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Novel methods of malignant brain tumor treatment utilizing the tumor microenvironment

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Novel methods of malignant brain tumor treatment utilizing the tumor microenvironment

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DOCTORAL DISSERTATION

Doctoral dissertation for the degree of Doctor of Philosophy (PhD) at the Faculty of Medicine at Lund University to be publicly defended on the 15th of June at 13:00 in the Conference Room, Department of Neurosurgery, Skåne University Hospital, Entrégatan 7, 222 42 Lund, Sweden

Faculty opponent

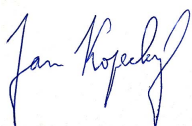
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Abstract <p>Malignant brain tumors constitute a disaster in the lives of patients, either in the form of extremely low survival in glioblastoma, or the serious long-term adverse effects of therapy in medulloblastoma. These two tumor types represent the most common malignant brain entities in adults and children, respectively. Ever since the early 2000's, no major improvement of patient outcomes occurred. Immunotherapy, which recorded revolutionary successes in several tumor types, has so far failed in brain tumors. This disappointing phenomenon is the result of intrinsic characteristics of glioblastoma and medulloblastoma and their microenvironment. Therefore, other treatment modalities that exploit the distinct attributes of malignant brain tumors are urgently needed.</p> <p>In this thesis, I describe the features of both tumor types and the development of their therapy until today. Moreover, general features of tumor microenvironment are contrasted to the unique aspects of the brain tumor counterpart. Next, I outline the underlying mechanisms of conventional immunotherapy and recount the natural features of both tumors that prevent its effective deployment. Finally, I suggest alternative approaches that circumvent the challenges encountered so far, such as avoiding the blood brain barrier via local treatment administration or focusing on macrophages as the principal agent of immunotherapy instead of T cells. Antisecretory factor (AF), a new agent in cancer treatment, as well as modulation of CD24/Siglec10 "don't eat me" signalling are examples of the latter.</p> <p>Following is a summary of the four projects. In publication I, the concept of intratumoral temozolomide treatment is investigated from the perspective of tumor immune microenvironment. Publication II describes the effects of AF16 on macrophages and glioblastoma cells. Publication III is a pilot clinical trial of an AF preparation in patients with newly diagnosed glioblastoma. Manuscript IV examines the modulation of CD24 and Siglec10 to reduce antiphagocytic signalling in glioblastoma and medulloblastoma.</p> <p>The impact of this research is represented by the first published immunological effects of local delivery of temozolomide through convection-enhanced delivery in murine glioblastoma; first investigation of AF16, macrophages and tumor cells; first Salovum human cancer trial; laying the groundwork for CD24-Siglec10 signaling modulation in human glioblastoma and medulloblastoma.</p>		
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2022

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MADE IN SWEDEN 

To Petra and Eva

“I want to be a healer, and love all things that grow and are not barren.”

Éowyn, The Lord of the Rings

“Sometimes in life there are events that you need to be a little foolish to handle.”

François de La Rochefoucauld

“Difficult things in the world must have their beginnings in the easy; big things in the world must have their beginnings in the small. Make something big by starting with it when small.”

Lao Tzu

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Abstract

Malignant brain tumors constitute a disaster in the lives of patients, either in the form of extremely low survival in glioblastoma, or the serious long-term adverse effects of therapy in medulloblastoma. These two tumor types represent the most common malignant brain entities in adults and children, respectively. Ever since the early 2000's, no major improvement of patient outcomes occurred. Immunotherapy, which recorded revolutionary successes in several tumor types, has so far failed in brain tumors. This disappointing phenomenon is the result of intrinsic characteristics of glioblastoma and medulloblastoma and their microenvironment. Therefore, other treatment modalities that exploit the distinct attributes of malignant brain tumors are urgently needed.

In this thesis, I describe the features of both tumor types and the development of their therapy until today. Moreover, general features of tumor microenvironment are contrasted to the unique aspects of the brain tumor counterpart. Next, I outline the underlying mechanisms of conventional immunotherapy and recount the natural features of both tumors that prevent its effective deployment. Finally, I suggest alternative approaches that circumvent the challenges encountered so far, such as avoiding the blood brain barrier via local treatment administration or focusing on macrophages as the principal agent of immunotherapy instead of T cells. Antisecretory factor (AF), a new agent in cancer treatment, as well as modulation of CD24/Siglec10 “don't eat me” signaling are examples of the latter.

