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'Pediatric glioblastoma multiforme (GBM) models for immunotherapy testing:

Incorporating models of the tumour immune microenvironment and the blood-brain-barrier (BBB).'

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Abstract

Pediatric Glioblastoma Multiforme (GBM), while not as common as adult GBM, exhibits poor prognosis despite the application of many conventional cancer treatment approaches. Cancer immunotherapy, including cancer vaccines, adoptive T-cell therapy, oncolytic virotherapy and checkpoint inhibitor therapy, has showcased promising treatment effects for many solid tumours and is currently explored for pediatric GBM treatment, as well. Patient response to immunotherapy is closely related to the composition of the tumour microenvironment and its immune components. Therefore, preclinical tumour models incorporating constituents of the tumour immune microenvironment (TIME) are essential as platforms for testing immunotherapy. For the testing and development of immunotherapeutic and other drug treatments for brain tumours, the treatment needs to cross the Blood-Brain-Barrier (BBB). *In-vitro* models of the BBB can be used to assess BBB permeability for a specific drug. Here we present an overview of immunotherapeutic options for pediatric GBM, in the stage of clinical-trial research, and analyze *in-vitro* and *in-vivo* mouse models as well as *in-vitro* BBB models for the testing of current or future immunotherapy against pediatric GBM. *In-vitro* 3D organoid models of pediatric GBM are an already-established model, showing early signs of promising results as a platform for adoptive T-cell therapy testing, with future steps entailing TIME representation for patient-specific immunotherapy testing.

Plain Language Summary

Pediatric Glioblastoma Multiforme (GBM) is an aggressive pediatric brain tumor responsible for many children deaths per year. Current approaches to treat cancer include surgery, chemo- and radiotherapy, as well as immunotherapy, which manipulates the immune system to fight cancer. The latter is gaining ground in the field of cancer therapy; however, is still not widely adopted in clinical trials for pediatric GBM treatment. To reduce time needed for immunotherapy to be applied in clinical practice, preclinical testing of immunotherapy needs to be accurate and to include parameters of the immune microenvironment of the tumor. Therefore, mouse models, human cancer cells and tissue-like structures can be manipulated to incorporate tumor immune components and function as platforms for immunotherapy testing. In comparison to other tumor types, drugs used to treat brain tumors need to cross a tissue structure, which controls substance exchange between the brain and the blood, called the Blood-Brain-Barrier (BBB). Immunotherapy permeability through the BBB can also be assessed in models of this tissue structure. In this review, we explore clinically tested immunotherapeutic options for pediatric GBM and traverse models of this tumor type as well as BBB models for immunotherapy testing of already established or future treatments. 3D tissue cultures called 'organoids' are the more promising models for testing immunotherapy so far. Further development of these models will take us a step closer to finding the best treatment for each patient with this aggressive tumour.

Introduction

Cancer is one of the prime causes of death among children worldwide and pediatric brain cancer is deemed the most lethal (Thorbinson & Kilday, 2021). While not as common as their adult counterparts, childhood high-grade gliomas result in over 40% of all childhood brain tumour deaths (Buccoliero et al., 2022). Previous histological classification of pediatric high-grade gliomas (pHGGs) according to the World Health Organization (WHO) led to the subdivision of high-grade gliomas into two categories: anaplastic astrocytoma (AA; WHO Grade III) and glioblastoma (GBM; WHO Grade IV) (Njonkou et al., 2022). However, recent molecular classification of high-grade gliomas identified three molecular subgroups: H3.3 mutant, IDH mutant, and H3.3/IDH wild-type, including their subgroups (Buccoliero et al., 2022).

Glioblastoma multiforme (GBM), the most aggressive high-grade glioma subtype, is a rare entity occurring in 3% of all childhood brain tumours (Singla et al., 2021). This brain tumour has a higher incidence in children younger than five and between 15 and 19 years old, showcasing a less favourable prognosis for the older children (Das & Kumar, 2017). Current treatment options encompass maximal safe resection of the tumour and subsequent radiation therapy in children older than three years old. Chemotherapeutic options - mainly used against adult glioblastoma already - have been implemented; however, the different molecular and immunological landscape of pediatric glioblastoma points to the need for other strategies (Njonkou et al., 2022). Present-day immunotherapy holds promise to treat various solid tumours, including pediatric GBM, causing milder side effects and aiming for complete remission of the tumour.

Developing effective immunotherapeutic options for treating pediatric GBM requires understanding the tumour immune microenvironment (TIME). The tumour microenvironment of pHGGs and GBM mainly includes microglia, macrophages, astrocytes, and vasculature, comprised of endothelial cells and pericytes infiltrating the tumour bulk. T cells and natural killer cells (NK) are also present in small numbers (Njonkou et al., 2022; Ross, Velazquez Vega, et al., 2021). It is significant to note that pediatric and adult GBM exhibit major differences in tumour immune microenvironment (TIME) characteristics. In the adult tumour, NK cell cytotoxic activity is increased and MHC I expression by tumour cells is low. Regarding immune checkpoint proteins, including CTLA-4, PDL-1 and TIM-3, they showcase overexpression in adult GBM (Z. Chen & Hambardzumyan, 2018; Njonkou et al., 2022). However, in both pediatric and adult GBM,

tumour-associated macrophages (TAMs) and microglia are highly occurring in the TIME and T-cells are more scarce (Z. Chen & Hambardzumyan, 2018). The TIME of pediatric GBM will be discussed later in the review.

With the recent galloping growth of immunotherapy, the need for accurate preclinical testing is growing accordingly. In preclinical cancer research, models are necessary to study the molecular basis and oncogenic events causing a tumour, the tumour heterogeneity, interactions between the tumour and the immune system and potential metastasis. Additionally, preclinical tumor models serve as platforms for testing potential therapeutic options (Sajjad et al., 2021); *in-vitro* and *in-vivo* tumour models can facilitate the evaluation of immunotherapy efficacy and the prediction of responses. *In-vitro* models such as cancer cell lines, 3D cultures, recently established brain organoids and *in-vivo* mouse models, including Xenografts and Genetically Engineered Mouse Models (GEMMs), are discussed in this literature review (Fig. 2; Li & Langhans, 2021). Before being adopted in clinical research, immunotherapies developed for pediatric GBM have been assessed in one or more of these preclinical models and conclusions can be drawn on which model is more suitable for testing each type of immunotherapy. Plain tumour models do not usually have a representation of the patient immune system, however, both *in-vitro* and *in-vivo* options for incorporating factors of the immune system are available (Akter et al., 2021). These more complex models are expected to be more accurate in immunotherapy testing, covering two significant aspects: tumour clearance and immune response by immunotherapy. Finally, as immunotherapy for pediatric GBM needs to access the tumour located in the brain parenchyma, it needs to cross the BBB, as discussed above. Therefore, models have been developed to mimic the BBB, including Transwell static models, organs-on-a-chip and organoids (Hajal et al., 2021). Testing of BBB-crossing is our proposed final line of testing to verify that a particular immunotherapy can reach the brain if administered through the bloodstream.

Here, we give an overview of current immunotherapeutic options for pediatric GBM and discuss *in-vitro* and *in-vivo* models for testing each type of immunotherapy. We aim to pair immunotherapy types with accurate models that each can be tested on to assess potential future discoveries in the field. Specifically, we propose testing of immunotherapy in three levels, assessing the efficacy of immunotherapy and the contribution of the immune system in tumour models enriched with TIME representation and testing the ability of immunotherapy to enter the CNS in BBB models. While not widely used for pediatric GBM immunotherapy testing yet, *in-*

vitro organoid models are emerging as the most promising model, since they accurately represent 3D tumour characteristics and can be co-cultured with immune and other cells of the tumour microenvironment.

