Immunological aspects of intratumoral chemotherapy and immunotherapy in malignant brain tumors

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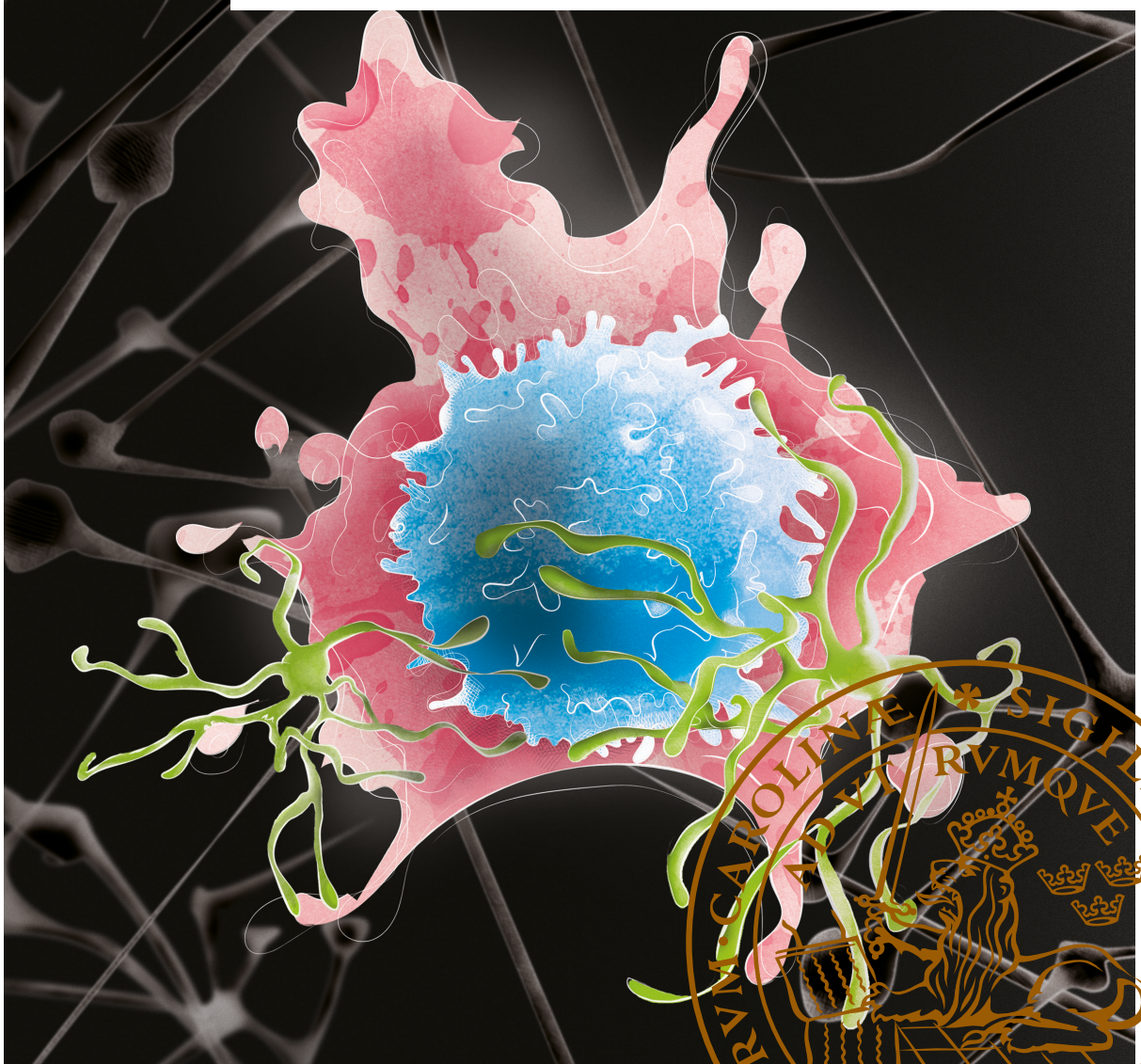

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Advances in surgery, chemo- and radiotherapy have only modestly improved survival rates of malignant brain tumor patients during the last decades. Emerging evidence suggests that an efficient treatment of malignant brain tumors will likely require the management of multiple aspects of tumor pathobiology in order to manipulate features as tumor heterogeneity and tumor immunosuppression. Immunotherapy based on peripheral vaccination of autologous tumor cells target both dividing and non-dividing tumor cells and lead to immunological memory. Moreover, intratumoral administration of chemotherapeutic drugs, also referred to as convection-enhanced delivery (CED), is a technique used to circumvent the blood-brain barrier (BBB) and increase the drug distribution within the tumor, while reducing the systemic side effects associated with systemically delivered chemotherapeutic drugs.

In this doctoral thesis, I propose intratumoral delivery of cytostatic drugs and immunotherapy as combined tools to treat malignant brain tumors. Thus, the treatment efficacy and the immune-related mechanisms of CED of clinically relevant cytostatic drugs and immunotherapy were investigated in glioma mouse models. CED of temozolomide (CED-TMZ) cured GL261-bearing mice and acted synergistically with wildtype cell immunizations. In addition, CED-TMZ was more effective and less toxic than single intratumoral injections of TMZ in the GL261 model. CED-TMZ prolonged survival in KR158-bearing mice but cure was only achieved with immunotherapy as a monotherapy and in combination with CED-TMZ. The immune dependence of the therapeutic effect of CED-TMZ was confirmed in immunocompromised mice bearing GL261. Infiltration of CD8+ and CD4+ T cells was increased in both models after CED-TMZ and immunization. CED of cisplatin (CED-CIS) induced cure in the GL261 model. As for CED-TMZ, the effect of CED-CIS was abrogated in immunocompromised mice. However, cell immunizations did not have any additive effect with CED-CIS. CED of mitoxantrone cured both GL261- and SB28-bearing mice. In addition, plasma samples from pediatric brain tumor patients were immune-profiled using cytokine multiplex arrays. We identified two patient groups with distinct preoperative inflammatory cytokine profiles that could be used as peripheral biomarkers to help design, predict or monitor the response of immunotherapy.

Altogether, these results have important implications for the future development and implementation of locally administered cytostatic drugs and immunotherapy against malignant brain tumors.

Key words: immunotherapy, convection-enhanced delivery, chemotherapy, mouse experimental glioma.

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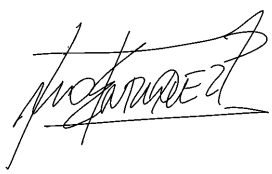
Signature  Date 2020-01-23

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To my family

Table of Contents

Abstract

Advances in surgery, chemo- and radiotherapy have only modestly improved survival rates of malignant brain tumor patients during the last decades. Emerging evidence suggests that an efficient treatment of malignant brain tumors will likely require the management of multiple aspects of tumor pathobiology in order to manipulate features as tumor heterogeneity and tumor immunosuppression. Immunotherapy based on peripheral vaccination of autologous tumor cells target both dividing and non-dividing tumor cells and lead to immunological memory. Moreover, intratumoral administration of chemotherapeutic drugs, also referred to as convection-enhanced delivery (CED), is a technique used to circumvent the blood-brain barrier (BBB) and increase the drug distribution within the tumor, while reducing the systemic side effects associated with systemically delivered chemotherapeutic drugs.

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Altogether, these results have important implications for the future development and implementation of locally administered cytostatic drugs and immunotherapy against malignant brain tumors.

Populärvetenskaplig sammanfattning

Hjärntumörer utgör omkring 2 procent av alla cancerdiagnoser och den vanligaste elakartade hjärntumörtypen är glioblastom (GBM). Patienter med GBM behandlas i dag med kirurgi, cellgifter och strålbehandling, men sjukdomen går sällan att bota och det krävs nya och mer effektiva behandlingsstrategier.

I min doktorsavhandling har jag använt tre olika musmodeller, kallade GL261, KR158 och SB28, för att utvärdera en kombinationsbehandling för patienter med GBM.

Behandlingen utgörs dels av cellgifter som ges direkt in i tumören via en läkemedelspump (kallat convection-enhanced delivery, CED, i avhandlingen), dels av ett tumörcellsvaccin vars syfte är att förmå kroppens eget immunförsvar att stöta bort tumören. Jag har undersökt effekten av tre olika cellgifter, temozolomid, cisplatin och mitoxantron, ensamt eller i kombination med tumörcellsvaccin.

CED av temozolomid, eller tumörcellsvaccin, botade en viss andel av möss med hjärntumören GL261, och vid kombinationsbehandling sågs en större effekt än den sammanlagda effekten av behandlingarna var för sig. Möss med den mer aggressiva hjärntumören KR158 botades inte av enbart temozolomid, men en viss andel kunde botas med tumörcellsvaccin. Vidare visade jag att CED av temozolomid var ett mer effektivt och mindre giftigt alternativ jämfört med att ge en injektion av samma mängd läkemedel vid ett enskilt tillfälle.

Även CED av cisplatin och mitoxantron botade en viss andel av möss med hjärntumören GL261, men till skillnad från temozolomid sågs med cisplatin ingen ytterligare effekt när behandlingen kombinerades med tumörcellsvaccin. Dock verkar både effekten av temozolomid och effekten av cisplatin vara kopplad till immunförsvaret, eftersom ingen behandlingseffekt sågs hos möss med ett dysfunktionellt immunförsvar. CED av mitoxantron hade även effekt hos möss med hjärntumören SB28, men inte KR158.

I avhandlingens sista del undersökte jag blodprover från patienter med olika typer av hjärntumörer, och kunde se att det finns viktiga skillnader i faktorer som är kopplade till immunförsvaret. Dessa faktorer skulle kunna användas som markörer för att visa hur samspelet mellan tumör och immunförsvar ser ut under en pågående behandling.

Sammanfattningsvis visar denna avhandling att CED av cellgifter, med eller utan tumörcellsvaccin, är en lovande behandlingsstrategi för patienter med GBM.

Resumen en español

Los tumores cerebrales representan aproximadamente el 2% de todos los diagnósticos de cáncer y el tumor cerebral maligno más común es el glioblastoma (GBM). El tratamiento de pacientes con GBM es con cirugía, quimioterapia y radioterapia, pese a ello, el pronóstico es desfavorable y todos los pacientes experimentan progresión tumoral con una mortalidad cercana al 100%. Por lo tanto, se necesitan nuevas y más efectivas estrategias de tratamiento.

En mi tesis doctoral, he usado tres modelos de GBM en ratones (GL261, KR158 y SB28) para evaluar el efecto terapéutico de dos terapias experimentales para pacientes con GBM. El tratamiento consiste en la administración de quimioterapia (temozolomida, cisplatino y mitoxantron) directamente dentro del tumor a través de una mini-bomba osmótica que transporta la droga (el procedimiento se llamada CED) y en vacunas compuestas de células tumorales atenuadas (llamada inmunoterapia) con el propósito de inducir una reacción inmune para eliminar el tumor.

CED de temozolomida e inmunoterapia curaron solo una proporción de ratones que portaban el tumor cerebral GL261, mientras la combinación demostró mejorar el efecto en comparación de los tratamientos solos. CED de temozolomida no tuvo ningún efecto en los ratones que portaban el tumor cerebral más agresivo KR158, pero cierta proporción de estos sí se curó solo con inmunoterapia. Además, se demostró que CED de temozolomida es una alternativa más efectiva y menos tóxica en comparación con una única inyección de la misma cantidad de medicamento. CED de cisplatino y de mitoxantron también curó una proporción de ratones con el tumor cerebral GL261, pero a diferencia de temozolomida, cisplatino no tuvo ningún efecto adicional cuando se combinó con inmunoterapia. Sin embargo, tanto el efecto de temozolomida como el efecto de cisplatino parecen estar relacionados con el sistema inmunitario, ya que no se observó ningún efecto terapéutico en ratones con el sistema inmunitario disfuncional. CED de mitoxantron también curó ratones con el tumor cerebral SB28, pero no con KR158.

Por último, muestras de sangre de pacientes pediátricos con diferentes tipos de tumores cerebrales se analizaron y se encontró que existen diferencias importantes en los factores relacionados con el sistema inmune. Estos factores podrían usarse como marcadores para evaluar la interacción entre el tumor y el sistema inmune antes, durante y después de estos tratamientos experimentales.

En resumen, esta tesis muestra que el CED con o sin inmunoterapia es una estrategia prometedora para el tratamiento de pacientes con GBM.

Original papers

This thesis is based on the following original papers:

Paper I. Convection-enhanced delivery of temozolomide and whole cell tumor immunizations in GL261 and KR158 experimental mouse gliomas.
Julio Enríquez Pérez, Jan Kopecky, Edward Visse, Anna Darabi, and Peter Siesjö.
BMC Cancer, 2020 Jan 3; 20(1):7–12.

Paper II. The effect of locally delivered cisplatin is dependent on an intact immune function in an experimental glioma model.
Julio Enríquez Pérez, Sara Fritzell, Jan Kopecky, Edward Visse, Anna Darabi, and Peter Siesjö.
Scientific Reports, 2019 Apr 4; 9(1):5632.

Paper III. Convection-enhanced delivery of mitoxantrone in GL261, KR158 and SB28 experimental mouse gliomas.
Julio Enríquez Pérez, Jan Kopecky, Edward Visse, Anna Darabi, and Peter Siesjö.
Manuscript.

Paper IV. Preoperative systemic levels of VEGFA, IL-7, IL-17A, and TNF-b delineate two distinct groups of children with brain tumors.
Emma Sandén, **Julio Enríquez Pérez**, Edward Visse, Marcel Kool, Helena Carén, Peter Siesjö, and Anna Darabi.
Pediatric Blood & Cancer, 2016 Dec; 63(12):2112–2122.

Related publications

Establishment and characterization of an orthotopic patient-derived Group 3 medulloblastoma model for preclinical drug evaluation.
Emma Sandén, Cecilia Dyberg, Cecilia Krona, Gabriel Gallo-Oller, Thale Kristin Olsen, **Julio Enríquez Pérez**, Malin Wickström, Atosa Estekizadeh, Marcel Kool, Edward Visse, Tomas Ekström, Peter Siesjö, John Inge Johnsen, and Anna Darabi.
Scientific Reports, 2017 Apr 18;7:46366.

Extracellular lipid loading augments hypoxic paracrine signaling and promotes glioma angiogenesis and macrophage infiltration.
Svenja Offer*, Julien Menard*, **Julio Enríquez Pérez***, Kelin Goncalves de Oliveira, Vineesh Indira Chandran, Maria C Johansson, Anna Bång-Rudenstam, Peter Siesjö, Anna Ebbesson, Ingrid Hedenfalk, Pia C Sundgren, Anna Darabi, and Mattias Belting. *Shared first authorship.
Journal of Experimental & Clinical Cancer Research, 2019 Jun 7;38(1):241.

Abbreviations

ACT	Adoptive cell therapy
AIC	5-aminoimidazole-4-carboxamide
APC	Antigen-presenting cells
ATP	Adenosine triphosphate
BBB	Blood-brain barrier
BCNU	Carmustine
CAR	Chimeric antigen receptor
CCL	Chemokine (C-C motif) ligand
CCR	C-C chemokine receptor
CED	Convection-enhanced delivery
CHI3L1	Chitinase-3-like protein 1
CIS	Cisplatin
CNS	Central nervous system
COX	Cyclooxygenase
CRT	Calreticulin
CSC	Cancer stem cell
CSF	Cerebrospinal fluid
CSF-1	Colony stimulating factor 1
CSFR	Colony stimulating factor receptor
CT	Computed tomography
CTL	Cytotoxic T lymphocyte
CTLA	Cytotoxic T lymphocyte-associated antigen
DAMP	Damage-associated molecular pattern
DC	Dendritic cells
DN	Double negative
DP	Double positive
ECM	Extracellular matrix
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Endoplasmic reticulum
GBM	Glioblastoma
GEMM	Genetically engineered mouse model
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GOF	Gain of function
HLA	human leukocyte antigen
HMGB1	High mobility group box 1
HSP	Heat shock proteins

ICD	Immunogenic cell death
ICI	Immune checkpoint inhibitor
IDH 1	Isocitrate dehydrogenase [NADP+]
IDO	Indoleamine 2,3 dioxygenase
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MAMP	Microbial-associated molecular pattern
MAPK	Mitogen-activated protein kinase
Mdm2	Murine double minute 2
MDSC	Myeloid-derived suppressor cell
MGMT	O6-methylguanine-DNA methyltransferase
MHC	Major histocompatibility complex
MRI	Magnetic resonance imaging
MTIC	5-(3-methyltriazen-1-yl)-imidazole-4-carboxamide
MTX	Mitoxantrone
MyD88	Myeloid differentiation factor 88
NF1	Neurofibromin 1
NFκB	Nuclear factor-κB
NK	Natural killer
NKG2D	Natural killer group 2 member D
NO	Nitric oxide
NS	Negative selection
PAMP	Pathogen-associated molecular pattern
PD1	Programmed death 1
PDGD	Platelet-derived growth factor
PDGFR	Platelet-derived growth factor receptor
PDL1	Programmed death ligand 1
PDX	Patient-derived xenograft model
PFS	Progression-free survival
PGE2	Prostaglandin E2
PI3K	Phosphoinositide 3-kinase
PLGA	Polylactide-co-glycolide copolymer
PRR	Pattern recognition receptor
PS	Positive selection
PTEN	Phosphatase and tensin homolog
RAGE	Receptor for advanced glycation end-products
Ras	Rat sarcoma
Rb	Retinoblastoma protein
rGBM	Recurrent glioblastoma

ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase
STAT	Signal transducer and activator of transcription
TAA	Tumor-associated antigen
TAM	Tumor-associated macrophage
T_{CM}	Central memory T
TCR	T cell receptor complex
T_{EM}	Effector memory T
TGF	Transforming growth factor
Th	T helper
TIL	Tumor-infiltrating lymphocyte
TLR	Toll-like receptor
TME	Tumor microenvironment
TMZ	Temozolomide
TNF	Tumor necrosis factor
Treg	Regulatory T cell
TSA	Tumor-specific antigen
VEGF	Vascular endothelial growth factor
WHO	World Health Organization

Introduction

Cancer

Cancer is a challenging disease for both physicians and patients. In 2017, 61 000 persons with cancer were registered in Sweden and it is estimated that one in every third person who lives in Sweden will get a cancer diagnosis during their life time [1, 2]. Cancer is a collective term that includes a group of diseases arising as a result of uncontrolled cellular growth. During cell division, the multiple DNA-repairing mechanisms are capable of repairing DNA damage, however, on certain occasions, the cell fails to repair the damage and normal cells eventually become "abnormal" or cancerous cells, leading to an uncontrolled cell division. The development and progression of cancer comprise a complex multiple-step process, illustrated by "The hallmarks of cancer" described by Weinberg and Hanahan in 2011 [3], see Table 1. A tumor is not only composed of cancer cells; the whole tumor microenvironment, including cancerous and non-malignant cells, such as immune, stromal, vascular cells, as well as soluble factors, are involved in a reciprocal communication supporting tumor progression. Hence, this thesis will focus on all of these aspects as well as on new potential treatments of glioblastoma.

Table 1.
The hallmarks of cancer. Adapted from Weinberg and Hanahan in 2011 [3].

Hallmarks of Cancer	
Tumor-promoting inflammation	Enabling replicative immortality
Avoiding immune destruction	Sustaining proliferative signaling
Resisting cell death	Deregulating cellular energetics
Evading growth suppressors	Inducing angiogenesis
Genome instability and mutations	Activating invasion and metastasis

Glioblastoma

Glioblastoma (GBM) (Fig. 1) is the most aggressive primary tumor in the central nervous system (CNS) of adults with a significant morbidity and invariable mortality. The latest advances in technology have improved the knowledge of the pathophysiology of GBM, which is mainly reflected by a new molecular classification and several novel experimental treatments.

Epidemiology and etiology

Primary brain tumors are classified according to the presumed cell of origin. Primary CNS tumors represent only 2% of all cancer diagnoses in adults in Sweden [1, 2]. Gliomas are tumors derived from glial cells and include astrocytic tumors (diffuse astrocytoma, anaplastic astrocytoma and GBM), oligodendrogliomas, ependymomas and mixed gliomas [4]. The global incidence rate of GBM is estimated to be less than 10 per 100 000 people and the incidence in Sweden was 1.2 per 100 000 habitants during the last 10 years [1, 2]. GBM accounts for approximately 80% of the gliomas and 14% of all brain tumors in adults. The most frequent location for GBM is the cerebral hemispheres and 95% of GBMs arise in the supratentorial region. Primary GBM can occur at any age but the median age for diagnosis is around 59 years and the incidence is higher in males than in females (1.58:1) [5].