Following is a summary of the four projects. In publication I, the concept of intratumoral temozolomide treatment is investigated from the perspective of tumor immune microenvironment. Publication II describes the effects of AF16 on macrophages and glioblastoma cells. Publication III is a pilot clinical trial of an AF preparation in patients with newly diagnosed glioblastoma. Manuscript IV examines the modulation of CD24 and Siglec10 to reduce antiphagocytic signaling in glioblastoma and medulloblastoma.

The impact of this research is represented by the first published immunological effects of local delivery of temozolomide through convection-enhanced delivery in murine glioblastoma; first investigation of AF16, macrophages and tumor cells; first Salovum human cancer trial; laying the groundwork for CD24-Siglec10 signaling modulation in human glioblastoma and medulloblastoma.

Populärvetenskaplig sammanfattning

Glioblastom hos vuxna och medulloblastom hos barn är två typer av elakartade hjärntumörer vilka orsakar många dödsfall samt ger långsiktiga besvärliga biverkningar framförallt hos barn. Behandling av båda tumörtyperna har inte ändrats på många år, och består av hjärnkirurgi, kemoterapi och strålning.

Immunterapi är en relativt ny behandling som utnyttjar kroppens egna immunceller till att bekämpa tumören med. Terapin har lyckats bota andra elakartade tumörer, tex malignt melanom eller njurcancer. Tyvärr har inte immunterapi varit lika framgångsrikt mot hjärntumörer. Det beror på att de biologiska mekanismer som behövs för att immunterapin ska fungera inte finns i lika stor utsträckning hos glioblastom eller medulloblastom.

I min avhandling beskriver jag de olika egenskaper hos hjärntumörer som gör att immunterapi inte fungerar. Samtidigt presenterar jag alternativa sätt att effektivt behandla hjärntumörer med – lokal kemoterapibehandling, som ges direkt in i tumören ensamt, eller i kombination med ett immunaktivt ämne. Vi ser att dessa terapier påverkar andra immuncellstyper dvs makrofager, än konventionell immunoterapi.

De studier som vi har utfört och som presenteras här representerar inledande steg där antisekretorisk faktor används som makrofag-fokuserad terapi mot glioblastom. Dessutom undersökte vi hur modulering av ”ät mig inte” molekylen CD24 och Siglec10, som tillsammans förhindrar tumörcellsuppätning av makrofager, kan bidra till behandlingseffekt.

Sammanfattningsvis konkluderar jag att det finns en stor efterfrågan av nya behandlingsmetoder mot elakartade hjärntumörer. Därför föreslår jag lokal behandling där läkemedel ges direkt till tumören, användning av antisekretorisk faktor samt hämning av ”ät mig inte” signaler som nya behandlingsalternativ av elakartade hjärntumörer.

Original publications

This thesis is compiled from the following publications:

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

Publication II. Intratumoral administration of the antisecretory peptide AF16 cures murine gliomas and modulates macrophage functions.
Jan Kopecky, Julio Enríquez Pérez, Håkan Eriksson, Edward Visse, Peter Siesjö and Anna Darabi.
Scientific Reports, 2022 Mar 17; 12(1):1-11.

Publication III. Antisecretory factor is safe to use as add-on treatment for newly diagnosed glioblastoma.
Erik Ehinger, **Jan Kopecky**, Anna Darabi, Edward Visse, Charlotte Edvardsson, Gregor Tomasevic, David Cederberg, Mattias Belting, Johan Bengzon and Peter Siesjö
submitted to *Neurooncology*, 2022

Publication IV. Mitoxantrone-induced immunomodulation of CD24 - implications for targeted treatment of malignant brain tumors.
Jan Kopecky, Julio Enríquez Pérez, Stevanus Jonathan, Edward Visse, Valeria Governa, Hugo Talbot, Mattias Belting, Peter Siesjö and Anna Darabi
Manuscript

Related publications

This publication is not included in the compilation thesis:

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

Julio Enríquez Pérez, Sara Fritzell, **Jan Kopecky**, Edward Visse, Anna Darabi, Peter Siesjö

Scientific Reports, 2019 Apr 4, 9(1):5632-10.