Current immunotherapeutic approaches for the treatment of pediatric Glioblastoma

Increasing interest in immunotherapy and extensive research have led to several immunotherapeutic options being proposed for the treatment of pediatric brain tumours, as well. For pediatric GBM, identifying critical components of the TIME is essential in order to develop successful treatments and trigger an immune response against GBM. The tumour microenvironment of pHGGs and GBM mainly includes microglia, macrophages, astrocytes, and vasculature, comprised of endothelial cells and pericytes infiltrating the tumour bulk. T cells and natural killer cells (NK) are also present in small numbers (Njonkou et al., 2022; Ross, Velazquez Vega, et al., 2021). Regarding features of the tumour immune microenvironment, there has been increased expression of MHC I peptides and HLA-G and HLA-E peptides promoting inhibitory pathways and elevated recruitment of T regulatory cells (T-reg). Additionally, low NK cell activation – due to reduced NKG2D ligand expression– and tumour infiltration have been noted, as well as CD47 up- and PDL-1 downregulation, immunosuppressive cytokine expression, hypoxia and other mechanisms of immune invasion discussed more thoroughly in other literature reviews (Njonkou et al., 2022). These components of the TIME take place in the immune response triggered by immunotherapy types discussed further, which include dendritic cell and peptide vaccines, checkpoint inhibitors, adoptive cell therapy and oncolytic virotherapy.
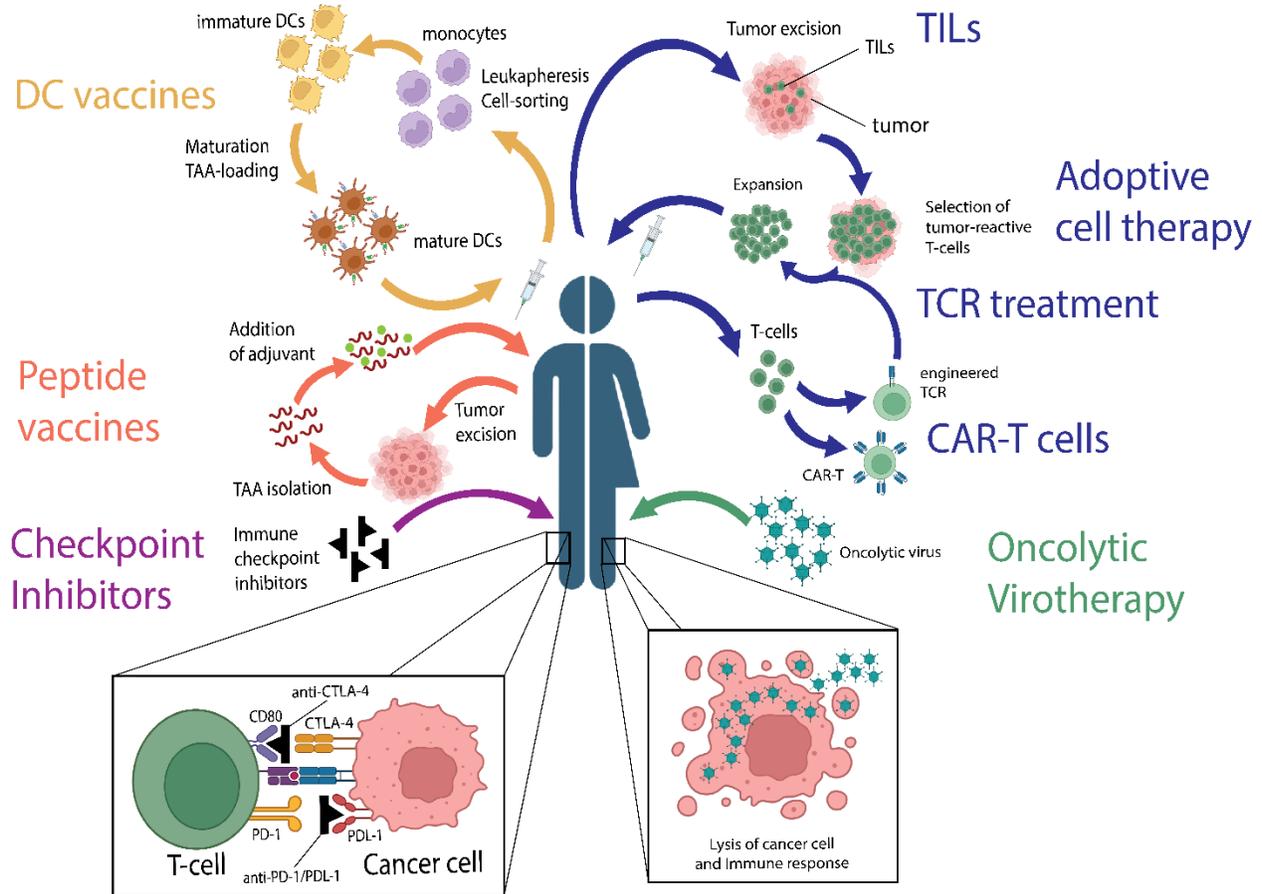
Figure 1. Graphic representation of immunotherapy types, engineering mechanisms and action

Current immunotherapy options include dendritic cell and peptide vaccines, checkpoint inhibitors, adoptive cell therapy and oncolytic virotherapy. To generate dendritic cell vaccines (top left), monocyte precursors are harvested, amplified and exposed to a specific antigen during the maturation process to become dendritic cells. These are injected back into the patient to trigger an immune response. Peptide vaccines (middle left) involve the harvesting of tumour-associated antigens (TAAs) or tumour-specific antigens (TSAs), isolation of peptides from these antigens and injecting them to the patients along with an adjuvant for them to be taken up by antigen-presenting cells (APCs). Checkpoint inhibitors (bottom left) are proteins designed to block the bond between cancer and immune cell receptors, leading to immunosuppression, thus increasing the intensity of immune cell responses. Oncolytic virotherapy (bottom right) is developed to cause lysis of tumour cells and inflammation in the tumour site by administration of natural or genetically modified viruses. For adoptive cellular therapy (top right), there are three methods, which involve tumour infiltrating lymphocytes (TILs), T cells with engineered T cell receptors (TCR) and chimeric antigen receptor T cells (CAR-T). For TIL therapy, T cells are isolated from patient tumours and exposed to tumour antigens. In contrast, T cells are harvested with leukapheresis in TCR treatment and CAR-T cell therapy. The protocol includes expanding T cells ex vivo and infusing them back into the patient to trigger an immune response against tumour antigens. Created with BioRender (accessed on the 23rd of November 2022).

Cancer cell vaccines

Peptide vaccines

Cancer vaccines aim to provoke an immune response by the patient's system; specifically, peptide vaccines function by introducing antigen peptides into the immune system. T-cells will recognise these peptides and trigger the immune response to eliminate the cancer cells. Tumour antigen peptides can be either tumour-specific antigens (TSAs), meaning proteins only expressed by the tumour cells due to a tumour-specific mutation and absent from normal tissue, or tumour-associated antigens (TAAs), which include proteins expressed in much higher concentrations by cancer cells compared to non- cancer cells (Foster et al., 2019). Peptides from these antigens can be generated *in-vitro* and delivered to the patient via injection into lymph nodes, where the most famous antigen-presenting cells (APCs) reside, the dendritic cells (DCs). The peptides are ideally presented on major histocompatibility complex (MHCs) molecules on the surface of the DCs. Short peptides (up to 12 amino acids) can bind directly to the MHC molecule, while longer peptides of up to 30 amino acids are endocytosed by the DCs, processed in the endoplasmic reticulum (ER) and then presented to CD8+ cytotoxic and/or CD4+ helper T-cells (Galluzzi et al., 2014). For antigens to be identified as TAAs or TSAs, they need to originate from oncogenic proteins and have differential expression in the tumour cells, either by being overexpressed or being unique to them. It is also significant that they are recognised by T-cells to trigger the immune response (Foster et al., 2019). While for TAAs, the peptides are general for most patients and sometimes common between different tumour types (Melief et al., 2015), in the case of the more personalised TSA peptide vaccines, patient tumour-specific antigens are analysed -to identify whether they bind to MHC molecules of the patients- and then synthesised and administered to the patient (Sahin & Türeci, 2018).

At a recently completed clinical trial (NCT02750891), a peptide vaccine (DSP-7888 - adegramotide/ nelatimotide) was tested on GBM and grade III or IV glioma patients younger than 19 years of age, however, no data have been posted yet. DSP-7888 includes three synthetic peptides of Wilms' tumour 1 (WT1), a transcription factor expressed in many solid tumour types. The vaccine is expected to activate helper and cytotoxic T-cell-mediated immune responses against these tumours (Suginobe et al., 2022). Another phase II -currently recruiting- clinical trial

(NCT0391675) is testing V-Boost immunotherapy, an oral tablet of hydrolysed antigens of GBM along with alloantigens, on patients younger than five years of age.

Dendritic cell vaccines

DC vaccines or DC-based immunotherapies are based on the ability of DCs to present antigens to naive T cells and generate an immune response towards a specific antigen. The method involves harvesting DC precursor cells from the patient or a donor (after leukapheresis), amplifying them and guiding their maturation towards DCs ex vivo, exposing (priming) them to the specific tumour antigens and then reinfusing them back into the patient (Foster et al., 2019; Palucka & Banchereau, 2012). Priming can be achieved with different methods, all of which aim at stimulating DCs by introducing the peptide antigens themselves, introducing DNA or RNA, which upon expression will generate these peptides or exposing them to bulk tumour lysates or tumour mRNA (Constantino et al., 2017; Foster et al., 2019). The DCs infused back into the patient can present these antigens, which they uptake, process and present to CD8+ and CD4+ T cells leading to the activation of an immune response.