Exposure to ionizing radiation and certain genetic syndromes are well-defined risk factors for development of GBM [6]. The time between radiation exposure and the development of GBM may range from years to decades [7]. However, there is no evidence of a link between the risk of developing GBM and routine exposure to diagnostic radiation, neither in children nor in adults [8]. Hereditary cancer syndromes, including Li-Fraumeni syndrome, neurofibromatosis type 1, tuberous sclerosis and Turcot's syndrome are associated with increased risk of GBM [9]. There are no identified carcinogenic factors that increase the risk of developing GBM; neither are there conclusive associations between GBM and environmental factors. The presence of allergic disorders and infections appear to be inversely associated with incidence of GBM [10]. Magnetic fields have been suggested as a risk factor but not proven for GBMs. Overall, until now, there is no firm manageable risk factor, no screening test or prevention concept available for GBM.

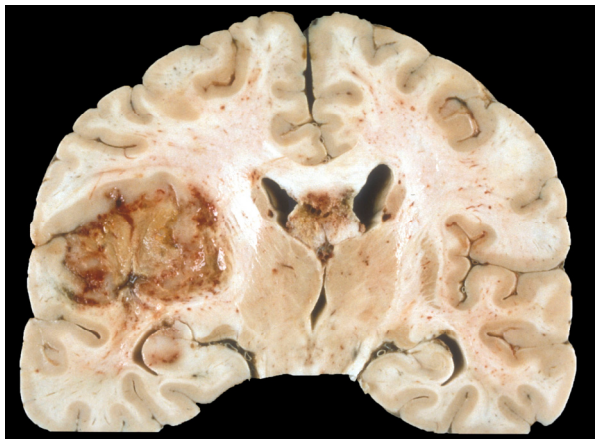

Figure 1.
Macrosection of GBM. Common features include a central core of necrosis and hemorrhages. This work is licensed under a Creative Commons Attributions-ShareAlike 3.0 Unported Licence.

Cell of origin

Although multiple genetic and epigenetic alterations are known to promote gliomagenesis, the cellular origin of gliomas remains uncertain. Several cell types have been suggested as presumed glioma-initiating cells; most evidence has been presented for neural stem cells and oligodendrocyte precursor cells [11]. Whether mature astrocytes and/or neurons are able to directly transform remains highly debated. Intratumoral differences in the mutational profile and clonality of tumor cells can be found in GBM. Distinct clonal populations within GBM can exhibit a hypermutated phenotype or treatment resistance [12].

Signaling pathways

Intratumoral heterogeneity indicates the presence of several epigenetically and genetically different cell subpopulations within a single tumor and have been shown to contribute to tumor growth, progression and treatment failure [13]. The heterogeneity could increase during the natural evolution of GBM [14]. Overexpression of signaling pathways could lead to uncontrolled cell growth. The main signaling pathways in GBM are three: the receptor tyrosine kinase pathway (*RTK/RAS/PI3K*) which is altered in almost 88% of GBMs, *TP53* pathway in 87% of GBMs and retinoblastoma protein (*Rb*) signaling pathway altered in approximately 78% of GBMs [15]. (Further discussed in *Immunosuppression mechanisms elicited by the tumor microenvironment.*)

Classification

Macroscopically, GBM usually appears as a heterogeneous, single, relatively large, irregularly shaped lesion which arises from the white matter with cystic and gelatinous areas and foci of hemorrhage and necrosis. GBM and other malignant gliomas are highly invasive but typically confined to the CNS and mostly do not metastasize [16, 17].

The World Health Organization (WHO) classification system groups primary brain tumors according to immunohistochemical and molecular features [4] and, recently, methylation profile [18]. Primary brain tumors are classified into four histological grades (I–IV), defined by increasing degrees of undifferentiation, anaplasia, and aggressiveness. Brain tumors are named by the histopathological name followed by the genetic features [4]. Histologically, GBM displays pleomorphic cell populations which range from small poorly differentiated tumor cells to large multinucleate cells with prevalent mitotic activity. Furthermore, it displays proliferation of endothelial cells, frequently with glomeruloid morphology and multifocal necrosis with a pseudopalisading pattern, *i.e.* tumor cells make a false border around the periphery of necrotic areas [16, 17]. The central tumor area consists of up to 80% necrotic tissue. Cancer cells with high proliferation rate are found in the peripheral zone. Another factor unique to GBM is the rapid invasion of the tumor into the surrounding brain tissue, but also later time to more distant locations, especially in the myelinated brain structures like the corpus callosum or perivascular spaces [19].

The presence or absence of mutations in the *IDH1* (isocitrate dehydrogenase [NADP], cytoplasmic) and *IDH2* (mitochondrial) genes distinguish primary and secondary GBMs [4]. Primary GBM or IDH-wildtype represents the majority of GBM. It arises rapidly from non-neoplastic brain cells and progress quickly. In addition, a subgroup of lower-grade gliomas may carry molecular features and signatures similar to GBM, with a similarly aggressive natural course [20]. Around 10% of GBMs are secondary, IDH-mutated. The mutation in the *IDH1* or *IDH2* genes is an early event during gliomagenesis (for IDH as an immunosuppressive factor, see *Immunosuppression mechanisms elicited by the tumor microenvironment)*. Thus, these tumors often arise from a prior low-grade glioma [21]; they preferentially arise in younger patients and are associated with a better outcome. Other common genetic alterations in these tumors include *TP53* and *ATRX* mutations [4]. Secondary GBMs are to be classified as a distinct biological and molecular entity for which different treatment strategies will ultimately be proposed [15].

The histologic features of the tumor often do not reflect the molecular diversity of these lesions. Thus, the molecular classification of GBM should be an integral part of the diagnosis of brain tumors. IDH-wildtype GBMs can be transcriptionally subclassified into three molecular variants, all defined by distinct genetic

alterations; classical, mesenchymal and proneural. Common features of the classical subtype include enhanced epidermal growth factor (EGF) receptor (EGFR) expression through amplifications or mutations (point and vIII mutations), as well as chromosome 7 amplifications, chromosome 10 deletions and *Ink4a/ARF* locus deletion. The mesenchymal subclass displays loss of neurofibromin 1 (NF1) expression due to *NF1* mutation/deletion and high expression of *CHI3L1*, *MET* and genes involved in the tumor necrosis factor (TNF) and nuclear factor–κB (NFκB) pathways. Proneural GBMs are characterized by e.g. enhanced platelet-derived growth factor (*PDGF*) receptor-α (*PDGFRA*) expression [18]. The originally described fourth subgroup, neural [22], has recently been removed as it is believed to have been the result of contamination by normal neuronal tissue [18]. Epigenetic profiling has suggested six GBM subgroups: IDH, mesenchymal, receptor tyrosine kinase (RTK) I (proneural), RTK II (classical), and the subgroups K27 and G34 which are characterized by histone 3 mutations and are almost exclusively found in children [23]. Molecular subclasses predict prognosis, delineate a pattern of disease progression, and resemble different stages in neurogenesis.

Clinical presentation

The clinical history of patients with brain tumors depends on the tumor growth rate and grade. The signs and symptoms of GBM may develop rapidly in a range of 3 to 6 months and are produced by the following mechanisms: Firstly, the tumor directly affects the parenchyma as a result of necrosis and growth, which gives rise to symptoms such as focal neural deficit, cognitive impairments, motor dysfunction and seizures. Seizures are present in 20 to 40% of patients with GBM and usually are with a focal onset, which could be simple partial, complex partial or generalized seizures [24]. Secondly, the gradual increase in size and the edema in the tumor-adjacent area may increase the intracranial pressure, resulting in hydrocephalus, visual disorders or headaches associated with vomiting and papilledema. Thirdly, there are unspecific signs and symptoms such as fatigue, drowsiness or insomnia [25, 26].

Diagnosis

Magnetic resonance imaging (MRI) and computed tomography (CT) are mainly used to scan the brain in order to identify a tumor [27] (Fig. 2). However, the conclusive diagnosis is based on histopathological assessment. MRI with contrast is the most sensitive and specific tool and is principally used to assess tumor extension and the degree of peritumoral edema. The mass has low-signal intensity on T1-weighted images, presenting as a central hypodensity surrounded by an enhancing rim of tumor with thick, irregular walls, corresponding to the cellular and

highly vascularized peripheral area of the neoplasm. Gadolinium enhancement denotes angiogenesis and vascular permeability. On T2-weighted images, the lesion appears as a high-signal intensity mass, however the area is broader, less well defined and overlaps with the surrounding vasogenic edema [28, 29]. CT is time- and cost-efficient in comparison with MRI, but it is less informative and its use is therefore limited to patients unable to undergo MRI [30]. With CT, the lesion appearance is variable and typically shows irregular borders with a peripheral ring-like zone of contrast enhancement around a dark, central necrotic area [27]. The surrounding edema has a hypodense or isodense appearance. In summary, MRI and CT may define the margins of the tumor for surgical resection and for planning of the radiation fields. Moreover, they can differentiate the tumor from other benign mass lesions, brain abscess, toxoplasmosis and help to refine the differential diagnosis [31].

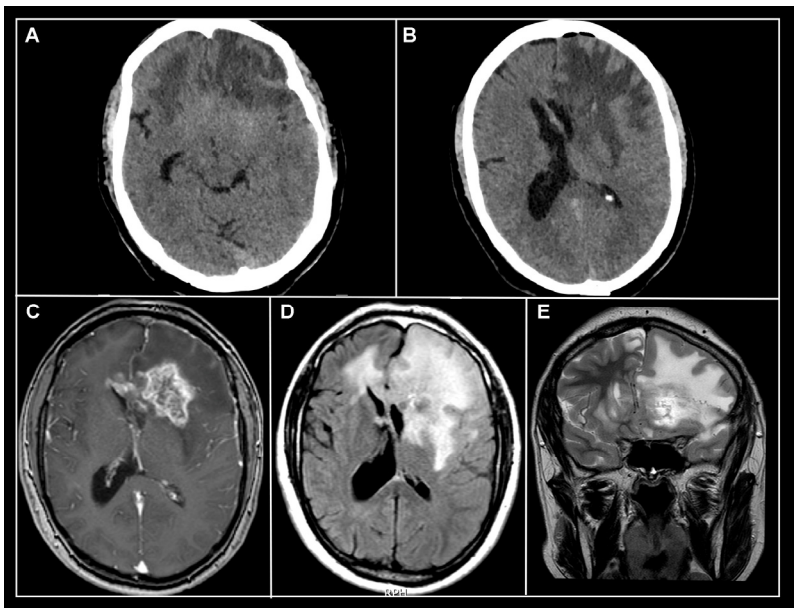

Figure 2.
Male patient with GBM in the left frontal lobe, shown by computed tomography (A–B) and magnetic resonance imaging with gadolinium contrast enhancement (C–E). Images were kindly provided by Dr Julio Enríquez Viteri.

Treatment

Current therapies can slightly prolong survival and enhance the quality of life of GBM patients, although GBM remains a deadly disease. The median survival time of treated GBM patients is < 10 months from the diagnosis and the five-year survival rate with treatment is less than 5% [5].

The therapeutic standard management of newly diagnosed GBM typically includes surgery, adjuvant radiotherapy, concomitant and maintenance chemotherapy and supportive care. In addition, corticosteroids are often used to manage cerebral edema. Due to the fact that corticosteroids dampen the immune system, they have been associated with shortened survival and may play a role in the outcome of immunotherapeutic approaches [32, 33]. In patients with seizures, levetiracetam is often prescribed because of its low toxicity profile and because there are no drug-to-drug interactions with chemotherapeutic agents [34].

Surgery

Surgical resection is completed to the maximal safe extent possible. Surgery reduces tumor load, rapidly relieves intracranial pressure and provides tissue for histological and molecular diagnosis [35]. Technological tools used to improve the safety and maximize the extent of resection during tumor surgery are intraoperative navigation, intraoperative MRI, fluorescent markers to maximize tumor visualization, electrophysiological monitoring and functional brain mapping [36-41]. Notably, due to the infiltrative nature of GBM, a tentatively macroscopically complete resection is not curative. However, gross total resection was significantly associated with a lower relative risk for mortality compared with subtotal resection [35, 42-45]. Furthermore, evidence supports the established association between supermarginal resection, where tissue beyond contrast enhancement is removed, and survival. When it is safely feasible, it may prolong survival without significant increase in overall or neurological postoperative morbidity [46]. Tumors in eloquent cortex, brain stem or basal ganglia are usually not candidates to surgical intervention and these patients usually have worse prognosis [42].

Systemic chemotherapy and radiation therapy

Temozolomide (TMZ) is the main chemotherapeutic agent used to treat GBM and it has been shown to significantly prolong overall survival and progression-free survival (PFS) [47]. It is administrated orally (75 mg/m^2 daily x 40–49 days) concomitantly with radiotherapy, followed by 6 cycles of maintenance TMZ (150–200 mg/m^2 x 5/28 days) [48, 49].

Radiotherapy has been shown to improve survival and aims to eradicate remaining tumor after surgery. Modern radiotherapy focally treats MRI-evident disease plus a margin and is usually initiated 3 to 4 weeks after surgery. The cumulative absorbed dose of 60 Gy is given in daily doses of 1.8 to 2.0 Gy fractions for approximately 6 weeks [50]. Radiotherapy and stereotactic radiosurgery are found to be helpful therapies where surgery has limited applications, such as the elderly patients and recurrent GBM (rGBM), respectively [51, 52].

Common side effects of chemotherapy include, among others, cumulative toxicity, nausea, fatigue, headache, constipation and myelosuppression [53]. The impaired

bone marrow reserve, as a result of treatment, may influence a subsequent second-line chemotherapy or immune interventions [54]. An increased risk of secondary malignancies with new mutations has been proposed after prolonged treatment with TMZ postulating the TMZ-induced hypermutation signature [55]. Several side effects are associated with radiotherapy such as lymphopenia, radiation necrosis, radiation-induced permanent neuronal damage with ensuing neurological and cognitive morbidity and radio-resistance in some tumors [50, 56].

Cytostatic drugs

Temozolomide

The gold standard chemotherapeutic drug for the treatment of gliomas is TMZ since Stupp et al. demonstrated a small but significant increase in mean survival following TMZ treatment [48]. TMZ is a second-generation imidazotetrazine, a pro-drug with 100% oral bioavailability and extensive tissue distribution, including penetration of the blood-brain barrier (BBB) [57].

In reaction with water under physiological pH conditions, TMZ spontaneously converts to the active alkylating agent 5-(3-methyltriazen-1-yl)-imidazole-4-carboxamide (MTIC) and further degrades into 5-aminoimidazole-4-carboxamide (AIC) and a methyldiazonium cation, a highly reactive DNA-alkylating compound [58]. This unstable cation transfers a methyl group to the DNA causing the cytotoxic effect of TMZ, inhibiting DNA, RNA and protein synthesis and leading to apoptosis in rapidly dividing cells. The methylation occurs at the purine bases of the DNA, at the O6 and N7 positions of guanine and the N3 position of adenine. The O6 methylation accounts for 5% of the total adducts caused by TMZ. O6-methylguanine in itself is not lethal to cells but it is the main cause of cytotoxicity [54]. Furthermore, during DNA replication thymine is incorporated instead of cytosine, opposite O6-methylguanine. The mismatch repair pathway of the cell recognizes this mismatch and removes the aberrant thymine residue in the daughter strand. Nonetheless, unless the methyl adduct is removed from the guanine, thymine is likely to be reinserted opposite the lesion. It is the repetitive rounds of the mismatch and repair pathway that result in a state of chronic strand breaks, which finally triggers the apoptotic response [59].

TMZ has been shown to induce tumor regression and remissions in patients with low-grade astrocytoma and GBM and may have some activity against melanoma. TMZ is considered somewhat less toxic and better tolerated than many other alkylating agents but does have side effects such as fatigue, nausea and vomiting, gastrointestinal upset and myelotoxicity, mainly lymphocytopenia [59, 60].

The inability of TMZ to generate endoplasmic reticulum (ER) stress and to subsequently induce translocation of calreticulin (CRT) to the cell membrane surface, has led to the classification of TMZ as a non-*bona fide* immunogenic cell death (ICD)-inducer drug (see *Immunogenic cell death*). However, it has been shown to induce the translocation of high mobility group box 1 (HMGB1) from the cell nucleus to the cytoplasm and induces secretion of adenosine triphosphate (ATP) [61]. Additionally, TMZ can act in an immunostimulatory or immunosuppressive way, depending on the timing and mode of delivery as well as the dosing strategy. It has been shown to impact T cell proliferation and the proportion of regulatory T cells (T reg), and to enhance cross-priming of dendritic cells (DC) [60]. For the mechanisms of TMZ resistance in GBM, see *Mechanisms of treatment resistance*.

Cisplatin

Platinum-based drugs, such as cisplatin (CIS) (cis-diamminedichloroplatinum-II), are described as alkylating-like drugs due to their ability to crosslink with the DNA. CIS becomes activated intracellularly and subsequently covalently binds to DNA. As a result, intrastrand-DNA adducts are made between purine bases, bending and unwinding the double helix. This produces a distortion of the DNA which can hamper cellular processes, such as replication and transcription, that require DNA-strand separation to different extents. Consequently, there is a prolonged G2 phase leading to activation of signaling pathways related to DNA-damage recognition and repair, and ultimately apoptosis [62].

CIS is among the most widely used chemotherapeutic agents. It is part of standard treatment of medulloblastomas [63-65] and has been part of the clinical treatment of patients with brain tumors as a single agent [66, 67] or in combinations with radiotherapy [68] and chemotherapeutic drugs such as carmustine (BCNU) [69, 70] and TMZ [71, 72]. CIS has mainly shown efficacy against solid neoplasms outside the CNS, for example testicular, ovarian, breast, colorectal, lung and head and neck tumors [73]. There is no clear explanation of the differences in the clinical efficacy of CIS between medulloblastomas and GBMs.

Systemically delivered CIS poorly penetrates into normal brain tissue due to the presence of the BBB. Less than 5% of the plasma concentration of CIS is detected in the brain after intravenous delivery [74]. Some studies have however detected therapeutic platinum levels in primary and secondary brain tumors and to a lesser extent in the edematous brain adjacent to the tumor after systemic delivery [75, 76]. The systemic toxicity of CIS is significant and is a restricting factor. It comprises severe nephrotoxicity and ototoxicity, as well as hematological toxicity and peripheral neurotoxicity [64, 73, 77].

CIS has been described as a non-*bona fide* or partial ICD-inducer drug. It induces ATP secretion and translocation of HMGB1 from the cell nucleus to the cytoplasm,

but its inability to induce ER stress prevents CRT translocation from the ER to the outer surface of the plasma membrane [61, 78]. CIS may also modulate antitumor immunity through other mechanisms, such as improving the recruitment and proliferation of immune effector cells and augmenting their lytic activity, up-regulating major histocompatibility complex (MHC) I and down-regulating immunosuppression in the tumor microenvironment (TME) [79].

Lastly, both intrinsic and acquired (during courses of therapy) mechanisms of resistance to CIS have been described, including decreased membrane transport of the drug, increased cytoplasmic detoxification, increased DNA repair and increased tolerance to DNA damage [73].

Mitoxantrone

Mitoxantrone (MTX) is an anthracenedione antineoplastic agent. It produces a delay in cell-cycle progression and, although not considered cell cycle-specific, is most cytotoxic to cells in the late S phase, affecting both healthy and cancer cells. It acts primarily on the DNA and induces DNA strand breaks by stabilizing the topoisomerase-DNA cleavable complex and by generation of free radicals, and also induces DNA aggregation and compaction via electrostatic cross-linking [80, 81].

MTX poorly penetrates the BBB because of its poor lipid solubility. Thus, it has a limited effect on brain tumors when delivered systemically [82, 83]. Even so, it has been found in therapeutic concentrations in brain tumor tissue from patients who were administrated systemic preoperative MTX [84]. Systemic MTX is associated with dose-limiting adverse reactions including nausea, vomiting, hair loss, cardiac toxicity and immunosuppression. Irreversible cardiomyopathy is a particularly concerning side effect [80]. MTX has been characterized as a *bona fide* ICD-inducing drug, as it induces CRT expression on the cell surface, HMGB1 translocation to the cytoplasm and extracellular release of ATP [61, 85].

MTX has been used clinically in primary and rGBM [84, 86, 87]. Moreover, attempts have been made to improve the therapeutic effect of MTX against GBM, for instance changing the administration route such as intratumoral MTX delivery combined with systemic TMZ [88] or intraarterial administration [83]. Additionally, a mix of Surgifoam® and MTX can be safely applied into the post-GBM resection cavity without any observable side effects [89]. However, MTX is principally used to treat certain types of non-CNS cancer, mostly acute myeloid leukemia, non-Hodgkin's lymphoma and metastatic breast cancer [80].

Finally, mechanisms through which tumor cells could generate resistance to MTX includes, among others, an increased P-glycoprotein expression, alteration of the levels or activity of topoisomerase II and enhanced DNA repair mechanisms. MTX has also demonstrated immunosuppressive, antiviral and potential anti-angiogenic activities in pharmacodynamic studies [81].

Mechanisms of treatment resistance

Over time, GBM cells become resistant to damage caused by TMZ. The principal mechanisms responsible for TMZ-acquired resistance are (i) DNA repair mechanisms, (ii) overexpression of EGFR, galectin-1 or murine double minute 2 (Mdm2), (iii) *TP53* or phosphatase and tensin homolog (*PTEN*) mutations [54, 58].

