Experimental models of pediatric brain tumors. Establishment, immunophenotyping and clinical implications.

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Experimental models of pediatric brain tumors

Establishment, immunophenotyping and clinical implications

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“Essentially, all models are wrong, but some are useful.”

George E.P. Box

Till Anne-Marie

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Abstract

Brain tumors are the most common solid tumors in children. Current treatment protocols fail in 25% of patients and are associated with significant long-term adverse effects in survivors. Experimental models of these tumors are scarce and will be crucial for the development of more efficient treatment strategies, including molecular targeting and immunotherapy.

In this thesis, I describe the establishment and characterization of novel *in vitro* and *in vivo* models of pediatric brain tumors. I initially define a standardized protocol for establishment of patient-derived cell cultures, based on the concept of serum-free monolayer culturing. In addition, I describe the generation of an orthotopic xenograft model of a high-risk Group 3 medulloblastoma (MB-LU-181) by cerebellar inoculation of low-passage tumor cells.

The newly established experimental models were phenotyped alongside patient samples, with emphasis on inflammatory mediators that could serve as future targets for therapeutic intervention. Tumor markers, cytokine signatures and components of the COX-2/mPGES-1/PGE2 pathway were generally preserved following propagation of tumor cells *in vitro* and *in vivo*, demonstrating the biological faithfulness of the models. CD24 was identified as a clinically and experimentally useful immunomarker for medulloblastoma cells, but additional detailed studies are needed to determine the prerequisites for targeted treatment. *PTGS2* (COX-2) and *VEGFA* were overexpressed in Group 3 medulloblastoma compared to other medulloblastoma subgroups; COX-2 was further evaluated as a therapeutic target in an immunocompetent high-grade glioma model, where simultaneous administration of COX-2 inhibitors and GM-CSF based immunotherapy cured >60% of tumor-bearing mice.

I finally performed a systemic immune characterization of children with brain tumors. Multiplex analysis of preoperative plasma samples identified patient groups with distinct cytokine profiles, which could have important implications for the development and clinical implementation of immunotherapies.

In brief, this thesis presents novel experimental models that recapitulate the phenotype of pediatric brain tumors and will serve as tools for future studies of tumor biology and preclinical drug evaluation. The results also implicate a role for immune intervention and monitoring in the treatment of children with brain tumors.

Populärvetenskaplig sammanfattning (Swedish summary)

Bakgrund

Hjärntumörer drabbar 80-90 svenska barn varje år och är därmed den näst vanligaste cancerformen hos barn efter leukemi. De drabbade barnen behandlas med kirurgi, oftast i kombination med strålbehandling och kemoterapi. Behandlingen fungerar bara hos ¾ av patienterna och ger dessutom i många fall svåra långsiktiga biverkningar hos de barn som botas. Det övergripande syftet med vår forskning är därför att utveckla effektivare och mer specifika behandlingsstrategier för barn med hjärntumörer. Vi är speciellt intresserade av *immunterapi,* en behandlingsmetod vars huvudsyfte är att förmå kroppens eget immunförsvar att stöta bort tumören.

Arbetet med att utveckla nya tumörbehandlingar kräver forskningsmodeller, där det undersöks om en behandling är effektiv och säker innan den ges till patienter. Exempel på sådana forskningsmodeller är tumörceller som växer i odlingsflaskor (*in vitro,* vilket ordagrant betyder ”i glas”) respektive tumörceller som växer i djur, oftast möss eller råttor (*in vivo,* vilket betyder ”i liv”). Det är viktigt att komma ihåg att dessa modeller är just modeller – en god effekt av en behandlingsmetod i en forskningsmodell betyder inte nödvändigtvis att samma effekt kommer att uppnås hos patienter. För att förbättra förutsägbarheten försöker forskare ständigt att förbättra sina modellsystem, eller utveckla nya modeller som så nära som möjligt liknar motsvarande tumörformer hos människor.

Syftet med avhandlingen

I nuläget finns endast ett fåtal forskningsmodeller av hjärntumörer hos barn, och dessa representerar tillsammans bara en bråkdel av alla tumörformer som förekommer. Huvudsyftet med min avhandling var därför att bygga upp nya *in vitro*- och *in vivo*-modeller av hjärntumörer hos barn.

Delarbete I-III

Första delen av avhandlingen är en beskrivning av de nya forskningsmodellerna. Jag beskriver dels processen med att bygga upp modellerna, dels vilka egenskaper de har och hur pass väl de återspeglar patienternas tumörer.

Delarbete I handlar om *in vitro*-modeller. Att studera tumörceller som odlas i cellodlingsflaskor är en vanligt förekommande forskningsmetod. Av bekvämlighetsskäl använder sig forskare oftast av cellinjer, som har odlats i flaskor sedan många år tillbaka i tiden. Cellinjer är lätta att arbeta med eftersom de växer snabbt och är väl anpassade till den artificiella miljön utanför kroppen. Denna anpassning gör dock att cellernas egenskaper skiljer sig från tumören de en gång isolerades ifrån, och de återspeglar därför dåligt verkligheten.

Ett alternativ till cellinjer är att isolera tumörceller direkt från tumörpatienter, och odla dessa *in vitro* under en kortare tid. Vi har använt en relativt ny metod för att isolera och odla tumörceller från patienter. Metoden går ut på att använda minimalt med tillsatser i odlingsmiljön, och samtidigt tvinga cellerna att växa på en plan yta. Detta odlingssätt har tidigare bara använts för elakartade hjärntumörer från vuxna, och vi har nu förenklat och anpassat metoden för att passa de tumörformer som förekommer hos barn.

I delarbete I visar vi att 1) vi kan få tumörcellerna att överleva i flaskor efter att ha opererats ut ur patienter; med vår metod har vi lyckats odla celler från ungefär 70% av tumörerna, vilket är ett bra resultat, 2) vi får cellerna att föröka sig så att det går att få fram det antal celler som behövs för analyser under en rimlig tidsperiod, och 3) cellkulturerna liknar ursprungstumören med avseende på olika proteiner, både de som finns inuti och de som utsöndras av cellerna.

Trots att det finns fördelar med att odla celler i flaskor (såsom att det är en förhållandevis praktisk och billig metod), så är en stor nackdel att celler i odling (*in vitro*) trots allt befinner sig i en konstgjord miljö. De påverkas till exempel inte av förändringar i blodflöde och syresättning, och saknar den ständiga samverkan mellan tumörceller och friska celler som finns *in vivo*. I **delarbete II** har vi därför skapat en *in vivo*-modell, som ska komplettera *in vitro*-modellerna i delarbete I.

In vivo-modellen representerar ett *medulloblastom av grupp 3*. Denna tumörtyp drabbar främst yngre barn, bildar ofta metastaser, och trots aggressiv behandling överlever bara omkring 50% av barnen som drabbats. Vi isolerade celler från en sådan tumör och injicerade dem i hjärnan hos möss. Efter ett par månader hade cellerna bildat stora tumörer, och från dessa tumörer kunde vi isolera nya tumörceller och återinjicera dem i andra möss. Genom att kontinuerligt upprepa denna procedur kommer vi att ha en ständig tillgång till tumörutvecklande möss, som vi kan använda för att undersöka nya behandlingsstrategier.

På samma sätt som med *in vitro*-modellerna undersökte vi noggrant tumörerna från möss, och kunde konstatera att tumörerna växte på samma sätt och innehöll samma proteiner som fanns i patientens ursprungliga tumör. Ett av de proteiner vi utvärderade kallas CD24.

I **delarbete III** beskrivs i detalj förekomsten av CD24 i våra forskningsmodeller, men också i ursprungstumören och i ett stort antal andra hjärntumörer av olika typer. Vi konstaterade att CD24 finns i flera olika tumörtyper, men dess mönster ser så pass annorlunda ut i medulloblastom att man skulle kunna använda

det vid diagnostik av hjärntumörer. Det kommer dock att krävas ytterligare studier innan vi kan avgöra om det går att använda sig av CD24 i behandlingssammanhang.

Delarbete IV-V

Många av de proteiner som studerades under beskrivningen av modellerna i delarbete I-III är relaterade till immunförsvaret. I andra delen av avhandlingen har vi undersökt hur dessa proteiner skulle kunna användas till nytta för patienterna.

I **delarbete IV** har vi studerat ett protein som var vanligt förekommande i både forskningsmodellerna och de ursprungstumörer vi använde som jämförelse. Detta protein heter COX-2, och är ett enzym som styr produktionen av ett annat protein, kallat PGE2. COX-2 och PGE2 reglerar en rad olika funktioner som bidrar till tumörutveckling, bland annat blockering av immunförsvarets angrepp på tumören. I våra *in vitro*-modeller kunde vi sänka produktionen av PGE2 genom att behandla cellerna med ett läkemedel som blockerar funktionen av COX-2.

Baserat på dessa fynd ville vi se vad som händer om funktionen av COX-2 blockeras *in vivo*. För denna studie använde vi en forskningsmodell av ett *höggradigt gliom*, som är en annan typ av elakartad hjärntumör som förekommer hos både barn och vuxna. När vi blockerade funktionen av COX-2, samtidigt som vi gav en immunstimulerande behandling, kunde vi bota så många som 60-70% av de möss som bar på ett gliom.

Sammantaget har vi sett i våra forskningsmodeller att tumörceller producerar ett flertal faktorer som har koppling till immunförsvaret. I **delarbete V** har vi gjort en analys av blodprover, för att se om det går att upptäcka motsvarande faktorer hos patienter. Vi har totalt undersökt ett femtiotal blodprover från barn som drabbats av olika hjärntumörtyper och hittat intressanta skillnader i immunprofiler. Baserat på fyra faktorer går det att separera patienterna i två olika grupper, och vi har även hittat enstaka patienter som verkar ha en kraftig pågående aktivering av immunförsvaret. Vi tror att dessa skillnader reflekterar olika stadier i samspelet mellan tumör och immunförsvar, och att de kan ha betydelse för hur patienten kommer att svara på behandling.

Slutsatser

I min avhandling beskriver jag ett antal nya forskningsmodeller av hjärntumörer hos barn. Vi har inte bara byggt upp ett bibliotek av modeller för vår egen framtida forskning, utan också beskrivit våra metoder på ett sådant sätt att andra forskare kan använda sig av informationen för att bygga upp sina egna modeller. Vi visar också att det skulle kunna finnas en fördel med att blockera funktionen av COX-2 hos tumörpatienter. Slutligen visar vi att det finns förutsättningar att använda sig av blodprover för att övervaka hur patienter skulle reagera på en sådan, eller annan, immunterapibehandling.

Original papers

This thesis is based on the following original papers:

Paper I — A standardized and reproducible protocol for serum-free monolayer culturing of primary paediatric brain tumours to be utilized for therapeutic assays.
Emma Sandén, Sofia Eberstål, Edward Visse, Peter Siesjö, Anna Darabi. *Scientific Reports.* (2015) Jul 17;5:12218

Paper II — Establishment and characterization of an orthotopic patient-derived Group 3 medulloblastoma model for preclinical drug evaluation.
Emma Sandén[*], Cecilia Dyberg[*], Cecilia Krona, Malin Wickström, Julio Enríquez Pérez, Marcel Kool, Edward Visse, Peter Siesjö, John I Johnsen[**], Anna Darabi[**]. *Manuscript*
[*]shared first authorship [**]shared senior authorship

Paper III — Aberrant immunostaining pattern of the CD24 glycoprotein in clinical samples and experimental models of pediatric medulloblastomas.
Emma Sandén, Cecilia Dyberg, Cecilia Krona, Edward Visse, Helena Carén, Paul A Northcott, Marcel Kool, Nils Ståhl, Annette Persson, Elisabet Englund, John I Johnsen, Peter Siesjö, Anna Darabi. *Journal of Neuro-Oncology.* (2015) May;123 (1):1-13

Paper IV — Intratumoral COX-2 inhibition enhances GM-CSF immunotherapy against established mouse GL261 brain tumors.
Sofia Eberstål, **Emma Sandén,** Sara Fritzell, Anna Darabi, Edward Visse, Peter Siesjö. *International Journal of Cancer.* (2014) Jun 1;134 (11):2748-53

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

Related publications

Potential therapeutic targets in pediatric tumors

Absence of Epstein-Barr and cytomegalovirus infection in neuroblastoma cells by standard detection methodologies.
Sehic D, Forslund O, **Sandén E,** Mengelbier LH, Karlsson J, Bzhalava D, Ekström J, Warenholt J, Darabi A, Dillner J, Øra I, Gisselsson D.
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Enhancer hijacking activates GFI1 family oncogenes in medulloblastoma.
Northcott PA, Lee C, Zichner T, Stütz AM, Erkek S, Kawauchi D, Shih DJ, Hovestadt V, Zapatka M, Sturm D, Jones DT, Kool M, Remke M, Cavalli FM, Zuyderduyn S, Bader GD, VandenBerg S, Esparza LA, Ryzhova M, Wang W, Wittmann A, Stark S, Sieber L, Seker-Cin H, Linke L, Kratochwil F, Jäger N, Buchhalter I, Imbusch CD, Zipprich G, Raeder B, Schmidt S, Diessl N, Wolf S, Wiemann S, Brors B, Lawerenz C, Eils J, Warnatz HJ, Risch T, Yaspo ML, Weber UD, Bartholomae CC, von Kalle C, Turányi E, Hauser P, **Sandén E,** Darabi A, Siesjö P, Sterba J, Zitterbart K, Sumerauer D, van Sluis P, Versteeg R, Volckmann R, Koster J, Schuhmann MU, Ebinger M, Grimes HL, Robinson GW, Gajjar A, Mynarek M, von Hoff K, Rutkowski S, Pietsch T, Scheurlen W, Felsberg J, Reifenberger G, Kulozik AE, von Deimling A, Witt O, Eils R, Gilbertson RJ, Korshunov A, Taylor MD, Lichter P, Korbel JO, Wechsler-Reya RJ, Pfister SM.
Nature. (2014) Jul 24;511(7510):428-34

Wnt/beta-catenin pathway regulates MGMT gene expression in cancer and inhibition of Wnt signaling prevents chemoresistance.
Wickström M, Dyberg C, Milosevic J, Einvik C, Calero R, Sveinbjörnsson B, **Sandén E,** Darabi A, Siesjö P, Kool M, Kogner P, Baryawno N, Johnsen JI.
Nature Communications. (2015) Nov 25;6:8904

Immunotherapy of malignant brain tumors

IFNγ in combination with IL-7 enhances immunotherapy in two rat glioma models.
Fritzell S, Eberstål S, **Sandén E,** Visse E, Darabi A, Siesjö P.
Journal of Neuroimmunology. (2013) May 15;258(1-2):91-5

Intratumoral temozolomide synergizes with immunotherapy in a T cell-dependent fashion.
Fritzell S, **Sandén E,** Eberstål S, Visse E, Darabi A, Siesjö P.
Cancer Immunology, Immunotherapy. (2013) Sep;62(9):1463-74

Aluminium based adjuvants and their effects on mitochondria and lysosomes of phagocytosing cells.
Ohlsson L, Exley C, Darabi A, **Sandén E,** Siesjö P, Eriksson H.
Journal of Inorganic Biochemistry. (2013) Nov;128:229-36

Immunizations with unmodified tumor cells and simultaneous COX-2 inhibition eradicate malignant rat brain tumors and induce a long-lasting CD8(+) T cell memory.
Eberstål S, Fritzell S, **Sandén E,** Visse E, Darabi A, Siesjö P.
Journal of Neuroimmunology. (2014) Sep 15;274(1-2):161-7

Inspirational literature

Sofias nya höstkappa.
Sandén E.
Hemmets veckotidning. (2015) 46:40-1

Abbreviations

ADCC	antibody-dependent cell-mediated cytotoxicity
APC	antigen presenting cell
AT/RT	atypical teratoid/rhabdoid tumor
BBB	blood brain barrier
CNS	central nervous system
COX	cyclooxygenase
CSC	cancer stem cell
CSF	cerebrospinal fluid
CT	classical type, chemotherapy
CTL	cytotoxic T lymphocyte
CTLA-4	cytotoxic T lymphocyte-associated antigen-4
DAMP	danger-associated molecular pattern
DC	dendritic cell
dCLN	deep cervical lymph node
DIPG	diffuse intrinsic pontine glioma
DN	desmoplastic/nodular
EGF	epidermal growth factor
EPN	ependymoma
ETMR	embryonal tumor with multilayered rosettes
FDA	US Food and Drug Administration
FFPE	formalin-fixed paraffin-embedded
FGF	fibroblast growth factor
GBM	glioblastoma multiforme
GDSC	granulocyte-derived suppressor cell
GEMM	genetically engineered mouse model
GL-GM	GM-CSF-transduced GL261 cells
GM-CSF	granulocyte macrophage-colony stimulating factor
GNP	granular neuron precursor
H&E	hematoxylin&eosin
HGG	high-grade glioma
i.c.	intracranial
IFN	interferon
IL	interleukin
iNOS	inducible nitric oxide synthase
i.t.	infratentorial

IT	immunotherapy
L	ligand
LCA	large cell/anaplastic
LGA	low-grade astrocytoma
LGG	low-grade glioma
MB	medulloblastoma
MBEN	medulloblastoma with extensive nodularity
M-CSF	macrophage-colony stimulating factor
MDSC	myeloid-derived suppressor cell
MGMT	O6-methylguanine-DNA methyltransferase
MHC	major histocompatibility complex
MN	meningioma
MNP	mononuclear phagocyte
mPGES-1	microsomal prostaglandin E synthase-1
MRI	magnetic resonance imaging
MTD	molecularly targeting drugs
NIReg	neuroimmune regulatory protein
NK	natural killer
NSC	neural stem cell
PA	pilocytic astrocytoma
PD-1	programmed cell death protein-1
PDGF	platelet-derived growth factor
PDX	patient-derived xenograft
PF	posterior fossa
PG	prostaglandin
PNET	primitive neuroectodermal tumor
PRR	pattern recognition receptor
R	receptor
RL	rhombic lip
RT	radiotherapy
s.c.	subcutaneous
s.t.	supratentorial
TCR	T cell receptor
TGF	transforming growth factor
Th cell	T helper cell
TIC	tumor-initiating cell
TLR	Toll-like receptor
Tmem cell	T memory cell
TMZ	temozolomide
TNF	tumor necrosis factor
Treg	T regulatory cell
VEGF	vascular endothelial growth factor
WBC	white blood cell

Introduction

Pediatric brain tumors

A brief overview

Brain tumors affect 80-90 Swedish children each year, making it the second most common cancer in children after leukemia. Current treatment approaches comprise surgical resection, usually in combination with radiotherapy (RT) and chemotherapy (CT). Around 75% of the patients are cured, however cure rates vary between 10 and 90% depending on diagnosis and risk factors. In addition, many children suffer from severe long-term adverse effects due to treatment-associated damage to the developing nervous system. The most common brain tumor entities in children are medulloblastoma (MB), ependymoma (EPN) and low-grade astrocytoma (LGA) including pilocytic astrocytoma (PA) (see Figure 1); however, there are numerous molecular variants of each tumor type.

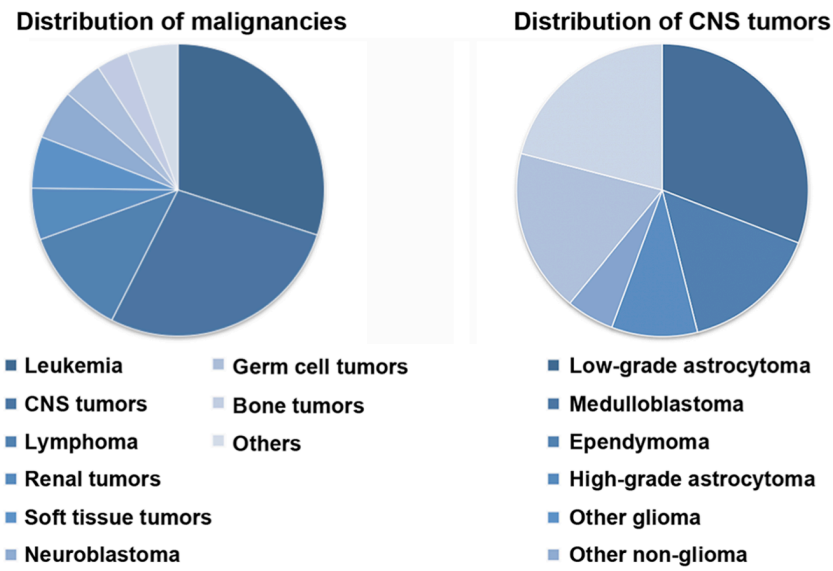

Figure 1. Distribution of malignancies and CNS tumors in Swedish children (<15 years of age) 1984-2010[1].