Current trials for pediatric GBM include a tumour lysate DC vaccine (NCT01808820) and a CMV RNA-Pulsed DC vaccine (NCT03615404) trial. For the first trial, after surgical tumour resection, pediatric patients with anaplastic astrocytoma and glioblastoma multiforme undergo leukapheresis. Following, peripheral blood mononuclear cells are isolated and DCs deriving from them are injected into the patients periodically. Three days after each DC-injection, tumour lysate is also introduced to the patient and DCs are expected to present these antigens to T cells for the immune response against the tumour cells to be activated. For the second trial, the same procedure is followed; however, DCs are loaded with ribonucleic acid (RNA) encoding the human CMV matrix protein pp65 fused with the LAMP protein (pp65-LAMP) plus the proinflammatory cytokine GM-CSF as adjuvant (Zhao et al., 2018). Pp65 protein is an antigen overexpressed in GBM cells, and LAMP is a lysosomal protein, which in fusion with pp65 is responsible for the transport of the fusion protein to the lysosome of the DCs (Eskelinen, 2006; Lucas et al., 2011). There pp65 is loaded on MHC II molecules, which will then present the antigen to T cells to activate the immune response against GBM cells. Apart from adult and pediatric GBM, patients with Malignant Glioma and Medulloblastoma from 0-35 years of age were also eligible for this clinical trial.

Oncolytic virotherapy

Oncolytic virotherapy is developed to cause lysis of the tumour cells using natural or genetically modified viruses. These viruses specifically target cancer cells and cause their lysis and inflammation in the area due to excessive viral replication. Furthermore, an immune response is activated after lysis of the tumour cells because of the cell death and subsequent release of tumour antigens into the extracellular space (Njonkou et al., 2022; Russell et al., 2012). Most often herpes simplex virus (HSV), poliovirus, adenovirus or measles virus are genetically manipulated for oncolytic virotherapy to specifically target tumour cells, by inserting tissue-specific promoters or silencing genes that affect tropism (Hamid et al., 2017). Additionally, genes can be inserted in the virus genome that encode proteins activating organised cell death pathways, immunostimulatory cytokines, or inhibiting cell survival proteins in the cancer cells (Galluzzi et al., 2014).

Several clinical trials testing oncolytic virotherapy against pediatric glioblastoma are currently active and/or recruiting, including trials for HSV G207 (NCT03911388, NCT04482933, NCT02457845), polio/rhinovirus recombinant PVSRIPO (NCT03043391), Adenovirus (Adv)-mediated herpes simplex virus thymidine kinase AdV-tk (NCT00634231) and Wild type reovirus (NCT02444546) in combination with other adjuvants or immunostimulators. HSV is a neurotropic DNA virus used in oncolytic virotherapy after being genetically engineered to only retain replication ability in glioma and other brain tumour cells, and therefore potentially cause lysis (Markert et al., 2000). Similarly, RVSRIPO has a tropism for most solid tumours, including GBM, as they express the universal CD155 antigen, to which RVSRIPO binds, transfers the viral RNA genome to the cell and induces oncolysis. The same CD155 molecule is expressed by many APCs, which are also targeted upon RVSRIPO virotherapy, leading to inflammation, and increased antigen presentation and therefore have an antitumour effect (M. C. Brown & Gromeier, 2015). In the case of Adv-tk, administered along with the antiviral pro-drug valacyclovir, herpes simplex virus thymidine kinase leads to the phosphorylation of valacyclovir, same as ganciclovir, to induce inhibition of DNA-polymerase function, leading to the target-cell death. The gene-transfer vector used is an adenovirus, which is efficient as it allows for short-term expression, is useful to destroy tumour cells, and causes an immune response (Sandmair et al., 2002). Finally, reovirus (respiratory enteric orphan virus) is a double-stranded RNA virus with a tropism for Ras-activated cells, including glioma cells. Ras-activated cells are susceptible to reovirus infection, as their double-

stranded RNA-activated protein kinase (PKR) is inhibited, therefore allowing for the synthesis of viral proteins, reovirus replication and cell lysis (Wilcox et al., 2001).

Checkpoint Inhibitors

Checkpoint regulator blockade allows for the easier stimulation of T cells against tumour cells and has been incorporated into immunotherapy options for several cancer types. Checkpoint regulators are T cell surface proteins, which bind to ligands on APC cells or others and lead to immunosuppression. In the tumour microenvironment, binding of the T cell receptor to the ligand leads to decreased proliferation and activation of the T cells against tumour cells, as intracellular inhibitory signals from the binding outmatch stimulatory ones (Foster et al., 2019). Checkpoint inhibitors have been developed to block this bond and tip the balance towards intracellular stimulatory signals, thus increasing the duration and intensity of T-cell responses (B. Huang et al., 2021). Several checkpoint inhibitors have been developed, which are used as combination therapy for treating advanced melanoma and other adult tumour types.

Emerging options in checkpoint inhibition include blocking of programmed cell death protein 1 and (PD-1) and ligand PDL-1, cytotoxic T-lymphocyte antigen 4 (CTLA-4), macrophage immune checkpoint tumour cell receptor CD47 and others, such as indoleamine 2,3-dioxygenase-1 (IDO-1) and mucin domain 3 (TIM-3). PD-1 is expressed on activated T cells and other immune cells, such as B and NK cells and binds to ligand PDL-1, inhibiting functions of immune cells, for instance, activation, proliferation of T-cells and cytokine secretion (Sharpe & Pauken, 2018). Pembrolizumab and Nivolumab are two widely used antibodies targeting PD-1. These antibodies have also been tested in combination with Ipilimumab, targeting CTLA-4. CTLA-4 receptors in T cells impede T-cell-mediated immune activation by binding to CD80/CD86 ligands on tumour cells. Similar to PD-1, TIM-3 is also expressed in many immune cells, including T cells, Tregs and macrophages, leading to immunosuppression upon binding. Other checkpoints expressed on tumour cells include CD47, which blocks phagocytosis by macrophages of the tumour cells when bound to its ligand (W. Zhang et al., 2020), and IDO1 upregulation in tumours, resulting in T cell immunosuppression. IDO1 is a metabolic enzyme involved in the catabolism of tryptophan, found upregulated in the cytoplasm of cells in multiple tumours, including adult GBM (Prendergast et al., 2017). Known inhibitors targeting TIM-3, CD47 and IDO1 are BMS-986258, CC-9002 and Indoximod, respectively.

These options have been used to treat several adult tumours and have uncovered essential features of tumours responsive to immune checkpoint inhibitors. These functions include high mutational load or microsatellite instability. Highly mutant tumours are more prone to be recognised by T cells; therefore, checkpoint inhibitors are more efficient as an immunotherapeutic treatment for them (Yarchoan et al., 2017). Microsatellite instability, a result of mismatch repair, is also characterised by increased mutational burden and has been identified as a feature of a subset of pediatric glioblastomas (Viana-Pereira et al., 2011), despite the overall low mutational burden of GBM (Hoffman et al., 2019). Furthermore, cancer predisposition syndromes, specifically constitutional mismatch repair deficiency syndrome (CMMRD), are characterised by the inability to repair mutations during DNA replication, resulting in high mutational load as well (Henderson et al., 2022). Therefore, CMMRD-positive, microsatellite instable and hypermutant pediatric glioblastomas are expected to be benefitted from checkpoint inhibitor immunotherapy (Henderson et al., 2022; Hoffman et al., 2019).

Currently, clinical trials targeting pediatric GBM using immune checkpoint inhibitors are either ongoing or have been completed. In most cases, immune checkpoint inhibitors have been used in combination to chemotherapy, radiotherapy or surgery. Specifically, current trials use the Nivolumab and Ipilimumab combination (NCT04323046) -targeting PD-1 and CTLA-4 respectively- pre and post-surgery and Indoximod, which is an IDO-pathway inhibitor, in combination with chemo- and radiotherapy (NCT02502708, NCT04049669). Further treatment options include CD40 agonists, like APX005M (NCT03389802). CD40 binds to its ligand CD154 on T cells, allowing the interaction and activation of DCs, monocytes and B-cells. The use of immunostimulatory CD40 agonists enables direct activation of APCs against TAAs and stimulates the immune response, mainly since CD40/CD154 are highly expressed in glioma patients with more favourable prognosis (Ceglia et al., 2022; Chonan et al., 2015).

Adoptive T cell therapy (mention all: TCR, CAR-T, TILs)

Adoptive cell therapy includes the harvesting, ex vivo manipulation, and delivery back into the patient of lymphocytes, mostly T cells and recently also natural killer (NK) cells. Adoptive T cell therapy can be subclassified into treatment using tumour-infiltrating lymphocytes (TILs), T cells with engineered T cell receptors (TCR), and chimeric antigen receptor T cells (CAR-T). In the case of TILs, to harvest them, patient tumour parts are resected, and autologous T cells are exposed

to specific tumour antigens and expanded ex vivo before being injected back into the tumour site. TIL-adoptive cell therapy has been very effective in the treatment of metastatic melanoma (Feldman et al., 2015). In another approach, synthetic TCRs can be encoded by patient T cells through viral transduction. These engineered TCRs target a specific tumour peptide presented on an APC. After manipulating ex vivo expanded patient T cells to express these TCRs, they are infused back into the patient, where they trigger an immune response against tumour antigens (Foster et al., 2019). The same concept is applied to CAR-T cell therapy for the harvesting, manipulating, and delivering CAR T cells (Sterner & Sterner, 2021).