DNA repair is mediated through the enzyme O6-methylguanine-DNA methyltransferase (MGMT) and DNA mismatch repair enzyme activity. MGMT repairs the lesion caused by TMZ by removing the methyl adduct from the O6-guanine position in a single step, independently of any other protein or cofactors. Accordingly, high levels of MGMT activity in tumor cells directly repair DNA and are associated with poor TMZ response [90-92]. About 45% of GBM patients present an epigenetically silenced *MGMT* gene [90]. This is ensured by hypermethylation of promoter CpG islands [91]. TMZ-treated patients carrying tumors with epigenetic silencing of the *MGMT* promoter have a longer survival than those with an unmethylated *MGMT* and those treated with radiotherapy alone [90]. Studies of paired primary GBM and rGBM samples show that the *MGMT* promoter methylation is unstably conserved from the newly diagnosed to progressive disease settings and GBMs have the ability to recur with a newly reprogrammed epigenetic status [93]. DNA mismatch repair is a system that corrects errors in nucleotide base matching generated during DNA synthesis [54]. In mismatch repair-deficient conditions, the O6-guanine methyl adduct is tolerated and can be mutagenic. This may be an important mechanism in the development of mutations secondary to TMZ treatment and has been described in secondary GBM as well as in the potential development of a hypermutated phenotype [55, 94]. A number of pharmacological approaches have been explored to modulate MGMT activity and enhance drug response, mainly by inhibition of MGMT [95].

Alternative administration route – intratumoral chemotherapy

Most chemotherapeutic drugs lack efficacy against GBM since molecules with a molecular weight greater than 180 kDa cannot cross the BBB. Additionally, the diffusion of a compound in a given tissue depends principally on its free concentration gradient and its diffusivity in the tissue. Hence, high-molecular weight compounds are unable to diffuse over large distances and pass the BBB, limiting the distribution of certain drugs in the CNS when they are administered systemically [96]. Intratumoral administration aims to improve the pharmacokinetic and toxicity profiles of the administered agents, which translates into a reduced amount of drug dispersed throughout the body and opens the opportunity to use other cytostatic drugs or compounds with potential antitumor features that have not been tested before due to their pharmacokinetic limitations.

Intratumoral drug delivery approaches include the injection and infusion of the therapeutic agents, the use of implantable polymers that slowly release drugs and convection-enhanced delivery (CED) [97].

Manual injection and implantable reservoirs

The general injection approach for the treatment of GBM is to inject the therapeutic agent into the remaining cavity after the resection of the tumor or intratumorally if the tumor is inoperable. Unfortunately, injection approaches did not become a breakthrough in the treatment of brain tumors since, despite their benefit, they are generally associated with a high risk of side effects, such as infections, edema and backflow of the solution along the catheter [98]. The Ommaya reservoir has been extensively used for intracavitary delivery. It is composed of a mushroom-shaped reservoir, which is implanted subcutaneously and connected to an outlet catheter positioned within the tumor area through a burr hole in the skull. The Rickham is similar to the Ommaya reservoir but its smaller size reduced the risk of infection although it makes it more difficult to locate the reservoir under the skin [98].

Biodegradable drug carriers

These controlled-release systems use biodegradable polymers that carry a therapeutic agent and are placed into the remaining cavity after the resection of the tumor. The release features are based on the composition of the polymers. They deliver a high drug load locally into the tumor area in a sustained manner over prolonged periods of time. The release of a drug from the biodegradable systems is influenced by the degradation of the polymer, diffusion and the erosion processes. The components commonly used for the biodegradable polymeric systems are polylactide-co-glycolide copolymer (PLGA), polyanhydride-poly[bis(p-carboxyphenoxy)] propane-sebacic acid and fatty acid dimer–sebacic acid [98]. During the last two decades a wide range of polymeric delivery systems such as wafers, microspheres, nanospheres, gels and microchips have been developed [99]. Gliadel® wafer (BCNU-PCPP:SA polymer, 1,3-bis(2-chloroethyl)-1-nitrosourea, carmustine) is the only polymeric system that is approved by the FDA for the treatment of brain tumors [100, 101]. Gliadel® was reported to be safe, however, hemiplegia, brain edema, confusion, seizures, serious intracranial infection and intracranial hypertension were the most frequent neurologic adverse events recorded in Gliadel® clinical trials [99]. In addition, if not implanted properly, the wafer could block the flow of cerebrospinal fluid (CSF) and might cause obstructive hydrocephalus.

Convection-enhanced delivery

CED is a technique that generates a pressure gradient to deliver a solution directly into the interstitial spaces of the CNS. It offers solutions to many limitations of other

techniques and allows the therapeutic agent to pass the BBB in a targeted and safe manner, thereby improving the tissue concentrations of the agent. CED is a broadly applicable technique that can be used to deliver many therapeutic agents for diseases such as brain tumors, Parkinson's disease and Alzheimer's disease. One cannula containing one single- or multi-lumen catheter is placed stereotactically into the targeted tumor area of the brain tumor through burr holes in the skull. The catheter is proximally connected to an infusion pump, which generates a positive pressure gradient for a certain time [97]. The cannula has to be positioned safely away from the ventricles to prevent leakage of chemotherapeutic agents into the CSF, which could cause aseptic chemical meningitis [102]. CED offers several advantages. First, CED improves intratumoral spatial distribution because the pressure gradient allows agents to be infused over a larger volume, more evenly and at higher quantities. Second, CED lacks the steep concentration gradients associated with diffusion-mediated delivery, thus the dose can be reduced. Third, unlike diffusion, CED occurs independently of an agent's molecular weight or diffusivity [97].

CED has been widely studied in GBM treatment with many therapeutic agents, such as conjugated toxins [103], liposomes containing drugs or nanoparticles [99], oncolytic viruses [104, 105] and agents unable to penetrate the BBB. The chemotherapeutic drugs that have been administrated to GBM patients in phase I and II clinical trials are, among others; carboplatin [106, 107], CIS [108], MTX [109], nimustine hydrochloride [110], paclitaxel [111] and topotecan [112]. In addition, mathematical modelling can be employed to investigate the suitability of chemotherapeutic drugs from the perspective of intratumoral transport and predict the drug transport processes, such as convection/diffusion-driven drug migration in the interstitial fluid, binding to proteins, absorption onto the cell membrane, accumulation in cell´s interior, as well as elimination caused by metabolism, degradation and blood drainage [113].

Challenges associated with CED include backflow, air bubbles, the choice of the agent, the type and position of the cannula, limitations surrounding flow within brain tissue, white matter edema, target heterogeneity, active tumor/BBB disruption, challenges in the ratio of volume of infusion to the volume of distribution and, finally, flow rate [114, 115].

We have used ALZET® osmotic pumps in our CED studies. ALZET® minipumps can be used for systemic administration when implanted subcutaneously or intraperitoneally or they can be attached to a catheter for intravenous/intra-arterial or intracerebral infusion. ALZET® pumps use the difference of osmotic pressure between the osmotic layer compartment within the pump and the tissue where the pump is implanted. The high osmolality of the osmotic layer causes water to flow into the pump through a semipermeable membrane. As the water enters the osmotic layer, it compresses the flexible reservoir which leads to a release of the solution

from the pump in a controlled and predetermined rate. ALZET® pumps can deliver many commonly used compounds with certain limitations on viscosity and chemical structure. Thus, the delivery profile of the pump is independent of the drug formulation. The rate of delivery is controlled by the water permeability of the pump's outer membrane. The volume delivery rate is fixed at manufacture and varies between 0.11 and 10 μL/hr. Delivery durations vary between 1 day and 6 weeks. Since the volume delivery rate of the pump is fixed, the dosing rates are managed by the concentration of agent in the solution used to fill the pump reservoir [116, 117].

In conclusion, all intratumoral chemotherapy techniques bypass the BBB and reduces systemic toxicity. Thus, antitumor drugs that are often toxic systemically can be delivered at higher concentrations with excellent intratumoral distribution. It has been shown to be safe and somewhat effective in preclinical and clinical studies.

Modeling glioma

In vitro models

Tumor-derived cell lines are obtained from a subset of cells from surgically removed human or experimental gliomas. Cell lines are often the initial tools employed for drug screening since they are easy to grow and are cost-effective. Clonal experiments or single-cell analyses are straightforward, providing rigorous information without the complexity of extrinsic signals. A major risk of working with cultured cells is however that they may diverge, genetically or epigenetically, to the point of being non-relevant to the human disease. Thus, *in vitro* discoveries should be complemented with *in vivo* experiments [118-120].

In vivo models

The application of animal models in glioma research is necessary in order to develop novel therapeutic strategies. The use of mice to create suitable models has clear advantages. Firstly, manipulation of the mouse genome to create specific genetic changes is relatively easy compared with other mammalian species [121]. Secondly, there is a great availability of inbred strains, and thirdly, mice have extensive molecular and physiological similarities to humans [122]. In addition, mouse models are often reproducible. The requirements for modelling GBM-immune interactions are rather different to those for understanding other aspects of tumor biology. In our current research, we need to assess the interaction between GBM

and the immune system, thus, we use both immunocompetent [123] and immunosuppressed [124] strains.

Glioma models

Tumor models differ in their immunogenicity, growth patterns and invasiveness [120]. No currently available model reproduces exactly all of the major characteristics found in human GBM and this should be taken into consideration when analyzing preclinical results and translating them into the clinic. The experimental glioma model should preferably mimic as many of the main characteristics of human gliomas as possible. Firstly, the tumor cells should be of glial cell origin and be tumorigenic. Tumors should preferably form within a time frame that let the animal survive long enough to allow for investigation of the treatment aims. Moreover, the tumor model should have a glioma-like infiltrative growth pattern and preferably be as weakly immunogenic as possible but at the same time, express tumor-associated antigens (TAA) and/or tumor-specific antigens (TSA). A number of different approaches have been employed for developing animal glioma models, including chemically or virally induced models, xenograft transplantation models and genetically engineered mouse models (GEMM) [122].

Chemical carcinogen-induced models

Gliomas have been induced by treating animals with DNA-alkylating agents, resulting in models with undefined genetics. Tumor inductions occur through a non-random alkylation of bases which gives rise to base mispairing and point mutations [120, 125]. As a result, the time of induction, incidence, malignancy type and location varied greatly within each study. An advantage of these models is that the tumors present in a syngeneic immunocompetent host allowing the immune system to interact with the developing tumor. The most common chemically induced glioma mouse model is GL261 [126].

Genetically engineered mouse models

GEMMs recapitulate relevant genetic and molecular characteristics of the human tumors [120]. They are created by introducing defined genetic alterations in the germline and using breeding strategies that generate compound mutants with alterations in both oncogenes and tumor suppressors. Several methods have been developed for this aim, such as retroviral and adenoviral vectors or recently by CRISPR ribonucleoproteins [120, 121]. Consequently, GEMMs are relatively simple to generate, easy to use and would often include a built-in mechanism to assess therapeutic effects, such as a bioluminescent reporter [127].

GEMMs provide a platform to evaluate the tumorigenic capacity of oncogenes and tumor suppressors. GEMMs spontaneously develop tumors that recapitulate human

gliomas in several aspects. They form *in situ* and appear to harbor many cellular subpopulations, including putative cancer stem cells (CSCs). Moreover, GEMMs give rise to tumor–stroma interactions resembling those found in native tumors [121]. Although spontaneous tumor models are mimicking primary glioma in patients much more closely than engrafted models, the main drawbacks are the poor reproducibility, low tumor penetrance, prolonged latency for tumor formation and the need to add *in vivo* imaging techniques or another method to find and assess tumor growth [127].

Transplantable models

The engrafted glioma mouse models can be either xenografts from human primary tumor cells or cell lines, so-called patient-derived xenograft models (PDX) or allografts from rodent tumor cell lines [120]. The grafts are usually orthotopic, transplanted into the brain with stereotactic procedures, or heterotopic, typically subcutaneous. Due to the fairly good reproducibility of engrafted tumor models and the possibility to control spatial and temporal tumor initiation, they are widely used for evaluating therapeutic strategies [122]. Large cohorts of tumor-bearing mice can be generated with consistent tumor sizes and sites. Monitoring of the transplanted tumor cells using bioluminescence *in vivo* is also possible. The downsides are that this approach typically requires large numbers of cells for injection, and there is limited ability to control events during engraftment and seeding steps. Also, the injection procedure itself inevitably creates an injury, thereby disrupting normal tissue architecture and physiology [128].

PDX models cannot be used when studying immune-mediated antitumor therapies, thus, syngeneic models are frequently used for that purpose. Transplantation of orthotopic grafts into syngeneic hosts have the advantage of modelling immune interactions although there is the possibility of rejection [122].

Mouse glioma cell lines

GL261

The GL261 murine glioma model harbors several characteristics of most gliomas and is probably the most frequently used mouse glioma model. It was originally induced in the 1930's by the injection of carcinogenic 20-methylcholantrene pellets into the brain of a male C3H mouse [129]. The cancer cells were then maintained by serial intracranial and subcutaneous transplantations into syngeneic recipients until it became established as a cell line. The cell line is tumorigenic with a rapid growth rate, has an invasive but non-metastatic growth pattern and the *in vivo* tumors display necrotic areas, features found in human GBM [125]. GL261 cells implanted orthotopically gave rise to lethal tumors within 30–40 days in our lab.

The cell line was later characterized and defined as chemo- and radiosensitive. Moreover, it does not express the most important glial differentiation markers, such as glial fibrillary acidic protein (GFAP) [126, 130]. With regards to genetic alterations, GL261 cells carry an extremely large number of somatic mutations, 4 978 in total, due to the carcinogenic method used for the induction [131]. It has a homozygous point mutation in the *Trp53* tumor suppressor gene and a one-point mutation in the *K-ras* oncogene. It has elevated p53 and c-myc expression [126]. The number of unique TAAs is limited. With regards to immunogenicity, GL261 has no basal expression of MHC II, but has an elevated basal MHC I expression and low basal levels of CD80 and CD86 expression, which might be responsible for the moderate immunogenicity described in the cell line. However, MHC I and II expression can be up-regulated after the exposure to recombinant interferon-gamma (IFN-γ) or IFN-γ transduction *in vitro* but neither marker was up-regulated after the exposure to irradiation [126].

The GL261 model has been widely used in immunotherapy interventions such as adoptive cell transfer, serologic treatment with monoclonal antibodies, immunization and gene therapy [132] showing significant results. Additionally, it is also possible to find reports with GL261 cells expressing transfected firefly luciferase [133]. The major limitations of the model are its clearly distinct growth pattern from spontaneously arising glioma in humans and the moderate immunogenicity. The intrinsic immunological features of the cell line and the immune reactivity of the mouse strain might have varied during time and by handling by different research groups.

KR158

The KR158 cell line presents the characteristics of a grade III astrocytoma. KR158 cells arose spontaneously in the olfactory bulb, midbrain and medulla of a C57BL/6 female mouse from a double heterozygotes *Nf1*+/-;*Trp53*+/- cis (NPcis) mutant mother [134].

To generate the cell line, the brain tissue was harvested when the mouse developed signs of tumor growth, approximately at 8 months of age. Next, the cells were cultivated, established *in vitro* and expanded subcutaneously in nude mice [134] and orthotopically in C57BL/6 mice [135]. In our lab the cell line is tumorigenic and give rise to lethal tumors within 25–35 days.

The cell line has lost the wildtype alleles of *Nf1* and *Trp5*3 and expresses GFAP, nestin, Olig-1 and S-100 [134, 135]. Moreover, it generates tumors with a range of histological features from diffuse cells with atypical elongated astrocytic nuclei with irregular contours, similar to low-grade diffuse astrocytomas (WHO II), to large and aggressive tumors with increased mitotic activity and atypical blood vessels, like human anaplastic astrocytomas (WHO III). Additionally, it could present tumors

containing multinucleated giant cells and formed “secondary structures” indicating the potential progress to GBM [134]. Studies report that it expresses EGFR and PDGFRA [135]. A KR158 cell line expressing luciferase has been developed [136, 137].

KR158 has been shown to be resistant to radio- and systemic chemotherapy [136], although sensitive to oxaliplatin *in vitro* [133]. Multiple studies have been conducted evaluating immunotherapy interventions, for example carbon nanotubes carrying CpG oligodeoxynucleotides (SWCNT/CpG), intratumoral immunotherapy, improving the sensitivity to systemic TMZ [137] and adoptive cell transfer with DC vaccine [136]. Generally, the KR158 model has been shown to resist treatment including immunotherapy.

The KR158 model represents the loss of tumor suppressors rather than overexpression of transgenic oncogenes. It has a well-defined genetic background; therefore, the biological behavior of the tumor can be predicted. The *Nf1* mutation and loss of *Trp53* function may facilitate the progression to malignancy in this model. Both features may together accurately model human GBM and preferentially the mesenchymal GBM subtype which is associated with increased M2 macrophages and poor prognosis [18].

SB28

SB28 is a relatively new cell line, recapitulating the features commonly found in the proneural subtype of human GBM that is driven by PDGF signaling pathway [23]. It is a genetically engineered *de novo* induced murine glioma cell line. It was generated in C57BL/6 neonates by injecting the transfection reagent *Sleeping Beauty* transposon-flanked carrying the oncogenes N-*Ras*, *PDGF,* a short hairpin *Trp53* and luciferase into the right lateral ventricle [138]. Later on, green fluorescent protein (GFP) has been added [139]. For establishment of the SB28 glioma cell line, the brain tissue was harvested at 7 weeks following the glioma induction, then, the cells were sub-cultured and a clone with the highest luciferase activity was selected and grown as the SB28 cell line [138]. The orthotopically implanted SB28 are tumorigenic in our lab, generating lethal tumors within 40–60 days.

There is an absence of constitutively expressed MHC I and MHC II in SB28 cells, however MHC I is induced after the exposure to recombinant IFN-γ. CD80, but not CD86, is constitutively expressed by the cell line. The immune regulatory molecule CD274 (PD-L1) is inducible by IFN-γ. There are only 108 somatic mutations in the SB28 cell line, principally in the PDGF signaling pathway, consistent with the strategy used to create the model [131]. Histologically SB28 tumors present an invasive but non-metastatic growth pattern and areas of hypervascularization. Immunotherapeutic interventions using the SB28 model are increasing. It has been shown that the cell line is resistant to programmed death 1 (PD-1)- and cytotoxic T

lymphocyte-associated antigen 4 (CTLA-4)-blocking antibodies [131]. The key advantage of the model is that it recapitulates the characteristics of most human GBMs exhibiting MHC loss and low mutational load.

Tumor microenvironment and tumor immunology

The tumor microenvironment

The TME is composed of the extracellular matrix (ECM), soluble factors, blood vessels, stromal cells and cancer cells. The TME of GBM is similar to other cancers, however also unique in several aspects. The brain tissue is insulated by the BBB and has resident specialized immune cells that together contribute to the brain being widely considered a relatively immune-privileged organ, meaning that there is a tightly regulated immune response with restricted trafficking or patrolling by peripheral immune cells, which leads to a naturally more immunosuppressive environment or "cold" phenotype. The majority of GBMs fall into in this category and it is currently believed that "cold tumors" are less responsive to immunotherapies. The interaction between stromal cells and tumor cells is known to play a major role in cancer growth and progression, and reduction of immunosuppression has the potential to improve the treatment of GBM [140]. In addition, the high proliferation rate of cancer cells produces an insufficient blood perfusion which creates an oxygen gradient between the core and tumor perivascular areas and increases the demand of nutrients [141]. Thus, hypoxia generates a metabolic change in cancer cells and a permanent switch to glycolysis, instead of oxidative phosphorylation of pyruvate, leading to secretion and accumulation of high amounts of lactic acid in the extracellular space – a phenomena coined the "Warburg effect" [142]. Consequently, the TME of GBM displays a hypoxic and acidotic state which is associated with the development of GBM [143] and increased tumor aggressiveness through angiogenesis [143], maintenance of stemness [144], metastasis and treatment resistance [141].

Glycoproteins, hyaluronic acid and heparan sulphate proteoglycans are the predominant proteins in the ECM of GBM [145]. The latter in particular are up-regulated in GBM [146] and cause retention of heparin-binding angiogenic growth factors such as fibroblast growth factor and vascular endothelial growth factor (VEGF), promoting angiogenesis [147]. Interestingly, the ECM produced by cancer cells inhibits the migration of T cells across glioma-associated blood vessels and promote recruitment of M2 macrophages through the secretion of tenascin C [148] and periostin [149], respectively.

The cellular components of the TME can be divided into immune (see *The immune system*) and non-immune cells. The non-immune cells in the TME of GBM include neurons, normal and reactive astrocytes, cancer cells, fibroblasts, vascular endothelial cells and pericytes. It has been shown that neurons contribute to the outgrowth of GBM through neuron-derived neuroligin-3, which increases the proliferation of tumor cells and inversely correlates with survival [150]. Furthermore, astrocytes are extremely important for the development of GBM. Astrocytes repair brain tissue during different forms of injury, also referred to as reactive gliosis, which is one mechanism of wound healing in the brain [151]. In addition to the structural and homeostatic properties of astrocytes, several mechanisms are involved in the crosstalk between reactive astrocytes and gliomas such as secretion of metalloproteinases [152], cytokines [153], and microRNAs [154] playing in favor of tumor proliferation, invasion and resistance to radio- and chemotherapy [155]. Cancer cells have intrinsic features, autocrine and paracrine mechanisms to change and maintain their environment and promote their own proliferation and development (further discussed in *Immunosuppression mechanisms elicited by the tumor microenvironment*). Pericytes and endothelial cell signaling networks have been considered to contribute to tumor angiogenesis [156, 157] besides their essential role in the neurovascular unit and in the function of the BBB.