Brain tumors in children and adults

Adult brain tumors vastly outnumber pediatric tumors and are therefore better studied. Findings in adult brain tumors can however not readily be extrapolated to pediatric tumors, due to their differences in profound features such as tumor initiation and progression, genetic profiles, immune activation and therapeutic response[2-5]. In this thesis, the term "children" refers to 0-19 yrs of age when not otherwise stated – although, as will be exemplified in the following sections, the prevalence and features of certain tumor types and molecular subgroups may also differ between infants, young children, older children and adolescents.

In relative frequency, brain tumors are in fact more common in children than in adults, tentatively due to higher proliferation and potential for mutational events in the developing brain. The prevalence of specific tumor types also differs significantly. MB, originating in progenitor cells of the developing cerebellum, is the most common malignant brain tumor type in children, but is rare in adults[6]. Conversely, glioblastoma multiforme (GBM) and meningioma (MN) are more common in adults than in children[6]. The majority of brain tumors in adults are secondary, for instance melanoma or lung cancer metastases, whereas secondary brain tumors are uncommon in children. In contrast, pediatric brain tumors are more prone to disseminate and form spinal cord metastases[7,8].

Mutations in pediatric tumor types are low in numbers compared to adult tumors in which the tumor may have evolved during many years and gained multiple mutations during its progression[9]. Tumor types of the same histology may have different genetic profiles in children and adults, and progress accordingly[4]. Moreover, molecular subgroups are usually overrepresented in a certain age group; for instance, the rare cases of adult MB almost exclusively belong to the MB SHH subgroup[10].

Compared to adults, children have an overall better prognosis[6] and tend to respond better to therapy. Pediatric tumors are generally sensitive to CT, and children tolerate higher relative doses than adults do[2]. Treatment decisions for children with brain tumors are however a delicate balance between complete tumor eradication and the risk of causing severe long-time adverse effects due to irreversible damage to the developing central nervous system (CNS). RT is especially troublesome since radiation may damage healthy brain tissue adjacent to the tumor, and RT is therefore reduced or avoided in younger children[2]. Subsequently, many clinical trials in children focus on reducing side effects, rather than increasing cure rate. In this context, clinical trials in children face the challenges associated with low patient numbers, and multicenter recruitment is usually needed for rare tumor types.

Histologic and molecular classification

Brain tumor diagnostics – today and tomorrow

Following presentation of clinical symptoms, a brain tumor is confirmed by contrast-enhanced magnetic resonance imaging (MRI), although MRI can seldom define the exact tumor type. A detailed postoperative diagnosis is obtained by microscopic evaluation of resected tumor tissue. Tumor sections are stained with hematoxilin&eosin (H&E) for histological evaluation, and labeled with antibodies for detection of proteins that are characteristic for certain tumor types (see *Brain tumor markers*). In addition, fluorescent in situ hybridization is commonly used to identify tumor specific chromosomal or copy number aberrations.

Current brain tumor diagnostics is dictated by the World Health Organization (WHO) classification system from 2007, in which tumors are classified solely based on histological features[11]. In addition to the diagnosis per se, tumors obtain a grade I to IV designation based on proliferation, infiltration, cytology and structural features. This malignancy scale is tentatively indicative of prognosis, with grade IV comprising the most malignant tumor types.

A tremendous progress in genetic characterization of brain tumors over the last decade has brought with it significant new knowledge and clearly demonstrated the limitations of the traditional diagnostic system. As an example, recent retrospective genetic analyses of so-called "primitive neuroectodermal tumors" (PNET) have revealed that most of these tumors represent a mixture of other diagnoses[12]. Moreover, the prognostic value of the traditional I-IV grading system has in some cases lost its significance, as will be exemplified in following sections.

Shortly before the printing of this thesis (May 2016), the WHO 2007 classification was updated in an attempt to incorporate novel genetic findings into the old classification system[13]. This is the first of presumably many steps towards integrating molecular parameters into brain tumor diagnostics. In the updated CNS WHO 2016 version, several new brain tumor entities (*e.g.* embryonal tumor with multilayered rosettes (ETMR), C19MC-altered) have been introduced, while others (*e.g.* PNET) have been excluded, based on genetic similarities or disparities between tumor types. In addition, some traditional histological diagnoses (*e.g.* MB and GBM) have been expanded to include newly acknowledged variants defined by distinct mutations. For convenience, pathologists are currently given the option to state an integrative diagnosis (combining histological and genetic features) or suffice with a histologic diagnosis, not otherwise specified.

The benefits of improved diagnostics for risk stratification, epidemiologic studies and therapeutic intervention are easily appreciated; however the clinical implementation of integrative diagnostics will be challenging for small centers with limited financial or technical capacity to conduct the required genotyping assays. Defining robust and reliable immunohistochemistry approaches to detect

certain genetic characteristics may be a way of circumventing these challenges. Methylation arrays also hold great promise as relatively cheap diagnostic tools, which require minimal amounts of formalin-fixed paraffin-embedded (FFPE) material to obtain reliable results that can be compared to established reference data sets[14].

A description of the most common brain tumor types in children will follow, focusing on current histologic and molecular classification; risk stratification and treatment strategies are extensively discussed in *Current risk stratification and treatment protocols*.

Medulloblastoma

MB is the most common malignant brain tumor type in children, constituting around 15% of CNS tumors in the age group 0-15 yrs[1]. MBs are classified as WHO grade IV tumors, and patients have an overall poor prognosis (~60% survival rate)[1]. All MBs are located in or adjacent to the cerebellum (Figure 2) and present histologically as a highly proliferative dense cell mass, consisting of small round cells with sparse cytoplasm and lack of cellular differentiation. A homogenous appearance of this histology is referred to as classical type (CT) MB, whereas subsets of MBs also display areas of nodular differentiation. Depending on how prominent the nodularity is, these MBs are histologically classified as desmoplastic/nodular (DN) or MB with extensive nodularity (MBEN). Finally, the histological subtype large cell/anaplastic (LCA) displays widespread cytologic anaplasia or areas of large cells. LCA is uncommon and considered a high-risk feature in MB patients.[11,15]

During the past few years, extensive transcriptional profiling efforts have demonstrated a broad molecular diversity within tumors defined as MB[16-19], suggesting that MB should no longer be regarded as a single tumor entity with uniform treatment protocols. The current consensus, established in 2012[20], describes four molecular subgroups of MB, designated WNT, SHH, Group 3 and Group 4. The characteristics of each subgroup are summarized in Table 1. The four subgroups differ significantly on a genetic level, but also in prevalence, age and gender distribution, and prognosis[8]. The WNT and SHH subgroups are genetically well characterized and have been included as diagnostic variants in the CNS WHO 2016[13]. In contrast, Group 3 and Group 4 are provisional designations until sufficient genetic evidence defines them as separate variants. Tentatively, additional subgroups or subtypes will arise as the biology of Group 3 and 4 tumors is further elucidated.

The WNT subgroup is, as the name suggests, characterized by persistent activation of the WNT signaling pathway, almost exclusively due to a somatic mutation in the *CTNNB1* gene encoding β-catenin. The other major genetic hallmark of WNT tumors is monosomy 6, found in 75% of the tumors[21]. WNT MBs represent the least common subgroup, constituting approximately 10% of all

Table 1. Demographics and characteristic features of medulloblastoma subgroups[8,13,20-30].

Subgroup	WNT	SHH	Group 3	Group 4
Frequency	~10%	~30%	~25%	~35%
Age*	Children	Infants, (children), adults	Infants, children	Children
Gender (F:M)	1:1	1:1	1:2	1:2
Histology	CT, (LCA)	DN, CT, LCA, MBEN	CT, LCA	CT, (LCA)
Recurrence	Uncommon	Local	Metastasis	Metastasis
Risk	Low	Low/standard/high	High	Standard
Suggested cells of origin	Lower RL/dorsal brainstem progenitors	Cerebellar GNP	Cerebellar stem cells	Upper RL progenitors
Frequent chromosomal changes	Monosomy 6	Loss of 9q and 10q	Many chromosomal aberrations, including tetraploidy and chromotripsis	Tetraploidy Iso 17q Gain of 7
Frequent somatic mutations or copy number changes	*CTNNB1* *DDX3X*	*GLI2* *MYCN* *PTCH1/SMO/SUFU* *TERT* *TP53*	*MYC* *PVT1* *SMARCA4*	*H3K27* *KDM6A* *MYCN* *SNCAIP*

CT, classical type; DN, desmoplastic/nodular; GNP, granular neuron precursor; LCA, large cell/anapalstic; MBEN, medulloblastoma with extensive nodularity; RL, rhombic lip. *Infants: <3. Children: 4-16. Adults: >16.

MBs and occur in children of all ages except for infants. Importantly, patients with this tumor type have an excellent prognosis; recurrence is uncommon and 95% of children are alive 10 years after their initial diagnosis[8].

Approximately 30% of MBs are driven by SHH signaling. Mutations or amplifications in SHH MBs are seen in different compartments of the SHH signaling pathway, such as *PTCH1, SMO*, *SUFU* or *GLI;* accordingly, the SHH subgroup contain subtypes, with therapeutic implications that will be discussed in *Molecular targeting of tumor cells.* Patients with the hereditary Gorlin syndrome (associated with a germline *PTCH1* mutation) are predisposed to develop MB, although they represent a minority of the total number of SHH MBs[31]. In contrast to WNT tumors, SHH tumors most commonly present in infants and adults. However, when SHH tumors do occur in children of other ages, these tend to be more genomically unstable and display high-risk features such as *TP53* mutation[28]. All histological subtypes are represented within the SHH subgroup, although it should be noted that all nodular MBs fall into this category. Overall, patients with SHH MB have an intermediate prognosis, and recurrence is usually local rather than distant[8].

MB is the brain tumor type that most frequently disseminate and form metastases in the spinal cords, and this feature is attributed Group 3 and Group 4 MBs. Group 3 accounts for ~25% of MBs and is associated with the worst prognosis. It is most common in children <3 yrs old, and the survival rate is below 40% in this age group. The incidence of Group 3 MB decreases with increasing age.[8] In contrast to the well-characterized WNT and SHH tumors, a common oncogenic driver of Group 3 has not been identified. Recurrent mutations are scarce; rather, Group 3 MBs have unbalanced genomes with numerous structural rearrangements, including tetraploidy and chromotripsis. The most characteristic genetic event is *MYC* amplification, which is found in ~15% of Group 3 tumors and considered a high-risk factor within this patient group.[8,21,27,29,32] Group 4 is the most common (~35%), but least understood, of the MB subgroups. It is most frequent in children between 4 and 16 yrs, and the prognosis is slightly better than for patients with Group 3 tumors[8].

Low-grade glioma

Gliomas are broadly divided into (i) diffuse astrocytic and oligodendroglial tumors, (ii) other astrocytic tumors, (iii) neuronal and mixed neuronal-glial tumors and (iv) other gliomas[13]. Several histological subtypes exist within these categories, and low-grade gliomas (LGGs) comprise the subtypes that are designated WHO grade I or II[13]. LGGs are low-proliferative lesions, with grade I tumors being well circumscribed and grade II displaying some infiltration into surrounding brain tissue[11]. PA (WHO grade I) is the most common diagnosis among LGGs, and the most common CNS tumor entity in children altogether. Since PAs rarely present in adults, they are also referred to as juvenile astrocytomas. PAs can arise anywhere in the CNS but are most frequently infratentorial (i.t.) (Figure 2), while other LGGs are more common in the cerebrum.

The extensive histological subdivision aside, many LGGs share basic genetic features. A majority of pediatric LGGs are driven by the oncogene *BRAF*, either by duplication of a truncated form of *BRAF* (found in 60-90% of PAs) or by a point mutation referred to as BRAF V600E, which leads to aberrant activation of the MAPK pathway[33-35]. In addition, around 50% of pediatric LGGs show activation of PI3K/Akt/mTOR[36]. Although virtually all PAs share MAPK pathway activation[37], some genetic differences have been reported between i.t. and supra-tentorial (s.t.) PAs[38,39] (Figure 2).

Treatment protocols are similar for all LGGs, and patients with LGG are usually cured unless the tumor is located at surgically inaccessible site. Moreover, pediatric LGGs have the unique feature of potential spontaneous regression as the patient gets older[40]. However, a minority of LGGs may also progress into more malignant tumor types[41].

High-grade glioma

High-grade gliomas (HGGs) are gliomas classified as WHO grade III and IV. In children, the group of HGGs historically comprises anaplastic astrocytic and oligodendroglial tumors and the grade IV astrocytic tumor types GBM and diffuse intrinsic pontine glioma (DIPG). HGGs constitute 5-15% of all pediatric brain tumors and are associated with the worst prognosis; median patient survival time is 1.7 years[1,6,42]. The poor prognosis of HGGs is mainly attributed the proneness of HGG cells to infiltrate normal brain tissue and migrate long distances, making complete surgical resection impossible to obtain.

Anaplastic tumors (WHO grade III) are defined as tumors displaying nuclear atypia and enhanced proliferation. Grade IV tumors also contain areas of necrosis and microvascular proliferation[11]. This historical subdivision based on grade may have limited prognostic value for pediatric HGGs; pediatric grade III tumors are rare and lack the genetic features (*e.g. IDH1* mutation and 1p/19q deletion) that separate adult grade III from the majority of their more aggressive grade IV counterparts, questioning the relevance of grade III diagnoses in children[43].

GBMs has been extensively genetically characterized and can be subdivided into six molecular subgroups based on characteristic mutations: K27, G34, IDH, RTK-I, RTK-II and mesenchymal subtype. Pediatric GBMs represent the majority of tumors in the first two groups; that is, they carry mutations in the histone gene *H3F3A* resulting in amino acid substitution at the K27 or the G34 position[44]. The H3K27 mutation does not occur in adult GBMs, and is tentatively associated with a poor prognosis[45]. In addition, a fraction of pediatric GBMs display *PDGFRA* activation (found in the K27 and RTK-1 subgroups)[44].

Brainstem glioma is a historically used term for gliomas arising in the brain stem. These tumors were histologically classified from WHO grade I to IV, comprising actual PAs arising in the brainstem and higher grade DIPGs. The DIPG diagnosis was recently exchanged for "diffuse midline glioma, H3K27-mutant", since all DIPG tumors were found to harbor this mutation[13]. In contrast, hemispheric pediatric GBMs commonly display the H3G34 mutation[44] (Figure 2).

Ependymoma

EPNs account for ~10% of brain tumors in children[1] and are histologically classified as WHO grade II or III EPN. Grade I EPNs also exist, but are rare[11]. The II-III distinction of EPNs has been shown to be of little clinical significance compared to molecular features; both grade II and grade III EPNs are represented in the patient groups with the best and the worst outcomes respectively. Rather, prognosis is dictated by the presence of high- and low-risk mutations[46].

Current consensus describes three major subgroups of EPNs: spinal, s.t. and posterior fossa (PF), and each of these subgroups contains three subtypes[47]. Almost all EPNs in children are of the PF or s.t. type (Figure 2). The pediatric s.t. EPNs are frequently anaplastic, but can be either low-risk (harboring a fusion

between *YAP-1* and *MAMLD1* or *FAM118B*) or high-risk (harboring a fusion between *RELA* and *C11orf95*, which drives an aberrant NF-κB signaling). Similarly, the pediatric PF EPNs are divided between high-risk (Group A, $CIMP^+$) and low-risk (Group B, $CIMP^-$). The prognosis for high- and low-risk patients differ dramatically; the 10 year overall survival rates are ~50 % and ~95% respectively[47]. The *RELA*-fusion positive tumors account for most s.t. EPNs in children and have been included as a separate entity in the WHO CNS 2016[13], as opposed to all other EPNs, which are still just classified as grade II or grade III. Likely, the clinical diagnostics of EPNs will be further modified before long.

Less common tumor types

Besides the common tumor types already described, the papers of this thesis briefly touch upon a few additional rare cancer forms, which deserve mentioning but will not be further discussed. Notably, the most common brain tumor type in adults, MN, is rare in children and tends to be more aggressive than its adult counterpart. ETMR and atypical teratoid/rhabdoid tumor (AT/RT) are two uncommon WHO grade IV diagnoses with particularly poor outcome. Both tumor types were previously included among the MB or PNET tumors, but can now be identified by aberrant expression of INI-1[48] and C19MC[49] respectively.

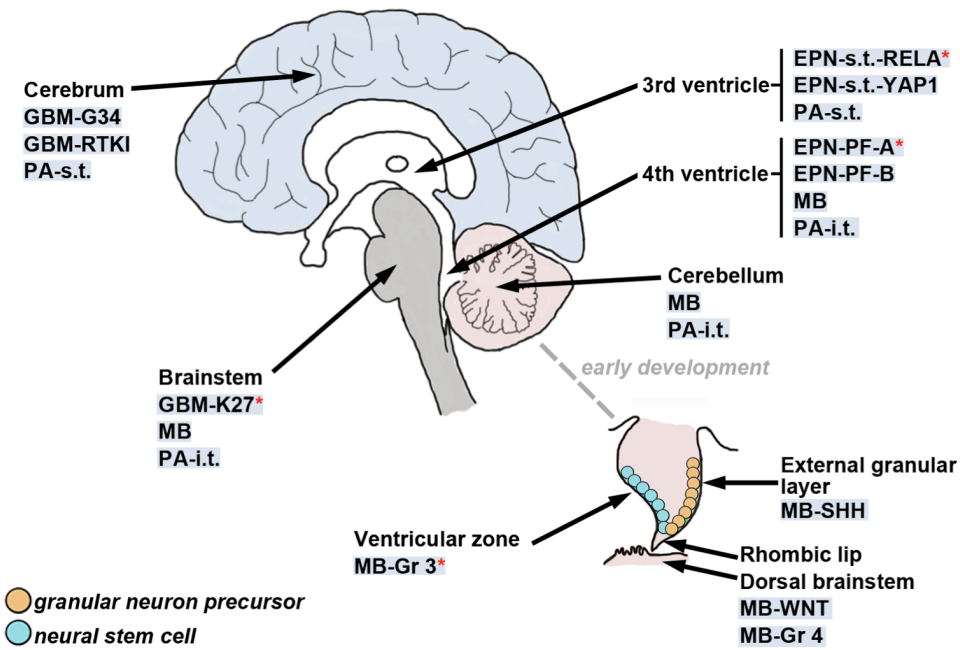

Figure 2. Overview of pediatric brain tumor subgroups by regional origin.
Red asterisk indicates subgroups with poor prognosis within tumor type.[8,23-26,38,44,45,47]. EPN, ependymoma; GBM, glioblastoma multiforme; i.t., infratentorial; MB, medulloblastoma; PA, pilocytic astrocytoma; PF, posterior fossa; s.t., supratentorial.

Brain tumor markers

Lineage-specific markers

Epitopes expressed in tissues of a specific tumor type can reflect its cell of origin, and lineage-specific markers are useful diagnostic complements to histologic evaluation. However, the dysregulated genome of tumors results in aberrant protein expression compared to the corresponding normal cell type, such as simultaneous or focal expression of diverse lineage-specific proteins. Clinical diagnostics therefore relies on panels of markers rather than a single universal marker for each tumor type.

Gliomas typically express markers of glial precursor cells, such as GFAP and SOX-2, and mature glial cells, such as GFAP, s100 (astrocytic tumors), Olig2 and CNPase (oligodendroglial tumors) and vimentin. EPNs can also express these markers, but generally have higher expression of EMA and nestin than astrocytic gliomas do.[50-53] EPNs are historically named after the belief that they arise in specialized glial (ependymal) cells, lining the brain ventricles. More recently, it has been suggested that EPNs arise in radial glial cells[54] or NSCs[55], but as EPNs are now known to comprise multiple subtypes, there is likely more than one specific cell of origin for these tumors[47].

MBs originate in neuronal stem/precursor cells (Table 1) and express markers of the neuronal lineage, such as nestin, neuron-specific enolase, synaptophysin, OTX1/2, neurofilaments and β-III-tubulin[56-59]. Distinct cells of origin has been suggested for the WNT, SHH, Group 3 and Group 4 respectively[23-26] (Table 1, Figure 2), and attempts have been made to identify specific IHC markers for each MB subgroup. Intracellular staining of β-catenin is a good indicator for WNT MBs[17,60], but may also appear in non-WNT MBs, and molecular verification of *CTNNB1* mutation is recommended. GAB1, GLI1 and SFRP1 has been suggested for the SHH subgroup, and NPR3 and KCNA1 for Group 3 and 4 respectively[17,60]. The clinical validation of these markers has however proved to be challenging.