To fully understand the potential of CAR T cell therapy, it is significant to understand their domains and functions. CAR T cells are constructed using a single-chain variable fragment (scFv), which is an antigen-recognition domain, along with a transmembrane spacer (hinge domain) and an intracellular signal transduction domain. The scFv is located on the outer surface of the plasma membrane and is a protein constructed by fusing - using a short linker peptide- the variable regions of both immunoglobulin chains of an antibody targeting a specific epitope. The spacer or hinge domain provides flexibility, as it connects the scFv domain and the T cell membrane, displaying a variable length, which influences the binding of the receptor (Sterner & Sterner, 2021). There are five generations of CAR T cells developed, depending on the parts comprising the intracellular signalling domain, which range from simple ζ chains of the TCR complex (first generation) and co-stimulatory molecules like CD137 and CD28 (second and third generation) to even IL-12 inducer regions (fourth generation) and STAT-3/5 binding receptors (fifth generation), to eliminate antigen-negative cancer cells at the site and generate memory T cells (Mehrabadi et al., 2022; Tokarew et al., 2019; H. Zhang et al., 2020). CAR T cell therapy has been effective in targeting B cell leukaemia, and its applications in solid tumour treatment are slowly increasing despite challenges (Sterner & Sterner, 2021).

Adoptive cell therapy is not as widely applicable in pediatric GBM as in other malignancies; however, CAR T cell therapy shows great promise. Anti-tumour immune response generated by autologous TIL cell therapy was not successful in GBM patients in a 1999 pilot study (Quattrocchi et al., 1999) and there have been no glioma or GBM clinical trials for testing engineered TCR T cell therapy (B. Huang et al., 2021). Regarding CAR-T cell therapy, CAR-T cells specific for IL13Ra2 or HER2 have been tested in adult GBM patients with promising outcomes; however, pediatric GBM has low cell surface antigens to be targeted (Sayour & Mitchell, 2017). Despite

that, two CAR-T cell clinical trials, which also apply to pediatric GBM patients, are currently recruiting. A CAR T cell therapy (NCT02208362) clinical trial started in 2015 uses T cells transduced with lentiviruses to express an IL13Rα2-specific 41BB-Costimulatory Chimeric Receptor. These modified T cells also express a truncated CD19 receptor so that transduced CAR T cells are marked, and the receptor can be used to conditionally induce cell death (Budde et al., 2013). The second clinical trial (NCT03170141) uses tumour targeting IgT cells expressing immune modulatory genes to target pediatric and adult GBM. In this approach, apart from targeting specific tumour antigens, CAR T cells are also manipulated to encode for immunostimulatory proteins, for instance, immune checkpoint inhibitors. The IgT cells are introduced intravenously or injected directly into the tumour location.

In-vivo and in-vitro models for testing pediatric glioblastoma immunotherapy and options for recapitulating the tumour immune microenvironment

As already discussed, pediatric brain cancer is one of the most significantly researched diseases, for which new types of combination treatment are proposed, gradually incorporating immunotherapeutic options. Generally, pediatric brain tumors are not as mutationally burdened as their adult counterparts, generating less neoantigens, which could function as targets for immunotherapy (Sayour & Mitchell, 2017). In order to understand tumorigenesis and to assess treatment options pediatric brain tumour models are necessary. Therefore, we discuss and compare common *in-vivo* and *in-vitro* pediatric GBM models, focusing on their ability to model the tumour immune microenvironment and to be utilised for immunotherapy testing.

In-vivo mouse models
Despite many *in-vivo* animal models -including Zebrafish and even Drosophila melanogaster- having been developed to recapitulate brain tumours and test therapies, we decided to focus on mouse models, as they represent the category with the highest number of brain tumour models developed. Different method-based categories of mouse (or sometimes rat) models include carcinogen-induced models, xenografts, and genetically engineered mouse models (GEMMs).

In carcinogen-induced models, we can instigate the tumour using chemical carcinogens or viruses known to induce the tumour type we aim to model. However, the tumour generated is naturally occurring, differs from the human counterpart and is not easily reproducible (Li & Langhans, 2021). After the tumour is generated, cancer cells can be harvested from the mice and cultured as a stable cell line. In this manner, multiple glioma cell lines have been developed (C6, 9L, T9, F98, RG2, BT4C, CNS-1) without the clear distinction of glioblastoma in most cases. In addition, the mouse brain has been observed to develop tumours with glioblastoma -or medulloblastoma-characteristics after injection of human adenovirus 12 (AD12) (Li & Langhans, 2021; K. Ogawa et al., 1969).
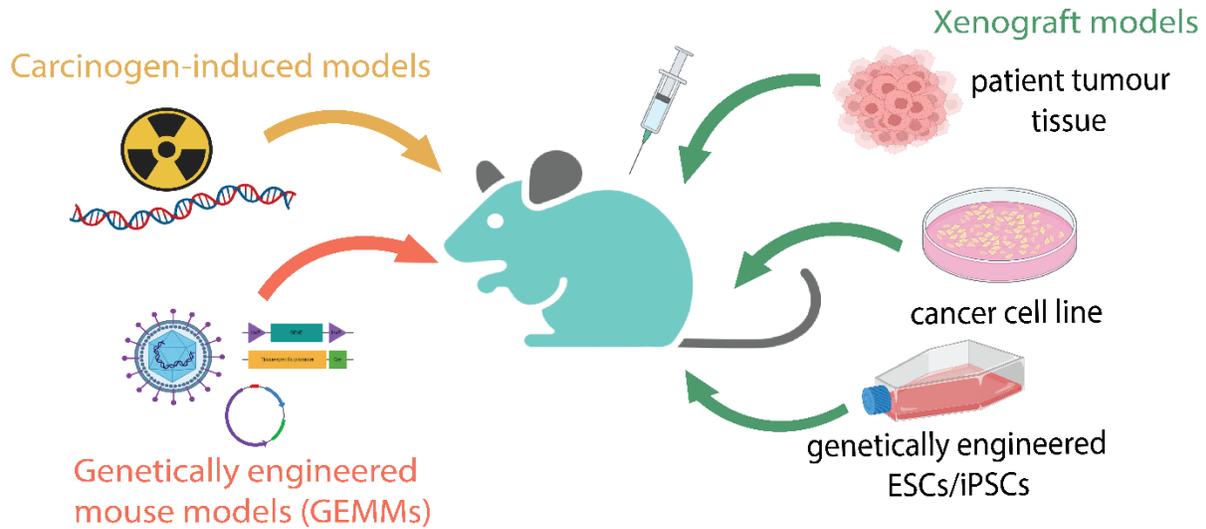
Genetically Engineered Mouse models (GEMMs) include transgenic and knockout mice, which can be 'global', meaning the oncogenic mutation is present in all tissues, or 'conditional', meaning the mutation is induced in a tissue-specific or time-specific manner (Li & Langhans, 2021). In both cases, the oncogenic mutation in introduced in zygotes or embryonic stem cells (ESCs). Transgenic mice are generated using the zygote technique by microinjection of the DNA transgene into the pronucleus (pronuclear injection technique; PNI) of fertilised eggs and these eggs are positioned into the oviducts of surrogate pseudopregnant female mice (Gordon et al., 1980; Liu et al., 2013). Generating knockout mice using the PNI technique in combination with the CRISPR-Cas9 system is also the preferred method, compared to the previous ESC-based approach. This approach includes 'deleting' a gene by inserting a drug selection sequence into the coding region, selecting for the ESC with the 'deleted' gene and subsequently introducing them into mouse blastocysts. Mice generated with this technique were chimeric, and breeding them with wild-type mice would lead to knockout mice among the offspring (Capecchi, 2005). Several methods are available to generate conditional knockout and transgenic mice, such as the Cre-loxP system, where Cre recombinase is expressed under a tissue-specific promoter and deletes the DNA fragment positioned between two loxP sites. Other widely known systems include the Tet-On system, RCAS/TVA system, CRISPR/Cas9 technology and the PiggyBac and Sleeping Beauty transposon systems. Already existing GEMMs of Glioblastoma multiforme (not distinguished between adult or pediatric) exhibit mutations observed in patient tumours, including p53, Cdkn2A, Nf1, Pten, Ras pathway mutations and EGFR, among others (Li & Langhans, 2021). Finally, GEMMs do not disrupt the native immune system of the mouse model (Simeonova & Huillard, 2014) and they are useful to study the onset of GBM, however since the tumor is from murine descendent, translating these studies and potential therapies to the human is more complicated.