The blood-brain barrier

A number of morphologic, functional and physiologic characteristics mediate the permeability of the BBB. The BBB is formed by capillary endothelial cells, surrounded by a basal membrane, pericytes and astrocyte foot processes. These cells form a diffusion fence, which protects the brain and severely restricts penetration of substrates into the brain parenchyma under physiologic conditions [96]. (Fig. 3)

Brain capillaries lack fenestrations and have continuous adhesion and tight junctions and low pinocytic activity compared with other peripheral tissue capillaries as well as high electrical resistance. Hence, paracellular transport of substances is negligible and polar and ionic substances are blocked from entering the brain. The astrocyte foot process covers over 90% of the endothelial cell surface and can release chemical factors and signals that modulate the permeability of the brain endothelium. The presence of nerve fibers from peripheral nerve ganglia and intrinsic brain neurons regulate the cerebrovascular tone which has an important role in maintaining a precisely regulated microenvironment for reliable neuronal activity [96].

Particularly, the BBB is compromised in GBM and a dramatic increase in the permeability of blood vessels is observed [158]. The development of edema is quantitatively related to the degree of break-down of the BBB. This loss of function can be seen with MRI where gadolinium crosses the dysfunctional BBB [159] which

has been described to be due to molecular alterations in the capillaries of GBM [160] and/or due to the loss of a heparan sulfate proteoglycan agrin via matrix metalloproteinases degradation [161, 162]. Consequently, molecules in the systemic circulation must either passively diffuse transcellularly or be actively transported across the BBB to enter the brain parenchyma. Passive diffusion is restricted to small molecules (< 400 Da), non-polar, and lipophilic compounds. Water-soluble or polar compounds can only penetrate by way of active transport systems like (i) adsorption-mediated transcytosis, (ii) receptor-mediated transport, (iii) inhibition of efflux pumps, (iv) cell-mediated endocytosis, and (v) use of peptide vectors [99]. Furthermore, the properties that a drug needs in order to efficiently cross the BBB are; fewer hydrogen bond donors, fewer positive charges, greater lipophilicity, lower polar surfaces, and reduced flexibility [163]. Chemotherapeutic drugs which normally cannot cross the intact BBB are able to reach the main bulk of GBM through the disturbed BBB [158]. Therefore, chemotherapeutic drugs administered systemically could more easily get into the main center of the GBM than into the tumor periphery with an intact or less altered BBB; the problem is reaching single tumor cells in the periphery zone where the BBB is not or only partly altered.

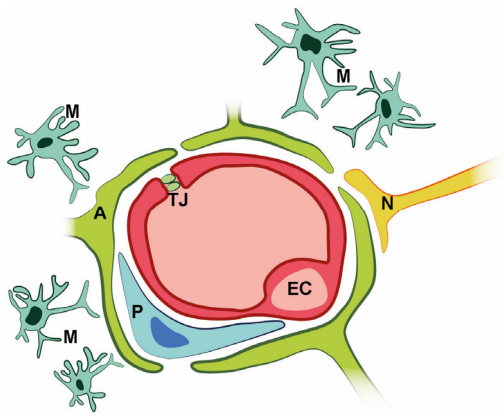

Figure 3.
Structural representation of the blood-brain barrier (BBB) components. The BBB is composed of cerebral endothelial cells (EC) with tight junctions (TJ), separated from pericytes (P) and the astrocytic foot (A) process by the basal lamina. Microglia (M) and neurons (N) are also part of the neurovascular unit. Adapted from Sharif et al. 2018 [96].

In order to improve the delivery of drugs across the BBB, many pharmaceutical approaches have been developed such as microspheres [164] which are small solid particles in the micrometer range (1–1 000 μm), and colloidal drug-carrier systems, e.g. liposomes [165], nanoparticles [166], nanogels [167] and exosomes [168] that are in the nanometer range (1–1000 nm) and entrap, dissolve or encapsulate the drug. These formulations have been reported to improve the delivery of drugs across the BBB in a significant manner and have been widely tested as monotherapies or

in combination in the treatment of GBM and experimental models [99]. Physical disruption of the BBB has also been tested clinically as an alternative to increase the drug distribution of chemotherapeutic drugs into the brain. This is commonly achieved by osmotic substances [169], biochemical vasoactive substances [170] or mechanically with ultrasound [171].

The immune system – an overview

The immune system is made up by biological structures and processes that protect the host against the invasion of external intruders, so-called pathogens, which could potentially cause an infection. The immune system distinguishes between self and non-self-molecules. Therefore, it maintains self-tolerance to the components of the organism but reacts against foreign molecules and corrupted self-antigens, like those produced by cancer cells. Antigens are defined as molecules or substances that bind to specific immune receptors and provoke an immune response. The immune response is mediated by leukocytes [172] and soluble factors such as cytokines, chemokines, antibodies and complement [173]. These soluble factors act as an autocrine, paracrine and endocrine signaling pathway that regulates the interactions between all the components of the immune system. The immune system comprises of two branches, the innate and the adaptive immune system, although their functions are complementary and closely connected (Table 2). In addition, the innate immune system activates and directs the adaptive immune response by cell to cell contact and cytokine secretion [172].

Table 2.
Components of the immune system

INNATE IMMUNE SYSTEM	ADAPTIVE IMMUNE SYSTEM
Non-specific response	Pathogen- and antigen-specific response
Composed of myeloid, NK, NKT and γδ T cells	Composed of antigens, B cells, T cells
Exposure leads to immediate maximal response	Long time between exposure and maximal response
Cell-mediated and humoral components	Cell-mediated and humoral components
No immunological memory	Exposure leads to immunological memory
Found in nearly all forms of life	Found only in jawed vertebrates

The innate immune system

The innate immune system mediates the initial immune response towards pathogen antigens and include epithelial barriers, complement and other proteins, and immune cells [172]. The cellular components mainly comprise cells of myeloid precursor origin such as mononuclear phagocytic cells (monocytes, which

differentiate into macrophages and DCs in tissues) and granulocytes (neutrophils, basophils and eosinophils), as well as natural killer (NK) cells, NKT cells, γδ T cells, which have a lymphoid origin [174]. All these cell types exert various functions such as direct killing of the pathogen through the release of antimicrobial compounds, pathogen phagocytosis, wound healing, etc. Most importantly for the scope of this thesis, the myeloid derived cells act as antigen-presenting cells (APC) [172, 175].

When a pathogen passes through the physical barriers it encounters the cellular components of the innate immune system. Myeloid-derived immune cells employ pattern recognition receptors (PRR) to identify invading pathogens and act as first line of defense by binding to molecular patterns (microbial-, pathogen- or danger-associated molecular patterns; MAMP, PAMP, DAMP). Next, the pathogens or pathogen-infected cells are phagocytized and subsequently an immune response is triggered [172]. PRRs are present on innate immune cells including monocytes, macrophages, DCs, neutrophils as well as epithelial cells. Toll-like receptors (TLR) are the most common and studied type of PRRs [176]. TLR expression and polymorphism have also been associated with the development and progression of human glioma, highlighting the role between TLRs and tumor progression [177-179]. TLRs are able to recognize an extensive variety of PAMPs among microbial species, including lipopolysaccharides (LPS) from Gram-negative bacteria, viral double-stranded RNA, flagellin and CpG motifs [180]. Upon binding, downstream signaling activates transcription factors of various pro-inflammatory mediators principally through the myeloid differentiation factor 88 (MyD88) pathway and activating NFκB and mitogen-activated protein kinase (MAPK) signaling pathways [181, 182].

TLRs can also induce inflammation in the absence of PAMPs, mainly after cellular injury. Thus, necrotic or stressed cells release endogenous molecules referred to as DAMPs. In addition, hypoxia and radiotherapy- or chemotherapy-induced cell damage lead to the release of DAMPs which consequently bind to TLR, thus provoking a sterile inflammation [85, 183, 184]. An increasing list of intracellular DAMPs have been recognized, including HMGB1, heat shock proteins (HSP), DNA, RNA, ATP and uric acid [185] and DAMPs have been described as fundamental parts of immune-mediated cell death [186].

TLR agonists have been studied extensively as adjuvants in vaccine-based immunotherapy. Vaccines are frequently poorly immunogenic and delivery of TLR agonists has been shown to enhance inflammatory responses. Several TLR agonists have achieved therapeutical effects in GBM models and have been tested in GBM clinical trials [187]. However, TLRs are also expressed on cancer cells so they might contribute to tumor-promoting activities instead, including cell growth, proliferation, invasion, migration, and even stem cell maintenance [187].

Monocytes

Monocytes originate in the bone marrow from progenitor cells of the myeloid lineage (CFU-GM). They are circulating cells and play important roles in the early inflammatory responses, host defense during infections, homeostasis and tissue repair [174]. Monocytes home into various tissues and differentiate into tissue-resident macrophages with more specific functions, e.g. microglia in the CNS. Monocytes have also been associated with cancer progression. They eventually infiltrate the tumor giving rise to tumor-associated macrophages (TAM) or are reprogrammed towards myeloid-derived suppressor cells (MDSC) [188].

The term MDSC describes the origin and the characteristic immunosuppressive function of these cells. They are an immature myeloid cell population that are further divided into two subgroups; granulocytic (G-MDSC) and monocytic (Mo-MDSC) that have the potential to mature into granulocytes or macrophages/DCs, respectively. However, the immature phenotype has been associated with tumor angiogenesis, metastasis and tumor burden both in cancer patients [189] and in mouse models [190]. GBM cells as well as stromal cells drive the expansion of MDSCs through a broad range of factors, mediated principally by the signal transducer and activator of transcription (STAT)3-signaling pathway, including granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin (IL)-6, VEGF, IL-1β and cyclooxygenase (COX)-2, whereas the activation of MDSCs is mediated by TLR ligands and different immunosuppressive cytokines [191, 192].

The phenotype of human MDSCs still remains poorly defined due to the overlapping of myeloid cell marker expression. However, in GBM patients, MDSCs express the common myeloid marker CD33 but lack the expression of the mature myeloid marker human leukocyte antigen (HLA)-DR. Accordingly, the major subpopulations of MDSCs in humans include a negative lineage (CD15- CD14- CD33+ HLADR-), granulocytic (CD15+ CD33+ HLADR-), which represents the largest population in GBM patients, and monocytic (CD14+ CD33+ HLADR-) [189]. In contrast to humans, the phenotype of MDSCs in mice is simpler; they are identified by the two markers Gr-1 and CD11b and subdivided into two different subsets, $CD11b^{+}$ $Ly\text{-}6G^{-}$ $Ly6C^{high}$ cells, which are termed monocytic MDSCs (M-MDSCs) and $CD11b^{+}$ $Ly6G^{+}$ $Ly6C^{low}$ cells, which are termed granulocytic MDSCs (G-MDSCs) and constitute the majority of MDSCs in mice [192, 193]. The fact that definition of MDSCs in mice depend on markers that are not found in humans has made predictions of MDSC biology from mouse models difficult.

Macrophages

During an infection, macrophages can be recruited from circulating monocytes. Macrophages are one of the most plastic immune cell types and are involved in many functions of the immune responses, predominantly immune surveillance, antigen presentation and tissue remodulation as well as dual features in cancer

evolution [188]. Depending on the signals received from the microenvironment, macrophages can either undergo a classical (M1) or alternative (M2) activation. The polarization towards classically activated M1 macrophages is induced by TLR ligands such as LPS and pro-inflammatory cytokines including IFN-γ and TNF. M1 macrophages drive Th1 immune responses, up-regulation of pro-inflammatory mediators such as TNF-α and IL-12, elevated antigen presentation as well as antimicrobial and antitumor qualities. Alternatively activated M2 macrophages are induced by IL-4, IL-10 and IL-13 and are specialized in tissue remodeling and wound healing and have anti-inflammatory qualities with an $IL\text{-}10^{high}$ $IL\text{-}12^{low}$ cytokine profile as well as a high expression of scavenger receptors [194, 195]. Nevertheless, it is valid to mention that M1 and M2 polarization represents a simplification of the wide spectrum of different polarization states that exists, primarily in M2 macrophages. However, contrary to current dogma, it has been proposed that glioma-associated myeloid cells exhibit distinct immunological functions, aligned close to non-polarized (M0) phenotype of macrophages [191].

Nonetheless, TAMs are one of the most important players in tumor progression. They represent the major leukocyte population within a tumor, predominantly with M2 attributions, and promote tumor proliferation and invasiveness [196]. TAMs and MDSCs are further discussed in *Immunosuppression mechanisms elicited by the tumor microenvironment*.

Neutrophils

Neutrophils are produced in the bone marrow from colony forming unit granulocyte/monocyte (CFU-G). Neutrophils are the predominant circulating immune cells with an essential role in the innate immune response, mostly because of their antimicrobial function. They are usually the first cells to arrive at sites of infection and employ a variety of defense mechanisms including phagocytosis of microbes, release of antimicrobial peptides and induction of neutrophil extracellular traps. Neutrophils have a short lifespan of approximately 8 hours in circulation in humans. Nonetheless, their lifespan can extend during inflammatory conditions enabling them to also participate in resolution of inflammation [174].

In the context of glioma, most of the glioma patients have a strong neutrophilia due to overproduction of G-CSF by tumor cells [197]. An elevated ratio of peripheral neutrophil-to-lymphocyte counts has been associated with poor prognosis when measured before treatment in patients with GBM [198, 199] and after TMZ chemotherapy and radiotherapy [200]. Circulating neutrophils promote tumor growth-inducing immunosuppression by production of arginase I [201]. The number of infiltrating neutrophils is correlated with glioma grade and with acquired resistance to anti-VEGF therapy in GBM [202].

Natural killer cells, natural killer T cells and γδ T cells.

This group of cells, owing to their invariant T cell receptors (TCR) and an innate immune receptor profile, are referred to as "innate lymphocytes". NK cells can kill target cells as cytotoxic T cells, but they recognize infected or stressed cells in the absence of MHC or by antibodies on the target cell (ADCC), allowing for a much faster immune reaction. Conversely, MHC functions as an inhibitor of NK cell cytotoxicity [172, 203]. The role in the antitumor response is especially important because tumor cells that are missing MHC I markers cannot be detected and destroyed by other immune cells, such as T cells. The cytotoxicity of NK cells is mediated by ligand-mediated killing (e.g. Fas/FasL) or cytotoxic release of perforin and granzymes [204]. NKT cells and γδ T cells are rare cell types that detect glycolipid antigens and phosphorylated stress-induced antigens respectively in the context of NKG2D and CD1d receptors. They produce vast amounts of cytokines such as IFN-γ and IL-17 and may also exert cytotoxic and phagocytic functions [172, 181, 204].

The adaptive immune system

The adaptive immune system has evolved to recognize both self- and non-self-antigens. Adaptive immunity involves a regulated interplay between APCs and T cells. Lymphocytes develop and become activated within lymphoid organs in the lymphatic system. The adaptive immune response harbors unique features compared to the innate response: it is pathogen/antigen-specific; it generates a long-term immunological memory and it regulates the host immune homeostasis. Adaptive immune cells recognize antigens through the antigen-specific TCR/CD3 and binding to either MHC class I or MHC class II molecules, or through B-cell receptors. T cells are involved in the cell-mediated immune responses, whereas the B cells are involved in the humoral responses. The T cell response is typically associated with the recognition of virus-infected and cancer cells; therefore, the discussion will focus on cellular immune responses. B cells on the other hand, when activated differentiate into antibody-producing plasma cells that respond through antibody-dependent cellular cytotoxicity, antibody-mediated neutralization of toxins, facilitating phagocytosis of bacteria as well as inhibiting the entry of bacteria into host cells [205, 206].

The maintenance and proliferation of peripheral naïve T cells is mainly controlled by IL-7, produced in the bone marrow, thymus and peripheral lymph nodes [207]. $CD3^+$ cells are further divided in cytotoxic $CD8^+$ T cells (CTL), which eliminate target cells expressing previously presented antigen, and $CD4^+$ T helper (Th) cells, which direct the adaptive immune response towards either a T cell or B cell response [172].

Th cells regulate the adaptive immune response and can differentiate into one of several Th subtypes (Th1, Th2, Th3, Th9, Th17, and Tregs) which are characterized by the type of cytokines they secrete in the context of antigen presentation or their localization. CD4 cells differentiate into Th1 in the presence of IL-2 and the activation of STAT4 and T-box (T-bet) transcription factor [208]. They support a CTL response and are characterized by secretion of IFN-γ, TNF-α and the T cell proliferation cytokine IL-12. Th1 cells are responsible for cell-mediated immunity, such as the delayed hypersensitivity reaction and are critical for the eradication of intracellular antigens and cancer cells. IL-4 signaling is required for differentiation of Th2 cells, which secrete IL-4, IL-5 and IL-13 [209]. Th2 cells are involved in the B cell response and optimal antibody production, particularly of IgE and IgG1 subtypes, and elicit allergic/humoral immune responses against extracellular pathogens. Tregs constitute a small portion of peripheral Th cells and are labeled as $CD4^+CD25^+FoxP3^+$ T cells. The elimination of these cells might result in autoimmune disease [210]. Tregs can be either thymically derived or natural Tregs (nTreg), that mediate peripheral tolerance to self and execute suppression via cell-to-cell contact, or Tregs that are generated via postthymic maturation (iTreg). The latter are further discriminated based upon the cytokines that cause their induction into T helper 3 $Foxp3^+$ cells (Th3), which are induced by transforming growth factor (TGF)-β and type 1 regulatory T $Foxp3^-$ cells (Tr1), which are induced by IL-10. While both subsets are generated in the presence of different cytokines, they exert their suppressive activity through secretion of the same cytokines that are responsible for their induction, IL-10 or TGF-β, respectively [211, 212]. Furthermore, signaling through IL-6 is important for differentiation into subsets Th17, Th22 and Th9. Th17 cells are characterized by IL-17A–F and IL-22 production [213]. Th17 cells mediate host immune responses against extracellular bacteria, some fungi and other microbes [214].

Antigen presentation

Throughout a process called antigen presentation, processed antigens can be presented to CD3 cells. The activation of the adaptive immune response is dependent on cell surface molecules known as MHC class I and II, which display a selection of peptides from the inside of the cell. MHC class I molecules are expressed on all nucleated cells in the body and present intracellular antigens to CD8 T cells, while MHC class II molecules are expressed only by specialized APCs and present phagocytosed and extracellular antigens to CD4 T cells.

Activated APCs migrate to secondary lymphoid nodes to present the antigen to T cells. An efficient antigen presentation requires specific and coordinated steps. Firstly, APCs present the antigen on the MHC molecule. The MHC/antigen complex binds to the TCR and activates naïve T cells. Simultaneously, CD28 on the T cell binds to CD80 (B7-1) or CD86 (B7-2) on the APC/DC giving a costimulatory signal

required for an appropriate T cell activation (Fig. 4A). Next, CD40 on the APCs needs to bind to CD40L on the T cell to further support the cell activation. Other costimulatory molecules on the T cell like OX40, 4-BBl and CD25 and their respective ligands also T cell activation (Fig. 4A). Once activated, cytotoxic T cells undergo clonal expansion, differentiation and leave the secondary lymphoid organs to infiltrate target tissues where they exert their effector mechanisms and become memory T cells once the inflammatory process is resolved. At the same time, T cells express inhibitory receptors. CTLA-4 is up-regulated shortly after T cell activation. It is analogous to CD28, so these receptors compete for binding to the B7 costimulatory molecules which initiates an inhibitory signal to avoid over-activation and consequently autoimmune diseases (Fig. 4B). Furthermore, effector T cells also express the PD-1 receptor during long-term antigen exposure. It binds to PD ligand (PDL) expressed in inflamed tissues and on cancer cells triggering an inhibitory signal as well [215] (Fig. 4B-C). Currently, monoclonal antibody-mediated CTLA-4 and PD-1 inhibition are used to treat GBM and are discussed in *Immunotherapy*.

CTLs directly lyse target cells through contact-dependent release of granules containing perforin, granzyme B and apoptosis-inducing proteases as well as the up-regulation of membrane-bound Fas ligand (FasL) which upon interaction of Fas receptor on the target cell induces apoptosis [205].

