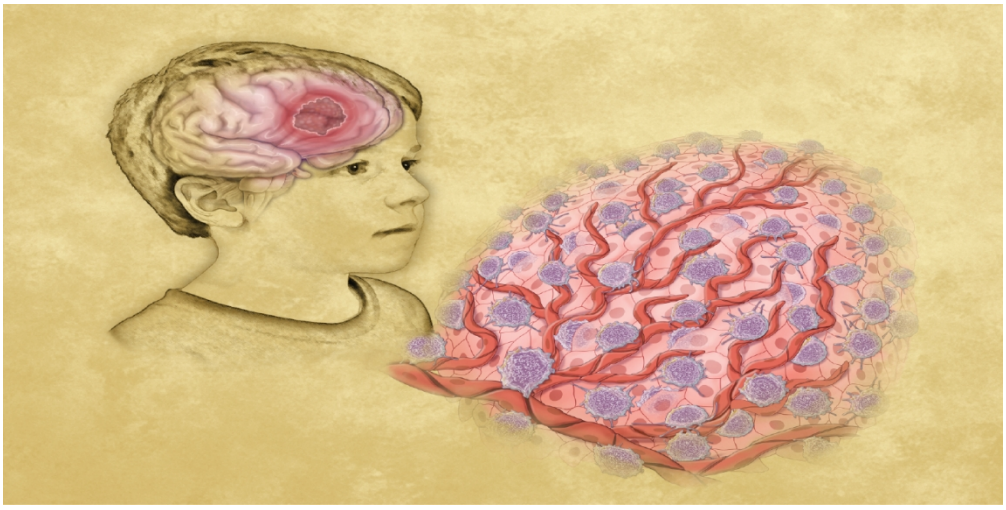
Table 1. Actively recruiting immunotherapy trials for pediatric high-grade gliomas as of March 2021.

NCT Number	Title of Study	Therapeutic Strategy	Phase	Objectives
NCT04196413	GD2CART for the Treatment of H3K27M Mutated Diffuse Intrinsic Pontine Glioma or Spinal Diffuse Midline Glioma	GD2 CAR-T cells	I	Determine feasibility of manufacturing autologous GD2 CAR-T cells. Assess safety, determine maximum tolerated dose, and establish phase II dosing. Assess expansion, persistence and phenotype of GD2 CAR-T cells.
NCT04185038	Genetically Engineered Cells (B7-H3-specific CAR T Cells) in Treating Pediatric Patients with Diffuse Intrinsic Pontine Glioma, Diffuse Midline Glioma, or Recurrent or Refractory Central Nervous System Tumors	B7-H3 CAR-T cells	I	Assess feasibility and safety of B7-H3 CAR-T cells. Establish dose tolerability and define maximum tolerated dose. Assess B7-H3 CAR-T cell distribution within CSF. Assess disease response to B7-H3 CAR-T cells.
NCT02442297	HER2-Specific T Cells in Treating Participants with Recurrent or Refractory HER2-Positive Glioblastoma	HER2 CAR-T cells	I	Evaluate safety, persistence, expansion, and function of HER2 CAR-T cells.
NCT03500991	HER2-specific CAR T Cell Locoregional Immunotherapy for HER2-positive Recurrent/Refractory Pediatric CNS Tumors	HER2 CAR-T cells	I	Establish safety and feasibility of HER2-specific CAR-T cell infusions. Assess HER2 CAR-T cell distribution and function in CSF and peripheral blood.
NCT03638167	EGFR806-specific CAR T Cell Locoregional Immunotherapy for EGFR-positive Recurrent or Refractory Pediatric CNS Tumors	EGFR806-specific CAR-T cells	I	Establish safety and feasibility of EGFR806-specific CAR-T cell infusions. Assess EGFR806-specific CAR T-cell distribution and function in CSF and peripheral blood. Assess expression of EGFR in relapsed treated tumors.
NCT02208362	Genetically Modified T-cells in Treating Patients with Recurrent or Refractory Malignant Glioma	Interleukin 13 receptor alpha 2 CAR-T cells	I	Feasibility and safety of ex vivo expanded CAR T-cells. Determine maximum tolerated dose schedule and phase II dosing. Assess post-immunotherapy responses.
NCT04099797	Genetically Engineered Cells (C7R-GD2.CAR T Cells) for the Treatment of Patients with GD2-Expressing High Grade Glioma or Diffuse Intrinsic Pontine Glioma, The GAIL-B Trial	GD2 CAR-T cells with constitutively active Interleukin 7 Receptor (C7R-GD2 CAR-T cells)	I	Determine safety of escalating doses of CAR-T cells. Estimate anti-tumor response. Evaluate fate and immunologic effects of CAR-T cells.
NCT03652545	Multi-antigen T Cell Infusion Against Neuro-oncologic Disease (REMIND)	Tumor multi-antigen associated specific cytotoxic T lymphocytes (TAA-T)	I	Determine safety and feasibility of rapidly generated TAA-T. Determine optimal dosing schedule of TAA-T.
NCT03911388	HSV G207 in Children With Recurrent or Refractory Cerebellar Brain Tumors	G207 Herpes simplex virus with or without radiotherapy	I	Determine safety and tolerability by frequency of grade 3 above adverse events. Assess immunologic response via HSV-1 antibody titers. Assess progression free survival and overall survival.
NCT02960230	H3.3K27M-specific Peptide Vaccine and Poly ICLC with or without Nivolumab in Treating Patients with Newly Diagnosed HLA-A2 Positive,	H3.3K27M-specific peptide vaccine and Poly ICLC with	I	Assess safety of H3.3K27M-specific peptide vaccine with or without Nivolumab in H3.3K27M+ DIPG or midline gliomas.