This limitation can be overcome by mouse models with human tumors. Mouse xenografts are models generated from the transplantation of cell lines, patient tissue or genetically engineered stem cells (neural stem cells or induced pluripotent stem cells) into a host mouse (Akter et al., 2021; Li & Langhans, 2021). Established glioblastoma cell lines, either mouse or human, can be transplanted to a host mouse and generate an allograft or xenograft, respectively. In the case of human cancer cell line transplantation, the host mouse needs to be immunocompromised. While tumours developed from cancer cell line transplantation in mice have been observed to lose the original tumour phenotypic characteristics overtime, patient-derived xenograft models (PDX) are known to retain these characteristics and therefore recapitulate the *in-vivo* tumour more accurately and provide a more reliable response to drugs (Day et al., 2015). PDX models can be generated by transplanting patient tissue in mice directly or after *in-vitro* culture of tumourigenic cells into neurospheres. Notably, 107 molecularly and phenotypically characterised patient-derived xenograft (PDX) models for pediatric glioblastoma have been developed by the Mayo Clinic Brain Tumour PDX National Resource, of which 105 were IDH-wild type and 3 were IDH-mutant samples (WHO 2016). Finally, xenograft mouse models can be generated by the transplantation of neural stem cells, genetically engineered to express GBM-driving mutations (Bachoo et al., 2002; Robertson et al., 2019), or neural stem cells generated by induced pluripotent stem cells, which harbour the patient mutational profile. The latter was reported to have a successful outcome in modelling SHH-medulloblastoma (Susanto et al., 2020). Mouse xenografts, generated with the available techniques, have many advantages; however, they usually present clonal outgrowth and demand immunocompromised mice for the tumour progression to be successful (Li & Langhans, 2021; van der Heijden et al., 2019).

Since the immune system plays a major role in glioma and GBM progression, it is essential to identify to what extent each type of *in-vivo* mouse model can mimic the tumour immune microenvironment and identify which immunotherapy options can be accurately tested on them. In chemically induced and genetically engineered mouse tumour models, in the case that the genetic manipulation does not lead to a lethal phenotype (usually occurs in 'global' transgenic or knockout models), the host immune system is intact and no surgical transplantation occurs (Fig. 2); therefore the TIME is, in principal, not disrupted (Hetze et al., 2021). However, chemically induced models are not ideal for immunotherapy testing, as the tumour mutational burden is high and the antigens expressed are different from the ones in human tumours, complicating the assessment of the immune response. Another critical factor shared between this model and

GEMMs is that the mouse immune system is different from the patient's (Fig. 2). As a result, immunotherapy types involving human patient cells, like DC vaccines, cannot be applied. However, GEMMs have been used for testing mouse TAA CAR-T cells to identify whether they exhibit on-target off-tumor toxicity (Pennell et al., 2018). The different immune system between human and mouse is also the reason why syngeneic mouse models are not translationally applicable in immunotherapy, except for testing mouse CAR-T cells and checkpoint inhibitors. In syngeneic mouse models, the cells from the tumour induced on a mouse model are introduced to an immunocompetent mouse of the same strain. Finally, regarding xenograft models, human glioblastoma cells are transplanted in an immunocompromised host, leaving the mouse immune microenvironment lacking significant components to trigger an immune response. Recently, humanised mouse models -mice with a human immune system- have been developed by transplanting human hematopoietic stem cells (HSCs; Fig. 2). While humanised xenograft models are expected to have better TIME recapitulation, some parameters must be considered. To avoid unwanted immune response in PDX models, the HSCs used to generate the humanised models and the tumour cells injected need to be from the same patient. Additionally, to depict the specific immune environment more accurately, injecting the cells orthotopically -in the original tumour site, here the brain- is preferred to injecting them in an unrelated site (heterotopically) (Hetze et al., 2021). As in humanised models human T, B, NK and dendritic cells are present in the circulation, these models can be manipulated to test all types of immunotherapy, including cancer vaccines, adoptive cell therapy and immune checkpoint inhibitors (Q. Chen et al., 2019). To take this approach even further, recently, human microglia derived from stem cells were successfully transplanted into immunocompromised mice (Mancuso et al., 2019). Since microglia play an important role as a component of the TIME in glioblastoma, combining these two protocols to generate mice with both humanised immune system and human microglia would be an ideal future step for glioblastoma modelling. However, several limitations still apply due to mouse and human physiological differences and the time needed to humanise the immune system of the host mouse.

In vivo mouse models

	Models	De novo carcinogenesis	Favourable characteristics towards GBM immunotherapy testing	Unfavourable characteristics towards GBM immunotherapy testing
In-vivo mouse models	**Carcinogen-induced**	Yes	• Intact immune system • Stability in tumour characteristics	• Usually histologically and mutationally different from human tumours
	Xenografts	No	• We can introduce a humanized immune system • Human stem cell-derived microglia can be introduced	• Requires immunodeficient mice (however, this can be addressed thanks to humanized mice)
	Allograft cell-line transplantation	No	• Intact immune system	• The tumour is of murine origin • Murine immune system
	Genetically engineered mouse models (GEMMs)	Yes	• More accurate tumour molecular recapitulation • Intact immune system	• The tumour is of murine origin • Murine immune system

Figure 2. Schematic presentation of common preclinical in-vivo mouse models (above) and advantages and disadvantages of these models towards GBM immunotherapy testing (below).

In carcinogen-induced models, the tumour is generated by chemical carcinogens or viruses. For the generation of transgenic and knockout genetically engineered mouse models, the tumour is induced through oncogene expression or tumour suppressor gene deletion. Other mouse models include xenografts. In xenograft models, cancer cell lines, patient tumour tissue or genetically engineered stem cells (neural stem cells or induced pluripotent stem cells) carrying the tumour mutations are transplanted into a host mouse. Created with BioRender (accessed on the 12th of December 2022).

Cancer cell lines

Pediatric glioblastoma cancer cell lines are *in-vitro* models conventionally derived from primary 2D cell cultures of pediatric glioblastoma samples from patients or animal models. Cancer cell lines can be grown in the lab and are expected to retain the original tumour characteristics (Fig. 3). Many cell lines have been generated from pediatric GBM patients. They can be used for drug testing and studying tumour characteristics, however, repeated passaging causes significant changes in the phenotypic and genotypic characteristics of the cells, thus influencing the accuracy of the model (Paolillo et al., 2021). In addition, 2D cell cultures maintained as a monolayer are not heterogeneous cell populations and have uniform access to nutrients and oxygen compared to tumour cells *in-vivo* (Fig. 3; Li & Langhans, 2021).

Cancer cell line models do not incorporate an immunological component, so testing immunotherapy is challenging. Generally, the contribution of cancer cell lines to immunotherapy research is mainly related to them being injected in mice to generate xenograft models or to identify novel TSAs and TAAs for the development of targeted immunotherapy (Okada et al., 2022). Recently, immune cell co-culture with cancer cell lines has been applied to model immunosuppression in breast cancer (Zheng et al., 2019, p. 1). Additionally, a method has been developed which could be employed to assess immunotherapy options quantitively. This model includes co-culturing lymphocytes and monocytes along with the cancer cells to identify changes in cytotoxicity. Despite the successful outcome when testing anti-PD-1 treatment in PC-3 prostate cancer cells, accurate modelling of the TIME is not achieved (Cerignoli et al., 2018). Therefore, co-culturing methods of immune cells with cancer cell lines are slowly discarded as a tool for translational immunotherapy testing and are mostly restricted to the early stages of the development of immunotherapeutic options (Mackenzie et al., 2022).

3D cultures

As monolayer cultures of cancer cell lines have showcased some weak points in modelling tumours and their microenvironment, 3D multicellular cultures have been developed to address current limitations. These models can be developed with multiple methods, some of which have also been applied to model pediatric GBM. Dissociation of tumour tissue from the patient led to the formation of 3D clusters of cancer stem cells, named 'tumoroids' or 'tumor spheres' (Boucherit et

al., 2020; Caragher et al., 2019) and short-term cultures called 'spheroids'. Spheroids can be generated from cancer cell lines and patient tumours (Stanković et al., 2021). While spheroids did not only include cancer stem cells, but also other tumour cell types, they were not able to recapitulate the tissue architecture and include the representation of various cell types. This was accomplished by 3D models called 'organoids'. Despite these two terms being used arbitrarily at times, depending on the author, spheroids are primarily referring to simple 3D cultures, in comparison to the more complex organoids, which can also be cultured long-term (Boucherit et al., 2020).