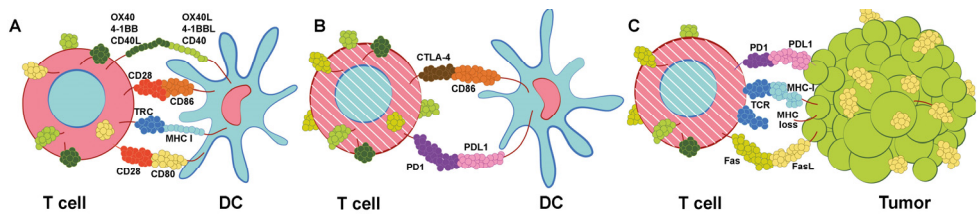

Figure 4.
Activation and inhibition signals of T cells. **A**, T cell activation by dendritic cell (DC); **B**, T cell inhibition by DC; **C**, T cell inhibition by tumor cell.

Immune memory

Additionally, adaptive immunity has the ability to generate an immunological memory. Naïve T cells access the lymph nodes via specialized high endothelial venules by expression of CD62 ligand (CD62L) and C-C chemokine receptor 7 (CCR7) [216]. Following a T cell response, most T cells die. However, a subset remains in the body as antigen-specific $CD8^+$ memory cells and have the ability to respond quickly and efficiently upon reinfection with the pathogen that has been previously encountered [172]. Memory T cells are divided into two subtypes based on the expression of the lymph node-homing molecules CD62L and CCR7. Central memory T cells (T_{CM}) are characterized as $CD62L^{high}$ $CCR7^{high}$. T_{CM} cells recirculates through secondary lymphoid organs as stem-like memory subsets. After

stimulation, they secrete IL-2 leading to proliferating and differentiation into effector cells producing mostly IFN-γ and IL-4. The other progenitor cell subtype is effector memory T cells (T_{EM}) characterized as $CD62L^{low}$ $CCR7^{low}$. T_{EM} display distinct sets of chemokine receptors and adhesion molecules required for homing into inflamed tissues or non-lymphoid organs. $CD8^+$ T_{EM} cells carry large amounts of perforin and both $CD4^+$ and $CD8^+$ T_{EM} produce high amounts of effector cytokines such as IFN-γ, IL-4 and IL-5 but present a limited proliferative potential [217]. Moreover, Th1 cells control the differentiation of $CD8^+$ and $CD4^+$ T cells into T_{CM} or T_{EM} throughout the transcription factor T-bet, where high amounts of T-bet predominantly induce T_{EM} cells [218].

Immune tolerance

Central tolerance is the capacity of the immune system to discriminate between self and non-self, preserving the integrity of the individual's own tissues. Thus, central tolerance is a balance between protecting the individual from autoimmunity while preserving responses against foreign antigens and cancer cells. T cells derive from $CD4^-$ $CD8^-$ (double negative, DN) progenitors and subsequently mature and give rise to a $CD4^+$ $CD8^+$ (double positive, DP) progenitor TCR cell population. Thereafter, DP T cell progenitor cells showing adequate affinity to MHC class I differentiate to single positive $CD8^+$ T cells and those showing affinity to MHC class II differentiate to single positive $CD4^+$ T cells [219].

During positive selection (PS), DP T cells are checked for their ability to bind the MHC complexes. PS occurs in the thymic cortex with the help of thymic epithelial cells that contain surface MHC molecules. If the TCR has appropriate affinity for peptide-MHC complexes, the cell is selected for survival but if the TCR cannot bind the MHC complex, then the cell does not receive a survival signal which leads to apoptosis. Subsequently, TCR cells are tested for their affinity to self, referred to as negative selection (NS), in the cortico-medullary junction and in the thymic medulla. The thymic epithelial cells display self-antigen to the TCR to test their affinity for self. If TCR binds a self-peptide it leads to clonal deletion by apoptosis. Altogether, T cells that do not bind self, but do recognize MHC complexes and are either $CD4^+$ or $CD8^+$, migrate to secondary lymphoid organs as mature naïve T cells [220].

On certain occasions, autoreactive T cells manage to escape the clonal deletion and enter the periphery where they might give rise to autoimmune or autoinflammatory diseases [221]. Central tolerance is also maintained in the periphery through regulatory processes including peripheral deletion, immunosuppressive Tregs, anti-inflammatory cytokines as well as up-regulation of inhibitory coreceptor regulators CTLA-4 and PD-L [219, 222]. The central tolerance process is in fact not exclusive for the clonal selection of T cells but can also be used by cancer for its own

advantage [223] (Fig. 4C), *see Immunosuppression mechanisms elicited by the tumor microenvironment*.

Tumor immunoediting

Immune surveillance is the ability of the immune system to detect and eradicate cancerous cells with both innate and adaptive effector mechanisms. A successful pathogen-mediated acute immune response is followed by immunosuppression, tissue healing and remodeling, and complete shutdown of the immune response, meaning homeostasis. However, in tumors the acute immune response fails to eradicate the tumor and a state of chronic low-grade inflammation is established, also referred as "the wound that never heals" [224]. This state not only prevents the eradication of tumor cells but can also drive further tumor progression. Immune surveillance does not consider that the immune system also drives tumor progressions, leading to the theory of tumor immunoediting, including three phases; elimination, equilibrium and escape [225]. It is challenging to determine these phases in patients because the tumor has usually already escaped immunoediting at the time of clinical presentation.

Elimination phase

In early stages, the elimination phase hampers the tumor development. Cancer cell growth demands an increased blood supply and generates local tissue damage due to local ischemia. Tumor and stromal cells undergoing stress start to produce soluble endogenous factors such as DAMPs, reactive oxygen species (ROS), ECM breakdown products, CD40L and cytokines such as TNFα, IL-1β, IFNs, GM-CSF and IL-15 [226, 227]. The release of danger signals initiates the first wave of inflammatory response including the recruitment and activation of innate immune cells such as macrophages, DCs, NK cells, NKT cells and γδ T cells. TNF-α, IL-1α, IL-1β, GM-CSF and IL-8 are the main recruiters of monocytes and neutrophils from the blood. Moreover, IFN-α, -β, -γ, TNF-α, IL-12 and GM-CSF boost APC functions such as up-regulation of MHC and costimulatory molecules and induce maturation and migration of DCs to draining lymph nodes. Macrophage-derived IL-12 activates NK/NKT cells which secrete the potent macrophage-stimulating factor IFN-γ [228-230].

The production of inflammatory cytokines such as IFN-γ, TNFα and nitric oxide (NO) may initially induce direct tumor cell cytotoxicity. Cells of the innate immune system can kill the tumor cells directly through (i) NK cells that recognize and eliminate cancer cells without MHC class I, (ii) the natural killer group 2 member D (NKG2D) receptor expressed on NK cells, NKT cells and γδ T cells recognize stress-induced proteins as tumor antigens and (iii) IFNs and RO secreted from innate immune cells that cause a direct toxic effect on the tumor cells [172, 231].

Notably, tumor clearance mediated by the innate immune system alone does not induce a long-term memory against secondary tumors.

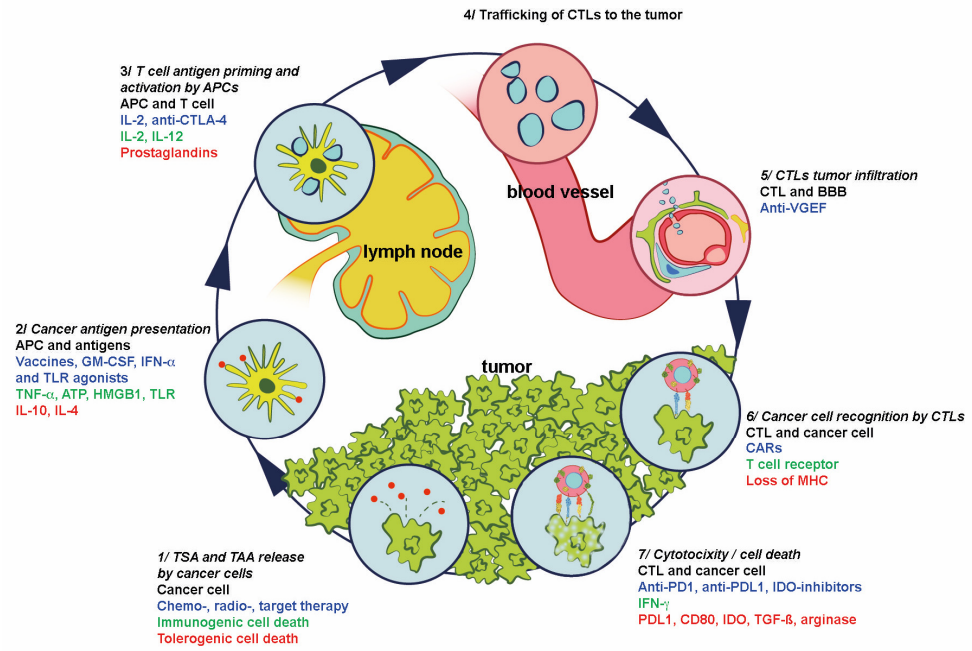

Figure 5.
The cancer-immunity cycle. The generation of immunity to cancer is a cyclic process which leads to an accumulation of immune-stimulatory factors (***shown in green text***), that in principle should increase and expand T cell responses, as well as inhibitory factors (***shown in red text***), that lead to immune-regulatory feedback mechanisms, which can limit the immunity. Although not illustrated, it is important to note that intratumoral T regulatory cells, macrophages and myeloid-derived suppressor cells are key sources of many of these inhibitory factors. This cycle can be divided into seven major steps, beginning with the release of antigens from the cancer cell and ending with the killing of cancer cells. For each phase the main cell types and anatomic locations involved (***shown in black text***) are listed alongside some examples of therapies currently under preclinical or clinical evaluation (***shown in blue text***). Notably, even though not developed as immunotherapies; chemotherapy, radiotherapy, and targeted therapies can primarily promote the cycle in step 1, and inhibitors of VEGF can potentially promote the cycle in step 5 by enhancing T cell infiltration into tumors. Moreover, vaccines can primarily promote the cycle in step 2; anti-CTLA4 can primarily promote the cycle in step 3 by inhibiting the development of an active immune response by acting primarily at the level of T cell development and proliferation; and anti-PD-L1 or anti-PD-1 antibodies can primarily promote the cycle in step 7 through an inhibitory function that modulates active immune responses in the tumor bed. Adapted from Chen et al. 2013. APC, antigen presenting cells; BBB, blood-brain barrier; CAR, chimeric antigen receptor; CTLA4, cytotoxic T cell antigen-4; CTL, cytotoxic T cell; GM-CSF, granulocyte macrophage colony-stimulating factor; IDO, indoleamine 2,3-dioxygenase; IFN, interferon; IL, interleukin; PD-L1, programmed death-ligand 1; TGF, transforming growth factor; TLR, toll-like receptor; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor. Adapted from Chen et al.[229] .

The cancer immunity cycle (Fig. 5) starts with cell debris of dying cancer cells being phagocytized by professional APCs. APCs carrying tumor antigenic material reach the lymph nodes and cross-present the TSAs or TAAs to naïve CD4 and CD8 T cells. Since the majority of peptides originating in tumor cells are endogenous proteins, the T cells will in many cases not be activated. However, when presented sufficiently antigenic TAAs or TSAs, tumor-specific CD4 T cells become activated

and start to produce IFN-γ that further facilitates the development of tumor-specific cytotoxic CD8 T cells [226]. The effector cells leave the lymph node and enter the circulation, eventually home to the TME and eliminate the remaining cancer cells [226].

In the TME, the early immune response favors differentiation towards Th1 cells. M1 macrophages and Th1 cells support each other's functions by feedback secretion of IL-12 and IFN-γ, and together strengthen both innate and adaptive effector functions. Consequently, incoming T cells become reactivated and start to proliferate and secrete IL-2, and IFN-γ to fully activate the CTLs, which eradicate cancer cells via perforin- or granzyme B-dependent mechanisms and by producing high levels of IFN-γ or by Fas/FasL. M1 macrophages, with a pro-inflammatory phenotype (IL-1α, IL-1β, IL-6, IL-12, IL-15, TNF-α), producing the effector factors ROS and NO and capable of inducing IFN-γ secretion in T cells and NK cells [232]. At this stage, Th17 cells may also contribute to antitumor immunity by promoting [233] or exerting [234] Th1-mediated antitumor functions.

Following the antitumor response, some T cells will survive the contraction phase and form a persistent pool of antigen-specific memory T cells, which can elicit an antitumor response upon tumor recurrence. Notably, most recurrent tumors usually display a new selection of cancer cells with a new antigenic repertoire and less immunogenic cancer cell clones. However, the memory T cells encompass specificity against an extensive amount of TSAs, thus raising the probability to respond against secondary tumor growth.

Equilibrium phase

The increasing tumor cell proliferation and immunosuppression lead to a balance between antitumor immune response and tumor growth, also referred to as the equilibrium phase [226].

During this phase, the immune system still controls tumor outgrowth although the antitumor response starts to attenuate. Furthermore, the constant pressure from immune cells will overtime select for cancer cells with immune-evading properties, and the inflammatory milieu will also facilitate additional mutations to occur. This selection promotes the generation of less immunogenic tumor variants and immune-resistant tumors [226]. Cancer cells employ several mechanisms such as down-regulation of TSAs, MHC or NK cell receptor ligands, thereby avoiding recognition and subsequent cytotoxic elimination [226] (Fig. 4C).

Even though the cancer cells may still be immunogenic, they can develop an immunosuppressive phenotype by up-regulating cell-bound molecules (such as PD-L1) or secreted factors (such as TGF-β, IL-10, indoleamine 2,3 dioxygenase (IDO) and prostaglandin E2 (PGE2)), which can inhibit cell-mediated killing, (Fig. 4C) prevent effector cell infiltration, suppress effector functions, induce and recruit

suppressive immune cell populations and subsequently skew the immune response from an active to a suppressed state [228, 229].

Escape phase

As new cancer cells are continuously arising, the selection pressure allows cancer cells to escape immune recognition. Consequently, the tumor fully engages in mechanisms avoiding the immune system. Thereby, surviving tumor cells that have acquired resistance to immune detection and elimination through genetic or epigenetic alterations, expand in an uncontrolled manner enhancing the immunosuppressive microenvironment which further inhibits immune effector functions while maintaining a chronic inflammatory state that foster tumor progression [226]. One obstacle of effective antitumor adaptive responses is T cell exhaustion. In the context of an infection, a persistent activation of T cells may result in the development of exhausted T cells. There are several mechanisms behind T cell exhaustion, including a reduced ability to produce IL-2 and IFN-γ as well as up-regulation of PD-1 [235, 236].

To further avoid antitumor immunity, tumors promote accumulation of immunosuppressive cells, generating an immunosuppressive environment. The most prominent immunosuppressive cells in a TME are the TAMs and MDSCs. Both cell types suppress T cell activity through the secretion of immunosuppressive mediators. Another immunosuppressing cell population is Tregs. The prevalence of Tregs is increased in various malignancies and they apply their immunosuppressive functions through e.g. expressing CTLA-4, affecting DC maturation, sequestering IL-2 and releasing anti-inflammatory IL-10 and TGF-ß [237]. Altogether, the immunoediting process develops tumor cells that avoid elimination by the immune system.

Immunogenic cell death

Apoptotic cells have long been considered as intrinsically tolerogenic or unable to elicit immune responses specific for dead cell-associated antigens. However, apoptotic cell death is often more efficient in eliciting a protective anticancer immune response than necrotic cell death [238]. Certain cytotoxic agents can trigger a functionally peculiar type of apoptotic death, denoted ICD, that has the ability to trigger an adaptive immune response. The concept of ICD comprises a dying cancer cell that releases a series of endogenous immunostimulatory DAMPs in a precise spatiotemporal configuration. This chronic exposure to DAMPs in the TME stimulates the innate immune system and can contribute to long-lasting protective antitumor immunity [239]. Three distinct intracellular events orchestrate ICD in dying tumor cells: (i) the cell surface translocation of CRT after ER stress, (ii) nuclear translocation and extracellular release of HMGB1 after plasma membrane

permeabilization and (iii) extracellular release of ATP secondary to autophagy. Interactions between the DAMPs and phagocytosis receptors, PRRs and purinergic receptors, respectively, on the surface of innate immune cells act as activators (Fig. 6). The inflammasome activation in APCs leads to secretion of IL-1β and IFN-γ that restimulate APCs to present antigens on MHC I and MHC II molecules to T cells and trigger a T cell immune response against TSAs and TAAs [239].

ICD has been described as a crucial process that may turn cancer cells into anticancer vaccines and mediate immune clearing of all cancer cells, which makes ICD unique and beneficial for cancer therapy. Several anticancer chemotherapeutic agents and radiotherapy have been successfully employed in the clinic for decades and have now emerged as novel immunotherapy tools. For a cytostatic drug to be classified as *bona fide*-ICD inducer, it has to release all the tree factors of ICD [61]. The gold standard approach to detect ICD relies on *in vitro* and specific *in vivo* vaccination experiments, and ICD can therefore not be proved in patients [240].

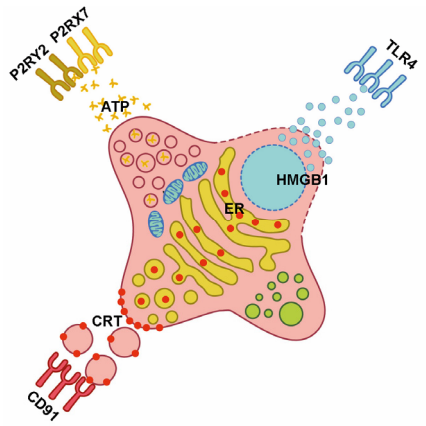

Figure 6.
Immunogenic cell death. Cancer cells responding to a lethal stimulus release danger-associated molecular patterns (DAMP) that trigger an adaptive immune response against tumor antigens. Such an immunogenic variant of regulated cell death is known as immunogenic cell death. It relies on the exposure of calreticulin (CRT) on the cell surface, on the secretion of ATP and on the release of high-mobility group box 1 (HMGB1), which accompanies cell death. When any of these DAMPs cannot be emitted in an appropriate spatiotemporal order, dying cancer cells cannot be perceived as immunogenic by the host immune system. ER, endoplasmic reticulum; TLR, toll-like receptor; P2RY2 and P2RX7, purinergic receptors. Adapted from Bezu et al. 2015 [61].

Calreticulin

CRT is an ER-resident protein that typically binds to misfolded proteins, thus preventing them from being exported from the ER to the Golgi apparatus. CRT also has a role as a second messenger. To trigger ICD, it is translocated to the plasma membrane of cancer cells by exocytosis following an anterograde ER-Golgi trafficking. CRT on the cell membrane has been found only on cells succumbing to

immunogenic apoptosis and acts as an “eat me” signal required for DCs to phagocyte dying cancer cells through the scavenger receptor CD91. The exposure of CRT during ICD is an early process occurring within a few hours after the stimulus. Consequently, it could be one of the determinants that distinguishes between immunogenic and non-immunogenic cell death. The capacity of ICD-inducing drugs has been shown to depend on the properties of ER stress and ROS production, considering that triggering ER stress can reinitiate an immune response suppressed by the TME [239]. Contrary to CRT, CD47 acts as a “don´t eat me” signal interacting with its receptor SIRP-α on macrophages to negatively regulate phagocytosis. CD47 is constitutively up-regulated on myeloid leukemias, and overexpression of CD47 favors disease dissemination by evading macrophage-mediated phagocytosis [241].

HMGB1

HMGB1 is a non-histone chromatin-binding protein that regulates transcription. HMGB1 overexpression is observed in almost all cancer cells and promotes cell cycle progression [183]. Although extracellular HMGB1 had been believed to be released mainly from the nucleus during necrosis, it was found to be excreted from cells undergoing late stages of apoptosis and autophagy assuming a DAMP-like roll. HMGB1 initiates potent inflammation by stimulating the production of pro-inflammatory cytokines from APCs via its binding to different surface receptors including receptor for advanced glycation end-products (RAGE), TLR2, TLR4, TLR9 and TIM3. It has been widely reported that the binding of HMGB1 to TLR4 is fundamental for activating DCs and facilitating antigen presentation by DCs to T cells. TLR4 activation subsequently triggers the TLR4/MyD88 pathway, which enhances tumor antigen processing by inhibiting fusion between phagosomes and lysosomes, which in turn promotes the processing of phagocytic cargo in DCs and accelerates the engulfment of antigenic components by DCs [242, 243]. Additionally, HMGB1 is an intrinsic sensor of oxidative stress. The immunomodulatory properties of HMGB1 might be determined by its redox status. Indeed, reduced HMGB1 production from dying cells was shown to trigger the immunogenic DCs, whereas oxidized HMGB1 during apoptosis fails to do so [239].

As part of ICD, the expression of HSP70 and HSP90 on dying cell membranes also has immunostimulatory properties. HSPs can also stimulate the immune system by acting on CD91 on the surface of DCs, thereby transmitting a maturation signal, or by chaperoning TSAs to MHC class I and II for efficient T cell activation [239].