Biomarkers

Biomarkers are defined as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention". Prognostic biomarkers indicate outcome for a patient group regardless of treatment, whereas predictive biomarkers reflect the response to therapy.[61,62] At the moment, there are no clinically used liquid biomarkers for brain tumors, as is the case for *e.g.* prostate and colorectal cancers. A number of cerebrospinal fluid (CSF)-, blood- and urine-derived biomarkers have been suggested for pediatric brain tumor patients (reviewed by Russell *et al*[63]), but none of them are clinically validated.

Tissue-derived brain tumor biomarkers are more frequent - although, as will be discussed in *Current risk stratification and treatment protocols*, brain tumor

patients are largely risk-stratified based on histological features rather than the prevalence of molecular tissue markers. This will likely change in the near future, as novel prognostic and treatment-indicating markers such as H3K27 and BRAF V600E are clinically implemented. The most widely validated brain tumor biomarker to date is O^6-methylguanine-DNA methyltransferase (MGMT) promoter methylation, which dictates response to temozolomide (TMZ) treatment in both adult and pediatric HGG patients[64,65]. *EGFR* variants, *IDH1/2* mutations and 1p/19q co-deletion are also useful for adult HGGs[66], but these alterations are rare in pediatric HGGs[44].

CD24

CD24 is a heavily glycosylated GPI-anchored protein that act as a regulator of proliferation and differentiation during neurogenesis. It is expressed in neuronal precursor cells in their transition from neuroblasts into fully differentiated neurons[67], and absence of CD24 or distinct CD24-associated carbohydrates cause enhanced proliferation and a lack of neurite outgrowth in neuron precursors[68-71]. In addition, CD24 has been reported to be up-regulated during neuroinflammatory conditions and have paradoxical roles in both autoimmunity[72] and immune suppression[73,74] within the CNS. Its diverse functions in the CNS, as well as in peripheral tissues, is likely attributed the absence or presence of distinct carbohydrates.

CD24 is an important tumor marker in several peripheral cancers, where it has been linked to proliferation, invasiveness, metastatic potential and poor prognosis[75-77]. Senner *et al*[78] was the first to report detection of CD24 protein in primary GBMs, and described increased invasion of $CD24^+$ rat glioma cells *in vivo*. Later studies have confirmed CD24 expression in GBM cell lines and tissues[79,80] and demonstrated how CD24 promotes invasiveness and migration of glioblastoma cells following induction by IGFBP-2[80] and tGLI1, a truncated splice variant of glioma associated homologue 1[81].

Less is known about the prevalence and function of CD24 in pediatric brain tumors. Overexpression of the CD24 gene has been demonstrated in 20 MBs of unknown subgroups[82], and protein expression has also been demonstrated in subsets of EPNs[83] and mixed gliomas[79]. In paper III of this thesis, we characterize the expression of CD24 in different types and subgroups of pediatric brain tumors, and discuss its potential utility as a diagnostic and prognostic marker in pediatric brain tumor patients. We also investigate the prerequisites for functional studies in our patient-derived experimental models.

Experimental brain tumor models

In vitro models

Established tumor cell lines have been extensively used for screening of therapeutic compounds and remain the cheapest and simplest way to study tumor biology and drug response. Although cell lines have significantly contributed to our understanding of tumor pathogenesis, the artificial milieu generates homogenous cell populations that contrast the heterogeneous mixture of differentiation states and molecular alterations seen in cells within a tumor tissue. Preclinical drug evaluations in traditional cell lines are consequently poorly predictive of patient response, and improvement of culturing conditions could tentatively improve clinical predictability[84].

Traditional brain tumor cell lines

The majority of all human brain tumor cell lines have been propagated in serum-containing medium for decades. It is now well established that the high-nutrient composition of serum induces irreversible cell differentiation and generates homogenous cell cultures that overtime acquire multiple molecular aberrations including copy number alterations and numeric and structural chromosome changes[85,86]. As a result, the gene expression profiles of serum-cultured cell lines differ significantly from those of the corresponding primary tumors[85]. Moreover, many serum-cultured cell lines are either poorly tumorigenic or give rise to *in vivo* tumors that are poorly representative of human tumors[85], as will be further discussed in *In vivo models.*

The concept of cancer stem cells

The cancer stem cell (CSC) theory states that a subset of tumor cells have the unique capacity to maintain tumor growth and progression and induce secondary tumors. As normal stem cells, CSCs are low-proliferative, carry drug resistance transporters and display high expression of DNA repair enzymes, making them resistant to conventional therapies[87]. These features together has led to the belief that they are responsible for tumor relapse, and CSCs are therefore of intense interest in the development of new drugs. Despite the similarities between CSCs and normal stem cells, it is not known if CSCs indeed arise in normal stem cells, or in differentiated cells that have regained stem-ness[88,89].

CSCs are defined as tumor cells that (i) display extensive self-renewal capacity, (ii) generate a large number of progeny, (iii) are capable of multi-lineage differentiation, (iv) form *in vivo* tumors that recapitulate the primary tumor, (v) are resistant to conventional therapies, and (vi) express stem cell/CSC-associated markers. The prevalence of such a cell is complicated to experimentally prove

without doubt; as an example, self-renewal capacity *in vitro* is not necessarily predictive of xenograft establishment *in vivo*[90]. Still, the term CSC is frequently applied to cells that have been identified by different methods and different criteria, resulting in confusion within the research field. Terms such as "tumor-initiating cell" (TIC) or "tumor-propagating cell" may be more accurate in different contexts.

It should be noted that the tentative CSC population within a tumor can harbor heterogeneous cell subsets with similar tumor-initiating capacity, and CSC and more differentiated tumor cells may also gain or loose stemness during tumor progression, resulting in a fluctuating rather than static CSC compartment[91]. Moreover, the CSC theory does not exclude that mutations occur in cells downstream of CSC, resulting in increased tumorigenicity or drug resistance through clonal selection of such cell populations.

Neurosphere cultures

Reynolds *et al*[92] first demonstrated that the neurosphere assay can be used to select for neural stem cells (NSC). By growing brain cells on a nonadhesive substrate in serum-free medium with continuous administration of growth factors, NSCs were isolated and kept in an immature proliferative state, generating spheres from single cells. Upon removal of growth factors the NCSs differentiated into mature neurons, astrocytes and oligodendrocytes. However, this simplified description has since then been revised, and it has repeatedly been demonstrated that NSC conditions also facilitate the growth of non-clonogenic progenitor cells, in addition to true stem cells[93-95].

Brain tumor CSCs share features with NSCs, and brain tumor researchers have adapted the concept of neurosphere culturing in attempts to preserve the CSC compartment within human brain tumors. The ability to form clonogenic spheres when plated at low density is the most common *in vitro* assay to demonstrate the prevalence of brain tumor CSCs (Figure 3A-B). A number of studies have demonstrated how patient-derived adult and pediatric brain tumor cells display a CSC-like phenotype when cultured in serum-free medium supplemented with epidermal growth factor (EGF) and fibroblast growth factor (FGF)[85,96-99].

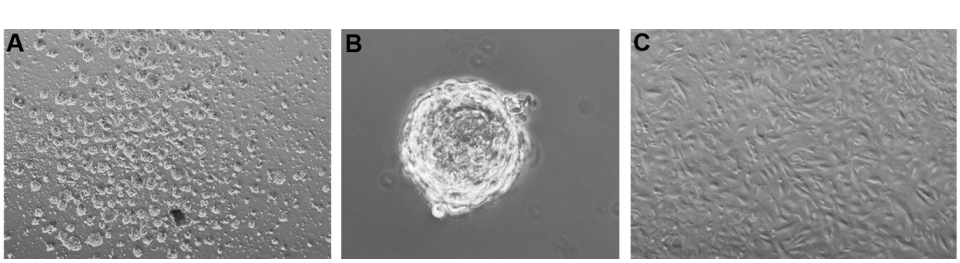

Figure 3. Patient-derived tumor cell cultures.
A, sphere culture; B, sphere in high magnification; C, monolayer culture. Modified image from paper I[100].

Adherent serum-free cultures

The neurosphere assay is however associated with several technical challenges. (i) Neurosphere cultures are complicated to establish and propagate, and are not optimal for all tumor types[101,102]. (ii) Spheres are heterogeneous in terms of viability, growth rate and differentiation state. Although this may be considered a true reflection of ongoing processes in primary tumors, it makes cultures difficult to propagate long-term[103,104]. In addition, it causes significant problems for standardization of experimental assays, both within and between laboratories. (iii) Evaluation of proliferation and sphere formation is complex, since formation (or fusion) of spheres is influenced by environmental factors[105].

Attempts have been made to establish CSC-enriched brain tumor cell lines as monolayers (exemplified in Figure 3C), by culturing tumor cells in NSC medium on a laminin-coated surface. Monolayer culturing enables homogenous cell exposure to growth factors, nutrients and oxygen, and could theoretically facilitate the progression of "pure" CSC cultures, in contrast to heterogeneous neurospheres. This strategy has been employed for adult GBMs[106-108], tentatively leading to increased success rate in culture establishment.

Concerns have been raised whether adherent glioma cell lines in serum-free conditions are equivalent to neurosphere culturing for propagation of brain tumor CSCs[109], but it has also been demonstrated that the two methods indeed generate similar cultures in terms of cell proliferation, differentiation, apoptosis and selected gene expression[110,111]. Importantly, both culture types show similar tumorigenic capacity and generate invasive tumors *in vivo*[110].

Modeling pediatric brain tumors in vitro

The majority of all brain tumor cell lines represent adult GBM. Pediatric brain tumor cell lines are scarce; around 60 cell lines have been reported in the literature (extensively reviewed and referenced by Xu *et al*[112]) and only a handful of these are used in the majority of publications. 2/3 of the published cell lines are derived from MB, and the rest represent HGG, EPN, "PNET" and AT/RT. LGGs are particularly difficult to model *in vitro* due to their low proliferation rate, and only 3 PA cell lines have been reported[112].

The most commonly used MB cell lines (such as DAOY, D283 and D341) were established in serum-containing medium several decades ago. The distinct prognoses and molecular characteristics of MB subgroups make it crucial to model these as separate disease entities, and attempts have been made to subgroup traditional MB cell lines. However, the results between different studies differ significantly depending on method applied, and for several of the cell lines more than one subgroup has been suggested. As an example, Gendoo *et al* developed a bioinformatic tool for classification of experimental MB models in 2015[113]. Although results sometimes differed between replicates, all analyzed cell lines (DAOY, D425, ONS-76, D283, D341, PFSK-1, D384, D458) were predicted to

Table 2. Experimental brain tumor models.

	MB-WNT	MB-SHH	MB-Gr3/4	EPN	HGG
GEMM*					
	Ctnnb1+Trp53	*Ptch*	*Myc +Trp53*	*Ephb2*	*Pdgfb*
	Ctnnb1+Trp53+ Pik3ca	*Ptch+Trp53*	*Mycn*	*C11orf95-RELA*	*V^{12}H-Ras+EgfrvIII*
		Ptch+Cdkn1b	*Myc + Gfi1*		*Pten+Trp53*
		Ptch+Cdkn2c			*Nf1+Trp53*
		Mycn+Trp53			H3K27+*Pdgfra*
		Smo			
		Sufu+Trp53			
		Shh+Myc			
Mouse cell lines / ST**					
				Vn19	CT-2A
					GL26
					GL261
					SMA-560
Human cell lines / PDX***					
		Daoy	D341	nEPN1	LN-18°
		ONS-76	D384	nEPN2	LN-229°
		UW228	D425	BDX-1425EPN	TN8G°
		UW426	D458	D528	U87°
			MED8A	D612	U251°
			HD-MB03		SF188
			MB002		KNS-42
			CHLA-01-MED		CHLA-200
					D212MG
					D456MG
					JHH DIPG1

GEMM, genetically engineered mouse models; PDX, patient-derived xenograft models; ST, syngeneic transplantable models.*GEMMs have been generated by modification of the listed genes[26,32,55,114-122]. **Transplantable mouse cell lines established from spontaneous or environmentally induced tumors[123,124]. ***Human cell lines for *in vitro* studies and generation of PDXs[112,125,126]. °indicate human cell lines established from adults, the remaining human cell lines are established from pediatric patients. 7/8 cell lines listed as MB-Gr 3/4 belong to Group 3, only CHLA-01-MED belong to Group 4.

belong to either WNT or SHH subgroup. In sharp contrast, a majority of these cell lines are *MYC*-amplified and are as such used to model Group 3 or 4 MBs *in vitro*[127-130]. Also in more recently established serum-cultured cell lines, subgroup affiliation may differ depending on method applied[131]. The discrepancy is therefore likely a combination of genetic aberrations acquired during long term culturing, and current classification tools being suboptimal for cell lines. In a

recent review of the field, Ivanov *et al* concluded that only 12 MB cell lines worldwide have strong evidence for a specific subgroup affiliation, *i.e.* classification was obtained with transcriptional profiling and have shown consistent results across analyses[125]. These cell lines are listed in Table 2, and all but one belong to SHH or Group 3.

In 2003, it was demonstrated that neurosphere cultures could be obtained from pediatric brain tumors, including EP, MB and astrocytomas[96,97]. Several studies have since then established neurosphere cultures in order to generate patient-derived xenograft (PDX) models (*e.g.* Zhao *et al*[132]); however, most novel pediatric brain tumor cell lines that have been established and used for *in vitro* assays during the last decade have utilized the old concept of serum-culturing[131,133,134], tentatively due to the technical challenges associated with neurosphere assays. A nice exception is the Group 4 MB sphere line CHLA-01-MED, which recently became available via ATCC[135].

So far, no comprehensive studies have evaluated the concept of serum-free monolayer culturing for pediatric brain tumors - this will be addressed in paper I of this thesis.

In vivo models

Animal models have significant advantages over cell lines, by enabling studies of tumor progression in a physiological environment. An optimal *in vivo* model should preferably recapitulate the full biology of the human tumor, have high tumor incidence and short latency time, be cheap, reproducible and technically simple to establish and use. Needless to say, no *in vivo* model meets all of these criteria. The distinct advantages and disadvantages of different animal models (summarized in Table 3) must be carefully considered when choosing the best strategy to answer a defined research question.

Pediatric brain tumors are almost exclusively modeled in mice, and the mouse models are broadly divided into genetically engineered mouse models (GEMM) and transplantable models (syngeneic or xenograft).

Genetically engineered mouse models

GEMMs are generated through introduction of a restricted number of genetic modifications, which ultimately leads to spontaneous tumor development in the mouse. The most common strategy to develop GEMMs is to replace segments of a gene of interest via homologous recombination in mouse embryonic stem cells, resulting in a knock-out or knock-in mouse[136]. A knock-in modification can be conditional (most commonly using the Cre/loxP system[137]), so that expression of the gene of interest is restricted to a certain tissue or time point. GEMMs have the advantage of a functioning immune system, and facilitate realistic interactions

between tumor and stroma. It should however be kept in mind, that while GEMMs are indispensable tools for studying functions of defined genes in tumor initiation and progression, they are simplified variants of the human disease and do not recapitulate the complex genetic events in human tumors.

Syngeneic transplantable mouse models

Syngeneic transplantable cell lines were for many decades the only option for tumor studies *in vivo*, and they are widely used even after the introduction of GEMMs because of their low cost, simple managing and predictive and reproducible behavior. Transplantable murine models were initially established from spontaneously arising or environmentally induced tumors. Since spontaneous tumors are extremely rare, tumors were commonly induced by intracranial (i.c.) injection of carcinogenic compounds[138]. Alternatively, carcinogens were injected intravenously or transplacentally into pregnant mice or rats, upon which a certain proportion of the offspring developed brain tumors[139,140]. In more recent years, transplantable mouse models have also been derived from GEMM tumors.

There are a limited number of murine tumor cell lines available, and they are poorly representative of the wide variety of tumor types and molecular variants that exist in humans. Moreover, it is well known that individual syngeneic models only partially recapitulate the molecular alterations and biological behavior of corresponding human tumors[141]. Specifically, cell lines tend to (but not always) grow as circumscribed cell masses, lacking the invasive and metastatic features of high-grade tumors. Even so, syngeneic models have certain biological advantages over PDX models, since they provide an immunocompetent species-matched stromal environment for tumor growth.

Patient-derived xenograft models

PDX models are generated by inoculation of human cell lines, low-passage tumor cells or freshly dissected tumor tissue into immunocompromised mice. The most common hosts are NOD/SCID and NOD/RAG mice, which lack mature T cells due to impaired somatic recombination of the T cell receptor (TCR). To further increase engraftment rates, these mice may be cross bread with mice harboring other immune deficiencies, *e.g.* IL-2rγ^{null}, generating NSG or NRG mice. The IL-2 receptor γ-chain directs proliferation of T cells, B cells but also NK cells and monocytes.

PDXs models are generally considered to be useful for mimicking the molecular complexity of human tumors, and their utility for preclinical drug evaluations has been demonstrated for a number of cancer forms, including pediatric brain tumors[142,143]. The preclinical results obtained in PDX models have however not led to any dramatic advances in clinical treatment strategies, and researchers have addressed two main aspects in order to improve clinical predictability.

(i) The site of tumor implantation. PDX models are commonly established at subcutaneous (s.c.) sites, to simplify the surgical procedure and enable easy monitoring of tumor growth, but s.c. xenografts typically lack invasive capacity. Orthotopically implanted xenografts more closely mimic histological features, invasiveness, metastasis and drug response of the primary tumors[144,145].

(ii) The origin of tumor material used for implantation. Cell lines are the most convenient way to establish PDX models, but similarly to murine cell lines, they generate tumors that do not represent the histopathological features of primary tumors[85]. In the context of brain tumors, the introduction of TIC-enriched brain tumor cell lines has been a step forward, since they generate tumors with more accurate biological and molecular phenotypes than tumors obtained from serum-cultured cell lines[85,108,146].

The optimal strategy for preserving the features of the original tumor is to implant freshly dissected tumor cells. This approach has generated brain tumor models of EPN, MB and GBM, which recapitulate the histology, transcriptome, genome and proteome of corresponding primary tumors[111,132,147,148]. However, many labs do not have the capacity to establish such models, due to complicated logistics and a high cost.

Humanized PDX models

While PDX models have demonstrated molecular faithfulness in a number of cancer forms, a limitation of these models is the lack of immune pressure and species–specific stromal interactions, which are crucial elements that may lead to false-positive or false-negative responses in drug screening. For instance, the cytostatic agent TMZ, which is the standard treatment of GBM patients, executes its effect in an immune-dependent manner[149]. It has been demonstrated that implantation of tumor tissue chunks will initially preserve human stromal elements, however these are replaced by their mouse counterparts over a few *in vivo* passages[150,151].

Humanized PDX models have emerged as a strategy to overcome the limitations of traditional PDXs and to enable immunotherapeutic studies in molecularly advanced tumor models. The methodology is complex and remains to be fully standardized, but broadly comprises immune reconstitution of severely immunodeficient mice before or after tumor implantation, using patient-derived peripheral blood mononuclear cells, $CD34^+$ immune precursor cells or infiltrating T cells isolated from the primary tumor[152].

***Modeling medulloblastoma* in vivo**

Of the four described MB subgroups, the majority of all available MB mouse models represents the SHH subgroup (exemplified in Table 3). Since the connection between the Gorlin syndrome and dysregulation of SHH in MB was discovered, numerous GEMMs of MB have been generated by modifying different

Table 3. Advantages and disadvantages of mouse models in brain tumor research.

	Advantage	Disadvantage
GEMM	Enable studies of specific mutations	Lack genetic complexity of human tumors
	Can be designed to mimic rare subgroups	Incomplete tumor penetrance
	Mimic tumor initiation and progression	Unpredictable growth
	Realistic microenvironment	Time-consuming to develop
		High cost
ST	Rapid growth	Homogenous
	Predictable	Rarely infiltrative
	Reproducible	Limited diversity of models
	Inexpensive	Does not mimic tumor initiation
	Realistic microenvironment	
PDX		
Human cell lines	Represent human biology	Homogenous
	Rapid growth	Rarely infiltrative
	Predictable	Limited diversity of models
	Reproducible	Does not mimic tumor initiation
	Inexpensive	Lack human stroma
		Deficient immune system
Fresh tissue/	Heterogeneous	Limited availability of material
Low-passage cells	Molecular fidelity	Variable engraftment rate
	Infiltrative and metastatic	Does not mimic tumor initiation
	Maintain human stroma?	Lack human stroma
	Possibility to make post-treatment models	Deficient immune system
		High cost
Humanized models	More realistic microenvironment	Higher cost
		Technically complicated

GEMM, genetically engineered mouse models; PDX, patient-derived xenograft models; ST, syngeneic transplantable models.

components of the SHH pathway (*e.g. Ptch1*, *Smo* or *Sufu*), commonly in combination with deletion of *Trp53* or cyclin-dependent kinases[114-116]. Mouse SHH tumors are frequently induced in cerebellar granule neuron precursors, but may also be induced in NSCs. The simple genetics of WNT tumors also make this subgroup suitable for modeling with genetic engineering; for instance, overexpression of *Ctnnb1* and a *Pik3ca mutation* in combination with *Trp53*

knockout in lower rhombic lip (RL) precursors give rise to WNT tumors with a low latency, high penetrance and molecular faithfulness of human WNT tumors[32].