Abbreviations

ACD	accidental cell death
AF	antiseecretory factor
APC	antigen-presenting cell
ARDS	acute/adult respiratory distress syndrome
ATP	adenosine triphosphate
BBB	blood-brain barrier
BCG	Bacillus Calmette-Guérin
BTB	blood-tumor barrier
CAF	cancer-associated fibroblast
CAR	chimeric antigen receptor
CCL	CC chemokine ligand (followed by a number)
CD	cluster of differentiation (followed by a number)
CED	convection-enhanced delivery
CNS	central nervous system
CRP	C-reactive protein
CSF	cerebrospinal fluid
CSF	colony-stimulating factor (followed by a number)
CTLA	cytotoxic T-lymphocyte-associated protein
DAMP	damage-associated molecular pattern
DC	dendritic cell
DNA	deoxyribonucleic acid
dsDNA	double-stranded deoxyribonucleic acid
ECM	extracellular matrix
EGFR	epidermal growth factor receptor
ESR	erythrocyte sedimentation rate
EV	extracellular vesicle
FDA	Food and Drug Administration
GBM	glioblastoma
GTR	gross total resection
HCC	hepatocellular carcinoma
HIF	hypoxia-inducible factor
HMGB1	high mobility group box protein 1
HSP	heat-shock protein
HSV	herpes simplex virus
IBD	inflammatory bowel disease

IBS	irritable bowel syndrome
ICD	immunogenic cell death
ICI	immune checkpoint inhibition/inhibitor
ICP	intracranial pressure
IDH	isocitrate dehydrogenase
IFN	interferon
IFP	interstitial fluid pressure
IIH	idiopathic intracranial hypertension
LMD	leptomeningeal dissemination
MAPK	mitogen-activated protein kinase
MB	medulloblastoma
MD	Ménière disease
MDSC	myeloid-derived suppressor cell
MGMT	O ⁶ -methylguanine-DNA methyltransferase
MHC	major histocompatibility complex
MTX	mitoxantrone
NK	natural killer
NKCC1	Na-K-Cl cotransporter 1
NPH	normal-pressure hydrocephalus
OC	ovarian cancer
OS	overall survival
PAMP	pathogen-associated molecular pattern
PD	programmed death
PFS	progression-free survival
pHGG	pediatric high-grade glioma
PSMD	proteasome 26S subunit ubiquitin receptor
RCD	regulated cell death
RCT	randomized controlled trial
RNA	ribonucleic acid
SHH	<i>sonic hedgehog</i> pathway-activated
Siglec	sialic acid-binding immunoglobulin-like lectin
siRNA	short interfering ribonucleic acid
SBS	short-bowel syndrome
SIRP	signal regulatory protein
SPC	specially processed cereal
TAA	tumor-associated antigen
TAM	tumor-associated macrophage
TBI	traumatic brain injury
TLR	toll-like receptor
TME	tumor microenvironment
TMZ	temozolomide
TNBC	triple-negative breast cancer
Treg	T regulatory cell

VEGF vascular endothelial growth factor
WNT “wingless-related integration site” pathway-activated

Introduction

Malignant primary brain tumors

Solid tumors generally are divided into benign and malignant entities. Benign tumors retain characteristics of well-differentiated cells, grow inside a clearly demarcated capsule, and do not form distant metastases, among other attributes. Cells of malignant tumors lost most features of differentiation, grow invasively into the surrounding tissue and form distant metastases. Central nervous system (CNS) neoplasms possess specific features given the highly specialized and isolated nature of the brain and spinal cord. For instance, exact location in the brain (i.e. an organ where specific areas are exclusively responsible for functions at distant body locations) can be equally important to a patient's prognosis as the tumor grade of malignancy.

Furthermore, CNS tumors are divided based on the organ of origin into primary – those that originate from the neuronal, glial or auxiliary tissue as part of the CNS, and secondary – tumors that began outside of the brain or spinal cord and disseminated there as part of their hematogenous spread. The main tumor types that are investigated further in this thesis are glioblastoma (GBM), in the past also called glioblastoma multiforme, and medulloblastoma (MB). Despite originating from different cells of origin and belonging to unrelated taxonomies, these diseases also possess some similarities, as described below.