ATP

Another important factor for induction of ICD is a reduction of intracellular and an accumulation of extracellular ATP concentrations from dying cancer cells treated with certain chemotherapeutic drugs. Chemotherapy affects ATP concentrations at

the pre-apoptotic level, before and during the process leading to apoptosis and secondary necrosis [243]. ATP interactions with the P2RX7 purinergic receptor on APCs triggers the NLRP3 or caspase-1 activation complex and subsequent secretion of IL-1β, thus, facilitating anticancer immune responses. Certainly, the endogenous ligand that binds with the highest affinity to the receptor P2RX7 is ATP [244]. ATP can also play the role as a "find-me" signal for the efficient clearance by APCs that express the purinergic receptor P2RY2, facilitating their migration into inflamed sites [239]. There has been an ample consensus that ATP levels would control the switch between apoptosis, which requires high ATP levels and necrosis, that occurs at low ATP levels [245]. It remains to be determined whether ATP is passively or actively released and if such ATP release may indeed represent a checkpoint to the immunogenicity of chemotherapy.

Altogether, CRT exposure, HMGB1 release and ATP secretion by human cancer cells appear to be the gold standard for accurately predicting the ICD-inducing capacity of chemotherapeutic agents. Unlike CRT induction, the release of ATP and HMGB1 is triggered by a range of death-inducing stimuli, and is not restricted to induction of ICD [243].

Immunosuppression mechanisms elicited by the tumor microenvironment

GBM has an immunosuppressive TME generated by the interplay between cancer cell intrinsic features and the other cellular components of the TME. As mentioned above, direct interactions between physiological factors such as constant cycles of hypoxia, acidosis, necrosis and angiogenesis as well as physical components such as the BBB, neurons and astrocytes are critical in determining the phenotype of the TME. However, cancer cells and tumor-infiltrating immune cells are the main drivers of the constant state of "chronic inflammation" that originally granted the term "the wound that never heals" [224].

Immunosuppression derived from cancer cells

Tumor cell-intrinsic mutations that contribute to "cold tumor" phenotype

The most common genetic or epigenetic cancer cell intrinsic factors that have been shown to modulate the immunosuppressive TME of GBM are the PI3K (phosphoinositide 3-kinase), Ras-MAPK, TP53, IDH, IDO and WNT/β-catenin pathways [246] that regulate proliferation, survival and invasion.

The most common pathways responsible for PI3K and Ras-MAPK activation in GBM are aberrant EGFR [247] and c-mesenchymal-epithelial transition receptor (c-met) [248] signaling. The loss of function of the tumor suppressor PTEN also results

in constitutively active PI3K signaling [249] which increases the expression of PD-L1 causing the suppression of T cells [250]. Moreover, Ras activation is induced in GBM due to mutations and loss of function on the *NF1* gene [251]. An active Ras-MAPK pathway has been shown to induce IL-6 production [252] and activation of p38 MAPK [253]. IL-6 is known to induce CCL2 expression on cancer cells and the activation of NF-κB and STAT-3 in infiltrating monocytes and glioma stem cells [252]. Thus, monocytes are recruited into GBM mediated by the presence of CCL2 while active STAT-3 promotes an M2 macrophage phenotype [252]. Moreover, the activation of p38 MAPK plays an important role in the induction of TGF-β [254]. The effects of TGF-β include inhibition of APC maturation and antigen presentation, impaired DC migration and cytokine secretion, induction of the M2 macrophage phenotype as well as inhibition of T cell activation and differentiation [255]. Notably, a phase II clinical trial in GBM with a TGF-β2 inhibitor report a trend towards prolonged survival [256]. The loss of function mutation of *TP53*, found in a large proportion of GBM, [257] is associated with tumorigenesis as it leads to increased proliferation, reduced cell death, and genetic instability [258]. However, *TP53* mutations in GBM are mostly point mutations that lead to a high expression of gain of function (GOF) oncogenic variants [259]. These GOF mutations promote GBM malignancy, probably acting as transcription factors on a set of genes other than those regulated by TP53 wild type [259] and have been associated with progression and shorter overall survival in GBM, specifically, by up-regulation of CCL2 and TNFα expression via NF-κB signaling, consequently increasing microglia and MDSC infiltration [260].

IDH is a ubiquitous enzyme responsible for catalyzing the conversion of isocitrate into α-ketoglutarate as part of the tricarboxylic acid cycle. Mutations can occur in either *IDH1* or *IDH2*. IDH mutations, specifically R132H, modulate the TME due to the production of the oncometabolite 2-hydroxyglutarate (2HG) from α-ketoglutarate [261], which directly suppresses immune surveillance and intratumoral T cell accumulation [262, 263] and limits the function of NK cells by down-regulation of NKG2D ligand expression [264]. Furthermore, IDO, a tryptophan catabolic enzyme that inhibits T cell function, is not normally expressed in the CNS [265] but has been found in GBM [266]. Clinical and preclinical data show that IDO expression is involved in immune tolerance and tumor immune escape functions by induction of T cell deactivation, apoptosis and immunosuppressive programming via the expression of FoxP3 [265]. Consequently, it increases the recruitment of immunosuppressive Tregs that lead to brain tumor growth [267]. Finally, the relevance of the WNT/β-catenin pathway in the TME of GBM is related to its role in the maintenance of the BBB integrity [268]. In addition, observations in melanoma show that it regulates IDO expression [269], promotes the secretion of IL-6 and TNF-α in infiltrating TAMs [140], limits DC infiltration and dampen both T cell priming and effector T cell recruitment [246].

Loss of HLA/MHC

One mechanism through which GBM cancer cells avoid the immune system is counteracting the correct antigen recognition. This can occur by down-regulation of costimulatory molecules, HLA class I molecules or proteins associated with the antigen presentation machinery such as tapasin [270]. Around 50% of GBM have lost the expression of HLA class I [270] (Fig. 4C). Cancer cells lacking MHC class I avoid the recognition by T cells during immune surveillance but can be cleared by NK cells. Additionally, HLA-G, which provides immune tolerance throughout the maternal-fetal interface by inhibiting NK cell activity, is also expressed in GBMs, and is tentatively involved in tumor escape [271, 272]. Accordingly, the presence of HLA defects in GBM may explain the relatively poor therapeutic effect reported in clinical T cell-based immunotherapy trials conducted in patients with GBM.

Immunosuppression derived from immune cells

Regulatory T cells

GBM cancer cells can express IDO [267] and secrete soluble factors such as CCL22, retinoic acid and IFN-α that together preferentially recruit Tregs to the TME and promote their survival and expansion [273]. Moreover, Tregs [274] are known to secrete IL-10 and TGF-β, down-regulate IL-2 production [214], express CTLA-4 and PDL-1 [275] and suppress the transcription of pro-apoptotic genes [273], which directly inhibits IFN-γ production and limits the function of intratumoral CD8 T cells [276]. Furthermore, Treg infiltration correlated with tumor grade in GBM [277]. Interestingly, low-dose TMZ before DC vaccination specifically reduced Tregs in advanced melanoma patients [278]. Similarly, metronomic TMZ but not standard dose significantly decreased Treg/$CD4^+$ ratios in a glioma rat model [279].

Tumor-associated macrophages and myeloid-derived suppressor cells

GBMs comprise two distinct macrophage populations, bone marrow-derived macrophages (BMDM) and microglia, which together are referred to as TAM [280]. TAM populations have been linked to the growth, angiogenesis and metastasis of cancer cells and can be described both spatially and functionally. BMDM infiltration increases from the periphery to the center of the lesion and may localize preferentially in the perivascular area where they produce a strong immunosuppression. On the other hand, resident microglia localize in the marginal peritumoral areas and display little or no suppression [281, 282]. CD45 expression levels distinguish microglia ($CD45^{low}$) from BMDMs ($CD45^{high}$) but in the GBM context CD49D/ITGA4 was identified as a marker for BMDM but not microglia [280]. At the functional level, microglia and BMDMs from GBM tissue have shown to be less efficient APCs [283, 284] whereas iron metabolism and phagocytosis only are characteristic of BMDM [282]. The M1 phenotype is characterized as $IL\text{-}12^{high}$, $IL\text{-}23^{high}$ and $IL\text{-}10^{low}$ and production of TNF-α, IFN-γ and ROS [276] and M1

macrophages are found in normoxic tumor regions. M2 macrophages are present in areas of hypoxia [285] and are defined as IL-12^{low}, IL-23^{low} and IL-10^{high}. GBM cells recruit TAMs by the overexpression of colony-stimulating factor-1 (CSF-1) [286] and TGF-β1 signaling pathways [287] leading to a pro-tumorigenic TME by the release of immunosuppressive factors such as IL-10 and overexpression of IDO, promoting an M2 phenotype [285]. M2 cells also lack expression of key costimulation molecules such as CD40, CD80 and CD86 [288] and drive both tumor angiogenesis [276] and resistance to anti-VEGF agents [289]. Moreover, ARG1, CD206, CD204 and CD163 are considered M2 markers and increase as tumor grade increases [286, 290]. CD163 has been associated with poor prognosis in GBM patients [285]. Notably, the mesenchymal GBM subtype, characterized by NF1 deficiency, promotes macrophages/microglia recruitment in GBM and has been associated with increased TAM/microglia infiltration where the M2 phenotype is significantly more abundant [18]. Finally, the plasticity and ability of TAMs to reprogram could have a therapeutic potential (discussed in Immunotherapy).

MDSCs, which varyingly are described as part of TAMs or as its own entity, inhibits the functions of T cells through various mechanisms. Mo-MDSCs suppress T cell function primarily by (i) up-regulating inducible nitric oxide synthases (iNOS) [291], (ii) secreting immunosuppressive cytokines such as IL-10 and TGF-β [292], (iii) promoting angiogenesis through the production of matrix metalloproteinase 9 [293] and (iv) producing arginase l which depletes the levels of L-arginine in the TME that leads to inhibition of CD3-zeta chain and IL-2 production impairing the GO-G1 cell cycle phase resulting in T cell apoptosis [294, 295]. In contrast, G-MDSCs primarily use ROS, which directly down-regulate the γ-chain of the T cell receptor, thus impairing their activity [296]. Additionally, MDSCs promote Tregs [297] which subsequently dampens T cell and NK cell function, and express COX-2 and PGE_2 [298].

GBM patients had a significant elevation of MDSCs in peripheral blood [299] but a decrease in absolute numbers of circulating Th cells; however, there is an increased proportion of immunosuppressive Tregs within the remaining $CD4^+$ cell pool in the blood [300]. A study on newly diagnosed GBM patients revealed that reduced MDSCs over time is accompanied by a concomitant increase of DCs in peripheral blood. In addition, a reduced number of MDSCs was found in GBM patients with extended survival, comparable to the levels of low-grade glioma patients. Lastly, tissue from rGBM showed an increased level of MDSCs, which is associated with poor prognosis [299].

Immunotherapy

GBMs, as many malignant tumors, display a state of chronic inflammation. Inflammation is part of tumor progression and elimination; therefore, it is rational to speculate that by externally manipulating the IS it could be possible to overcome tumor progression and promote tumor elimination. Emerging clinical data suggest that immunotherapy is an attractive treatment strategy for GBM and is likely to become an essential part of the clinical management of GBM. All immunotherapies share the common property of targeting the host immune system rather than the cancer itself [301]. Consequently, immunotherapy aims to amplify the natural dampened antitumor immune responses into an effective antitumor-specific response resulting in tumor eradication and long-lasting immunological memory, thus, preventing a probable tumor relapse.

The main advantages of immunotherapy are (i) the potential to generate a long-term cellular memory, and that it (ii) is less toxic for the normal brain parenchyma than chemotherapeutic agents, (iii) targets both dividing and non-dividing tumor cells, and (iv) has the ability to adapt to new mutations occurring during tumor progression. However, immunotherapy could also present unwanted effects such as induction of autoimmunity, including encephalitis [302] or a prolonged inflammatory status. There are also technical and logistical difficulties associated with immunotherapy and some of them have a time-consuming developing and manufacturing process [303]. The features of GBM that could potentially limit the action of immunotherapy are the low tumor mutational burden, the broad tumor heterogeneity, the location which leads to a restricted access of drugs and perhaps most significant, immunosuppression directly impacting the functionality of T cells.

Cancer immunotherapies can be categorized according to whether they (i) increase the existing immune response mediated by natural immune effectors, named *amplifiers*, e.g. tumor vaccines, checkpoint inhibitors or adoptive immunotherapy with tumor-infiltrating lymphocytes (TIL) or (ii) create non-natural immune effectors that initiate new immune responses directed toward tumor-expressed targets, named *synthetic immunotherapies*, e.g. monoclonal antibodies or adoptive immunotherapy with lymphocytes expressing either chimeric antigen receptors (CAR) or affinity-matured TCRs. Synthetic immunotherapies do not require inherent tumor immunogenicity to be effective; rather, they direct the immune system to recognize molecules predicted to manifest a favorable therapeutic index, based upon differential expression in cancer tissues versus normal vital tissues [301].

Vaccines

Vaccination is one of the earliest developed immunotherapeutic modalities. Immunization presents TAAs and/or TSAs at sites outside the tumor and CNS, usually subcutaneously. At the immunization site, the antigens are believed to be phagocytized by infiltrating APCs. The APCs mature and migrate to the secondary lymphoid organs, where they will present antigens and activate T cells. Vaccines can be boosted with adjuvants like aluminum or TLR4 agonist or by preconditioning the vaccine site with recall-antigen substances such as tetanus/diphtheria toxoid [304].

GBM vaccines can be divided into two main categories: peptide or protein vaccines and cell-based vaccines.

Peptide vaccines

Peptide vaccines are interesting immunotherapies for the treatment of GBM because they are easy to manufacture and administrate and have the capacity to induce tumor-specific immune responses in patients with primary and recurrent gliomas and stimulate early signs of clinical response [305]. Vaccines targeting the *EGFR* deletion mutation (EGFRvIII) [305, 306] or *IDH1* mutation [307, 308] have been tested clinically. EGFRvIII vaccines are the most widely studied TSA vaccines against GBM. One of them, Rindopepimut (CDX-110), despite showing promising results in phase I and II clinical trials, unfortunately did not increased survival in patients with newly diagnosed GBM when combined with standard TMZ treatment [309].

The heterogeneity of GBM and the relatively low mutational burden and subsequent absence of highly expressed TAAs and TSAs [310] are obstacles for peptide vaccines. The lack of antigen specificity carries the risk of leading to antigen escape, e.g. loss of expression of the targeted antigen and therefore treatment resistance [311]. Therefore, combining multiple neoantigen candidates can potentially overcome tumor escape. There is clinical evidence of personalized cancer vaccines targeting TSAs. Studies on TAAs/TSAs derived from whole exome sequencing [312] or neoantigens with unmutated TAAs to increase the number of actionable epitopes [313] have reported a good response, infiltrating tumor-reactive T cells with memory phenotypes and neoantigen-specific clonality [313].

Tumor cell vaccines

Autologous whole tumor cells have been used in several studies as a source of tumor antigens. Resected autologous tumor cells are expanded *ex vivo*, and injected back into the patient. The tumor cells are commonly inactivated by genetic modifications or high doses of irradiation [314]. Irradiation may also enhance the immunogenicity of the injected tumor cells [315]. In addition, tumor cells can be genetically

engineered to further increase cytokine production and boost the induced immune reaction. GM-CSF is the most commonly studied cytokine in this context. By using whole tumor cells, the immune response will be directed towards several tumor antigens, thereby diminishing the risk of therapy resistance. There is however a theoretical potential for selection of autoreactive T cells due to the lack of antigen specificity [316]. The limiting factor of whole tumor cell vaccines is that the development of the vaccine depends on the amount of tumor cells resected and their expansion *in vitro*. No toxic events have been recorded and longer survival was detected in some of the studies [317]. In the clinic, autologous irradiated tumor cells accompanied by an allogeneic tumor cell line (K-562) secreting GM-CSF was associated with systemic T cell activation and antitumor immunity [303, 317].

Dendritic cell vaccines

DCs are potent immunostimulatory cells that continuously screen the antigenic environment of the host and specifically activate $CD4^+$, $CD8^+$ T cells and B cells. Monocytes can be isolated peripherally and then cultured in the presence of GM-CSF and IL-4 to generate DCs [316, 318]. Then, immature DCs can be pulsed with autologous tumor-eluted peptides [319], tumor lysate/whole tumor cells [320] or tumor-derived RNA [321]. DCs can mature *in vivo* or *in vitro* with adjuvants like a-galactosylceramide, CD40-specific antibody, inflammatory cytokines or specific TLR ligands) and then pulsed with defined T cell epitopes [322].

With regards to GBM, a phase III clinical trial reports systemic and intracranial T cell response [319] but a large variety of clinical trials employing DCs have been published and report beneficial effects in terms of survival in non-randomized trials [323].

Adoptive cell therapy

Adoptive cell therapy (ACT) involves expanding autologous cytolytic lymphocytes *ex vivo* and reinfusing these tumor-specific T cells back into the patient [324]. Culturing peripheral lymphocytes *in vitro* in the presence of IL-2 produces unspecific lymphocyte-activated killer cells with cytolytic properties [316]. Treatment approaches differ in the types of cells administered, the route of administration and the activation status of the cells. ACT includes the transfer of a variety of immune populations such as (i) autologous mononuclear cells, (ii) lymphokine-activated killer cells, (iii) mitogen-activated killer cells, (iv) TILs and (v) antigen-specific cytotoxic T cell lymphocytes [316]. The routes of administration have generally been either systemic [325] or intralesional [326].

The application of ACT is limited by the relatively low frequency or lack of tumor antigen-specific lymphocytes that can be isolated from GBM, or one TSA that can

be suitably targeted. It is also limited due to a reduced trafficking of adoptively transferred cells to the tumor site and the highly immunosuppressive TME found in GBM [327]. To tackle ACT limitations, strategies have been designed to manipulate T cells to express specific TCR-αβ transgenes to generate a TCR capable of targeting a known tumor antigen [328]. Additionally, in order to bypass MHC restriction and to allow targeting of non-protein antigens, CAR T cells were developed [329].

Chimeric antigen receptor T cell

CAR T cells consist of an extracellular domain expressing a single chain Fv region. Thus, the TCR "resembles an antibody" and is capable to target a known tumor antigen. Remarkably, this tactic does not require the tumor antigens being targeted to be presented in the context of MHC and so bypasses one potential mechanism of tumor cell evasion. Firstly, CAR linked either the CD3 or FcγR signaling domains to the scFv recognizing tumor antigen, but their efficacy was limited due to the lack of costimulatory pathway activation [327]. Consequently, CARs have been designed to incorporate the CD28 or 4-1BB signaling domains, named "second generation CAR T cells" [330]. Then, additional signaling domains (4-1BB, CD28 and/or OX-40) have been added to the intracellular component of the CAR to make "third generation CAR T cells" [327]. The second generation CARs have had unprecedented success with remission in several hematological cancers through targeting the CD19 antigen [132]. A fourth generation of CARs have been proposed, equipped to overexpress cytokines that potentiate their function [331].

Most of the studies with CAR T cells in GBM target EGFRvIII [332], IL13Rα2 [326] or HER2 [333]. Since EGFRvIII [306], IL13Rα2 [334] and HER2 [335] are highly expressed in primary human GBMs but relatively absent in normal cells, they appear to be promising CAR targets. Clinical trials against these targets have adequate antitumor capabilities. They have been found safe based on the absence of off-target toxicities and lack of cytokine release syndrome [325], achieved demonstrable regression of tumors and [331] detectable levels of intratumoral CAR T cells after tumor resection [336]. Off-target toxicity by HER2-targeting CARs has been reported since HER2 is also expressed in healthy tissues like epithelial lung cells [337]. Unfortunately, none of the CAR target antigens discussed above are universally expressed in GBM, allowing the outgrowth of antigen-negative clones and subsequent recurrence. Of note, myeloconditioning prior to adoptive transfer has been shown to improve to CAR therapies. Lymphodepletion induces a massive T cell proliferation and amplifies tumor-specific immune responses, by replacing the T cell pool with only those T cells that can propagate an immune response against the targeted antigen [338, 339].

Immune checkpoint inhibitors

Cancer-immunity cycle refers to a series of steps that initiate and support an antitumor immune response which leads to efficient cancer cell death. Immune checkpoint molecules are crucial for self-tolerance and function as brakes that prevent the immune system from attacking cells indiscriminately. Immune checkpoints are receptors on the surface of activated T cells. When the receptors have been activated, they promote self-tolerance by suppressing T cell inflammatory activity and even inducing T cell apoptosis. Some cancers can protect themselves from T cell cytotoxicity by overexpressing and stimulating immune checkpoint pathways [229].

CTLA-4 has been the most extensively studied immune checkpoint receptor. It dampens T cell activation by competing with the costimulatory molecule CD28 for binding of B7 on APCs [340]. Ipilimumab (anti-CTLA-4, Bristol-Meyers-Squibb) treatment leads to increased availability of CD28, which amplifies T cell responses. Ipilimumab was first FDA-approved for metastatic melanoma in 2010 and is now an accepted therapy in several cancer types.