Patients with Group 3 and Group 4 MBs are the ones in most urgent need of new therapies, however the poor knowledge of the biology of these tumors has limited the generation of GEMMs. Based on the frequent amplification of *MYC* in patients, two genetic models of Group 3 MB have been generated by inoculating *Trp53*-deficient mice with NSCs or cerebellar progenitor cells displaying aberrant expression of *Myc*. Although *TP53*-deletion is a rare event in Group 3 MB patients, these genetic models replicate many features of the clinical phenotype[26,119]. Swartling *et al* created another genetic mouse model reminiscent of Group 3/4 MB, by overexpressing *Mycn* in the developing cerebellum[120]. Moreover, transduction of NSCs with retroviruses encoding either of the novel oncogenes *Gfi1* and *Gfi1b,* together with *Myc,* gave rise to MBs mimicking the clinical and molecular features of Group 3[121-bk].

Transplantable mouse MB models are historically scarce, compared to the vast amounts of environmentally induced murine HGG models. In more recent years, transplantable cell lines have been established from several of the GEMMs mentioned above, which will greatly enhance the practical utility of these models. Around 25 human MB cell lines, including the ones listed in Table 3, have been used for *in vivo* studies[112]. Four MB cell lines and corresponding PDX models have been used for drug screening within the Pediatric Preclinical Testing Program (PPTP)[142]. The subgroups of these cell lines are however not always known, as previously discussed in *Modeling pediatric brain tumor in vitro.*

Different research groups have made independent successful attempts to establish PDX models specifically for Group 3 and Group 4, by orthotopic inoculation of either primary cell cultures or single cell suspensions obtained from freshly dissected tumor tissue (*e.g.* Dietl *et al*[133]); one such model is described in Paper II of this thesis. These approaches have generated PDX models that recapitulate the histologic and molecular profile of primary MBs. Some studies have established panels of MB PDXs; one study specifically addressed molecular subgroup affiliation, and it was demonstrated that MB subgroup is maintained after 3 generations *in vivo*[132]. These results are tentatively confirmed by an ongoing study comprising ~30 MBs (abstract Brabetz *et al*, 2016[153]).

***Modeling high-grade glioma* in vivo**

The extensive characterization of human glioma during many years has resulted in a wide range of genetically modified HGG models (exemplified in Table 3)[117]. Many of them are based on mutations commonly found in adult gliomas, such as *EGFR, PTEN, TERT* and *CDKN2A*[44]. With the recent awareness of genetic events that are almost exclusive to children, new models are being developed to specifically mimic pediatric HGG. One example is a mouse model were *Trp53*-

deleted neural precursor-like cells were transformed to express *PDGFRA* and the H3K27 mutation, resulting in tumors closely mimicking pediatric DIPG[122].

In addition to GEMMs, there is an extensive number of human, mouse and rat glioma cell lines available for transplantation (examples are listed in Table 3). However, only a handful of them are explicitly representing pediatric tumors[112], reflecting the relative rareness of this diagnosis in children compared to MB. GL261 (used in paper IV of this thesis) is to date the most frequently used murine glioma model and has been well characterized. It was originally induced in the 1930s by i.c. implantation of pellets of the highly carcinogenic compound 3-methylcholantrene (MCA) into C57BL/6 mice[138,154]. Despite its extensive passaging *in vitro* and *in vivo* for almost a century, the GL261 model mimics many of the features of human grade III-IV glioma, such as aggressive growth, local invasiveness, nuclear atypia, angiogenesis and necrosis[155]. In addition, it displays enhanced *Myc* expression and carries point mutations in *k-ras* and *Trp53*, features that are seen in subsets of human GBMs[156].

Phenotyping of experimental models

The attempts to identify specific markers for the detection and isolation of brain tumor CSCs have been difficult. Normal glial cells and neurons originate in NSCs, which express *e.g.* CD15, CD133, nestin, BMI-1, SOX-2 and Musashi-1. CD133 was early on identified as putative CSC marker in human GBM and MB, based on the observation that $CD133^+$, but not $CD133^-$, human tumor cells initiated tumors in immunodeficient mice[157]. These results have later been contradicted by others demonstrating that also $CD133^-$ glioma cells have tumor-initiating capacity[158-161]. The NSC marker CD15/Lewis-X has also been widely suggested as a brain tumor CSC marker in mice and human MB and GBMs[162-165], but again, conflicting reports have been presented[158,166,167]. Other less studied putative brain tumor CSC markers include CD44[168], ALDH[169], A2B5[170] and integrin-α6[171].

A challenge with identification of CSCs in human/mouse tissues or *in vitro* cultures is the need for antibody-based cell sorting based on markers expressed on the cell surface. Such markers are commonly sensitive to enzymatic digestion[172] and may fluctuate depending on culture conditions[173] and number of passages *in vivo*[159]. Moreover, antibodies may be glycoform-specific and consequently target only specific variants of a defined protein[174,175]. These facts could partly explain the discrepancy between results obtained in different studies, and complicates the interpretation of phenotyping efforts in tissues and experimental models.

Tumor immunology

The primary function of the immune system is to defend its host against pathogens; however, its ability to react against what is perceived as "non-self" also has important implications for the development of a tumor. The immune system may both prevent and promote tumor formation and progression, depending on the extent and nature of the immune response.

Components and basic functions of the immune system

The immune system is broadly divided into the innate and the adaptive part, although their functions are closely connected. The immune response is mediated by ($CD45^+$) leukocytes and soluble factors; the ones that are closely linked to tumor immunology are depicted in Figure 4. Cytokines are small membrane-bound or secreted signaling molecules that are crucial regulators of the interactions both within and between innate and adaptive immunity.

The innate immune system mediates the initial response towards pathogens, and consists of epithelial barriers, complement proteins and immune cells. Innate immune cells mainly comprise cells of myeloid precursor origin and are defined as mononuclear phagocytes (MNP) (monocytes, which differentiate into macrophages and dendritic cells (DCs) in tissues) and granulocytes (neutrophils, basophils and eosinophils). Some cells with lymphoid precursor origin (natural killer (NK) cells, NKT cells and γδT cells) are also considered parts of the innate immune system.[176,177]

Innate immune cells patrol blood and tissues and are constantly replenished to ensure a rapid and strong response towards infecting pathogens. Monocytes and granulocytes recognize pathogens via the binding of large molecular motifs (microbial-, pathogen- or danger-associated molecular patterns (MAMPs, PAMPs, DAMPs)) to pattern recognition receptors (PRRs), and subsequently engulf (phagocytose) pathogens or pathogen-infected cells. NK cells function by recognizing infected or stressed cells based on their expression of activating or inhibitory receptor[178], and eliminate them by ligand-mediated killing (*e.g.* Fas/FasL) or cytotoxic release of perforin and granzymes. NKT cells and γδT cells are rare cell types that depend on CD1d and stress-induced molecules for their activation; they produce vast amounts of cytokines and may also exert cytotoxic and phagocytic functions[179,180]. Although the functions of innate cells are inherent, their effects can be greatly enhanced by cytokine stimulation, such as interferon (IFN)-γ, interleukin (IL)-12 and tumor necrosis factor (TNF)-α. In addition, the innate immune system activates and directs the adaptive immune response by cell-cell contacts and cytokine secretion.[176,177]

Adaptive immune cells are, in contrast to innate immune cells, few and quiescent in healthy individuals. However, upon activation they rapidly multiply and home to the site of infection. The adaptive immune response harbor two unique features compared to the innate response: it is specific for a certain antigen, and it generates a long-term immunological memory against the specific antigens it has encountered. Adaptive immune cells comprise B cells and T cells, which originate in lymphoid precursors and are referred to as lymphocytes (together with NK cells, NKT cells and γδT cells). B cells mediate an antibody-based response, designed to combat extracellular pathogens. In contrast, T cells mediate cell-associated killing, typically associated with viral infections. Of the two, only T cells are strongly implicated in brain tumor immunology and will be discussed in detail. All T cells express CD3, and are further subdivided into cytotoxic $CD8^+$ T cells (CTL), which eliminate target cells expressing a specific antigen, and $CD4^+$ T helper (Th) cells, which direct the adaptive immune response towards either a T cell or B cell response.[177] The maintenance and proliferation of peripheral naïve T cells is controlled by IL-7, produced in the bone marrow and thymus[181].

Professional antigen presenting cells (APCs), including migratory DCs, represent the main link between the innate and the adaptive immune response. The activation of the adaptive immune response is dependent on cell surface molecules known as major histocompatibility complex (MHC) class I and II, which display a selection of peptides located inside cells. MHC class I is found on all cells and presents intracellular peptides to CTLs, whereas MHC class II is only expressed by APCs and presents phagocytosed extracellular peptides to Th cells.[177,182]

Activation of a T cell initially requires recognition by the TCR of a specific MHC displaying a specific peptide, in combination with co-stimulatory molecules such as CD80/CD86 on APCs binding to CD28 on naïve T cells. Traditional antigen presentation comprises peptides on MHC II presented to Th cells, but APCs can also present engulfed material on MHC I to activate CTLs via cross-presentation – a crucial concept for tumor immunology. Importantly, T cells are, with few exceptions, not activated against self-peptides. Upon activation, T cells undergo clonal expansion, differentiate into functional effector cells and home to the site of infection, where they exert their effector mechanisms. When encountering the correct MHC/antigen combination at the site of inflammation, the activated CTL will eliminate the presenting cell in the same manner as NK cells. An important difference between the two cell types is however that MHC function as an inhibitor of NK cell cytotoxicity. Conversely, NK cells can be activated against antigens without the need for MHC presentation.[177,182]

Th cells contribute to directing the adaptive immune response by secretion of cytokines, and can be subdivided based on their cytokine profiles and subsequent functions. The differentiation of $CD4^+$ T cells into either of these subsets is dictated by the cytokine environment in the context of antigen presentation. The most profoundly defined Th subsets are Th1, Th2 and Treg ($CD4^+CD25^+Foxp3^+$

regulatory T cells). Th1 cells support a CTL response and are characterized by secretion of IFN-γ, TNF-α and the T cell survival/proliferation factor IL-2. Committed Th1 cells will express the transcription factor Tbet. Th2 cells produce IL-4, IL-5 and IL-13 and induce a B cell response while simultaneously counteracting a Th1/CTL response. Tregs are characterized by secretion of IL-10 and transforming growth factor (TGF)-β, and act as suppressors of other T cells in order to abolish or limit a CTL response; this function is important for the body to avoid autoimmunity due to prolonged CTL activity. The Th subsets Th17, Th22 and Th9 have also been described. Th17 cells are induced by IL-23 and characterized by IL-17 production. However, the exact nomenclature and functions (especially with regards to tumor immunology) of these less common Th subsets are still a matter of debate.[183]

Other safety mechanisms to avoid autoimmunity include up-regulation of the so-called "immune checkpoint" molecules CTL-associated antigen (CTLA)-4 and programmed cell death protein (PD)-1 on T cells. CTLA4 will at prolonged T cell activation outcompete CD28 for binding to CD80/CD86 on APCs. PD-1 binds PD-L1 receptors expressed on a number of non-hematopoietic cells. These interactions inhibit the activity of activated T cells.[184] Following a T cell response, most T cells die – however, a subset remains in the blood as antigen-specific memory cells (Tmem) and have the capacity to respond quickly and efficiently upon reinfection with the pathogen that they have previously encountered. The maintenance of Tmems is largely dependent on homeostatic levels of IL-7 and IL-15.[181]

From immune surveillance to immune escape

The immune system has a theoretical capacity to eradicate tumor cells with the same mechanisms used to eliminate pathogens. However, tumors are commonly referred to as "wounds that never heal". In a pathogen-mediated immune response, as described above, an acute inflammation solves the problem (*i.e.* eradicate the pathogen) and is followed by immune suppression, tissue healing and remodeling, and complete shutdown of the immune response. In tumors, the acute inflammation fails to solve the problem (*i.e.* eradicate the tumor) and a state of chronic low-grade inflammation is established. This state not only prevents tumor elimination, but also drives further tumorigenesis. The process has been described in three sequential phases known as elimination, equilibrium and escape[185], and its main players are depicted in Figure 4.

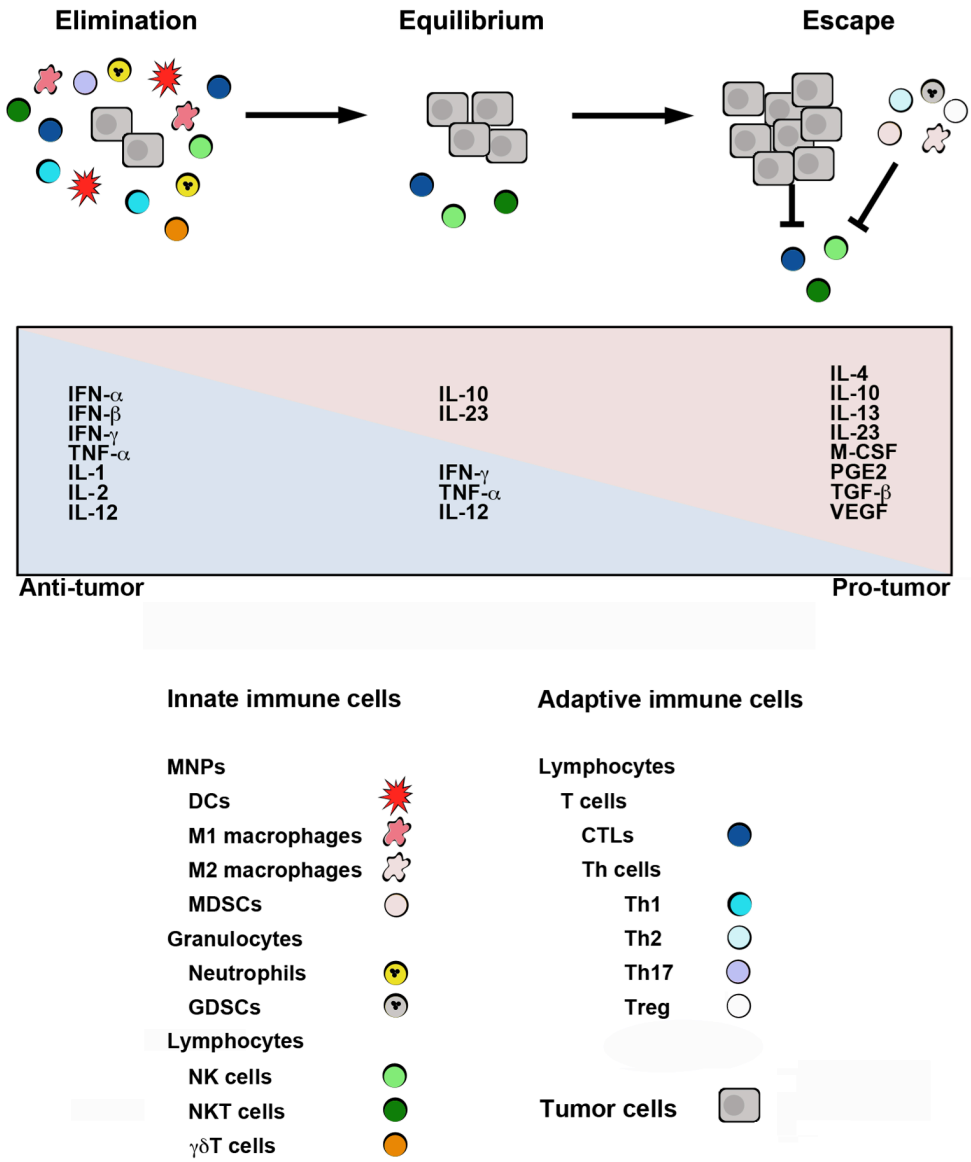

Figure 4. Cellular and soluble mediators of tumor immunity. Summary of references provided in *Tumor immunology*. CTL, cytotoxic T lymphocyte; DC, dendritic cell; GDSC, granulocyte-derived suppressor cell; IFN, interferon; IL, interleukin; M-CSF, macrophage-colony stimulating factor; MDSC; myeloid-derived suppressor cell; MNP, mononuclear phagocyte; NK, natural killer; PGE2, prostaglandin E2; Th, T helper; TNF, tumor necrosis factor; T reg, regulatory T cell; VEGF, vascular endothelial growth factor.

Immune surveillance / Elimination

The ability of the immune system to eliminate tumor cells is referred to as immune surveillance. At early disease stages, immune cells can hamper tumor development by both innate and adaptive effector mechanisms. The innate immune response is initially triggered by the local release of tumor- and stromal-derived factors, such as IL-1, macrophage-inflammatory proteins, TNF-α, and soluble DAMPs from necrotic tumor cells. Innate immune cells, including granulocytes, macrophages, DCs, NK cells, NKT cells and γδT cells, are recruited to the tumor site and may initially react towards tumor cells due to the absence or presence of cell surface molecules such as DAMPs, MHC and MICA. Tumor cells are cytotoxically eliminated and tumor debris is phagocytosed, as previously described.[185,186]

In this process, the innate cells at the tumor site produce pro-inflammatory cytokines, which recruit additional cells and further enhance the anti-tumor response. Macrophage-derived TNF-α, IL-1α and IL-1β are alongside granulocyte macrophage-colony stimulating factor (GM-CSF) and IL-8 the key recruiters of monocytes and neutrophils from the blood. Macrophages and NK/NKT cells signal in a positive feedback loop to activate each other: IL-12 activates NK cells, which in turn secrete the potent macrophage-stimulating factor IFN-γ. Macrophages are further activated by autocrine IFN-α,-β and IL-6 signaling. Several factors, including IFN-α,-β,-γ, TNF-α, IL-12 and GM-CSF enhance APC functions by *e.g.* up-regulation of MHC and co-stimulatory molecules, and induce maturation and migration of DCs to draining lymph nodes.[185-187]

In lymph nodes, DCs cross-present tumor-associated peptides to naïve T cells, but since the majority of peptides originating in tumor cells are normal body proteins, the T cells will in many cases not be activated. There are however several situations when it is possible to evoke an anti-tumor T cell response; for instance, mutated proteins are perceived as non-self, and the same may be true for proteins that are expressed in excessive amounts or are expressed in a tissue or during a developmental stage where the protein is not normally found. In those cases, tumor-specific CTLs may be activated, expanded and home to the tumor site, where they eliminate tumor cells expressing the tumor antigen on MHC I.[186]

The microenvironment in early inflammation (including the presence of IL-2, IL-12 and IFN-γ) favors differentiation of Th1 cells. Macrophages and Th1 cells continuously reinforce each other´s functions by feedback secretion of IL-12 and IFN-γ, and together strengthen both innate and adaptive effector functions.[183,187]. Macrophages with a pro-inflammatory phenotype (IL-1α,β, IL-6, IL-12, IL-15, TNF-α), producing the effector factors reactive oxygen species (ROS) and nitric oxide (NO) and capable of inducing IFN-γ secretion by in T cells and NK cells, are referred to as classically activated M1 macrophages.[188] At this stage, Th17 cells may also contribute to anti-tumor immunity by promoting[189] or exerting[190] Th1-mediated antitumor functions.

Immunoediting / Equilibrium

CTLs and NK cells are the main effector cells in tumor elimination, and IFN-γ, TNF-α and IL-12 are key mediators for maintaining an environment that promotes cytotoxicity. This milieu can temporarily keep the tumor in a dormant state. However, the constant pressure from these cells will overtime select for tumor cells with immune-evading properties, and the inflammatory milieu also facilitate additional mutations to occur. As new tumor cells are continuously generated, the selection pressure favors tumor cells that can escape immune recognition. This sculpturing of the tumor is known as immunoediting, and eventually results in a tumor that is decreasingly susceptible to immune attack[185,191]. Although the processes of immunoediting are known, the equilibrium phase, during which the tumor is neither eliminated nor progressing, is rather difficult to demonstrate in patients as the tumor has usually already escaped immune surveillance at the time of clinical presentation. Based on findings in mouse models it has been suggested that IL-12 and IL-23 could be the most important cytokines to maintain this phase[192].