GBM is the only Grade 4 glioma and the most common primary malignant brain tumor in adults. Since the incidence of brain tumors is higher in the adult than the pediatric populations, it is also the most common malignant brain tumor overall. The incidence in the US is 3.23 cases per 100,000 with median survival of 8 months after diagnosis and 5-year overall survival (OS) 6.8 % (ranging between 2-10% based on patients characteristics) [1]. Given its invasiveness and location in the brain, GBM is one of the deadliest tumor types overall.

The most important classification of malignant brain tumors at present is based on molecular biology and genetic changes. In this way, four subtypes of GBM were originally identified and later refined into three – proneural (also encompassing the original neural), classical and mesenchymal. The impact of these subtypes on patient characteristics and survival is small, though [2]. Aside from these, other genetic variations have been described, mainly the *IDH* gene and *MGMT*-promoter variants,

which bear a significant impact on treatment outcomes. IDH is a key enzyme in cell metabolism and its mutations cause accumulation of by-products and decreased energy production in cancer cells. MGMT neutralizes toxic agents, e.g. chemotherapy and when its promoter is silenced through methylation, glioblastoma cells become more sensitive to TMZ [3, 4].

GBM only rarely occurs in children, where it is classified under the umbrella category “pediatric high-grade glioma” (pHGG). This tumor entity is driven by different molecular mechanisms than in adults, which is reflected in the lower response rates to the adult treatment protocols [5, 6]. According to reports, pHGG carries a slightly better prognosis than in adults but the OS is still dismal [7]. Besides tumor-intrinsic factors, the higher functional reserve of the younger individuals as well as closer medical oversight in this population leading to earlier diagnosis may contribute to the longer OS.

GBM grows almost exclusively supratentorially, in the majority of cases in the temporal, parietal or frontal lobes. This gives rise to symptom syndromes resulting from both the increased intracranial pressure (ICP) and specific tumor location. In adults, peak incidence of glioblastoma occurs between the age 65 and 79, with age varying among reports [8], and the mortality increases with age, slowly in females and more exponentially in males (www.cancerresearchuk.org/health-professional/cancer-statistics). The higher incidence and lower survival in the elderly reflect the longer time to acquire oncogenic mutations, decreased intrinsic capacity to control the tumor growth and to withstand intense treatment protocols. However, due to the relative rarity of GBM and other malignant brain tumors compared to other cancer types in adults, brain tumors are not among the 5 most common cancers to cause death past the age of 50 [9].

The situation is different in the pediatric and young adult populations. Here, brain tumors are the leading cause of cancer-related death and a major source of mortality overall [10]. CNS tumors in general are also the most common solid tumor type that affects children, with MB being the most frequent pediatric malignant brain tumor [1]. Unlike GBM, which is of glial origin, MB is classified as embryonal, originating in subgroup-specific neuronal precursors in the cerebellum [11, 12]. Because of this, it grows infratentorially in the cerebellum itself or around the fourth ventricle in the posterior fossa, where it can cause obstructive hydrocephalus early in its course.

Molecular analysis revealed four basic subtypes of MB based on the main oncogenic drivers – WNT, SHH, Group 3 and Group 4, which all differ in the epidemiology, clinical course and prognosis [13, 14]. More recently, a subdivision was possible into 12 distinct subgroups – 2 in the WNT subtype, 4 in SHH and 3 in Group 3 and 4, each. These subgroups are referred to by Greek letters α - δ [15].

Unlike GBM, which forms distant metastases very rarely in cases where the tumor grows e.g., through the skull base and gains access to lymphatic and blood vessels of the head [16], MB can more readily form metastatic foci along the flow of CSF

and the pia mater, the so-called leptomeningeal dissemination (LMD). LMD eventually occurs in all untreated MB cases and is the cause of the majority of deaths, with metastases already present at diagnosis in about 40% of cases [17, 18]. Extracranial metastases of MB are rare [19].