PDL-1 (PD-1 ligand) is expressed on certain subsets of immune cells but is aberrantly expressed on tumor cells [340]. PDL1 expression in GBM has been correlated with worse prognoses in some studies [341]. PD-1 is a member of the CD28 family expressed on the surface of T cells and moderates the immune response under physiological conditions [340]. Binding of PD-1 leads to impaired T cell activation through decreased TCR signaling and decreased induction of key transcription factors such as activator protein 1 (AP-1) and nuclear factor of activated T cells [342]. Clinical trials with anti-PD-1 in other cancers promoted durable antitumor responses, which led to FDA approvals of nivolumab (anti-PD-1, Bristol-Meyers-Squibb) and pembrolizumab (anti-PD-1, Merck) [340].

PD-L1 induces and maintains Tregs in GBM, which are associated with decreased survival in glioma patients [343]. Anti-PD-1 therapy has been explored as a neoadjuvant in GBM. No clinical benefit has yet been observed, although it resulted in enhanced expression of chemokine and cytokine transcripts, higher immune cell infiltration and augmented TCR clonal diversity among tumor-infiltrating T cells, supporting a local immunomodulatory effect of the treatment [344, 345]. Safety studies with checkpoint inhibitors in GBM patients have shown toxicity comparable to that reported from other cancer types [346].

Oncolytic virotherapy

Originally, oncolytic virus therapies were considered a treatment strategy separate from immunotherapy [347]. However, oncolytic virotherapy is a very interesting tactic to overcome the immunosuppression of GBM. The therapy intents to infect

and lyse cancer cells aiming to initiate an endogenous secondary immune response to the cell lysis, prompting ICD pathways. Consequently, the immune response is triggered by the release of DAMPs and TAAs/TSAs. Alongside ICD, oncolytic viruses themselves induced an antiviral innate immune response mediated by PAMPs that potentially boosts the T cell response. Oncolytic viruses can thus be considered to function not only as direct cancer killing agents, but also as active anticancer vaccines [348].

Today, oncolytic viral approaches employ replication competent viruses, such as retroviruses, adenoviruses, herpes simplex viruses, polio viruses and measles viruses and are typically delivered intratumorally, often postsurgically into the resection cavity [105]. Oncolytic viruses been shown to synergize with radiotherapy or TMZ [349, 350]. Oncolytic viruses can also be used to transfer therapeutic payloads into tumor cells such as predrug components [351] and immunoregulatory IL-12 and OX40 ligand which are currently tested in clinical trials [348].

Two oncolytic viruses, PVS-RIPO and DNX-2401, have accomplished promising results and therefore been granted a fast-track designation by the FDA. PVS-RIPO is a replication-competent, live attenuated poliovirus vaccine/human rhinovirus chimera. PVS-RIPO infects the cell through the receptor CD155 which is up-regulated on malignant glioma cells and also expressed on APCs [352]. A phase I study in rGBM with PVS-RIPO showed a significant improvement in overall survival and is currently in phase II, although one possibly virus-related death and one dose-limiting toxic effect were reported [105].

DNX-2401 is a replication-competent adenovirus that was engineered with a specific mutation to restrict viral replication. Adenoviruses are relatively easy to genetically engineer and to maintain *in vitro*, however there are challenges associated with the identification of target receptors for the virus. This virus contains an arginine/glycine/aspartic acid design to target integrins on GBM, in order to increase infective specificity for tumor cells [104]. DNX-2401 has been investigated in a phase I trial in combination with TMZ and anti-PD1 antibody [346].

The gammaretrovirus Toca 511 (vocimagene amiretrorepvec) is a non-lytic, replicating retrovirus based on the amphitropic murine leukemia virus that has been engineered to encode a modified yeast cytosine deaminase that converts the prodrug 5-fluorocytosine (5-FC) to 5-fluorouracil (5-FU) in infected cancer cells [351]. Good tolerability and a significantly increased overall survival have been demonstrated in clinical studies. The proposed mechanism of action is not only a direct tumoricidal effect of the drug but also the antitumor immune response induced which has led to phase II trials in patients with rGBM [353].

Additional strategies

As previously described in this thesis, TAMs in GBM have an M0 or M2 phenotype and are genetically stable. The plasticity of TAMs makes them a good candidate treatment target. Repolarizing TAMs into an M1 phenotype without reducing their migration activity could tentatively reduce the degree of immunosuppression. TAMs are dependent on CSF-1 and its receptor CSF-1R for differentiation and survival [354]. TAMs secrete EGF and express CSF-1R, while GBM cells express EGFR and secrete CSF-1 to generate a paracrine loop [355]. Efforts to "re-educate" TAMs have been performed in preclinical animal models and tested in rGBM patients. Thus far, CSF-1R has been pharmacologically targeted and its inhibition has shown the ability to either deplete or reprogram TAMs in preclinical murine models. PLX3397, a CSF-1R inhibitor, reduced the number of TAMs and GBM invasion *in vivo* [355]. Similarly, BLZ945, another CSF-1R inhibitor, impeded the progression of intracranial xenografts by promoting TAMs [354]. However, rGBM patients treated with PLX3397 in a phase II clinical trial did not respond to treatment, although it was reported to be safe [356]. Nevertheless, even though the results of single agents are unsatisfactory, the combination of these inhibitors could modify the TME in ways that might permit the immune system to mediate tumor rejection when combined with other immune modulatory agents.

Aims of the thesis

The specific aims of the individual studies were:

Paper I. To assess the therapeutic effect of CED of temozolomide in combination with immunotherapy consisting of whole cell immunizations in the GL261 and KR158 glioma models.

Paper II. To assess the therapeutic effect of CED of cisplatin as a monotherapy or in combination with immunotherapy consisting of wildtype or GM-CSF-transfected cell immunizations in the GL261 glioma model.

Paper III. To assess the therapeutic effect of CED of mitoxantrone in the GL261, KR158 and SB28 glioma models.

Paper IV. To evaluate the feasibility of systemic immune profiling by characterizing the systemic cytokine profiles of pediatric brain tumor patients.

Results

Paper I.

Convection-enhanced delivery of temozolomide and whole cell tumor immunizations in GL261 and KR158 experimental mouse gliomas

In *paper I*, we investigated the therapeutic efficacy of CED of TMZ (CED-TMZ) and immunotherapy consisting of whole wildtype cell immunizations, combined and as monotherapies, in the GL261 and KR158 mouse glioma models. We also used immunosuppressed mice to explore if the effect of TMZ depended on the interaction between tumor cells and the immune system. In addition, we analyzed the changes in the intratumoral immune cell compartments following treatment of immunocompetent mice.

In the GL261 model, we found that all treatments significantly improved survival of tumor-bearing mice. The main finding was that CED-TMZ + immunotherapy synergized in this model. CED-TMZ as monotherapy cured 45% of mice bearing GL261 gliomas, while immunotherapy as monotherapy cured 15%. The cure rate was further enhanced to 93% when CED-TMZ and immunotherapy were combined. In addition, as a proof of concept, all mice treated with mini-osmotic pumps containing saline solution (NaCl 0.9%) succumbed to tumor growth. In the more aggressive KR158 model, both the combination of CED-TMZ + immunotherapy and immunotherapy as monotherapy cured 6% of mice bearing tumors. No mice treated with CED-TMZ as monotherapy were cured; however, there was a prolonged median survival compared with non-treated mice.

Treated GL261 mice that survived for more than 100 days after tumor inoculation were rechallenged with a new tumor in the contralateral hemisphere without further treatment. 100% of mice treated with CED-TMZ + immunotherapy and 90% of mice treated with CED-TMZ as monotherapy rejected the secondary tumor. Moreover, the effect of CED-TMZ in the GL261 model was totally abrogated in immunocompromised NOD-scid mice.

In a separate experiment conducted to analyze the intratumoral immune cell components after treatment, mice bearing tumors were sacrificed when the first animal in the experiment showed signs of tumor growth (day 33 for GL261; day 20 for KR158). Immunohistochemical analysis was performed to evaluate quantitative

and qualitative changes in intratumoral T cell ($CD4^+$, $CD8^+$) and macrophage ($F4/80^+$) populations, respectively. In the GL261 model, there was a significant reduction of tumor volume and an increase in the amount of $CD8^+$ and $CD4^+$ T cells in all treated groups, as well as an increase in F4/80 staining after CED-TMZ + immunotherapy compared with the non-treated group and monotherapies. In the KR158 model, only CED-TMZ + immunotherapy significantly reduced the tumor volume compared to monotherapies and non-treated mice. In addition, CED-TMZ + immunotherapy and immunotherapy as monotherapy significantly increased the amount of intratumoral $CD8^+$ and $CD4^+$ T cells per mm^2 compared to non-treated. CED-TMZ + immunotherapy as well as immunotherapy as monotherapy significantly decreased the percentage of stained area of $F4/80^+$ macrophages compared to CED-TMZ as monotherapy. Finally, we could not observe any differences in COX-2, mPGES-1 or iNOS staining between different therapeutic regimens in any model.

In the GL261 model, we also investigated whether a single intratumoral injection of TMZ could reach the same therapeutical effect as its corresponding pump dose. In brief, TMZ delivered by single injections resulted in lower survival and was less tolerated than the same dose delivered by pumps.

Paper II.

The effect of locally delivered cisplatin is dependent on an intact immune function in an experimental glioma model

In *paper II*, we investigated the therapeutic efficacy of CED of CIS (CED-CIS) and immunotherapy consisting of whole cell immunizations, with either GL261 wildtype cells (GL-wt) or GL261 cells transduced to produce GM-CSF (GL-GM). Changes in the intratumoral immune cell compartments after treatments were evaluated. In addition, we used immunosuppressed mice to explore if the effect of CIS depends on the interaction between tumor cells and the immune system.

We found that CIS is an effective drug against GL261 cells *in vitro* as well as *in vivo*. CED-CIS cured 40% of mice bearing GL261 glioma and the effect was abolished in immunocompromised NOD-scid IL2rγ null (NSG) mice. CED-CIS had no additive effect with neither GL-wt nor GL-GM immunizations. The combination of CED-CIS with GL-wt or GL-GM immunotherapy cured 33 and 22% of treated mice, respectively, whereas GL-GM immunotherapy as monotherapy also cured 22% of the mice. Notably, CED-CIS resulted in high levels of toxicity at the higher doses tested.

An immunohistochemical analysis was performed to investigate the qualitative changes of intratumoral T cell ($CD4^+$, $CD8^+$) and macrophage ($F4/80^+$) populations.

We found a significant increase in the percentage of stained area of $CD8^+$ cells in mice treated with GL-GM immunotherapy as monotherapy. No changes in $CD4^+$ T cell populations were observed. Furthermore, there was a significant reduction in the percentage of stained area of $F4/80^+$ macrophages in mice treated with GL-GM immunotherapy as monotherapy and CED-CIS + GL-GM immunotherapy compared with non-treated mice. The $F4/80^+$ macrophages also expressed CD206.

Paper III.

Convection-enhanced delivery of mitoxantrone in GL261, KR158 and SB28 experimental mouse gliomas

MTX is a *bona fide* ICD-inducing drug and has the potential to enhance antitumor specific cellular immunity but has been sparsely tested in GBM. We therefore hypothesized that MTX would have a therapeutic advantage compared with conventional drugs. In *paper III*, we investigated the therapeutic efficacy of CED of MTX (CED-MTX) in GL261, KR158 and SB28 mouse glioma models and whether MTX induced *in vitro* changes in MHC, costimulatory molecule expression and ICD-related proteins.

All glioma cell lines were sensitive to MTX exposure *in vitro* but at different half-maximal inhibitory concentration (IC_{50}). The lowest MTX concentrations that reached IC_{50} was 1 μM in KR158 cells and 2 μM in GL261 cells after 48 hours. IC_{50} in SB28 cells was between 2 and 5 μM and it was independent of exposure time. In the *in vivo* setting, we found that CED-MTX cured 16% of GL261-bearing mice (30 μM). There was a trend towards enhanced cure rate in the SB28 model (33% with 30 μM). No mice with KR158 tumors were cured.

Furthermore, MTX exposure enhanced expression of immunomodulatory proteins *in vitro*. There was no up-regulation in the expression of any marker after 24 hours of exposure to MTX in any sample. However, after 48 and 72 hours the expression of MHC I and MHC II was up-regulated in KR158 and SB28 cells whereas GL261 and SB28 cells up-regulated CD80 and CD86 after exposure to 1 μM MTX.

Moreover, we validated the ability of MTX to induce ICD, although not previously shown in these cell lines. First, CRT was up-regulated in all cell lines after the *in vitro* after exposure to 1, 2 and 5 μM of MTX during 24, 48 and 72 hours. The up-regulation was dependent on the dose and exposure time; however, the up-regulation was most prominent in the KR158 cells at the highest dose, up to 95%, while at lower doses the up-regulation was between 36 and 47% only after longer exposure time. GL261 cells up-regulated CRT by 14 to 36%, and there were no major changes depending on exposure time or dose. In contrast, SB28 cells showed a similar up-regulation between 17 and 46%, but only at longer exposure time with

no change at short exposure time. Secondly, we investigated HMGB1 translocation from the nucleus to the cytoplasm after the exposure to 1 μM of MTX. Immunofluorescence microscopy showed that non-treated GL261 cells were largely negative for HMGB1, except for a few clusters with positive nuclear staining. After exposure to 1μM of MTX all GL261 cells displayed a positive HMGB1 nuclear staining. HMGB1 was translocated from the nucleus in SB28 cells after exposure to 1μM MTX, (Fig. 3, bottom panels). KR158 cells exposed to MTX did not display any change (data not shown).

Paper IV.

Preoperative systemic levels of VEGFA, IL-7, IL-17A, and TNF-β delineate two distinct groups of children with brain tumors

The clinical response to immunotherapy and intratumoral chemotherapy could tentatively be reflected in the blood circulation. As an initial step to evaluate the feasibility of systemic immune monitoring, we performed a comprehensive systemic immune profiling of preoperative plasma samples from brain tumor patients in *paper IV*.

We analyzed 20 cytokines in samples from 45 children with brain tumors (medulloblastoma, ependymoma, sarcoma, high-grade glioma, pilocytic astrocytoma, and other low-grade gliomas) and identified four factors that separate the patients into two groups defined as group **A**, $VEGFA^{high}/IL\text{-}7^{high}$, $IL\text{-}17A^{low}/TNF\text{-}\beta^{low}$ and group **B**, $VEGFA^{low}/IL\text{-}7^{low}$, $IL\text{-}17A^{high}/TNF\text{-}\beta^{high}$. Overall, most patients with medulloblastomas, GBMs and healthy controls were found in group A, whereas the other tumor types were equally distributed between the two groups.

When comparing tumor patients with healthy controls, we found trends towards increased IL-10 and decreased IL-12/23 and TNF-α in several tumor types. Four patients in this cohort displayed evidence of enhanced systemic immune activation, including increased levels of GM-CSF, IFN-γ, IL-6, IL-12/23 and TNF-α.

In addition, as a proof of concept that the cytokine modifications were related to tumor burden, we collected blood samples from one medulloblastoma patient at the day of primary surgical resection and 1 month after surgery. At time of surgery, the cytokine profile of plasma was consistent with most other samples from medulloblastoma patients; that is, low levels of IFN-γ, TNF-α, and IL-12/23, and high levels of IL-7, VEGFA, and IL-10. One month later, cytokine values had increased or decreased to approximately the values of healthy controls, except for IL-7, which was lower than that in healthy controls 1 month after surgery.

Discussion

The treatment of GBM presents a major challenge and combined therapeutic approaches are necessary to improve the outcome of GBM patients. I propose CED as an alternative drug delivery method combined with whole cell-based vaccine immunotherapy to overcome tumor growth and generate immunological memory against glioma cells and their respective TAAs and TSAs. In this thesis, GL261, KR158 and SB28 syngeneic mouse glioma models were used to evaluate the therapeutic effect of CED-TMZ, CED-CIS and CED-MTX by Alzet® mini-osmotic pumps, alone and in combination with immunotherapy consisting of wildtype or cytokine-transduced glioma cells. Further, the intratumoral immune cell infiltration following the different treatments was analyzed. Additionally, I explored the *in vitro* changes of immunomodulatory protein expression and ICD markers on glioma cells after the exposure to some chemotherapeutic drugs.

Convection-enhanced delivery of chemotherapeutic drugs in syngeneic murine glioma models

TMZ is the most clinically effective drug against GBM and has been established as part of the gold-standard care for newly diagnosed GBM patients [48], despite the fact that systemic administration of TMZ has not shown proof of concept in any animal model of GBM. However, intratumoral administration of TMZ has achieved cure and prolonged survival in several reports, including our own. Therefore, intratumoral drug delivery of TMZ is a valid option for clinical therapy. In this study the therapeutic effect of CED-TMZ was prominent, curing mice bearing GL261 gliomas. In the KR158 model, CED-TMZ prolonged median survival compared to healthy controls but did not cure any animal. The weaker effect of CED-TMZ in the KR158 model could be due to tumor-intrinsic factors such as a high proliferation rate and a reduced susceptibility to immune cell-induced tumor lysis or due to the profound immunosuppressive features of the model [136]. To our knowledge, intratumoral delivery of TMZ has not been clinically tested yet, but intracerebral infusion of TMZ in a glioma rat model increased median survival compared with systemic intraperitoneal TMZ [357]. Furthermore, intratumoral delivery of TMZ with biodegradable polymers (polyanhydride CPP:SA) was superior to oral administration in a rodent glioma model [358]. CED of liposomal TMZ resulted in greater tumor inhibition and significantly higher survival in a rat glioma model, although there were no advantages compared with the drug solution [359, 360].

Liposomal TMZ delivery was also effective in U87MG tumor-bearing mice, significantly inhibiting tumor growth without evidence of systemic toxicity [361]. Moreover, a single intratumoral injection of slow-releasing microspheres containing 10% TMZ led to significant reduction of both subcutaneous and orthotopic human glioma xenografts [164].

The therapeutic effect of CED-CIS found in the GL261 model is in accordance with previous studies. The survival rate following CED-CIS therapy in this study was higher and obtained with a lower dose (0.72 μg) than in other reports, where intratumoral CIS did not have a therapeutical effect as monotherapy (3 μg) [362], or led to a slightly increased life span (~1 mg) [363] or up to 13% survival (3–6 μg) [108, 364] in rat glioma models. Additionally, biodegradable polymers [365, 366], hydrogels and solid slow-release rods [367] have been shown to be effective for intratumoral delivery of CIS in several experimental glioma models as a single agent or in combination with radiotherapy [108, 362]. A more recent study showed that CED-CIS consisting of nanoparticles loaded with CIS reduced the inherent toxicities associated with CIS and delivered higher concentrations of CIS throughout the tumor bulk, yielding improved therapeutic efficacy [166]. Loco-regional CIS has been tested clinically as intracarotid artery infusion of CIS in patients with brain tumors [66, 76], as biodegradable carboxyl-cellulose polymer in GBM patients [368] and as CED-CIS [369] in patients with rGBM. All delivery systems were proven feasible, well tolerated and there was evidence of therapeutic effect without systemic or local toxicity.

CED-MTX significantly cured mice bearing established GL261 tumors at the concentration of 30 μM. Moreover, we observed a trend to prolonged survival in mice bearing established GL261 and SB28 tumors at concentrations of 10 and 30 μM, respectively. There have been other efforts for locoregional administration, e.g. intra-arterial transient cerebral hypoperfusion in a glioma rat model [83] and new formulation of MTX for intratumoral delivery in glioma rat models, as well. For instance, MTX biodegradable wafer matrix implanted at day 5 after tumor inoculation reached therapeutic drug concentrations in the brain for at least 35 days and significantly improved survival compared with controls but reported signs of significant toxicity at high drug concentrations [370]; MTX EVAc–biopolymers administered at day 2 significantly prolonged survival, however, an important dose related morbidity was described [371]; poly(D,L-lactide-co-glycolide) (PLGA) microspheres loaded with MTX administered at day 7 significantly reduced tumor size compared with controls [82]. Overall, MTX displays a therapeutic effect in experimental glioma models, despite toxicity occurring even in slow-release formulations of MTX. Future studies of intratumoral MTX should be directed toward the investigation of the CNS toxicity associated with the drug and the technique. MTX is poorly lipid-soluble and thus has limited efficacy for brain tumors when delivered systemically [83, 372]. Despite this MTX has been tested in

clinical trials of GBM patients. Green et al. assessed MTX in tumor tissue after preoperative systemic delivery of 5 to 6 mg/m^2 MTX in 10 patients and concluded that MTX reached therapeutic concentrations [84]. Intratumoral delivery of MTX has been reported by Boiardi el al. and was found to be safe and feasible with preservation of good quality of life in two pilots studies. It prolonged progression-free survival in patients with recurrent GBM when combined with radioimmunotherapy [109] or combined with the Stupp protocol in comparison to the patients usually treated with second line chemotherapy treatments [88]. Then, a non-randomized study in recurrent GBM patients reports that the addition of local MTX significantly reduced the risk of death by 50% [86]. In addition, other attempts of intratumoral chemotherapy with MTX was performed by Ferroli et al. with a mix of surgifoam® and mitoxantrone. It was proven safe when applied intra-operatively into the resection cavity without any observable side effects [89].