Mechanisms that may be acquired by the tumor are down-regulation of tumor antigens, MHC or NK cell receptor ligands, in order to prevent recognition and subsequent cytotoxic elimination. Even when tumor cells retain their immunogenicity, they may acquire an immunosuppressive phenotype, including up-regulation of cell-bound molecules (such as PD-L1) or secretory factors (such as TGF-β, IL-10, indoleamine 2,3 dioxygenase and prostaglandin (PG)E2), which inhibit cell-mediated killing, 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[185-187].

Immune suppression / Escape

Without therapeutic intervention (as will be discussed in *Immunotherapy*), the tumor will eventually escape immune attack and firmly establish an immunosuppressive microenvironment that inhibits immune effector functions while preserving a chronic inflammatory state that favors tumor progression. Recruited and induced suppressive immune cells, including Tregs, Th2 cells, M2 macrophages and immature myeloid/granulocytic cells (myeloid-derived suppressor cells, MDSC, granulocyte-derived suppressor cells, GDSC), are increasingly contributing to maintaining this milieu.

Macrophages are highly plastic cells compared to Th cells, and their phenotypes may drift along a scale with M1 and M2 as extreme endpoints depending on the microenvironment. The M1 phenotype has been described previously; M2 macrophages are induced by *e.g.* macrophage-colony stimulating factor (M-CSF), IL-4, IL-10 and IL-13, and characterized by secretion of potent immunosuppressive mediators such as IL-10, TGF-β and PGE2. Similarly to the relationship between M1 and Th1, M2 and Th2 reinforce each other´s functions -

while at the same time inhibiting the M1/Th1 response.[188] Several markers has been suggested for identification of macrophage subsets, such as inducible NO synthase (iNOS) (mouse), CD64 (human) and CD80 (mouse/human) for M1, and CD163 (human), Arg-1 (mouse) and CD206 (mouse/human) for M2[193,194]. However, while the M1/M2 phenotypes are easily defined following induction *in vitro*, their explicit identification *in vivo* is challenging due to the a heterogeneous cell population with less clear polarization than what is observed *in vitro*[195]. Also, as exemplified above, suggested markers may be species-specific.

The COX-2/mPGES-1/PGE2 pathway

Several tumor- and M2/Th2-associated factors are not only immunosuppressive, but also contribute to tumor progression through enhanced tumor proliferation, angiogenesis and metastasis. One such factor is PGE2, which is produced by the conversion of arachidonic acid into PGH2 by cyclooxygenase (COX) enzymes, followed by conversion of PGH2 into PGE2 by microsomal PGE synthase-1 (mPGES-1). COX-2 and mPGES-1 are highly induced in pro-inflammatory conditions, and induce a Th2 response, limit and counteract IFN-γ, IL-2 and IL-12 production, inhibit NK cell effector functions and promote the development of Tregs and MDSCs.[196] In addition, PGE2 has been shown to promote proliferation, survival, angiogenesis, invasiveness and chemoresistance of tumor cells[196-199]. The expression of COX-2 and PGE2 in pediatric brain tumors is demonstrated in paper I and II of this thesis, and the therapeutic effect of COX-2 inhibition *in vivo* is then evaluated in paper IV.

Angiogenesis and VEGF

Tumor growth and invasion is dependent on blood vessel generation from pre-existing blood vessels, *i.e.* angiogenesis, to maintain sufficient access to oxygen and nutrients as the tumor increases in size. The "angiogenic switch", induced by tissue hypoxia and activation of hypoxia-inducible factors, is a key step in tumor progression and triggers a pro-angiogenic program that drives tumors into a malignant phenotype with the potential for systemic dissemination[200,201]. Tumor-derived vessels are disorganized and less functional than normal vessels, resulting in leakage, edema, poor drug administration and the maintenance of hypoxic tumor regions that facilitate evolvement of features such as drug resistance[202]. Angiogenesis is strictly controlled by the balance between pro- and anti-angiogenic factors, such as endostatin (anti-angiogenic) and vascular endothelial growth factors (VEGF), angiopoietins, FGF, PDGF (pro-angiogenic), secreted by tumor and stromal cells[203].

Hypoxia and angiogenesis are tightly linked to immune suppression. The pro-angiogenic microenvironment facilitates recruitment of suppressive cell types such as Tregs and MDSCs, and skews infiltrating monocytes into a suppressive M2 phenotype[204-206]. Hypoxic factors extend their effect systemically and may

interfere with the maturation and induction of anti-tumor cells[207], and the intratumoral infiltration of anti-tumor cells is further dampened by hypoxia-induced down-regulation of endothelial molecules that are required for CTL trafficking[208]. Conversely, tumor-associated suppressive immune cells secrete pro-angiogenic factors[204,205], and this crosstalk maintains a constant hypoxic immuno-suppressive microenvironment that drives tumor progression.

Special considerations in CNS and pediatric immunology

CNS immunology

An extensive inflammation in the brain could be lethal due to an inflammatory edema and increased i.c. pressure, and the CNS has therefore evolved specialized functions to limit the immune response. Still, the CNS is by no means immunologically silent, and the historical view of the CNS as an immunologically privileged site was recently nicely described by Dunn & Okada[209] as a "conceptual albatross that has likely attenuated enthusiasm for CNS immunotherapies over decades". With that said, a couple of important differences between CNS and peripheral immunology should be considered.

(i) The brain is scarcely populated with resident immune cells. The main defense consists of stationary macrophages, referred to as microglia, located in the brain parenchyma. Microglia are functionally versatile cells and direct CNS development and homoeostasis in addition to their phagocytic capacity[210]. Moreover, other brain locations such as the meningies and the perivascular spaces are inhabited by specialized macrophage and DC populations[211].

(ii) Brain entry of peripheral immune cells and blood molecules such as antibodies is restricted by the blood brain barrier (BBB), comprising specialized endothelial cells with low levels of the adhesion molecules that are required for leukocyte trafficking. The BBB endothelium is connected by tight junctions that largely prevent molecular influx. Macrophages, DCs and activated/memory T cells) may however cross the BBB in healthy individuals, and an inflamed BBB up-regulates leukocyte adhesion molecules, further increasing immune cell entrance[212]. Importantly, the BBB in immature vessels of high-grade brain tumors is dysfunctional[213], which may both facilitate and prevent cellular trafficking.

(iii) Resident brain cells constitutively express membrane-bound or secreted neuroimmune regulatory proteins (NIRegs) such as CD47, which polarize and inhibit the function of both resident and infiltrating innate immune cells[74]. CD24 is believed to function as one such NIReg, by binding and neutralizing the DAMP high-mobility group box 1[214]. In addition, factors such as TGF-β are constitutively expressed in different parts of the healthy CNS, maintaining an immuno-suppressive environment[215].

(iv) The brain lacks a conventional lymphatic system and there are no i.c. lymph nodes. It has been demonstrated in mice that CSF, tentatively including brain antigens and immune cells, drain into extracranial deep cervical lymph nodes (dCLN) via meningeal lymphatic vessels located in the dural sinuses – suggesting that lymphatic drainage in the CNS is more similar to peripheral tissues than was previously believed[216].

(v) The brain lacks professional APCs. It has been proposed that professional phagocytes (microglia, macrophages, DCs) or amateur phagocytes (endothelial cells, pericytes, astrocytes, ependymal cells, neurons) act as APCs, but no conclusive evidence has been presented. It is therefore not known if antigen presentation occurs i.c. or in dCLNs.[209]

Pediatric immunology

A detailed description of the maturation of the immune system is provided by Simon *et al*[5]; key references are mentioned here.

It is well known that infants are born with an immature immune system and therefore more susceptible to infectious pathogens. Innate immune cells are present at birth, but are not fully functional; they have impaired phagocytic capacity, and respond to stimuli only with low cytokine secretion[217-219]. The T cell compartment of the infant is not only naïve, due to lack of antigen encounters, but activated Th cells tend to develop a Treg phenotype, resulting in a predominantly suppressive Th2-skewed response to non-self antigens[220,221].

Immune cells gradually mature during childhood, and the pool of Tmem cells is increased with every antigen encounter, including vaccinations. Since an immunological memory is generated also in adulthood, it is somewhat misleading to state that an immune system is "fully mature" at a certain age. Rather, the absolute and relative numbers of immune cell populations fluctuate over life, from infancy to old age[222].

Very young children clearly have an immunological disadvantage in dampening tumor development. Still, older patients can be attributed the same disadvantage, but for different reasons. With increasing age, the immune system gradually looses its effectiveness and both innate and adaptive functions deteriorate[223]. Although old patients have a large pool of Tmems, the production of naïve T cells are low, and the response to new antigens is therefore limited[224]. In addition, older patients acquire a pro-inflammatory body environment, which further facilitates tumor development and progression[222,225].

The immunome of pediatric brain tumors

Adult GBM is the primary model system for characterization and therapeutic modulation of the immune response against brain tumors[226,227] and its prominent

immunosuppressive features are well known[228]. However, these findings are not necessarily applicable to pediatric brain tumor patients. First, pediatric brain tumors comprise a diverse group of tumors with distinct genetic and biologic features that may influence an immune response. Second, the median age for diagnosis of GBM is 64 years[6]. As previously described, the immunological status and subsequent ability to evoke an anti-tumor immune response depends on age.

Few studies have comprehensively investigated the immune response against different brain tumor types in children. Only recently, a combined flow cytometry and gene expression study demonstrated distinct immunophenotypes (including disparate myeloid and lymphocyte infiltration patterns, as well as expression of markers of immune activation and inhibition) of the most common brain tumor forms in children[229]. PAs and EPNs display evidence of ongoing immune surveillance, with increased frequencies of both myeloid cells and lymphocytes compared to MB and GBM[229]. Tumor-infiltrating myeloid cells in these tumor types predominantly exhibit an M1 phenotype, including high expression of CD64 and MHC-related genes[229,230]. An M1/CTL profile has also previously been linked to good prognosis in children with EPN[231].

In contrast, pediatric GBM and MB display lower immune cell infiltration, and immune cells are enriched in T cell-suppressive/inactive markers (PD-1, Foxp3, CD163, CD206)[229,232]. Even so, a study comprising both pediatric and adult HGGs showed an enrichment of immune-related genes and IHC markers in the few long-term survivors that are found within the HGG population. Long-term survivors displayed an enhanced myeloid M1 profile (*e.g.* up-regulation of CD86 and MHC-related genes) and CTL response (*e.g.* increased CTL infiltration and up-regulation of granzyme B)[233]. These results highlight that immune surveillance is not restricted to LGG patients, but can also occur in a subset of HGG patients.

MBs have been attributed a number of suppressive mechanisms to escape cytotoxic elimination, including defects in components required for antigen presentation on MHC I[234], modified expression of ligands to prevent NK cell cytotoxicity[235] and high production of TGF-β[232], VEGF[236] and PGE2[237]. In a SHH MB model, it was demonstrated that inhibition of TGF-β limited Treg activity and promoted the expansion and activation of a potent CTL response, indicating that TGF-β is a key CTL resistance mechanism for MB cells (given that MHC class I expression is maintained)[232]. PGE2 has been linked to immunosuppression, as well as to survival, proliferation, angiogenesis and radioresistance of MB cells[237,238].

Importantly, these results are not necessarily representative of all patients with a specific tumor type. Significant differences in immune signature (defined as expression of lymphoid- and myeloid-associated genes and infiltration of diverse immune cell populations) have been detected in distinct molecular subgroups or patients groups with MB[239], EPN[240] and GBM[241], which complicates the interpretation of historical data.

The distinct genetic profiles of MB subgroups could have direct implications on tumor immunity, however very few studies have so far addressed this question. Human SHH and WNT tumors have an increased macrophage compartment compared to Group 3 and Group 4, with enhanced expression of M2-associated genes (*CD163, CSF1R, PTX3*)[239]. These results were corroborated in a comparison between a SHH mouse model and a *Myc*-driven Group 3 MB model, where it was shown that both myeloid and lymphocyte infiltration was higher in the SHH model[242]. Interestingly, the Group 3 mouse model responded better to anti-PD-1-treatment, suggesting that this T cell-inhibiting pathway could be of importance specifically for Group 3 patients[242]. The clinical applicability of this finding is however not clear, since PD-L1 has not yet been identified in human MBs[243,244].

While EPNs overall display enhanced immune surveillance compared to MB and GBM[229], this is not true for all patients. As previously described, pediatric EPNs are found within the high-risk subgroups PF-A and s.t.-RELA, and the low-risk subgroups PF-B and s.t.-YAP1. The immunomes of PF-A and PF-B have been thoroughly studied during the past few years, and it has been demonstrated that the high-risk PF-A tumors are characterized by a pro-tumor phenotype including dysfunctional T cells, constant activation of IL-6 and increased expression IL-8, VEGF, TGF-β and COX-2[240,245]. This profile is not observed in PF-B tumors. Interestingly, the immune profiles also differ significantly at relapse; recurrent PF-B tumors display an antigen-specific adaptive immune response, which is likely facilitated by the lack of profound inflammation at diagnosis[240]. The s.t.-RELA tumors are characterized by aberrant NF-κB signaling, which is a key regulator of the cellular inflammatory program – however, the immunological signature of this tumor subgroup has not been directly compared to PF EPNs.

Immune monitoring and biomarkers

The close link between the immune response and tumor progression provides a strong rationale to search for immune-associated biomarkers for cancer patients. The extent or signature of intratumoral immune infiltrates has shown prognostic value in patients with peripheral and CNS tumors[231,233,246], but further clinical validation is required. In 2012, a global effort was initiated to evaluate a standardized clinical "immunoscore" as a complement to the traditional TNM (tumor size, lymph node, metastasis) staging for peripheral tumors with an emphasis on colorectal cancer[247]; this study can hopefully pave the way for future clinical implementation of prognostic immune biomarkers. Immune-associated markers may also predict response to specific immunological therapies[248], discussed in the next section.

Systemic biomarkers such as cytokines are particularly valuable for monitoring of an ongoing treatment response. Although cytokines usually act at

short distances, their effect can extend into the systemic circulation and a prognostic value of systemic cytokine levels have been demonstrated in multiple cancer forms[249]. Adult GBM patients display profound systemic immunosuppression, including increased levels of IL-10[250], and decreased levels of Th1-associated cytokines (IL-12[251], IFN-γ and TNF-α[252]). Given the differences within and across the local immunome of pediatric brain tumor types, systemic immune signatures could be hypothesized to be equally heterogeneous and subsequently useful as systemic biomarkers. In paper V of this thesis, we have performed a systemic immune profiling of patients with PA, EPN, MB, HGG and LGG, and searched for distinguishing factors within the cohort.

Current and future treatment of children with brain tumors

The overall survival rates for children with brain tumors have slightly improved over the last decades[1], but are still poor for high-risk patients. Current treatment comprises surgical tumor resection, alone or in combination with RT and CT. As previously discussed, children with brain tumors have different prognoses depending on tumor features other than histology alone; still, current risk stratification and treatment protocols are largely based on histological diagnosis. Recent data suggest that subsets of children may be over-treated with current therapies at the cost of long-term adverse effects, while in other cases the treatment strategies of today are insufficient. Future treatment options, currently evaluated in clinical trials, can be divided into three main approaches: (i) de-escalation of current therapies, including reduction of RT or replacement of RT with combinations of CT; (ii) targeting of tumor-specific molecules using small molecule inhibitors or antibodies; (iii) modification of the tumor microenvironment with anti-angiogenic intervention or immunotherapies (IT).

Current risk stratification and treatment protocols

International treatment guidelines of pediatric brain tumors are summarized below. Although treatment (such as choice of cytostatic drug) may vary slightly between institutions worldwide, the general concepts remain. In addition to targeting of tumor cells, the treatment of children with brain tumors includes symptom-relieving drugs such as corticosteroids to prevent and reduce tumor-induced edema, and anti-epileptic drugs.

Medulloblastoma

The treatment protocol for MBs has been roughly the same for the past 50 years, and comprises surgical resection, RT and CT for all children >3 years old. Because of the metastatic nature of MB, RT is delivered both to the brain and to the spinal cord. The RT dose has been gradually reduced since the 50s, but is still associated with significant long-term adverse effects including neuro-cognitive dysfunctions, impaired fine motor skills, delayed growth and radiation-induced secondary tumors[253-255], and a further decrease in dose is warranted. The brains of infants and young children, especially cells in the hippocampus, are particularly vulnerable and RT has therefore been completely abandoned in treatment of this age group.

MB patients are currently stratified as high-risk, standard-risk or infant-risk patients based on tumor histology, age and the presence of metastases. High-risk patients are those presenting with significant residual tumor and/or metastases and/or a tumor with LCA histology. These are treated with CT (*e.g.* cisplatin, vincristine, cyclophosphamide, etoposide, methotrexate or carboplatin) and higher dose RT of the brain and spinal cord. Standard-risk patients are those exhibiting none of the features described above. They are treated in much the same manner as high-risk patients, but the RT dose is reduced. Treatment of infants, in this context defined as children <3 years old, comprise surgical resection with or without CT. Infants, high-risk patients >3 years and patients with recurrence may also be considered for inclusion in clinical trials.

Molecular MB subgroup affiliation has limited influence on the choice of up-front treatment. Some clinics use the presence of *MYC* amplification[29] to include patients in high-risk protocols. In contrast, *CTNNB1* mutation is an indication of lower risk[256] and may justify a decrease in RT dose and maintenance CT.

Low-grade glioma

LGGs are slow growing and associated with good outcome, and surgery of non-symptomatic tumors may be postponed under observation. Following surgery where the tumor is radically removed, patients are not given further treatment. If a portion of the tumor remains, the patient may still be observed until significant progression occurs. At that point, CT administration is considered (most commonly carboplatin with vincristine), but usually not RT.

High-grade glioma

Treatment of HGG comprises surgery, RT and CT, although it should be emphasized that this treatment fails in the majority of patients. Brain stem HGGs are especially difficult to treat since they are surgically inaccessible, and in many cases CT is the only treatment option for these patients. The standard CT for adult GBM is currently the alkylating agent TMZ, after it was shown to prolong (albeit moderately) survival in GBM patients in a phase III trial reported in 2005[257]. In

children, no such effect was seen[65]. However, the alkylating effect of TMZ can be counteracted by MGMT, and the expression or epigenetic silencing of MGMT therefore dictates therapeutic response. The promoter of MGMT is methylated in a subset of HGG patients, resulting in improved survival following TMZ treatment of both children and adults[64,65]. Patients with recurrent HGGs are commonly included in clinical trials.

Ependymoma

The extent of surgical resection is the most important factor to consider in the treatment of EPN[258]. Unless the entire tumor is completely removed, it usually recurs. EPNs are generally insensitive to CT[259] and patients are therefore commonly treated with RT alone following surgery. The exception is children <3 years old, which are treated with CT instead of RT. Despite the significant differences in prognosis between EPN variants, all grade II and III EPNs have the same treatment protocols. The rare cases of grade I EPNs are surgically removed without further treatment.

Molecular targeting of tumor cells

Molecularly targeting drugs (MTD) include small molecule inhibitors and molecule-specific antibodies that are designed to biologically modify drivers of tumor progression, or deliver toxins/radionucleotides to the tumor. The rationale for using molecular targeting is to avoid the broad unspecific activity and subsequent side effects of standard CT. A challenge specifically associated with development of MTDs against brain targets is that the drug must be able to penetrate the BBB, without causing significant neurotoxicity. So far, no MTDs have been included in standard treatment protocols for pediatric brain tumors, but several promising candidates are currently under clinical evaluation (summarized and continuously updated at www.clinicaltrials.gov). The only MTD approved by the US Food and Drug Administration (FDA) for treatment of pediatric brain tumors is everolimus (discussed below) for nonoperable subependymal giant cell astrocytoma.

MTD treatment of MB is within reach, specifically for tumors within the SHH subgroup. Preclinical and clinical investigations have evaluated multiple MTDs targeting different components of the SHH pathway, but the SMO-inhibitors are so far the most popular ones and have been investigated in several phase I and II trials. Given the range of molecular variants within the SHH subgroup, it is however crucial to take into account the individual genetics of patients before inclusion in clinical trials[28]. For instance, the SMO-inhibitor vismodegib showed variable efficacy in a phase II trial comprising MB SHH patients[260], but non-responders within the patient cohorts were been found to

harbor mutational events downstream of SMO. These patients may instead have responded to other drugs such as GLI inhibitors.

Molecular targeting specifically for Group 3 and Group 4 MBs are more challenging. MYC-inhibition has been suggested for treatment of Group 3 MBs, but direct molecular targeting of MYC is technically difficult to accomplish. Rather, attempts have been made to shut down gene expression by epigenetic regulation[261]. Of note, the poor prognosis of these two patient groups is attributed the metastatic capacity of the tumors, and children are usually included in clinical trials upon recurrence. Although MBs maintain their molecular subgroup affiliation at recurrence, the metastases differ significantly from the primary tumor in other genetic aspects[22] - rendering the risk that successful treatment of primary tumor models in a preclinical setting will prove less efficient in clinical trials. Metastatic models for these tumors are therefore highly warranted for MTD studies.