Adults can also be affected by MB, although about 70% of cases are children, most commonly between 0-4 and 5-9 years old [1]. Overall survival has improved with the implementation of intensive treatment protocols and is now at around 72% of all cases, with the youngest infants having significantly worse prognosis, which is subgroup dependent [20].

Compared to GBM, which has been the subject of a substantial volume of research, among other reasons due to the historically well-established selection of preclinical models, MB is studied less, partially due to low availability of animal models that would faithfully mimic the characteristics of primary human tumors. It is possible to use cells derived from human tumor samples but to establish *in vivo* experiments with these cells, immunocompromised mice must be used, which precludes any studies of the tumor-immune interactions. There are options, such as syngeneic mouse models [21] or mice that contain genetically engineered human immune cells. These could more closely reflect the human tumor microenvironment (TME) but their practical usability remains low. Lastly, designing clinical trials with children and infants diagnosed with MB faces a multitude of obstacles, not the least of which are ethical.

Research on GBM, which arises from neural stem cells and adult glial precursors [22], on the other hand, benefits from a range of animal models that better resemble the primary human tumor. However, no preclinical animal model is perfect, and none possesses all characteristics of the original cancer – invasive growth, immune “coldness” or the exact features in the TME of the human counterpart.

In the projects presented in this thesis, we used the murine malignant glioma cell lines GL261, KR158 and the relatively new SB28, together with primary human cells. The syngeneic GL261 murine glioma cell line was created by exposing mice to carcinogenic substances and harvesting the resulting tumors. It has been widely used as an easily accessible model of GBM, despite not faithfully representing the primary tumor’s characteristics (low mutational load and “cold” immunogenicity). KR158 originated spontaneously in the brain of NF1+/Trp53+ mice as Grade III anaplastic astrocytoma. It is resistant to chemoradiotherapy and characterized by aggressive growth. SB28 is the newest of these three models, being created by transfecting murine neurons with NRAS, PDGF and Trp53 oncogenes. Therefore, it closely approximates the proneural GBM, driven by the PDGF pathway [23, 24].

As for MB, there are no relevant murine cell lines currently at our disposal. The GBM cell line SB28 shares some characteristics with primary human MB cells, namely the large amount and pattern of CD24 expression, therefore we used it in our pilot CD24 experiments besides primary human MB cells.

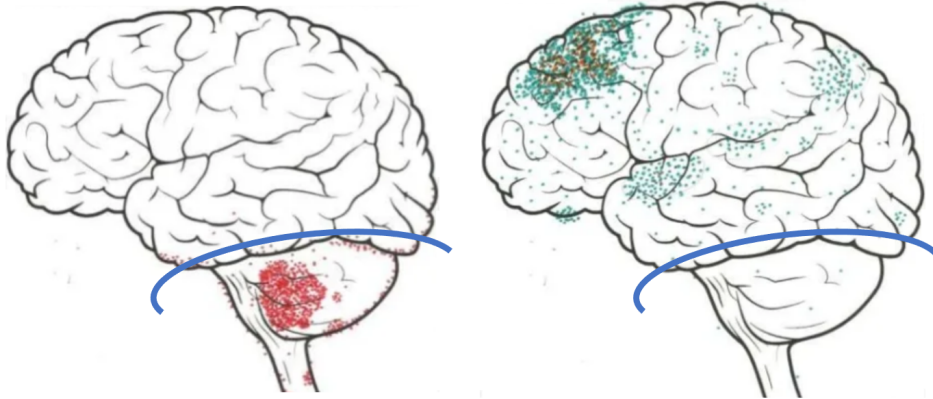


Figure 1. Typical location of malignant brain tumor growth

Medulloblastomas (red) are found in the posterior fossa underneath the tentorium cerebri (simplified, blue) around the 4th ventricle. Glioblastomas (green) occur in the cerebral hemispheres, in the frontal, parietal and temporal lobes. Adapted from Herbert Engelhardt, MD, PhD

(Next page) Table 1. Basic characteristics of MB and GBM

* available only to certain patient populations; ** subtype dependent; *** based on positive and negative prognostic factors