Some organoids can self-organise into 3D cultures without the need of a scaffold to recapitulate the extracellular matrix (ECM), while others need scaffolds, like Matrigel, to surround and support the cells *in-vitro* (Kaur et al., 2021; Li & Langhans, 2021). The ECM also regulates proliferation, differentiation, migration and stemness due to connections of stem cells to integrins (Cooper & Giancotti, 2019; Frantz et al., 2010). Scaffolds can be generated using natural (collagen I, Matrigel, e.t.c.) or synthetic (PEG, MAX-8 β hairpin hydrogel, e.t.c) hydrogels (Li & Langhans, 2021) and organoids can be encapsulated in them either after their formation or in the form of cell suspension (Orcheston-Findlay et al., 2021). Apart from hydrogel scaffolds, which are microporous, highly water-absorbing materials, there are also fibrous and porous scaffolds -named after the network structure of the scaffold (Cha & Kim, 2017; Stanković et al., 2021). The cells in 3D models are polarised, having a basal and an apical pole and therefore showcasing differences in expression, more representative of the *in-vivo* situation (Boucherit et al., 2020), compared to 2D cell cultures. 3D model applications for immunotherapy testing will be discussed at the end of this part.

Organoids

Organoids are organ-like 3D cultures, which can be manipulated to study pediatric brain tumours as they better represent the cellular heterogeneity of the tissue (Andreatta et al., 2020). Both healthy and cancer organoids can be patient-derived or generated from embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs). Healthy patient-derived organoids can be generated using specialized media supplemented with significant growth factors, which retains adult stem cells. In the same concept, primary patient or xenograft tumor tissue can be manipulated to form patient-derived tumour organoids (PDTOs) (Drost & Clevers, 2017), by retaining the cancer stem cells. Glioblastoma organoids (GBOs) have been developed from patient tumors and they were shown

to mimic histological features, diverse cell types, mutations and gene expression of the parental tumors (Jacob et al., 2020).

On the other hand, a tumour can be recapitulated in organoids, by generating healthy ESC or iPSC-derived organoids and then manipulating them to form tumors. As pediatric GBM arises mostly supratentorially in the cerebral hemispheres, cerebral organoids are generated to be used as platforms for tumor formation. To generate cerebral organoids, the differentiation of stem cells is guided towards the neuroectodermal lineage (Kim et al., 2020). Cerebral organoids display ventricles, neural rosettes, neural stem cell expression and cell types of the developing brain, including neuron and astrocyte progenitors (Lancaster & Knoblich, 2014; Orcheston-Findlay et al., 2021). Therefore this platform is expected to include astrocytes and other glial progenitors, which according to the current theory are proposed to be the cell-of-origin of adult and pediatric GBM (Suri et al., 2009). Healthy cerebral organoids can be genetically manipulated to form tumours by introducing oncogenes through vectors or using gene editing techniques (Ballabio et al., 2020). Alternatively, patient-derived brain tumour cell lines can be introduced into healthy cerebral organoids and lead to *in-vitro* tumour formation using co-culture methods or via injection (Linkous et al., 2019; J. Ogawa et al., 2018). These organoid models recapitulate the mature organ structure, and include representation of multiple cell types and their interactions (Kim et al., 2020).

All in all, cancer organoids are a very compelling *in-vitro* 3D model for studying brain tumors and testing therapy. The ability to be cultured for a more extended time period than typical spheroids, as well as their recapitulation of the brain niche and accurate representation of tumour cell heterogeneity and expression, are reasons that render organoids a valuable model to recapitulate brain tumours *in-vitro* and test potential therapeutic options (Fig. 3; Langhans, 2018). Additionally, it is significant to point out that they can be cultured in high-throughput screening microplates, allowing for better drug screenings' visualization (Fan et al., 2019).

However, cancer organoid models also present some shortcomings. They cannot represent all of the cell subtypes of the *in-vivo* cerebrum and neuronal markers are often co-express by multiple cells. This could be a disadvantage for modelling other brain tumors, however the potential cell-of-origin of glioblastoma is expected to be present. Stress pathways are also activated in cerebral organoids generated with all known protocols, which might affect gene expression, response to drug treatments e.t.c (Fig. 3; Bhaduri et al., 2020). In addition, organoids do not incorporate a vasculature or factors of the TIME, in contrast to some *in-vivo* models. To address these

limitations, organoids can be positioned in microfluidic chips and form 'organs-on-a-chip', which have the additional benefit of a continuous culture with medium perfusion to mimic blood flow and can be supplemented with cells of the immune system, as will be discussed later (Fig. 3; Boucherit et al., 2020; Lovett et al., 2020; Sontheimer-Phelps et al., 2019). Organs-on-a-chip are very promising models, however they are still in the early stages of their development. Additionally. recent technological advances in 3D bioprinting have enabled the development of highly complex 3D cultures with precise control of the scaffold stiffness, porosity e.t.c. These scaffolds include different cell types and ECM characteristics to accurately recapitulate the tumour microenvironment. In bioreactors, we can also control other conditions of the culture, such as temperature, nutrient supply, oxygen and CO_2 concentration, pH, shear stress, e.t.c. (Stanković et al., 2021). These models take the modelling method one step further to include the ECM and develop tumours, more similar architecture- and expression-wise to the patient tumours.

3D model co-culture systems to study the TME and TIME of GBM and test immunotherapy: food for thought from adult GBM

3D models of GBM can be helpful in testing specific types of immunotherapies, depending on the aim of the testing, even without adding components of the immune system. Specifically, CAR-T cells targeting EGFRvIII antigen have been tested on patient-derived adult GBM organoid models by co-culture to identify EGFRvIII antigen loss, invasion of the tumour by T cells and tumour cell death with multiple assays (Jacob et al., 2020). Such techniques can be used for pediatric GBM as well, in order to test the efficacy of CAR-T cell therapy or other adoptive T cell immunotherapy options.

As GBM is known to interact with many stromal cells -components of the tumour microenvironment- incorporating them in models would make the study and testing of immunotherapeutic options more accurate, not to mention that immunotherapy is in most cases responsible for instigating an immune response against tumour antigens. Therefore, approaches include co-culturing 3D adult GBM models with endothelial cells , astrocytes and mesenchymal stem cells (Avci et al., 2015; Breznik et al., 2017; Grodecki et al., 2015). Regarding the immune components of the tumour microenvironment, it has been noted that pediatric GBM differs from adult GBM.

Ignoring the differences between adult and pediatric GBM, therapeutic approaches to the adult tumour have long been proposed for children, in many cases showcasing low efficiency, as the TIME of pediatric brain tumours has not sufficiently been researched (Ross, Velazquez Vega, et al., 2021). Pediatric GBM and high-grade-gliomas (pHGG) have a primarily immunosuppressive microenvironment, exhibiting low infiltration of the tumour by myeloid cells, CD4+ and CD8+ T cells and PD-1+ cells. However, despite low infiltration of the tumour by T cells, pHGG and pGBM seem to be recruiting tumour-associated macrophages (TAMs) and microglia to the tumour site (Engler et al., 2012; Lieberman et al., 2019; Ross, Chen, et al., 2021; Ross, Velazquez Vega, et al., 2021). As a result, pediatric GBM models incorporating microglia and macrophages would be more representative of the TIME and ideal for immunotherapy testing.

Although such models have not yet been published for this particular pediatric tumour type, we discuss known co-culture methods of 3D adult GBM or other gliomas with immune cells correspondingly, focusing on microglia and macrophages as representative of the TIME. Such models include co-cultures of patient-derived neurospheres (from glioma-initiating stem cells) with microglia (Wei et al., 2019) and a mixture of GBM and microglia co-cultured in a scaffold of hyaluronic acid-gelatin hydrogel (Leite et al., 2020). The latter was also used for drug testing and is proposed to have applications in clinical testing (Leite et al., 2020). Additionally, another 3D model of a matrix chamber embedded with a glioma and macrophage/microglia co-culture (Coniglio et al., 2016) was recently established. Apart from co-culture methods, tri-culture of GBM, endothelial and immune cells (mainly macrophages and microglia) has been successful in a microfluidic organoid model to study the interactions between these cell types as well as angiogenesis (Cui et al., 2018). Furthermore, a healthy brain organoid model containing microglia has been developed by co-culturing primitive neural precursor cells (pNPC) -derived from human pluripotent stem cells (hPSC)- and primitive macrophage progenitors (PMPs) (R. Xu et al., 2021). This model could be manipulated to generate pediatric GBM tumours with a controllable microglial ratio. Finally, newly developed patient-derived micro-organospheres are able to retain most of the original immune cells from the tumor biopsy and the immunosuppressive environment. This automatic microfluidics platform is able to retain these cells in the Matrigel droplet, allowing them to penetrate the organoid and has proven successful as a potential potency assay for adoptive T cell therapy (Ding et al., 2022). We propose that employing similar techniques, as presented for the models mentioned above, could lead to pediatric GBM 3D co-culture models, to efficiently

test immunotherapy and study immunotherapy-induced tumour-immune interactions in pediatric GBM.

All in all, it is imperative to discuss current immunotherapeutic types that could be tested using *in-vitro* models. So far, cancer vaccine testing cannot be recapitulated *in-vitro*; combining patient-derived organoids with humanised mice could be one future approach (Shelton et al., 2021). Regarding the rest of the immunotherapy types, adoptive T-cell therapy (Dijkstra et al., 2018), immune checkpoint inhibitors (Neal et al., 2018) and oncolytic virotherapy (Hamdan et al., 2021) methods have been tested in complex immune-organoid cancer models so far, not only to validate efficacy but also to instigate more effective anticancer immune responses (Wang et al., 2022). Despite such testing not having been applied in pGBM, there is a lot of potential for the testing of immunotherapeutic options of these types in pediatric GBM models with TIME representation in the future.