Aberrant BRAF and MAPK signaling is a driver of the majority of LGGs. A proportion of tumors carry the BRAF V600E mutation[262] and clinical efficacy has been demonstrated for the BRAF V600E inhibitors dabrafenib in a phase I trial[263]. Another treatment strategy that have shown efficacy, and which is also suitable for treatment of BRAF-duplicated LGGs, is MAPK inhibition with selumetinib[264]. The other major pathway activated in LGGs is PI3K/Akt/mTOR, which may preferentially be targeted with mTOR inhibitors such as everolimus[265].

10-25% of HGGs also display the BRAF V600E mutation[44], and treatment with dabrafenib has shown effect in several HGG patients[263,266]. No EPN-specific MTDs have so far been evaluated in clinical trials. The high-risk EPN subgroups PF-A ($CIMP^+$) and s.t.-RELA are of specific interest in preclinical evaluation of new treatment strategies. DzNEP is a compound that diminishes the function of H3K37. It has shown efficacy in orthotopic PDX models of PF-A[267], and could also be a treatment option for HGGs of the H3K37 subgroup, as well as for subsets of patients with Group 3 and Group 4 MB.

The concept of using MTDs targeting single tumor-associated antigens faces a number of challenges (in addition to induction of drug resistance, discussed later). The relatively low numbers of pediatric brain tumors worldwide, especially when singled down to subgroup level, makes development of MTDs for these patient groups expensive. Also, clinical trials require international collaborative efforts that are logistically complicated. A number of clinical trials are therefore evaluating general cancer targets for treatment across pediatric brain tumor types, typically those for which there are already approved drugs for peripheral cancers. Such targets include for instance EGFR, histone deacytolases, human epidermal growth factor receptor-2 and anaplastic lymphoma kinase. In addition, micro-environmental features (*i.e.* vascularization, extracellular matrix interactions and immunity) are important for maintenance of tumor growth across tumor types and

may therefore be targets for MTDs, as will be discussed in the two following sections.

Anti-angiogenic intervention

Drugs targeting angiogenesis aims at switching the pro-/anti-angiogenic balance of tumors, and subsequently normalize the tumor vasculature. Theoretically, this could reduce hypoxia, dampen metastasis, facilitate drug delivery, increase drug sensitivity and skew the immunosuppressive tumor milieu[268,269]. Several anti-angiogenic strategies have been evaluated in clinical trials of pediatric brain tumor patients, however the clinical benefit has been very moderate[270]. The most common way to target angiogenesis in tumors is MTDs against VEGF or VEGFR. Overexpression of VEGF has been repeatedly demonstrated in pediatric brain tumors[236,271-273], and MTDs for VEGF/VEGFR have been evaluated for treatment in numerous clinical trials. The VEGF-antibody bevacizumab is of particular interest, since it is approved for treatment of adult GBM. Phase II trials of bevacizumab have been conducted for pediatric HGG and EPN, however these have failed to show improvement in survival[274,275]. Other anti-angiogenic approaches, such as low-dose CT alone or in combination with inhibitors of IFNs, EGFR or PDGFR, have been evaluated in phase I-II trials for treatment of HGG, EPN and MB, but also for these agents the clinical efficacy was limited[270].

The targeting of single tumor-associated antigens commonly leads to development of resistance, as the selection pressure favors tumor cells that are able to withstand treatment by down-regulation or modification of the target, or up-regulation of compensatory mechanisms. SMO-inhibitors, discussed previously, have for instance been shown to induce a resistance mutation in SMO[276] or downstream mutations that maintain SHH signaling[277]. Similarly, treatment with VEGF inhibitors can lead to up-regulation of Ang2 or FGF, and this is likely one of the explanation for clinical failure of VEGF inhibitors (other potential mechanisms are nicely reviewed by Sie *et al*)[270]. Simultaneous targeting of multiple angiogenic pathways could be a way of circumventing this problem, and several ongoing and recruiting clinical trials evaluate combinations of anti-angiogenic drugs and other MTDs and/or different CT combinations. In a recently finalized phase II trial, the combination of bevacizumab and an EGFR/ErbB2-inhibitor was well tolerated, but did not improve outcome in children with EPN[278]. Bevacizumab and the CT combination TMZ/ irinotecan have so far shown promising results in single cases of recurrent HGG and MB[279,280] and are currently evaluated in phase II trials for recurrent CNS tumors.

Another strategy to circumvent the problem of tumor-associated drug resistance is modification of stromal cells, since their stable genomes are far less capable of developing resistance mechanisms. One such approach is IT.

Immunotherapy

IT has two major theoretical advantages over the previously described treatment strategies: the ability to adapt to new mutations arising during tumor progression, and the potential to generate a long-term biological memory that could prevent tumor recurrence. IT comprises multiple technical strategies, all with the basic aim to enhance anti-tumor immunity by increasing immune surveillance mechanisms and/or decreasing chronic inflammation and immune suppression. Treatment design is however a delicate balance between obtaining clinical efficacy and avoid unwanted induction of autoimmunity or pro-longed inflammation that favors tumor progression.

Broadly, ITs are divided into passive or active therapies. Passive IT includes transfer of immune effectors, including T cells or antibodies. In this respect, many of the MTDs described previously are included in the definition of IT (although the primary aim of antibody delivery is not necessarily to induce an immune response). Active ITs aim at modifying and supporting the endogenous immune response by *e.g.* immunizations, cytokine therapy or pharmacological inhibition of suppressive targets.

Enhancing immune surveillance

Immune surveillance can be enhanced by systemic or local delivery of cytokines such as IL-2, IL-7, IL-15, IFN-γ, TNF-α or GM-CSF.[281,282] Such therapies boost a general immune response, and are associated with significant side effects. A more tumor-specific CTL response can be induced by (i) adoptive T cell transfer; this strategy includes passive transfer of T cells that have been isolated from the patient and expanded *in vitro*. T cells may also be re-educated or modified *in vitro*, for instance by co-culturing with APCs presenting tumor antigens[283], or genetic modification[284], (ii) vaccination with tumor antigens, either as single peptides[285] or inactivated whole tumor cells that have[286] or have not[287] been transfected to express immune-stimulatory molecules, or (iii) adoptive DC transfer (so called DC vaccines), where blood monocytes are matured *in vitro* and primed with tumor peptides[288], cell lysates[289] or RNA[290]. Notably, therapies that induce T cell responses against a single tumor antigen may lead to development of resistance; a well cited example is a clinical phase II trial of GBM patients, where a DC vaccine against an EGFRvIII-peptide showed initial clinical efficacy. However, 82% of the tumors had lost expression of EGFRvIII at recurrence[285].

Most of these approaches have shown limited therapeutic efficacy as monotherapies, but the use of adjuvants or combinations of different treatment strategies may boost the effect. Local vaccine adjuvants such as aluminum[291], tetanus toxoid[226] and PRR agonists[292] increase innate immune functions, which facilitates and strengthens the generation of a CTL response at the vaccination site. Moreover, the effect of adoptive T cell transfer can be enhanced by simultaneous

delivery of intratumoral IFN-α/IFN-γ/IL-2[293], chemokines[294] or PRR agonists[295] that enhances leukocyte infiltration[294] and makes the tumor microenvironment more favorable for infiltrating effector cells[293,295].

It has repeatedly been shown that treatment strategies aiming at enhancing immune surveillance mechanism can indeed induce an anti-tumor response – this response is however limited by the induction of immunosuppressive counter mechanisms, and combinatory treatments with agents inhibiting immuno-suppression are highly warranted.

Combatting immunosuppression

Attempts to inhibit immunosuppressive pathways include the use of MTDs to (i) inhibit immune-inhibitory receptors, (ii) deplete, or block the differentiation or recruitment of suppressive immune cell populations, or (iii) inhibit immuno-suppressive enzymes and soluble factors.[296]

The first strategy most commonly includes prevention of T cell tolerance/ apoptosis induced by prolonged antigen stimulation, by blocking CTLA-4 and PD-1/PD-L1 respectively. The CTLA-4 inhibitor ipilimumab and the PD-1 inhibitors nivolumab and pembrolizumab were recently approved by the FDA for treatment of malignant melanoma. The reported clinical efficacy is encouraging[297,298] - however, the effect of the drugs is not restricted to T cells directed against tumor antigens, and significant side effects have been reported, especially for ipilimumab[299].

There is a number of strategies aimed at reducing the immunosuppressive effects of MDSCs, M2 macrophages and Tregs (reviewed and extensively referenced by *e.g.* Devaud *et al*[296]): intratumoral neutralization of the chemokines CCL2, CCL17 or CCL22 reduces tumor recruitment of Tregs and MDSCs; tumor-associated myeloid cells can be depleted or inhibited with clodronate liposomes, CSF1R inhibitors or Gr-1 antibodies, while CD25-targeting appears to diminish the effector T cell population in addition to Tregs. In addition, direct targeting of immunosuppressive enzymes and factors such as TGF-β, IL-10 and arginase, can skew the microenvironment to facilitate re-programming of intratumoral suppressive cells into anti-tumor effectors.[296]

COX-2 inhibition has been experimentally demonstrated to induce a Th1 switch, reduce induction of immunosuppressive cell types, promote tumor elimination by adaptive effectors[300,301] and increase the therapeutic efficacy of whole cell vaccines[302] and DC vaccines[303]. Pharmacological inhibition of COX-2 can be obtained with general COX inhibitors (non-steroidal anti-inflammatory drugs) such as aspirin and diclofenac. Selective COX-2 inhibitors are associated with a decreased risk of severe side effects such as gastrointestinal bleeding. Still, the risk of side effects from long-term use has been considered significant and led to the withdrawal of several drugs (including valdecoxib) from the market. The only FDA-approved selective COX-2 inhibitor currently on the market is

celecoxib. The European Medicines Agency has also approved etoricoxib and parecoxib for clinical use.

Combining immunotherapy with other therapies

Although conventional therapies are intuitively non-compatible with ITs due to their induction of lymphopenia, IT and CT/RT may in fact be mechanistically synergistic in carefully designed treatment protocols. The state of systemic lymphopenia favors immune reconstitution by for instance increased systemic levels of IL-7, suggesting that such a time point could benefit the induction of a CTL response by vaccination. Furthermore, CT agents such as cyclophosphamide appear to predominantly deplete immunosuppressive cell populations – again providing a treatment window for IT[304]. CT and RT may also induce direct changes in the tumor microenvironment that facilitates anti-tumor immunity. Gemcitabine, oxaliplatin and RT may up-regulate MHC on tumor cells and induce immunogenic cell death that triggers an immune response against tumor antigens[304]. Conversely, IT may increase tumor cell sensitivity to CT and RT[305,306].

The combination of IT and novel MTDs against tumor targets also have a potential to act in synergy. In addition to biological modification and toxin delivery, antibodies against tumor targets can contribute to tumor cell elimination by antibody-dependent cell-mediated cytotoxicity (ADCC)[307]. Such effect can be enhanced by simultaneous IT that recruit and activate innate immune cells. Both antibodies and small molecule inhibitors may also effectively shut down immunosuppressive signaling pathways downstream of their molecular target, or up-regulate components required for antigen presentation[308]. Finally, MTDs augment an anti-tumor response by direct reduction of tumor burden. Also, anti-angiogenic drugs may act in synergy with ITs by *e.g.* facilitating immune effector trafficking following vaccination[309].

Immunotherapy for treatment of pediatric brain tumors

Immune cell infiltration has been associated with improved outcome in adult GBM patients[250], and numerous ITs have been evaluated for treatment of this patient group. So far, the immunological responses in individual patients have been encouraging, but the overall clinical efficacy has been moderate[310]. Children and adolescents could theoretically respond better to IT than older patients do, due to their enhanced ability to generate a CTL response against new antigens[222-225]. On the other hand, the risk or nature of side effects cannot readily be extrapolated from adult studies, and safety concerns largely dictate the introduction of new immunotherapeutic strategies in children.

The prerequisites for immune intervention differ between pediatric brain tumor types (reviewed by Griesinger *et al*[311]). A subset of tumors (in particular LGGs and EPNs) evokes an M1/CTL response and thus provides a permissive microenvironment for CTLs generated by vaccinations during early treatment.

Adoptive T cell transfer may also be an alternative for these patients, given the increased number of T cells available for initial isolation. MBs on the other hand display poor T cell infiltration and a strongly immunosuppressive environment. CT and RT may provide a boost for antigen presentation of antigens from non-resected tumor cells, but will also limit subsequent T cell proliferation. Patients with MB could therefore potentially benefit from vaccinations in the recovery phase following conventional treatment, although adjuvant treatments targeting immunosuppression is strongly implicated.

The majority of clinical IT trials involving children with brain tumors have been conducted in patients with HGGs, typically at relapse following the failure of standard treatment. HGGs are intuitive targets for IT, since the immune system has the theoretical ability to eliminate single tumor cells within normal tissues – cells that would be very difficult to target with conventional therapies. Exploratory/ phase I studies have evaluated DC vaccines primed with tumor cell lysate[312-314] or RNA[315], peptide vaccines[316] and adoptive T cell transfer primed with autologous tumor cells[317], for the treatment of primary and recurrent HGGs, and a few cases of recurrent EPNs and MBs. Importantly, these treatment strategies were considered safe and well tolerated. Clinical and immunological responses were indicated, but the number of enrolled patients was too small to draw certain conclusions about treatment efficacy. Interestingly, children and adolescents tend to respond better than adults to DC vaccination[314,318]. Several ongoing or recruiting pediatric clinical trials will further evaluate the effect of DC or peptide vaccines in combinations with the PRR agonists imiquimod (toll-like receptor (TLR)7/8 agonist) and Poly-ICLC (TLR3 agonist).

In addition to cell-based ITs, a number of immune-modifying antibodies are currently under preclinical development for treatment of pediatric brain tumors; one example is an inhibitor of the NIReg CD47[319]. After the clinical implementation of PD-1 and CTLA-4 inhibitors for peripheral cancers, both drug types are under clinical evaluation for treatment of brain tumors. PD-1 antibodies alone or in combination with ipilimumab have shown therapeutic efficacy and induction of a long-term memory in preclinical studies of CNS tumors[320,321], and will shortly be evaluated for treatment of adult GBM in phase III trials. In addition, children with recurrent brain stem glioma are treated with pembrolizumab in a recently initiated phase I trial.

Molecular subgroup affiliations will likely influence the future design of preclinical and clinical IT studies. This could be particularly relevant for EPN patients, where it is clear that different subgroups of tumors have different immunological profiles. The improved outcome of patients carrying low-inflammatory PF-B tumors suggests that patients with immunosuppressed PF-A tumors could benefit from ITs modifying their pro-inflammatory micro-environment by *e.g.* inhibition of the IL-6 receptor or downstream targets of IL-6/STAT3 signaling such as VEGF and COX-2[245].

Summary of the field

If this thesis were written five years ago, it would have been a very different story. Over the past decade, tremendous progress has been made in the molecular understanding of pediatric brain tumors, resulting in a reorganization of classifications schemes and identification of novel potential biomarkers and therapeutic targets. While this progress has changed the focus of the research society, clinical diagnostics, risk stratification and treatment indication is still largely based on traditional pathology.

It is increasingly clear that pediatric brain tumors comprise a diverse group of tumors with substantial heterogeneity within each tumor type. Around 75% of the patients are cured with current treatment strategies, however cure rates vary between 10 and 90% depending on diagnosis and risk factors. The current treatment of children with brain tumors is fairly uniform and is clearly insufficient for some patients, whereas others may be over-treated with current therapies at the cost of long-term adverse effects.

The development and implementation of more efficient and specific treatment strategies require clinically relevant experimental models; however, current experimental models are few and poorly representative of the wide variety of tumor types and molecular variants that exist. Less than 60 pediatric brain tumor cell lines have been presented and most of them have been cultured for decades in serum-containing medium, with the risk of considerable genetic drift. Moreover, there is a lack of mouse models representing the most aggressive tumor subsets such as pediatric HGG and Group 3 MB, for which novel treatment strategies are urgently needed.

ITs have a theoretical potential to cure brain tumors with less side effects than conventional treatment and also induce a long-term immunological memory that could prevent tumor recurrence. The development of such therapies is however dampened by the poor knowledge of the immunological pathways involved in distinct pediatric brain tumor subsets. Immunological findings, as well as results from IT trials, cannot readily be extrapolated from adult brain tumor patients because of differences inherent to both tumors and immune systems.

The overall aim of this thesis was to establish and characterize novel patient-derived *in vitro*- and *in vivo*-models of pediatric brain tumors, to be used in future development of novel treatment strategies. Models were phenotyped alongside patient samples with an emphasis on immune-related pathways, and the prerequisites for immune intervention and systemic immune monitoring of children with brain tumors were investigated.

Aims of the thesis

The specific aims of the individual studies were:

Paper I. To evaluate the novel concept of serum-free monolayer culturing for the establishment and propagation of primary pediatric brain tumor cell cultures.

Paper II. To establish a novel mouse model of high-risk Group 3 MB, by orthotopic transplantation of patient-derived tumor cells.

Paper III. To perform a detailed characterization of CD24 in pediatric brain tumors.

Papers I-III. To describe the features of the newly established models, including expression of tumor markers, CSC markers, components of the COX-2/mPGES-1/PGE2 pathway and cytokine signatures.

Paper IV. To evaluate the therapeutic efficacy of combining COX-2 inhibition with a GM-CSF based whole cell vaccine in an immunocompetent brain tumor model.

Paper V. To characterize the systemic cytokine profiles of children with brain tumors.

Results and discussion

Establishment and immunophenotyping of patient-derived experimental models

A standardized method for culturing pediatric brain tumors (Paper I)

In vitro models of pediatric brain tumors are scarce[112], and there are no comprehensive cell culturing protocols that are specifically developed for these tumor types. The aim of this study was to increase the number of relevant *in vitro* models both at our laboratory and others, by defining a culturing protocol that is (i) based on the concept of serum-free monolayer culturing, as has previously been demonstrated for adult GBM[106]; (ii) suitable for establishment of multiple pediatric brain tumor types, since diagnosis is usually not known at the time of surgical resection and subsequent primary cell culture establishment; (iii) simple, both in terms of practical performance and components of the medium, to facilitate reproducibility; and (iv) cost-efficient, to further increase the utility of the protocol.

Our early attempts to establish and propagate primary pediatric brain tumors included cell culture mediums developed for NSC cultures (unpublished data); however, such mediums either had a poorly defined composition, or required the addition of a large number of external components to the medium base. In our current protocol we use a simple, defined serum-free medium (Ultra Culture™) that has been shown to preserve brain tumor antigens for IT studies[322]. We initially supplemented the medium with the growth factors EGF and FGF, which are required for sustaining the growth of NSCs[323], but as FGF may also antagonize SHH signaling and inhibit proliferation of SHH-activated tumors[324] we have over time excluded FGF from the medium altogether, and several cell cultures in Paper I have been successfully expanded with EGF only. It should however be noted that we have retrospectively found that only one of our cell cultures represents a SHH MB, and this tumor could be propagated also in the presence of FGF (abstract Darabi *et al*[325]).

The extracellular matrix molecule laminin promotes survival and proliferation of NSCs *in vitro*[326] and is the routinely used substrate for propagation of adherent NSC and GBM cultures[106,108]. In the current study, we have propagated

Table 4. Protocol for culturing primary pediatric brain tumor cells[100].

Establishment of monolayer cell cultures
1. Transfer fresh tissue into a petridish containing TrypLE™ Express
2. Cut tissue into small pieces using a sterile scalpel-blade
3. Incubate at 37° for 15 min
4. Mechanically dissociate the enzymatically treated tissue in UC using a 5 ml serological pipet
5. Mince cell solution through a cell strainer (75 µm) using a 2 ml syringe plunger
6. Rinse the cell strainer with cell culture medium
7. Centrifugate cell solution and discard supernatant
8. Count cells
9. Seed in 6-well plates (Ultra-Low™) at a density of approximately 400.000 cells/well
10. Upon sphere formation (typically 1-5 days), transfer spheres to Cell-Bind™ plates
Propagation of monolayer cells
1. Maintain cultures at 37°, 21% O_2, 6% CO_2
2. Feed with fresh medium every 3-4 days, until cells reach high confluence
3. Detach cells by incubating with TrypLE™ Express at 37° for 5-15 min
4. Re-suspend cells in 2/3 fresh UC and 1/3 conditioned medium
Cryopreservation
1. Pellet cells and re-suspend in freezing medium
2. Freeze vials in Mr.Frosty™ containing isopropanol for 24h
3. Transfer vials to liquid nitrogen
4. For thawing, hold vial in running warm water until pellet disappears
5. Transfer solution to 2 ml cold UC and keep on ice for 5 min
6. Add 2 ml UC and keep on ice for 2 min
7. Add another 2 ml UC and keep on ice for 2 min
8. Add 5 ml UC
9. Centrifugate cell solution and discard supernatant
10. Seed cells in fresh UC
UC medium
UltraCULTURE™ cell culturing medium
2 mM L-glutamine
1% Penicillin-Streptamycin
EGF (20 ng/ml)
FGF (0-40 ng/ml)

cell cultures on plates and flasks with adhesive plastic (Cell-Bind™), which is significantly cheaper than biological coating. During culturing, we have been able to transfer cells between laminin-coated plates and adhesive plastic plates without obvious changes in cell proliferation, suggesting that the two surfaces are equally efficient for propagating brain tumor cells (preliminary data).