	H3.3K27M Positive Diffuse Intrinsic Pontine Glioma or Other Newly Diagnosed Gliomas	or without Nivolumab (anti-PD1)		Determine overall survival at 12 months. Assess H3.3K27M epitope-specific cytotoxic T lymphocyte response.
NCT03396575	Brain Stem Gliomas Treated With Adoptive Cellular Therapy During Focal Radiotherapy Recovery Alone or With Dose-intensified Temozolomide (BRAVO)	Total tumor RNA loaded dendritic cell vaccine (TTRNA-DC) with or without chemotherapy	I	Assess safety and feasibility of TTRNA-DC vaccines with or without chemotherapy. Determine the maximally achievable dose or maximum tolerated dose. Assess post-immunotherapy anti-tumor immune response in lymphocytes.
NCT01130077	A Pilot Study of Glioma Associated Antigen Vaccines in Conjunction With Poly-ICLC in Pediatric Gliomas	HLA restricted glioma antigen peptides with Poly ICLC	I	Assess safety and tolerability. Determine glioma-associated antigen-specific T-cell responses via IFN-gamma activity.
NCT03389802	CD40 Agonistic Monoclonal Antibody APX005M in Treating Pediatric Patients with Recurrent or Refractory Brain Tumors	CD40 agonistic monoclonal antibody	I	Evaluate safety of CD40 agonistic monoclonal antibody. Determine pharmacokinetics, maximum tolerated dose schedule, and phase II dosing.
NCT02359565	Pembrolizumab in Treating Younger Patients with Recurrent, Progressive, or Refractory High-Grade Gliomas, Diffuse Intrinsic Pontine Gliomas, Hypermutated Brain Tumors, Ependymoma or Medulloblastoma	Pembrolizumab (anti-PD1)	I	Establish safety and adverse effects with the administration of adult recommended dose. Estimate sustained objective response rate. Determine changes in immunophenotypic profile of CD8+ T cells.
NCT04323046	Immunotherapy (Nivolumab and Ipilimumab) before and after Surgery for the Treatment of Recurrent or Progressive High Grade Glioma in Children and Young Adults	Nivolumab (anti-PD1) and Ipilimumab (anti-CTLA4)	I	Measure changes in the genetic signature of the tumor microenvironment after treatment. Safety and tolerability.
NCT03690869	REGN2810 in Pediatric Patients With Relapsed, Refractory Solid, or Central Nervous System (CNS) Tumors and Safety and Efficacy of REGN2810 in Combination With Radiotherapy in Pediatric Patients With Newly Diagnosed or Recurrent Glioma	Cemiplimab (anti-PD1) with radiotherapy	I/II	Evaluate safety and anticipated phase II dose of Cemiplimab. Determine pharmacokinetics of Cemiplimab. Confirm safety and phase II dose of Cemiplimab with radiotherapy.
NCT04049669	Pediatric Trial of Indoximod With Chemotherapy and Radiation for Relapsed Brain Tumors or Newly Diagnosed DIPG	Indoximod (indoleamine 2,3-dioxygenase inhibitor) with chemotherapy and/or radiation	II	Improve antitumor immune responses and measure outcomes according to "immune-related response assessment for neuro-oncology" (iRANO) criteria.



Ross *et al.* provide a comprehensive review of the immune landscape of paediatric high-grade gliomas. They explore how different genetic alterations lead to the creation of specific tumour subgroups with distinct immune profiles, and discuss current and emerging immunotherapies.