An alternative method to simplify the procedure and eliminate the risks of the subcutaneous pump implantation would be to perform single intratumoral injections of cytostatic drugs. When testing injections with TMZ, we found that this method was less effective with a narrower therapeutic window than CED. Furthermore, TMZ bolus injection induced neurotoxicity and lethality at doses that the corresponding dose in the pump did not. We cannot determine whether the increased mortality was due to toxicity of the drug or due to the procedure itself, however, the latter is unlikely according to our experience with the procedure. Our results suggest that CED-TMZ was more effective in reducing tumor progression and provide better drug tolerance than a single intratumoral injection of the equivalent TMZ dose. On the other hand, the initial toxicity of CED-CIS toxicity was very high. After dose reduction, we found that local neurotoxicity was diminished but still present even at the lowest dose. Thus, the therapeutic window for CIS is narrow, as evident in both C57BL/6 and NSG mice. Moreover, early deaths of mice following CED-MTX without macroscopic evidence of tumor growth could be due to toxicity from MTX or an injury produced while implanting/removing the pump. Local toxicity of cytostatic drugs most probably depends on both dose and distribution and might therefore be reduced by encapsulation of the drug for slow release. Our observations speak against dose escalation for CED-CIS and CED-MTX but opens the possibility to increase the length of the treatment with a mini-osmotic pump with longer release time.

Convection-enhanced delivery offers an improved alternative for current therapies by reducing the dose of drug required to reach a therapeutic effect. In addition, this method may be used in combination with other anticancer drugs or conventional therapies in order to improve therapeutic efficacy and improve the outcome of the patients. Despite the advantages of CED described here, there might be other practical obstacles that need to be solved before the method can be applied for clinical management of brain tumors. The efficacy and safety of treatment observed

in brain tumor models suggests that the CED is a potential alternative treatment modality for human glioma patients and warrants further development.

Convection-enhanced delivery and whole cell-based immunotherapy in syngeneic murine glioma models

Despite the challenges of tumor cell-based vaccines, they have shown activity against high-grade gliomas. It has been shown that the combination of ionizing radiation and peripheral vaccination results in long-term survival in the GL261 model [373-375] demonstrating long-lasting remission. In this work, CED-TMZ synergized with immunotherapy with wildtype cells in the GL261 model as it did with immunotherapy using GM-CSF-transduced GL261 tumor cells (GL-GM) [376]. Immunotherapy with non-transduced cells is encouraging since removing the step of cell transduction simplifies the clinical translation of this therapy. However, immunotherapy with wildtype cells as monotherapy had a modest therapeutic effect compared to immunotherapy with GL-GM cells in the GL261 model. On the other hand, in the more immunosuppressive KR158 model, immunotherapy was the main factor responsible for prolonging median survival, even though it was not curative. CED-CIS did not have an additive effect with immunotherapy neither with wildtype cells nor with GL-GM cells, demonstrating differences in the ability of various chemotherapeutic drugs to boost an immune response. Belmans et al. found that the combination of immunotherapy with subcutaneous autologous tumor lysate and oral TMZ significantly improved the median survival with 80% compared with mock treated mice and 20% compared with TMZ monotherapy in the GL261 model, however no mice were cured [377]. Thus, the immunomodulatory features of TMZ [60] seems to be more prominent when it is administrated intratumorally. Furthermore, a phase I/IIa clinical trial with autologous formalin-fixed tumor vaccine added to the Stupp protocol was well tolerated and resulted in favorable PFS and OS in 24 newly diagnosed GBM patients [378]. In another phase I study examining the safety of two doses of GM-K562 cells mixed with autologous cells, the vaccine was found to be feasible, well tolerated, and active in patients with recurrent malignant glioma. The study also demonstrated activation of T cells with significantly increased expression of CTLA-4, PD-1, 4-1BB, and OX40 in CD4 T cells and PD-1 and 4-1BB in CD8 T cells [317].

Whole cell-based immunotherapies are less time- and cost-efficient than some other immunotherapies and the promising results suggests that they are valid treatment option, preferably in a combinatory setting. However, further research is required to refine this treatment strategy and to improve therapeutic effects on gliomas.

Intratumoral infiltration of immune cells following CED-TMZ, CED-CIS and immunotherapy

The ability of the immune system to eliminate tumor cells mainly relies on the capacity of the $CD8^+$ effector cells to home to and accumulate within the TME. Intratumoral $CD4^+$ and $CD8^+$ T cells are a predictor of clinical outcome in GBM patients, and a high level of CD4 combined with low CD8 was associated with unfavorable prognosis [379]. CED-TMZ as monotherapy and combined with immunotherapy increased the intratumoral influx of both $CD8^+$ and $CD4^+$ T cells in the GL261 and KR158 models, with the exception of CED-TMZ as monotherapy in the KR158 model. These results strengthen the finding that T cells and their intratumoral influx are essential for the therapeutic effect of CED-TMZ and immunotherapy, also supported by the lack of therapeutic effect of CED-TMZ in the NODScid mice.

Furthermore, it has been reported that systemic delivery of CIS promotes recruitment and proliferation of $CD8^+$ effector cells into the tumor as well as improves their lytic activity [79]. However, our results in the GL261 model do not confirm this statement, since CED-CIS did not increase $CD8^+$ T cell influx, while immunotherapy with GM-CSF-transduced cells as monotherapy did. Moreover, CED-CIS combined with immunotherapy showed a tendency towards less $CD8^+$ T cell intratumoral influx compared to animals receiving immunotherapy with GM-CSF-transduced cells as monotherapy, suggesting that CED-CIS either reduces intratumoral influx or is lethal to the T cells. The fact that CED-CIS did not increase neither $CD8^+$ nor $CD4^+$ T cell infiltration and that the effect was abolished in NSG mice, lacking T cells but with reduced macrophage function, suggests that macrophages could be partially responsible for the antitumor effects. Contrary to the findings of CED-CIS, CED-TMZ as monotherapy or combined with immunotherapy improved the intratumoral infiltration of T cells, reflected in a better cure rate and probably explaining the synergy between these two treatments. It also validates differences in the ability of various chemotherapeutic drugs to boost an immune response. Additionally, CIS has also been used to precondition immunotherapy, consisting of adoptive cytokine-induced killer cells in a murine lung carcinoma model. This treatment regimen increased the accumulation of T cells and reduced the percentage of Tregs intratumorally [380].

In GBM, the intratumoral expansion of immunosuppressive cells represents a cardinal strategy deployed by tumors to escape from detection and elimination by the immune system. The major components of these inhibitory cellular networks are Tregs, suppressive TAMs and MDSCs, thus representing the main obstacle for anticancer therapies, particularly for immune-based interventions [226]. However, recent data imply that MDSCs are the main immune suppressive cells in the GBM TME [191, 299]. CED-TMZ combined with immunotherapy showed an inverse trend in macrophage infiltration in the GL261 and KR158 models. Macrophages

were increased in the GL261 model after treatment but decreased in the KR158 model, albeit from a higher absolute value and heterogeneously. We speculate that the KR158 tumors contain a larger proportion of immunosuppressive myeloid cells and that these are reduced by therapy but not sufficiently enough to generate a positive therapeutic effect. Additionally, we have observed in preceding experiments that macrophages also increased after immunotherapy in the GL261 model [314]. Notably, we could not observe any immunohistochemical difference in COX-2, mPGES-1, iNOS, galectine-3 and pSTAT-1 between the therapeutic regimens, leaving the possibility of other qualitative differences in immune cells between the treatments and models. On the other hand, immunotherapy with GL-GM cell as monotherapy or in combination with CED-CIS reduced intratumoral macrophages. In addition, all $F4/80^{+}$ cells were predominantly $CD206^{+}$, suggesting a suppressive phenotype [381, 382]. Macrophages can both boost or inhibit chemotherapy by immune modulation depending on tumor type and type of chemotherapy [298], so their phenotype and activation status might differ between the treatments and models which needs to be further investigated. The effect of CED-TMZ and CED-CIS may not be dependent on quantitative but rather qualitative changes in macrophages.

Generation of an immunologic memory after CED-TMZ and CED-MTX and lack of therapeutic effect in immunocompromised mice of CED-TMZ and CED-CIS

Most of the surviving mice in the GL261 model, previously treated with CED-TMZ or CED-TMZ + GL261 immunotherapy, and one of two surviving mice bearing SB28 glioma treated with CED-MTX rejected a secondary tumor in the contralateral hemisphere. These results suggest that CED-TMZ as monotherapy and combined with immunotherapy, as well as CED-MTX, induced adaptive antitumor immunity and an immunologic memory.

In addition, our results show that the effect of CED-TMZ was abolished in NODScid mice bearing GL261 tumors. NODScid mice have deficient T and B lymphocytes and impaired NK-cell function [124]. In our previous report, the effect of CED-TMZ was totally abolished by T cell depleting antibodies [376]. Therefore, T cells are the fundamental effector cells in CED-TMZ. On the other hand, the therapeutic effect of CED-CIS was also abolished in in GL261-bearing NSG mice. Since the innate and adaptive immune system in NSG is highly compromised, with depletion of T, B lymphocytes, NK cell and reduction of myeloid cell function, the results suggest that the curative effect of intratumoral CIS could be dependent on both the innate and the adaptive immune system.

In vitro half-maximal inhibitory concentrations of TMZ, CIS and MTX in murine glioma cells

The *in vitro* IC_{50} of TMZ in GL261 cell has been previously reported by our group as 0.35 μM [376]. Lee at al. have published an extensive report of the sensitivity or resistance of human-derived glioma cell lines to TMZ. They report a wide variation of IC_{50} in the cell lines [92]. IC_{50} values for sensitive glioma cells are usually below 75 μM. In this work, GL261 cells were sensitive to CIS *in vitro* with an IC_{50} of 0.81 μM. This is consistent with a previous *in vitro* report in 23 cell lines, including GBM, with a median IC_{50} of 0.87 mM (0.24–4.29 mM) [65]. MTX is cytotoxic *in vitro* to the three tested glioma cell lines. The most sensitive cell line by dose was KR158 followed by GL261 and SB28. Judging by exposure time the SB28 cell line was most sensitive as it reached IC_{50} after 24 hours. These findings are consistent with previous studies showing high *in vitro* sensitivity to MTX in GBM patient-derived cell lines. Jordan et al. showed that human GBM cell lines exhibited intermediate sensitivity to MTX (1 μM) after 4-day incubation [383]. In addition, Senkal et al. found that MTX produced a linear increase of DNA strand break in a dose dependent manner at doses between 0.01 and 1.0 μg/ml in cell cultures derived from five human gliomas [384]. In a recent study, Yu et al. tested seven CSC lines derived from patients with GBM and the established cell line U87-MG. They found that MTX was the most effective drug with an IC_{50} ranging between 2.7 nM and 87.5 μM. Additionally, MTX (12 mg/kg, single dose i.v.) had a strong effect on tumor growth in U87-MG subcutaneous xenografts, proving MTX as one the most effective drugs in the study [385]. However, there is also a report where the viability of the U87MG glioma cell line was not affected, even after treatment with 5 μM of MTX [386]. These *in vitro* studies demonstrate that MTX is potentially useful for the treatment of patients with malignant brain tumors.

To use the *in vitro* IC_{50} to estimate the adequate dose that could reach a therapeutic effect *in vivo* and within the therapeutic window is challenging, since the relation between *in vitro* IC_{50} concentrations and the therapeutic dose *in vivo* is unclear. The CED-TMZ dose was between 60 and 200 times higher than the *in vitro* IC_{50}, depending on the cell line model. For CED-CIS, the therapeutic concentration was 41 times higher than the *in vitro* IC_{50} in the GL261 model, while in CED-MTX the therapeutic concentration was 15 and 6 times higher than the *in vitro* IC_{50} in GL261 and SB28 models, respectively. However, in the KR158 model, the relation was greater, 30 times higher than the *in vitro* IC_{50} concentration which did not have a therapeutic effect.

Expression of immune-related cell surface markers in murine glioma cells after exposure to CIS and MTX

Tumor eradication and the immunogenicity of dying tumor cells depend on immune mechanisms such as the expression of MHC [229], costimulatory molecules [387, 388] and other immune-related proteins. After *in vitro* exposure of GL261 cells to CIS, we detected a minor up-regulation of MHC I expression, however non-significant. Neither MHC II nor CD80 or CD86 was up-regulated. Furthermore, we performed an immunohistochemical analysis of MHC I, MHC II, CD80 and CD86 expression on cryosections of GL261 tumors and we could not detect any difference between untreated tumors and tumors treated with CED-CIS as monotherapy or in combination with immunotherapy. In contrast, it has been shown by others that CIS exposure can up-regulate MHC I in tumor cells of various cancer types and in tumor-associated APCs *in vitro* and *in vivo* [79]. Likewise, the curative effect of CIS was abrogated in mice lacking expression of CD80 and CD86 on APCs in preclinical models of human papillomavirus-associated cancer [387]. The minor MHC I up-regulation found in this study is probably not enough to enhance antigen presentation *in vivo*. The absence of up-regulation of MHC I and II, CD80 and CD86 on the tumor cells following CIS antagonizes the notion of the immunomodulating features of CIS [79].

In contrast, MTX was able to up-regulate these cell markers *in vitro*. SB28 cells are constitutively negative to MHC I [131] which probably reflects the characteristics of human GBMs exhibiting MHC I loss [270]. In this study, MHC I expression was enhanced on SB28 cells after exposure to MTX, and to a minor degree on the KR158 cells. MHC II was up-regulated to a small extent only on KR158 cells. CD80 was up-regulated on GL261 and SB28 cells; this up-regulation was greater with longer exposure time only on the GL261 cells. CD86 was modestly up-regulated only in GL261 cells after *in vitro* exposure to MTX. In the case of MTX, the up-regulation of CD80 and CD86 in GL261 and of MHC I and CD80 in SB28 may partly explain the *in vivo* therapeutic effect of CED-MTX. Thus, the up-regulation of these molecules will facilitate the recognition of tumor cells by T cells and also improve the priming of naïve T cells [389].

Expression of immunogenic cell death markers in murine glioma cells on tumor cells in vitro after the exposure to MTX

The *bona fide* ICD-inducing features of MTX makes it an interesting candidate for intratumoral administration. However, there is limited experience with loco-regional treatment of GBM with these kinds of drugs. It has to be noted that the concept of ICD has not been explored in the setting of intratumoral delivery of cytostatic drugs. MTX is the gold standard drug for detection of CRT expression, HMGB1 translocation, and ATP release [85], steps that together have been described to generate a cellular specific immune response against TAA or TSA

[239]. MTX generated an important up-regulation of CRT in the three murine glioma cell lines after exposure to MTX *in vitro*, ensuing phagocytosis by APCs, thereby promoting tumor antigen presentation and stimulation of antigen-specific cytotoxic T cells [390]. Furthermore, HMGB1 was translocated only in SB28 cells, which theoretically lead to an enhanced activation of APCs through TRL4 receptor, thus facilitating antigen presentation to T cells[391]. To the best of our knowledge, there are no previous reports on ICD marker expression in the SB28 cell line, and the expression of ICD markers following MTX treatment has not previously been investigated in GL261. However, *in vitro* ICD markers have been previously detected in the GL261 cell line, using photodynamic therapy [392] and oncolytic virus [393]. Moreover, *in vitro* exposure to oxaliplatin induced ICD markers in GL261 and KR158 cell lines [133].

Taken together, the up-regulation of MHC and costimulatory molecules combined with the up-regulation of ICD-related proteins after treatment with MTX might boost the intratumoral activity of antigen-specific cytotoxic T cells and therefore improve survival.

Paper IV. Preoperative systemic levels of VEGFA, IL-7, IL-17A, and TNF-b delineate two distinct groups of children with brain tumors.

The clinical success of immunotherapy requires extensive understanding of immune pathways in patients. In *paper IV* we showed that systemic immune monitoring of children with brain tumors is feasible and two groups (A and B) with distinct preoperative cytokine profiles were identified. The results have implications for the implementation of immunotherapy, for monitoring of tumor progression or regression and the treatment response, and to a lesser extent for the diagnostics of patients with brain tumors.

The biological interpretation of the A/B subdivision is not clear at this point. VEGFA and IL-7 are both associated with the regeneration of leukocytes and restructuring of tissues after infection which would not be in favor of immune surveillance, and is consistent with reports of established immunosuppression in GBM [299] and MB [394]. High IL-7 could reflect lymphopenia [395] In contrast, systemic IL-17 and TNF-ß could represent cytotoxic activation. In line with this reasoning, IL-17 has previously been associated with improved outcome in GBM patients[396]. Unrelated to A/B affiliation, we found trends towards increased IL-10 and decreased IL-12/23 and TNF-α when comparing tumor patients with healthy controls. Similar patterns have previously been reported in the plasma of GBM patients [397, 398].

Blood-derived biomarkers including systemic cytokine profiles might assist in the decision choice of an immunotherapeutic strategy and could tentatively indicate which patients are suitable candidates for immunotherapy since a certain systemic

cytokine milieu could counteract or boost the treatment. For example, we identified a small group of patients with an enhanced Th1 profile for which tumor vaccines could be a successful approach. In accordance with this reasoning, a previous study by Lasky et al. described a child with GBM displaying high systemic levels of IFN-γ, IL-6 and TNF-α, who responded well to subsequent DC immunotherapy [399]. In a single case, we showed that the systemic profile oscillates with tumor burden and that systemic immunosuppression is overturned following tumor removal, providing a window for immunotherapies. Moreover, this finding suggests that systemic immune monitoring could be useful to follow tumor regression and possibly progression, although it remains to be confirmed in a larger data set.

Conclusions and future perspectives

Concluding remarks

- CED is a proven efficient method to deliver chemotherapeutic drugs intratumorally in brain tumors. Overall, all the chemotherapeutic drugs tested reached, to some extent, a similar therapeutic effect. Since CED-CIS and CED-MTX showed a certain level of toxicity, the doses have to be refined, both experimentally and clinically.
- The therapeutic effect of CED-TMZ and CED-CIS is more dependent on the immune system than by their ICD features, indicating that the immune effects of chemotherapeutic agents are determined by multiple factors such as the drug specificity, dosing, distribution, tumor model and type of immunotherapy.
- Wildtype cell-based immunotherapy cured mice bearing GL261 and KR158 gliomas, and GM-CSF-transduced cell-based immunotherapy cured mice in the GL261 model. In addition, the intratumoral influx of T cells was increased.
- In the GL261 model, CED-TMZ synergized with wildtype cell-based immunotherapy whereas CED-CIS did not, not even with GM-CSF-transduced cell-based immunotherapy.
- CED-MTX and CED-TMZ as monotherapies and the latter combined with immunotherapy generated an immunological memory capable of eradicating secondary tumors in the SB28 and GL261 models, respectively.
- Single bolus administration of TMZ had a therapeutical effect, however with a narrower therapeutic window than CED-TMZ.
- Systemic immune monitoring of children with brain tumors is feasible and we identified patient groups with distinct preoperative cytokine profiles. The method can also be used to determine and monitor an ongoing immune response during immunotherapy and possibly predict clinical outcome.

Future perspectives

In this thesis, CED with three chemotherapeutic drugs and tumor cell-based immunotherapy were investigated in three experimental murine glioma models. These results are intended to form a basis for the translation to clinical therapy against GBM. Local chemotherapy by CED can both reduce the side effects of systemic treatment but and, as proven for temozolomide, synergize with immunotherapy.

The main long-term goal is to implement these experimental treatments to efficiently treat GBM patients in an individual or personalized manner. As a first step, tumor burden has to be reduced trough surgery and then complemented with intratumoral chemotherapy. Intratumoral chemotherapy can be delivered as CED or by local deposition at surgery. The latter would preferentially be done by a slow release formulation oriented against the line of resection/brain adjacent to tumor. Theoretically, tumors that are only biopsied could be treated by CED through single or multiple catheters. The present work demonstrates that both CIS and TMZ and probably also MTX depend on an intact immune system, thus additive or synergistic effects could be achieved by concomitant immunotherapy. TMZ is probably the best candidate since we did not detect any toxicity in the animals and immunotherapy had a synergistic effect but it is necessary to determine the optimal timing for administration and investigate the interactions of CED-TMZ and the immune system in the clinical setting. Nonetheless, other chemotherapeutic drugs may also have a potential, like those presented in this work and other not tested. Moreover, the combination of chemotherapeutic drugs and other therapeutic agents, especially immunotherapeutic agents such as checkpoint inhibitors or oncolytic virus, would perhaps improve the therapeutic effect. In addition, concomitant radiotherapy could support tumor reduction and also induce ICD, to some extent.

Realistic therapeutic efforts against GBM have to both enhance antitumor immunity and counteract tumor-elicited immunosuppression. Our results indicate that intratumoral chemotherapy encompasses elements of both these features. However, intratumoral chemotherapy will most probably not succeed as monotherapy and has to be combined with immunotherapies such as whole cell-based or DC vaccines or CAR T cells. However, the optimal dose, administration route and timing of the immune intervention in the clinical setting needs to be evaluated as well. At the same time, plasma cytokine levels would help to monitor the treatment.

Finally, the preclinical models are still necessary to elucidate the details of the mechanisms of CED and immunotherapy.

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