The culture protocol described in Paper I is summarized in Table 4. Briefly, monolayer establishment comprises mechanic and enzymatic dissociation of tumor tissue, sphere formation to exclude cell debris and most stromal cells, transfer of spheres to Cell-Bind™ culture flasks or plates, surface attachment, and subsequent expansion of monolayers in culture medium detailed above. With this procedure, we have to date established >15 monolayer cell cultures, representing MB, ATRT, EPN and HGG. Importantly, the MB monolayer cohort comprises four Group 4 MBs; only one verified cell line exists for this subgroup[125,134] and the current protocol could facilitate the generation of additional ones. The primary goal of our cell culturing project has been to obtain short-term cultures for immunoassays and generation of whole cell vaccines, and passage 3 (for spheres) and 5 (for monolayers) is used to define successful establishment of a cell culture. However, AA-LU-74, AEP-LU-149, AEP-LU-158, MB-LU-69, MB-LU-70, MB-LU-140, MB-LU-159 (Paper I) and MB-LU-338 (unpublished data) have been propagated for >20 passages *in vitro* and could thus be regarded as cell lines.

In parallel to monolayers, we have attempted to generate traditional sphere cultures from the same primary tumors. The success rate for cell culture establishment was higher for monolayer cultures (78% vs 65% for sphere cultures), but the two methods appear to complement each other, since a small subset of tumors can only be propagated as monolayers or spheres respectively. Monolayers were specifically suitable for tumor subsets that can be regarded as less aggressive (some EPNs, AA and beta-catenin-activated MB), while some Group 3 MBs generate sphere but not monolayer cultures (Paper I and unpublished data).

Following the establishment of cell cultures, we characterized the expression of a panel of lineage and putative CSC markers in cultures alongside primary tumor tissue. The prevalence and frequency of positive cells were similar in monolayer and sphere cultures for most markers (the exceptions being CD15 and CD24, further discussed in Papers I and III), in line with results obtained in laminin-based cultures of GBM[110]. Although, both spheres and monolayers were enriched for CD44 and nestin when compared to the primary tumors, suggesting a either selection of immature cells or up-regulation by *in vitro* conditions.

The concept of serum-free brain tumor cell culturing was initially introduced in order to maintain the population of CSCs *in vitro*. The prevalence of CSCs has been demonstrated by proliferation assays, single cell cloning, multi-lineage differentiation, tumor-initiating capacity and expression of CSC-associated markers[96-99]. In our experience, both spheres and monolayers exhibit long-term

proliferative capacity *in vitro*, however neither spheres nor monolayer cells are clonogenic when seeded as single cells but require high confluence for growth. Still, both sphere (Paper II) and monolayer (unpublished data, Table 5) cultures harbor cells tumor-initiating capacity. In addition, both spheres and monolayers display heterogeneity within cultures in terms of putative CSC (CD29, CD133) and mature lineage markers (nf-200, β-III-tubulin), suggesting that neither method can be used to sort out a strictly defined CSC subset from primary brain tumors.

With the intention of using the newly established cell cultures for future immunoassays, we conducted an analysis of secreted inflammatory factors in monolayer cultures. Serum-containing medium can interfere with cytokine detection because of its high protein content. Exclusion of serum shortly before analysis facilitates detection of low-abundant proteins, but may also induce stress-related changes in the secretory profile of cells that are acclimatized to growing in serum[327,328]. Such interference is avoided in serum-free cell cultures. Still, all *in vitro* cultures lack external stimuli provided by surrounding cells in the tumor tissue, and their secretory profile is likely only partly representative of the secretome *in vivo*.

Without added stimuli, all of our analyzed MB and EPN cultures secreted IL-6, IL-8, IL-15 and VEGFA. IL-8, IL-15 and VEGFA were also detected in two tissue lysates from matched primary tumors (one MB and one EPN), suggesting that these factors are indeed produced by tumor cells *in vivo* - although stromal cells could also contribute to the production. The IL-6 secretion *in vitro* should be interpreted with caution, since significant amounts of IL-6 were only detected in one of the corresponding primary tissues. *In vitro*-induced IL-6 signaling is a common phenomenon in cell cultures and is tentatively associated with cellular stress[328]. In future studies, we intend to study if CT agents, immune-modulating drugs and the presence of polarized immune cells affect cytokine profiles of monolayers. Preliminary data suggests that this is indeed the case.

All primary tumor tissues and derived cell cultures displayed positive immunofluorescent staining for COX-2, suggesting that this is a general therapeutic target for pediatric brain tumor patients. Production of PGE2 was confirmed in monolayer cultures and could be reduced upon treatment with the COX-2 inhibitor valdecoxib. Interestingly, one HGG culture secreted lower levels of PGE2 than EPN and MB cultures, and was also negative for other cytokines except VEGFA. This HGG culture is the only one in our cohort that is derived from the tumor of a newborn child, which could be an explanation for its immunologically silent behavior.

In summary, we show that the concept of serum-free monolayer culturing is feasible for pediatric brain tumors. We present a standardized, reproducible and cost-efficient protocol, with the potential to increase the availability of pediatric *in vitro* models for preclinical drug evaluation.

A xenograft model of Group 3 medulloblastoma (Paper II)

Animal models have significant advantages over cell lines, by enabling studies of tumor progression in a physiological environment. The biological complexity of Group 3 and Group 4 MBs has limited the generation of GEMMs, and there are few cell lines of these subgroups available for transplantation[125]. In this study, we have established an orthotopic PDX model representing a high-risk *MYC*-driven Group 3 MB (MB-LU-181), intended for future drug evaluation. Briefly, we inoculated low-passage tumor spheres (20.000 cells) into the cerebellum of NOD-*scid* mice and serially transplanted xenografts for three generations. Second generation xenografts have been characterized with H&E staining, immuno-fluorescent labeling and cytokine analysis (Figure 5).

We have attempted to establish PDX models of all four MB subgroups (unpublished data, Table 5), with MB-LU-181 being the most successful, likely due to its highly proliferative features. First generation PDXs of MB-LU-181 were generated in 2/2 mice with a latency of 17-18 weeks. In comparison, a Group 4 MB did not generate xenografts in any mice (n=4), and two mice inoculated with SHH MB developed tumors (n=4) with longer latency than the Group 3 MB. In these three tumors, we used low-passage cells (spheres) for inoculation. The beta-catenin-activated MB-LU-159 was poorly proliferative as spheres, but generated a robust monolayer culture (Paper I). Passage6 cells were used for transplantation; 1/4 inoculated mice in the first generation developed tumors, and analyses of second generation tumor-bearing mice are ongoing. PDX establishment is predictive of poor outcome in glioma patients[111]; this may be true for our small cohort as well, since patient MB-LU-181 died from disease <220 days after diagnosis, while the other three patients are currently free from disease (3-7 years after surgery).

MB-LU-181 xenografts mimicked the phenotype of the primary tumor, including MB histology, expression of *MYC*, lineage markers (nestin$^+$, nf-200$^+$, GFAP$^-$) and putative CSC markers (CD24$^+$, CD133$^+$, CD15$^-$, CD44$^-$). The human cytokine profile of xenografts was the same as for cultured spheres (VEGFA$^+$, IL-8$^+$, IL-16$^+$) but differed significantly from the primary MB-LU-181 tumor, where a range of other cytokines was detected in addition. Although the cells of origin of tissue cytokines were not consistently investigated, it is likely that factors such as IL-1, IL-12/23 and TNF-α originated in tumor-infiltrating immune cells rather than tumor cells. This reasoning is supported by preliminary data. VEGFA was on the other hand robustly expressed by tumor cells in both primary tissue and xenograft. Interestingly, we noticed that the tissue levels of VEGFA in MB-LU-181 were up to 4x higher than in other MBs (shown in Paper I and II). This observation was validated in a dataset of >400 MBs, where expression levels of *VEGFA* was significantly higher in Group 3 than in other subgroups.

Table 5. Establishing PDX models of MB subgroups.

Patient ID	Subgroup	Cells		Tumor	Latency*	Passages**
MB-LU-159	WNT?	p6 monolayer	20.000	1/2	22	2
			200.000	0/2	-	-
MB-LU-187	SHH	p<3 spheres	20.000	1/2	25	
			200.000	1/2	13	
MB-LU-181	Group 3	p<3 spheres	20.000	2/2	17-18	3
MB-LU-69	Group 4	p<3 spheres	20.000	0/2	-	-
			200.000	0/2	-	-

*Latency in weeks. **Number of passages *in vivo* as of to date. Serial passaging has not been attempted for MB-LU-187.

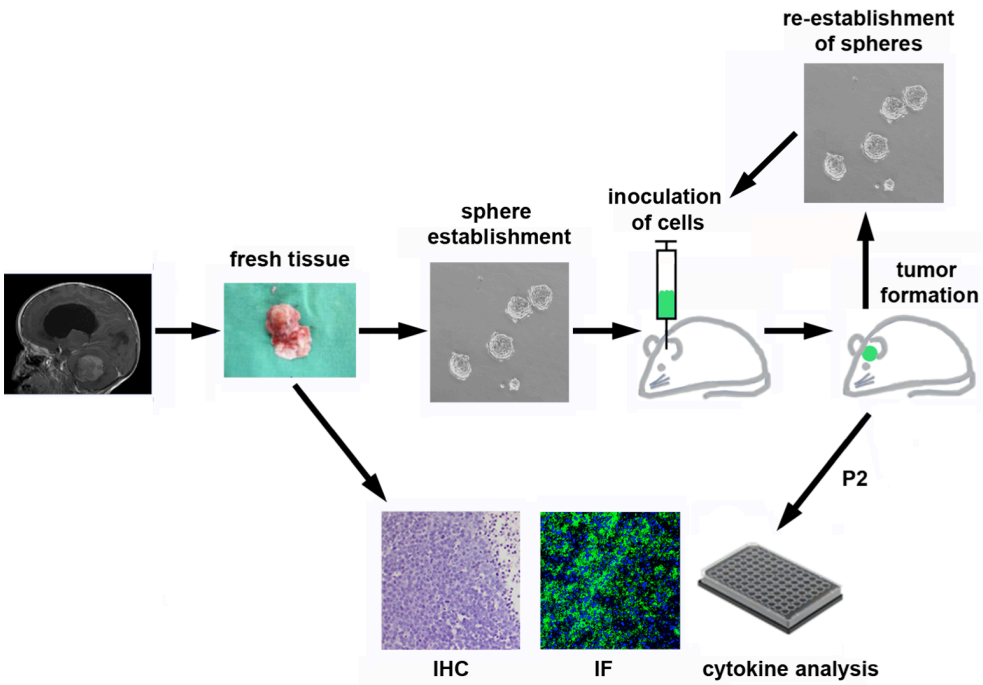

Figure 5. Experimental setup for the establishment and characterization of the MB-LU-181 model. Spheres were established from MB-LU-181 tissue. 20.000 cells were orthotopically inoculated into 2 NOD-*scid* mice. Upon tumor formation, spheres were re-established och serially inoculated. Second generation xeonografts were analyzed with H&E (IHC), immunofluorescent labeling (IF) and cytokine arrays. Representative MRI image of a medullo-blastoma is shown, licensed under the Creative Commons Attribution-Share Alike 3.0 Unported License.

In line with the result obtained in Paper I, both MB-LU-181 tissue and spheres were positive for COX-2. In a large data set we confirmed COX-2 (*PTGS2*) expression in tumors within all MB subgroups – although again, the expression was higher in Group 3 MBs than in other subgroups. A detailed characterization showed that COX-2 was found in both tumor cells and stromal cells (immune cells and vessels) in the primary tumor, and in stromal cells only in xenografts. To investigate the prerequisites for PGE2 production, we evaluated the expression of mPGES-1, which is the downstream enzyme of COX-2 in PGE2 synthesis. Both primary tissue and xenografts displayed mPGES-1 positivity in tumor cells. The distinct cellular origin of COX-2 and mPGES-1 in xenografts does not exclude (nor necessitate) PGE2 production *in vivo*, as the intermetabolite PGH2 can be transferred between cell types[329]. We have recently initiated studies to confirm PGE2 production in xenografts, and it therefore remains to be determined in which context the MB-LU-181 model is suitable for studies of COX-2.

We further characterized the mouse-derived components of MB xenografts and detected intratumoral myeloid cells, but no NK cells. Mouse IL-6 and TNF-α was up-regulated upon xenograft establishment, suggestive of macrophage activation. By IF staining, myeloid cells were relatively few and displayed a suppressed phenotype ($COX\text{-}2^+$, $CD206^+$). In comparison, we have found that transplantation of the slightly immunogenic mouse glioma cell line GL261 into NOD-*scid* induces a stronger macrophage response, with enhanced intratumoral macrophage infiltration and relatively lower frequency of $CD206^+$ cells (preliminary data). This suggests that macrophages in NOD-*scid* mice are indeed capable of responding to stimuli, and that the weak response in MB-LU-181 is related to either species-specificity or a poor immunogenicity of MB-LU-181 cells. Future studies will elucidate whether the macrophage population in MB-LU-181 can be increased and functionally improved by therapeutic intervention. Such a treatment strategy could potentially benefit MB patients.

Group 3 MBs represent a major therapeutic challenge due to their proneness for metastasis, but also because they predominantly present in children <3 yrs old – a patient group for which RT should be avoided due to potentially devastating side effects. Alternative treatment options are few, and there are no MTDs on the horizon for this patient group, as is the case for *e.g.* SHH MBs. PDX models such as MB-LU-181 will be important for the development of such drugs, as monotherapies or in combination with other therapies.

In this study, we specifically highlight VEGFA and COX-2 as two potential therapeutic targets for patients with Group 3 MBs. Both molecules have previously been implicated in MB pathogenesis[236,237], but we are the first to demonstrate that therapeutic intervention may be particularly beneficial for a specific subgroup. So far, VEGFA inhibitors have had little overall impact on the survival of children with brain tumors[270], but new combinatory treatment approaches have shown encouraging results[280]. Historically, clinical trials have not

considered molecular MB subgroups, as this concept is relatively recently introduced and not clinically implemented. We advise that subgroup affiliation is routinely included in future clinical trials, also if the therapeutic target is not known to be directly linked to a specific subgroup.

The clinical potential for COX-2 inhibition is further discussed in Paper IV, where the effect of COX-2 inhibition *in vivo* is evaluated. Interestingly, it was recently shown that VEGFA and COX-2 can act as independent regulators of angiogenesis[330]. COX-2 may therefore be partly responsible for sustained angiogenesis following VEGF-targeting therapies, and combined VEGFA and COX-2 inhibition as a treatment strategy could be of particular interest for children with Group 3 MB.

In summary, we present the establishment and characterization of a novel orthothopic mouse model of a *MYC*-driven Group 3 MB, and suggest VEGFA and COX-2 as suitable therapeutic targets for this patient group.

CD24 immunostaining for rapid identification of medulloblastoma (Paper III)

Of the lineage and CSC markers evaluated in papers I and II, we found it feasible to conduct a more thorough investigation of CD24 in pediatric brain tumors, as this protein have not only been linked to neurogenesis but also to neuroinflammation[72-74,214] and GBM invasiveness[78,80,81]. In addition, it has been attributed prognostic value in numerous peripheral cancer forms[76,77,331].

In an initial screen of >800 brain tumors, we found that CD24 is overexpressed in pediatric MB, EPN and HGG. Gene expression in EPN and HGG was similar to that of adult GBM, while expression in MBs of all subgroups was higher than in all other tumor types. However, there was no prognostic value of CD24 expression within the MB cohort. In a confirmatory IF study of ~40 tumors, we found increased stained area fraction in MB compared to other tumor types. These results corroborate a previous study reporting CD24 as a MB antigen[82] as well as previous observations in a small IHC cohort, where CD24 staining was more prevalent in MB than in other tumor types[83].

Detailed IF characterization revealed that CD24 is expressed by most tumor cells in MB tissues, but only observed in a small subset of the tumor cells in EPN and GBM, typically cells expressing nestin. CD24 expression thus coincides with an immature neuronal phenotype, as expected by its expression in neuronal precursors during CNS development[67]. The expression of CD24 was however not restricted to tumor cells, but was also observed in immune cells and on astrocytic and neuronal protrusions in both tumors and epileptic brain tissue. We hypothesize that the latter pattern reflects an inflammatory response, as the same protrusion-like staining has also previously been demonstrated in the CNS following

traumatic brain injury[73]. Still, it is possible that CD24 also has immune-related functions in tumor cells, in addition to its other reported functions.

Although CD24 expression was by no means restricted to MB, its immunostaining pattern in MB cells (*i.e.* membranous and cytoplasmic granules) was unique, and therefore had diagnostic value. In our tumor cohort (biobank samples collected between 2007 and 2014, published and unpublished data), we have been able to use CD24 immunostaining alone to distinguish MBs from other tumor types. In one case (unpublished data), the lack of granular CD24 staining pinpointed a tumor that had been clinically diagnosed as an MB based on traditional IHC markers; however, retrospective molecular analysis revealed that the tumor was indeed not an MB, suggesting that CD24 is a more useful MB marker than *e.g.* synaptophysin.

The value of a novel diagnostic IHC marker for MB can be debated, as IHC diagnostics is increasingly being complemented with molecular analyses. Still, a rapid and accurate MB diagnosis is crucial to dictate the nature of additionally required analyses, and can shorten the time to initiation of adjuvant treatment following surgery. In our hands, the immunostaining pattern of CD24 is evident by both IF labeling of cryosections and IHC labeling of FFPE tissue, when using the antibody clones ML-5 and ALB9. In contrast, the historically used CD24-Sn3b clone showed inconsistent staining patterns across and within tumor types, in line with a more recent report stating that this antibody is not CD24-specific[332].

It is widely appreciated that CD24 has different functions in different cell types and tissues due to its numerous glycoforms and ability to bind a large variety of ligands[333,334]. It can therefore be expected that CD24 is associated with more than one specified function in brain tumors. We evaluated our newly established experimental models and confirmed granular CD24-staining in MB-derived spheres and MB cells in the MB-LU-181 model. These models could thus be useful for future functional studies. Interestingly, CD24 was not detected by IF staining of monolayer cultures, but was expressed in secondary spheres generated from monolayers (preliminary data). Whether the discrepancy is due to altered gene expression, or glycosylation changes (preventing the antibody from binding to its epitope), is not clear at this point.

Although the function of CD24 in MB is not clear at this point, its expression in virtually all MB cells makes it interesting as a therapeutic target. Preclinical tumor studies have demonstrated how *in vivo* delivery of CD24-antibodies can antagonize CD24-associated functions such as proliferation[335], facilitate ADCC of tumor cells[336] and enhance the delivery of CT agents to the tumor[337]. As our study shows that CD24 is prevalent also in normal cell types in the brain, a potential targeted therapy must be carefully designed, for instance by the identification of MB-specific glycoforms.

In summary, we show that CD24 has diagnostic, but not prognostic, value for patients with MB, and requires further evaluation as a therapeutic target.

Modulating and monitoring the immune response against pediatric brain tumors

COX-2 inhibition potentiates a cell vaccine against glioma (Paper IV)

Children with HGG have an immensely poor prognosis[42], and treatment options are scarce. IT is an intuitive therapeutic approach for these patients, given the theoretical ability of immune cells to target infiltrating tumor cells while sparing normal brain tissue. Small-scale studies have indicated that children with HGG respond better to active IT than adults do[314] and immunological response has been reported in a subset of patients following adoptive transfer of tumor-primed DCs[312,314,316]. The immunosuppressive milieu of pediatric HGGs however provides a barrier for immune effectors[229], and simultaneous targeting of suppressive pathways could tentatively enhance therapeutic response of immune activation.