In vitro models

2D cancer cell line Spheroid Organoid Scaffold-supported organoid Organ-on-a-chip

	Models	De novo carcinogenesis	Favourable characteristics towards GBM immunotherapy testing	Unfavourable characteristics towards GBM immunotherapy testing
In-vitro models	**Cancer cell lines**	Yes	• Retain original tumour characteristics	• Non-heterogeneous cell population • Do not incorporate vasculature • Not an organ-like structure • Passaging causes changes in cells' phenotypic characteristics • Co-cultures with immune cells did not accurately recapitulate the TIME
	Spheroid cultures	Yes (when they are patient-derived) and No (when the tumour is introduced with genetic manipulation or NSC/iPSC cell transplantation)	• Retain original tumour characteristics • Co-cultures with immune cells can mimic the TIME	• Short-term culture • Do not incorporate vasculature • Not an organ-like structure
	Cerebral organoids		• Long-term culture can be achieved • Cellular heterogeneity is represented • GBM cell-of-origin is present • 3D tumour architecture similar to *in-vivo* tumour • Co-cultures with immune cells can mimic the TIME • High-throughput screening is possible	• Do not incorporate vasculature • Batch-to-batch variability • Neuronal markers are often co-express by multiple cells • Stress pathways are activated
	Organs-on-a-chip		• Organoid characteristics apply • Mimic blood flow • Incorporate vasculature • Co-cultures with immune cells can mimic the *in-vivo* TIME • Can integrate organotypic models for maximal tissue organization	• Surface effects

Figure 3. Schematic presentation of common preclinical in-vitro models (above) and advantages and disadvantages of these models towards GBM immunotherapy testing (below).

In-vitro cancer models include 2D cancer cell lines derived from patient tumours or mouse models and 3D models, either scaffold-supported or scaffold-free. Cancer spheroids are simple short-term cultures, while more complex long-term cultures are called organoid models. These can mimic the in-vivo tumour 3D architecture and might need the addition of a scaffold to allow growth and further recapitulation of ECM characteristics. For the generation of organs-on-a-chip, cancer spheroids and organoids can be positioned in microfluidic chips, which further allow fluid flow recapitulation and co-cultures with immune cells. Created with BioRender (accessed on the 12th of December 2022).

Successful immunotherapy crossing of the Blood-brain-barrier can be assessed with in-vitro Blood-brain-barrier models
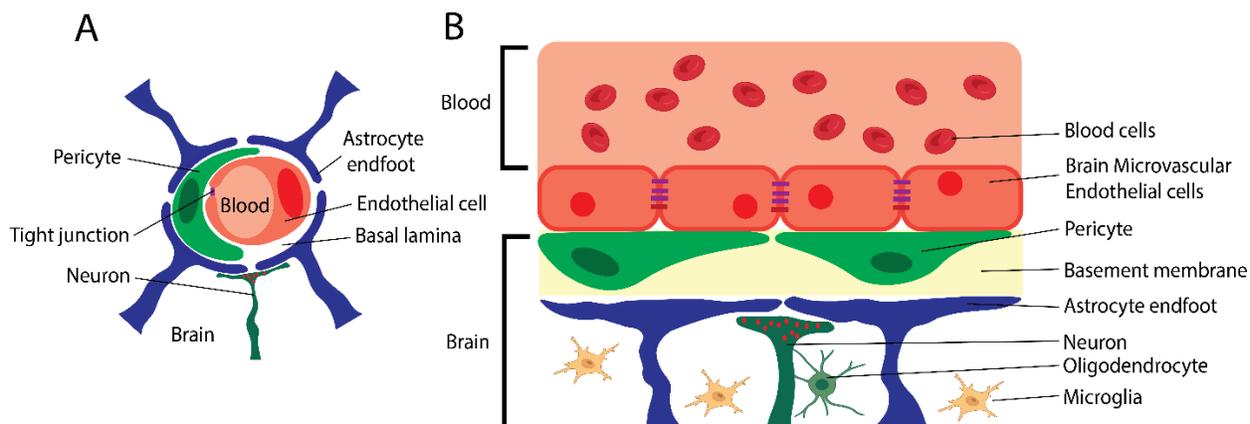

Figure 4. Schematic representation of the Blood-Brain-Barrier (BBB).

Horizontal (**A**) and vertical (**B**) cross-section of the BBB representing the anatomy of this histological barrier and supportive cell types. Brain microvascular endothelial cells (BMEC; endothelial cells) are connected with tight junctions forming the lining of the capillaries of the brain. These endothelial cells interact with multiple cell types in their surroundings, including pericytes and astrocytic endfeet -incorporated in a basal membrane-, neurons, oligodendrocytes, microglia, e.t.c. Functions of the BBB are fulfilled by these cells and their interactions. Created with BioRender (accessed on the 23rd of November 2022).

The Blood-brain-barrier (BBB) and the neurovascular unit consist of physical components (Fig. 4), forming a semipermeable structure on the walls of capillaries, which maintains the homeostasis of the brain and controls the supply of factors (Wasielewska et al., 2020). The BBB consists of a lining of endothelial cells, called the Brain Microvascular Endothelial cells (BMEC), closely

connected to pericytes, extracellular matrix elements and the end-feet processes of astrocytes (Fig. 4). Other cell types, like neurons, microglia and oligodendrocytes lie in close proximity and along with the other components regulate BBB functions and properties (Bhalerao et al., 2020; Williams-Medina et al., 2021). BMECs are connected with tight and adherent junctions, proteins which determine the permeability and electrical resistance of the BBB (trans-endothelial electrical resistance; TEER), the latter being kept in humans between 1500-8000 Ωcm^2 (Aday et al., 2016). In addition, BMECs recruit multiple specific transporter proteins to enable or prevent the entry of nutrients, waste and other components (Aday et al., 2016; Sweeney et al., 2018). Specifically, the BBB controls access to the brain by selectively blocking molecules larger than 400 Daltons from entering the cerebrospinal fluid (CSF), keeping it intact from bacteria, viruses, and potentially hazardous molecules (Upton et al., 2022). It simultaneously allows access to nutrients, ions, plasma macromolecules, and other essential molecules (Bhalerao et al., 2020) while permitting the exit of toxins and metabolism waste (Chow & Gu, 2015). Consequently, for a drug or immunotherapy to access the tumour bulk, its molecular size and lipophilicity must allow it to cross the BBB and remain active and in sufficient concentration to exercise its therapeutic effect (Upton et al., 2022). As for the delivery of immune cells through the BBB, the CNS is -to a certain degree- considered an 'immune privileged' organ for multiple reasons: Immune cells can cross the BBB selectively, T cells can enter the brain through the CSF, following their activation in the cervical lymph nodes, and microglia - the immune cells of the brain - are responsible for antigen presentation in the CNS. In addition, the lymphatic system does not participate in the immune response (B. Huang et al., 2021). Therefore, not only the ability of immunotherapy to cross the BBB but also the fact that selected immune cells can enter the brain are valuable tools for developing immunotherapeutic strategies. However, it is important to point out that in some cases, immunotherapy might not be necessary to possess BBB-crossing characteristics, as it can be delivered to the tumour area with other methods, bypassing the BBB. Such approaches include intrathecal administration (delivering therapeutics directly into the cerebrospinal fluid of the spinal cord) and intraventricular administration (delivering directly into the ventricular CSF). Other available methods are intranasal (via the olfactory and trigeminal nerve route), intratumoral and intracavitary delivery, and others (Li & Langhans, 2021). This literature review does not discuss to what extent these methods of bypassing the BBB are successful in delivering immunotherapy to pediatric GBM tumours.

The Blood-brain-barrier (BBB) is known to control the transport of drugs and other factors to the brain; however, brain tumours can alter its physical properties, forming the Blood-tumor-barrier (BTB). When a brain tumour-occurs, BBB characteristics, like permeability, efflux and effective drug concentration, are usually compromised and exhibit non-uniform values. Therefore, in GBM and other brain tumours, the compromised BBB is renamed into blood-tumour-barrier (BTB) (Stanković et al., 2021). Increased barrier permeability in these tumours facilitates GBM cell migration, directly affecting metastasis and tumour progression (Jia et al., 2014).