In this study, we have evaluated the treatment efficacy of a GM-CSF-based whole tumor cell-vaccine (GL-GM) in combination with COX-2 inhibition in the experimental HGG mouse model GL261. There are very few transplantable mouse models available that explicitly mimic pediatric HGGs[112], and GL261 is the most commonly used glioma model since it mimics many of the biological features of human GBMs[155]. Treatment of mice was initiated on day7 following tumor inoculation; s.c. immunizations with irradiated GL-GM cells were administered three times with two weeks interval, and COX-2 inhibitors were delivered with 28-days micro-osmotic pumps implanted on day7 (Figure 6).

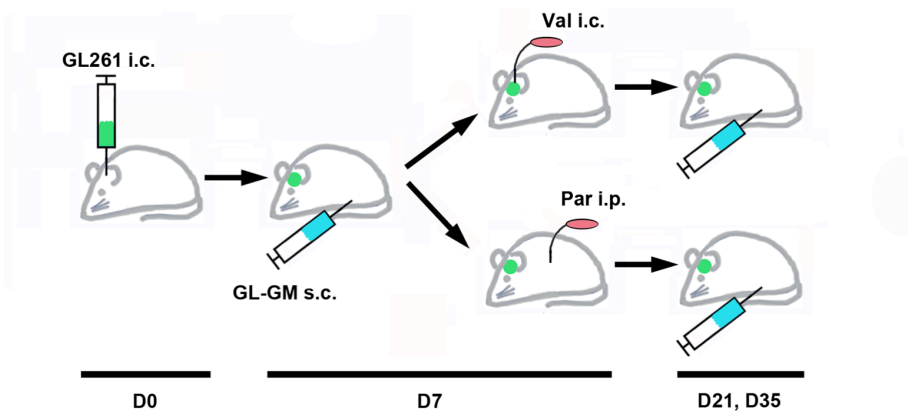

Figure 6. Experimental setup for combined COX-2 inhbibition and a GM-CSF-based whole tumor cell vaccine in experimental glioma.
On day0, 5.000 GL261 were inoculated intracerebrally (i.c.). On day7, 21 and 35, mice received subcutanous (s.c.) vaccinations comprising $2x10^6$ irradiated GL261 cells transduced to produce GM-CSF (GL-GM). Micro-osmotic 28-days pumps containing valdoxib (Val) or parecoxib (Par) were implanted i.c. (s.c. pump with ic catheter) or intraperitoneally (i.p.) in conjunction with the immunization on day7.

The rationale for choosing a whole cell vaccine for immune activation is the possibility to evoke a tumor-specific CTL, while avoiding side effects associated with general immune activators. In addition, a whole cell vaccine maximizes the amount of tumor antigens available for immune priming, reducing the risk of acquired resistance. Finally, whole cell vaccines are easier to produce in a clinical setting compared to more technical procedures as *in vitro* expansion and modification of patient-derived DCs/T cells.

We have previously obtained limited therapeutic effect using non-transduced whole cell vaccines in murine glioma models[338,339]. GM-CSF is a common preclinical and clinical vaccine adjuvant[340], used to enhance the recruitment and maturation of monocytes and DCs at the immunization site and its draining lymph nodes, and subsequently enhance phagocytosis, antigen presentation and T cell priming[187]. By combining the whole cell GL261 vaccine with GM-CSF, we have reported ~50% cure in the GL261 model when first immunization was administered on day1 (as opposed to day7 in the current setting) following tumor inoculation[339]. Our experimental vaccine constitutes a syngeneic tumor cell line transduced to produce GM-CSF (referred to as GL-GM); however, to further simplify a clinical protocol, it would be possible to use recombinant GM-CSF in conjunction with the vaccination[341], or a bystander GM-CSF-producing cell line[342].

PGE2 is temporarily important for antigen presentation by promoting the migration of DCs; however, it also has strong systemic and intratumoral immunosuppressive functions such as inhibition of systemic T cell proliferation, down-regulation of a Th1 response while promoting a Th2 response, and induction and recruitment of suppressive immune cells[300,301,343]. We therefore hypothesized that COX-2/PGE2 inhibition could further enhance treatment efficacy of GL-GM vaccinations. In line with this reasoning, we have previously reported an IFN-γ-skewed immune response and enhanced survival when combining COX-2 inhibition with a non-transduced whole cell vaccine for the treatment of rat glioma[302]. Indeed, we detected an enhanced survival rate in the current study when GL-GM was combined with systemic delivery of the selective COX-2 inhibitor parecoxib (5 mg/kg/day); cure rate for the combinatory treatment was 69%, vs 10-14% for monotherapies.

Systemic toxicity has limited the clinical use of selective COX-2 inhibitors. We therefore next investigated the feasibility of local drug delivery. Since parecoxib requires liver metabolism for exerting its pharmacological effect, we used its metabolite valdecoxib for intra-tumoral delivery. With a dose of 5.3 μg/kg/day (approximately 10x lower than the expected CSF levels following the parecoxib delivery)[344], we obtained approximately the same treatment efficacy in combination with GL-GM (63% vs 13-14% for monotherapies). This suggests that local COX-2 block is more important than peripheral, and that systemic side effects may be avoided while maintaining clinical efficacy if the COX-2 inhibitor is administered locally. Furthermore, both local and systemic COX-2 block

potentiated the generation of a T cell response, indicated by increased numbers of proliferating $CD4^+$ and $CD8^+$ cells 6 days following initiation of treatment. We also saw a trend towards increased Th1 commitment (determined by T-bet positivity) of systemic T cells in long-term survivors following combination therapy.

Interestingly, we found that GL261 cells did not express COX-2 *in vivo*, neither did they produce PGE2 *in vitro* – in sharp contrast to the results obtained in human cell cultures in paper I. However, the potent synergistic effect of local COX-2 inhibition and vaccination suggests that PGE2 is indeed produced at the tumor site. We did detect expression of COX-2 in tumor-infiltrating macrophages as well as expression of the downstream enzyme mPGES-1 in tumor cells *in vivo*, suggesting that PGE2 production occurs through intercellular metabolite transfer, as previously demonstrated by others[329]. Our results in the GL261 model strengthens the theory that PGE2 is produced in xenografts in paper II, where the same expression pattern was seen; *i.e.* $COX\text{-}2^+$ stromal cells and $mPGES\text{-}1^+$ tumor cells.

The most therapeutically challenging HGGs in children are non-operable brainstem gliomas. An autologous whole cell vaccine would not be possible to produce for these patients. In the current study, we noticed limited efficacy of COX-2 inhibition as a monotherapy (13-14% survival), suggesting that modification of the immune response is the most important mechanism by COX-2 in the GL261 model. Still, COX-2 inhibition may exert other tumor-antagonistic functions in patients, alone or in combination with other therapies. For instance, we have recently shown that celecoxib restores sensitivity to TMZ in MGMT-unmethylated glioma cells by down-regulation of PGE2-mediated WNT-signaling[306].

The expression of COX-2 in multiple tumor types (as demonstrated in Paper I and II) suggest that patients with brain tumors other than HGG could benefit from the treatment strategy outlined in this study. This could be particularly true for patients with Group 3 MBs (Paper II). While COX-2 inhibition in the context of tumor-promoting functions that are not related to a T cell response can tentatively be evaluated in the MB-LU-181 model (Paper II), we also intend to establish a transplantable immuno-competent Group 3 MB model for future IT studies.

In summary, we show that COX-2 inhibition augments the therapeutic effect of a GM-CSF-based whole tumor cells vaccine by enhancing the systemic T cell response. This treatment strategy could be feasible for children with operable brain tumors.

Systemic immune profiles of children with brain tumors (Paper V)

There is a general lack of soluble biomarkers for children with brain tumors[63]. The benefits of using blood-derived biomarkers for brain tumor patients are easily appreciated: the inaccessible location of brain tumors can present a challenge for obtaining preoperative biopsies, and current imaging techniques are suboptimal for accurate monitoring of tumor progression or regression[345]. The distinct intra-tumoral immune signatures of different pediatric brain tumor types and subgroups suggest that immunological parameters could be useful as diagnostic or prognostic markers. Moreover, systemic immune profiles could indicate suitable candidates for specific ITs, and be used to predict or monitor subsequent treatment response. As a first step towards such implementation, we have characterized the pre-operative systemic immune profiles of children with brain tumors.

In this study, we used a multiplex protein assay to evaluate the levels of plasma cytokines in 50 children, including 45 children with brain tumors (MB, EPN, HGG, PA and other LGG) and 5 healthy controls. We initially searched objectively for cytokine associations within the cohort. Quantitative correlation analyses are not optimal for small sample cohorts; instead, we categorically classified all values as positive or negative and evaluated the level of agreement between each cytokine pair (number of double-positive and –negative samples out of total number of samples). Associations were further tested with Cohen´s κ, which takes into account agreement by chance. Among a panel of 20 cytokines, we identified four factors that separated 45/50 patients into two groups, defined as $VEGFA^{high}/IL\text{-}7^{high}/IL\text{-}17A^{low}/TNF\text{-}\beta^{low}$ (referred to as Group A in the paper) and $VEGFA^{low}/IL\text{-}7^{low}/IL\text{-}17A^{high}/TNF\text{-}\beta^{high}$ (referred to as Group B in the paper). Most patients with MB, GBM and healthy controls were found within Group A, whereas other tumor types were equally distributed between the two groups.

The biological interpretation of this subdivision is not clear at this point, although the distinct pattern suggests that these two groups represent either sequential or parallel steps in the immunoediting process. VEGFA and IL-7 are both associated with the late stages of a normal infection, that is, regeneration of leukocytes and restructuring of tissues. Such a stage would not favor immune surveillance, and is consistent with reports of established immunosuppression in GBM and MB[229,232,236,237]. In contrast, we speculate that systemic IL-17 and TNF-β represents cytotoxic activation. In line with this reasoning, IL-17 has previously been associated with improved outcome in GBM patients[346].

In the paper, we present the hypothesis that high IL-7 levels reflect lymphopenia, as previously demonstrated by others[347]. This could however not be confirmed in the current study due to lack of clinical data. White blood cell (WBC) counts were available from 14/50 samples only, and specific lymphocyte counts were not available from any of the samples. WBC counts did not differ significantly between groups (median 8.9 million cells/ml for Group A; 12.7

million cells/ml for Group B; p=0.2593 (unpublished data, Figure 7)), but the trend towards decreased WBC numbers in Group A encourages further studies in a larger data set. Interestingly, T cells appear to become unresponsive to IL-7 during prolonged inflammation, resulting in impaired survival and homeostatic expansion of the T cell population, despite high systemic levels of IL-7[348,349].

Unrelated to A/B affiliation, we then compared the levels of cytokines between healthy controls and tumor patients. Overall, we found trends towards increased IL-10 and decreased IL-12/23 and TNF-α in several tumor types. Similar patterns have previously been reported in the plasma of GBM patients[250-252]. In a case study, we found that these alterations were reversed following removal of tumor tissue – however this pattern could not be confirmed in other patients due to lack of sequentially sampled blood. Notably, the modifications of Th1/Th2 cytokines rarely reached statistical significance, likely due to the low number of included samples. Our quantitative analyses may thus underestimate biological differences; specifically, the small number of samples from patients with LGG and HGG is likely not enough to detect potential biological differences associated with these 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. This response appeared to be completely individual, as no common feature (tumor type, WHO grade, age, gender, tumor location or preoperative steroid treatment) could be identified. Interestingly, three of the four patients fell outside of the A/B subdivision. Again, it is not clear if the Th1-skewed activation in this small group of patients is sequential or parallel to the A and B cytokine profiles.

Finally, we performed a direct comparison between blood samples in EDTA- and heparin-tubes respectively. We found that cytokine values differed significantly in absolute numbers, although qualitative differences and relative trends were maintained regardless of anti-coagulant – with the exception of IL-1α, which could be detected only following blood sampling in EDTA-tubes.

In this study, we included three plasma samples that are matched to *in vitro* cultures (MB-LU-69, MB-LU-70 and MB-LU-140) presented in Paper I. All three monolayer cultures secreted VEGFA, IL-6, IL-8 and IL-15. In one case (MB-LU-140) we also had access to primary tissue for cytokine analysis; the tissue was positive for VEGFA, IL-8 and IL-15, but also for IL-1α, IL-16 and TNF-α that were not found in the *in vitro* culture and interpreted as stromal-derived. The three corresponding plasma samples were more or less identical to each other – and to the primary tissue from MB-LU-140: all were positive for VEGFA, IL-8, IL-15, IL-16 and TNF-α (IL-1α not determined). IL-7 (3/3), IL-10 (2/3) and IL-12/23 (3/3) were found in the plasma samples but not in the corresponding *in vitro* cultures (only IL-7 was found in the MB-LU-140 tissue at levels close to the lower limit of quantification of the assay), suggesting that these factors have a predominantly systemic origin.

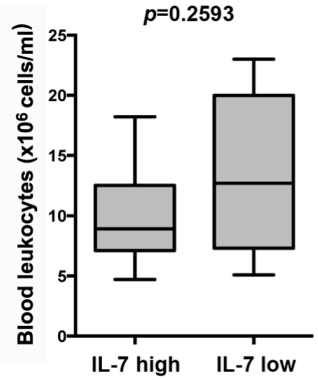

Figure 7. Systemic IL-7 and blood cell count in children with brain tumors. Total blood leukocyte count does not differ significantly between patients with high (*n*=7) and low (*n*=7) systemic levels of IL-7.

Implications for diagnostics. Despite the distinct intratumoral immune profiles reported for PA, GBM, MB and EPN respectively, we found that systemic immune profiles were not associated with a distinct tumor type. Even though cytokine profiles in plasma from MB and GBM patients were fairly homogeneous, their profile was also found in subsets of patients with low-grade tumors and the diagnostic value is therefore clearly limited. Notably, our MB cohort includes SHH, Group 3 and Group 4 MBs, and all but one (a SHH-activated MB) displayed similar cytokine profiles. Both EPNs and PAs were equally distributed between groups A and B, and is it tempting to suggest that such sub-division is related to molecular subgroups. However, this was not the case for PAs: the majority of PAs in both A and B were infratentorial. The EPNs in our cohort have not been subclassified, but the distinct immune profiles found among patients with i.t. EPNs are possibly related to EPN-PF-A and EPN-PF-B affiliation.

Implications for immune monitoring. Our study shows that systemic immune monitoring of children with brain tumors is feasible. In a case study, we showed that the systemic profile fluctuates with tumor burden. If this finding can be generally applicable to all patients remains to be determined; however, these results are very promising and suggest that the systemic immune profile can be used to follow tumor regression and tentatively relapse. Our study also demonstrates the importance of individual immune monitoring, since tumor type does not strictly predict the cytokine profile of a single patient.

Implications for IT. The diverse cytokine profiles suggest that optimal IT strategies differ between patients, and that systemically delivered ITs may be counteracted or boosted depending on the prevalent systemic cytokine milieu. The small group of patients with an enhanced Th1 profile may be suitable candidates for active ITs. In line with this notion, a previous study described a child with GBM displaying high systemic levels of IFN-γ, IL-6 and TNF-α, who responded well to subsequent DC IT[313]. The current study also demonstrates that systemic immunosuppression is reversed following tumor removal, providing a window for

IT delivery. Optimal timing with regards to other conventional treatment however remains to be elucidated in detail.

Implications for biobanking. Our study highlights key issues to be considered for future biobanking efforts, at ours and other institutions. We suggest that (i) blood is sampled with at least two different anti-coagulants in parallel, to ensure optimal detection of factors that may be retrospectively defined, (ii) blood is sequentially sampled during treatment following surgery, *e.g.* in proximity to routine blood sampling during CT, and (iii) WBC count and differential analysis of immune cell populations is routinely performed in blood collected for biobanks.

In summary, we show that systemic immune monitoring of children with brain tumors is feasible, and we identify patient groups with distinct preoperative cytokine profiles. Our results have implications for the future development and implementation of IT in children with brain tumors.

Summary of results

In this thesis, I describe the establishment and characterization of novel *in vitro* and *in vivo* models of pediatric brain tumors. I initially demonstrate the feasibility of serum-free monolayer culturing for the generation of patient-derived cell cultures, and describe the establishment of an orthotopic xenograft model of high-risk Group 3 MB by cerebellar transplantation of low-passage tumor cells into NOD-*scid* mice. Besides generating these models per se, I provide a detailed methodological description that is intended to facilitate the future generation of additional experimental models by others.

Tumor markers, cytokine signatures and components of the COX-2/mPGES-1/PGE2 pathway were generally preserved following propagation of tumor cells *in vitro* and *in vivo*. The neuroinflammatory and neurogenesis-regulatory protein CD24 was identified as a clinically and experimentally useful immunomarker for MB of all subgroups, but additional detailed studies will be needed to determine the prerequisites for targeted treatment. *VEGFA* and *PTGS2* (COX-2) were overexpressed in Group 3 MB compared to other subgroups; the effect of COX-2 inhibition *in vivo* was further evaluated in an immunocompetent HGG model, where simultaneous administration of COX-2 inhibitors and a GM-CSF based whole cell vaccine cured >60% of tumor-bearing mice. Finally, I demonstrate distinct systemic cytokine profiles in children with brain tumors, which could have important implications for the development and clinical implementation of ITs.

In brief, I present novel experimental models that recapitulate the phenotype of pediatric brain tumors and will serve as tools for future studies of tumor biology and preclinical drug evaluation. I also implicate a role for immune intervention and monitoring in the treatment of children with brain tumors.

Conclusions and future perspectives

Altogether, primary pediatric brain tumor tissues were used to establish 14 monolayer cell cultures and one orthotopic Group 3 MB model, intended for studies of tumor biology and preclinical drug evaluation.

The conclusions of the individual studies were:

Paper I. Serum-free monolayer culturing is suitable for *in vitro* propagation of most pediatric brain tumor subsets, and preserves the immunophenotype of the primary tumor.

Paper II. Serial orthotopic transplantations of low-passage tumor cells generate MB xenografts that replicate the phenotype of the primary tumor.

Papers I-II. Experimental models express clinically relevant immune-related factors, including components of the COX-2/mPGES-1/PGE2 pathway.

Paper II. *VEGFA* and *PTGS2* (COX-2) are differentially expressed in MB subgroups, and the highest expression is found in Group 3 MB.

Paper III. CD24 is a clinically and experimentally useful immunomarker for rapid identification of MB cells, but requires further detailed evaluation as a therapeutic target.

Paper IV. COX-2 inhibition enhances the therapeutic efficacy of a whole cell vaccine against brain tumors.

Paper V. Systemic immune monitoring of children with brain tumors is feasible, and identifies patient groups with distinct preoperative cytokine profiles.

In this thesis, I have established novel experimental models of pediatric brain tumors. Here, I present protein characterization of the models, but further studies will be (or are currently being) conducted in order to determine the molecular fidelity of the models. Also, the tumor-initiating capacity of monolayers will be confirmed for additional tumors.

Our long-term goal is to implement IT for treatment of pediatric brain tumors, primarily MB and HGG. Based on preclinical data, our general IT strategy comprises whole cell vaccination in combination with local cytokine treatment and simultaneous COX-2 inhibition[139,302,338,339,350,351]. GM-CSF-based IT, as presented in this thesis, is complicated to translate into a clinical therapy, as recombinant GM-CSF is currently not approved for clinical use within the EU. Rather, we intend to administer IFN-γ at the immunization site, as this setting has also been successful in our preclinical models[338,350].

Local COX-2 inhibition shows promising preclinical efficacy in our hands, but systemic administration will likely be the primary clinical choice due to lack of clinically available drugs or drug formulas that are optimal for local delivery. By further strengthening our preclinical data, for instance by testing the concept of local COX-2 inhibition in additional tumor models (both HGG and MB), we can justify the development or re-introduction of alternative COX-2 inhibitors that are suitable for local delivery in humans.

As a first step, the treatment outlined above will be used in compliance with current treatment. It will therefore be crucial to determine the optimal timing for administration of the different treatments. We have recently shown that the effect of a whole cell vaccine can be boosted by intermittent TMZ treatment[149], and we will next add COX-2 inhibition to this preclinical protocol in order to determine if and how these therapies synergize with each other.

For clinical implementation of IT in children with brain tumors, we intend to further characterize the local immunome of different tumor types and search for biomarkers that can indicate suitable patients or additional treatment strategies. The *in vitro* models described in this thesis are expected to contribute to such understanding, by enabling immune profiling of distinct tumor subsets following standard treatment (CT and RT) and immune-modulating drugs.

Finally, systemic biomarkers will be needed to predict and follow treatment response and to monitor potential side effects in treated patients. As I show in this thesis that systemic immune monitoring is feasible, we will next expand our analyses to include sequential monitoring during standard treatments. In this context, our results highlight a number of factors that will be considered for improvement of biobank sampling in the future.

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"Do. Or do not. There is no try."

Master Yoda

The Empire Strikes Back