To model BBB and BTB characteristics in the lab for drug and immunotherapy testing before proceeding to patient testing, we need preclinical *in-vitro* models. *In-vivo* mouse models are not as efficient since species differences occur. Despite mouse BBB having the same cell types as human, differences in morphology, architecture, the function of transporters, and gene expression have been identified, leading to altered drug responses (O'Brown et al., 2018). Different *in-vitro* models have been developed to overcome this, providing more accurate and controllable responses. Current guidelines for immunotherapy mention that it should ideally begin after five years of age (Finegold, 2007), while in some instances, even after the first year of age (Ragoonanan et al., 2021). While not enough research has been targeted towards comparing the adult and pediatric BBB in terms of permeability and other characteristics, in-vitro BBB systems have been used to test both adult and pediatric drugs. The quality of these models can be assessed by measuring BBB parameters, such as markers of the BBB, TEER and permeability for specific substances (Wolff et al., 2015). For BBB *in-vitro* modelling, original Transwell 2D systems and co-cultures have been evolved into more dynamic 3D models like organs-on-a-chip with the introduction of microfluidic approaches and BBB organoids in order to more accurately recapitulate the structure, cell interactions and ECM of the BBB (Aday et al., 2016; Wasielewska et al., 2020; Williams-Medina et al., 2021).

2D Transswell static models

Cells are cultured *in-vitro* in widely used Transwell static 2D culture systems to recapitulate BBB function. Transwell inserts are permeable devices which allow easy measurement of TEER and permeability. As a result, they have been widely applicable in studying and testing drug delivery (Wasielewska et al., 2020). In Transwell 2D models, BMECs (or brain endothelial cells; BECs) are co-cultured with other BBB cell cultures. Secondary cultures include astrocytes, pericytes,

neurons or others (Stanković et al., 2021). BECs can be isolated directly from the brain (primary), from (immortalised) human BEC cell lines or generated from ESCs and iPSCs. In Transwell models, BECs are cultured on the luminal/blood side of the insert (the top), while secondary cultures can be grown on the abluminal/brain side (the surrounding) or at the bottom of the insert (Stanković et al., 2021). When grown on the abluminal side, they are in contact with the endothelial cells; therefore, the co-cultures are termed 'contact' co-cultures, compared to the 'non-contact' co-cultures, which are grown at the bottom of the plate (Wolff et al., 2015). Non-contact co-cultures were not as efficient at maintaining high TEER values and BBB characteristics as contact cultures, while permeability was solely dependent on the BEC monolayer (Al Ahmad et al., 2009; Nakagawa et al., 2007).

While they have been used for immunotherapy testing, Transwell systems are still characterized by certain limitations that led to the discovery of more complicated models. Regarding applications of these models for immunotherapy testing, while not applied for pediatric GBM yet, transwell models were used for the study of EGFRvIII CAR-T immunotherapy targetting U87MG human GBM. In the same study, microfluidic BBB-on-chip (SynBBB) - which will be discussed further - was also tested (J. Huang et al., 2022). However, Transwell 2D models exhibit some limitations regarding accurate modelling of the BBB; the cells are not exposed to the blood flow or shear stress, 2D morphology is not representative of the BBB 3D architecture, and TEER values are lower than the ones measured *in-vivo* (Williams-Medina et al., 2021). Therefore, rapidly-developing 3D models aim to recapitulate the complex BBB cellular structure and its interactions with the ECM and blood flow (Aday et al., 2016).

BBB-on-a-chip

To mimic conditions occurring *in-vivo* more accurately, dynamic BBB organ-on-a-chip models have been developed using microfluidic technology. 2D BBB-on-a-chip models could incorporate the additional factor of shear stress through fluid flow in BBB modelling and also allow for the integration of sensors for measuring TEER and other parameters (B. Zhang et al., 2018). These models were generated by co-culturing BBB cells in microfluidic chips with hydrogels of a thickness below 100 μm (T. D. Brown et al., 2019). 2D BBB-on-a-chip models were followed by 3D *in-vitro* BBB microfluidics models (or 3D BBB-on-a-chip). These models include a supporting matrix, inside which BBB cell types are cultured in contact. Better microenvironment modelling,

organ architecture and the ability to measure TEER, permeability and shear stress are some of the advances that these models exhibit, as well as the possibility of easier imaging (Chin & Goh, 2018).

Modelling the transport of drugs and -gradually- immunotherapy through the BBB has been promising using BBB-on-a-chip models (Adriani et al., 2017; Griep et al., 2013), despite remaining restrictions. For example, a recently developed microfluidic device by Xu et al. combined rat BMECs and astrocytes with a 3D ECM and fluid flow. U87 glioma cells co-culture with astrocytes in the same model resulted in the intermix of the cells in one layer, and additional anticancer drug testing was performed to identify the ability of known drugs to cross the BBB and to drive GBM cell apoptosis. The testing results identified that several drugs, which in cell cultures were proven cytotoxic against glioma cells, had low BBB permeability (H. Xu et al., 2016). Regarding immunotherapy testing, however, while no methods have been used for pediatric GBM, a microfluidic BBB-on-chip (SynBBB) was recently applied to study EGFRvIII CAR-T immunotherapy targeting U87MG human GBM (J. Huang et al., 2022). Still, despite their great potential, *in-vitro* BBB-on-a-chip models can still evolve to include patient-derived BECs from iPSCs in co-culture with other cells of the BBB.

BBB Organoids

BBB organoids are an alternative platform to study the BBB and test immunotherapeutic options. 3D BBB spheroids are multicellular structures of iPSC-derived BECs and other cell types in ultra-low attachment (ULA) plates (Cho et al., 2017), while a recent human cortex spheroid model is known to have incorporated BECs, pericytes, astrocytes, oligodendrocytes, neurons and microglia (Nzou et al., 2018). Several *in-vitro* characteristics have been adopted in these models, despite lacking a microfluidic device for vessel formation and shear stress generation (Wasielewska et al., 2020). These include cell interactions and more *in-vivo*-like junctions in both spheroid models, with additional high BBB-marker expression and long-term culture survival in the cortical spheroid/organoid (Cho et al., 2017; Nzou et al., 2018). Finally, combining organoids and microfluidics might be the ideal future step to include all *in-vivo* BBB characteristics in one *in-vitro* patient-specific model system. Multilevel *in-vitro* immunotherapy testing would then be available for identifying both the permeability of the BBB for it, as well as the ability of immunotherapy to kill tumour cells and interact with the TIME of pediatric GBM.

Conclusion and future perspectives

Present-day immunotherapy promises to overcome limitations of conventional treatment options by being an arrow in the quiver of combination therapy for pediatric glioblastoma. Current immunotherapeutic treatment options include cancer vaccines, adoptive T-cell therapy, oncolytic virotherapy and checkpoint inhibitor therapy. To assess immunotherapeutic applications we need to use preclinical models for testing how the immune system responds to the treatment and if immunotherapy can cross the BBB and reach the tumour site. Both *in-vivo* and *in-vitro* approaches exhibit characteristics that would make them suitable for immunotherapy testing, however the best option currently available seems to be 3D *in-vitro* organoid models.

The more promising *in-vivo* mouse model approach for this multiple testing is humanised mouse xenograft models, which can recapitulate the tumour, including the vasculature and human immune cells, for studying immune responses. However, no humanized xenograft models have been generated for pediatric GBM yet and therefore assessing the accuracy of this model, as well as results from immunotherapy testing is not possible. Regarding the BBB, translating results generated in the mouse BBB to results useful for the human BBB is still challenging. As a result, immunotherapy testing can only be performed in these models using other techniques of delivering immunotherapy to the tumour site, excluding intravenous administration. In principle, all known types of immunotherapy could be tested in these *in-vivo* models; nevertheless, further understanding of these models, the immune microenvironment and comparison with human tumours is necessary.

In-vitro 3D organoid models exhibit multiple advantages compared to 2D and *in-vivo* models; they can better mimic tumour heterogeneity, architecture, and ECM interactions. Future perspectives for *in-vitro* 3D organoid pediatric GBM models involve incorporating microfluidics and 3D bioprinting techniques to take these models one step further and form organ-on-a-chip models. These can include co-cultures of multiple cell types -including immune cells-, fluid flow, and vasculature and allow for testing of BBB and BTB drug permeability. While not developed for pediatric GBM immunotherapy testing yet, these models are expected to enable testing of adoptive T cell therapy, immune checkpoint inhibitors and oncolytic virotherapy in the future. In addition, to allow high-throughput screening, they should be coupled with real-time tumour monitoring techniques, such as MRI and MALDI imaging (Stanković et al., 2021).

As immunotherapy options for pediatric GBM are still lacking and continue to be based on adult GBM, further research into the TIME of pediatric GBM is necessary. In parallel, 3D in-vitro organoid models are likely the way to go for immunotherapy testing so far, with future steps leading towards micro-organospheres for pediatric GBM, which incorporate the immune microenvironment from patient material in their matrix. From testing adoptive T cell therapy and immune checkpoint inhibitors to hopefully even cancer vaccines and oncolytic virotherapy within few weeks after the biopsy is taken, these organoid models are a promising innovation to achieve patient-specific combination therapy for better pediatric GBM treatment.

Acknowledgements

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