	Peak incidence age	Overall M:F ratio	Typical CNS location	Standard-of-care therapy*	Median OS	Metastasis tendency	Molecular classification
Medulloblastoma	0-9 years**	1.44 : 1**	Infratentorial; around the 4 th ventricle, in the cerebellum**	Age-dependent; radical surgery, followed by craniospinal RTx and multi-agent ChTx	± 72% at 5 years**	± 40% present with LMD**, mets to other loci are rare	4 subtypes – WNT, SHH, Group 3, Group 4; subdivided into 12 subgroups
Glioblastoma	65-79 years	1.6 : 1	Supratentorial; frontal, parietal, temporal lobes	Radical surgery, followed by concomitant TMZ and RTx	1-6% at 5 years ***	rare	3 subtypes – classical, proneural, mesenchymal; MGMT promoter methylation and IDH mutation variants

Tumor immune microenvironment

All cells and structures present inside and around a tumor are collectively called the tumor microenvironment (TME), which can be broadly divided into a cellular and acellular component (structural compounds of the extracellular matrix (ECM), soluble signaling molecules, blood and lymphatic vessels, etc.). It can further be classified as the tumor itself or “parenchyma” composed of cancer cells, and tumor-associated stroma, i.e. the wide array of supportive cells and ECM.

While knowledge of the existence of tumor stromal tissue and TME has remained since the early years of histology and pathology as a so-called “seed and soil theory” [25, 26], it was cancer cells that the treatment efforts mainly targeted until recently. Our understanding of the complicated communication between invading tumor cells and the host tissue microenvironment was limited until 2010’s. At this time, the role of TME was acknowledged as one of the hallmarks of cancer, resulting in tumor immune tolerance and support [27]. It became clear that tumor cells employ complex strategies to adapt to the hostile conditions while also changing the components of TME to support further tumor growth [28].

One of the early key needs of a rapidly growing tumor is sufficient blood supply bringing oxygen and nutrients. Therefore, starving cancer cells express hypoxia-inducible factors (HIFs) and vascular endothelial growth factor (VEGF) to cause neoangiogenesis, i.e. growth of new blood vessels, along the factor gradient into the tumor core. Such newly formed vessels, however, are immature, fragile, and prone to bleeding and degeneration, which sets a balance of intermediate-to-severe hypoxia in the tumor core with adequately supplied periphery [29-31]. Thus, in part due to the large hypoxic areas and rapid division, cancer cells switch their metabolism to anaerobic glycolysis, contributing to the generally acidotic environment [32, 33]. Several therapeutical attempts have been targeted at tumor neoangiogenesis with anti-VEGF antibodies recording marginal success at improving the prognosis of first-line treatment-resistant tumors [34].

Another large population in the TME are resident tissue supportive cells, i.e. fibroblasts in most cases of solid tumors. Under physiologic conditions, they maintain tissue integrity while providing essential signals to the organ parenchyma. A mounting volume of evidence shows that cancer-associated fibroblasts (CAFs) promote cancer growth by expressing pro-tumorigenic cytokines and signaling molecules such as growth factors, including VEGF, TGF β and others [35, 36]. CAFs also produce varying amounts of ECM, especially different types of collagen. In this way, CAFs contribute to ECM remodeling where it becomes a barrier to immune cell infiltration but also enables cancer cell escape and metastasis [37]. Activation of normal fibroblasts by tumor growth and their transformation into CAFs supports the approach to cancer as a “wound that never heals” [38].

Similarly, while an ordinary wound triggers inflammation as a normal process of healing, also cancer gives rise to pro-inflammatory signal in the resident tissue. With time and subsequent tumor growth and remodeling, the initial response becomes biphasic, with a protracted low-grade chronic inflammation ensuing [39]. At first, tissue resident innate immune cells, such as macrophages and dendritic cells (DCs) are engaged by the tumor. As the inflammatory signaling intensifies, populations of neutrophils, natural killer (NK) cells and peripheral blood-derived monocytes are recruited into the site. In time, adaptive immune cells like T cells migrate from local lymph nodes, primed by antigen-presenting cells (APCs) re-circulating from the tumor [40]. The evolutionary purpose of all these cell populations is to control the tumor growth and eliminate cancer, thus putting selection pressure on the surviving tumor cells.

Granulocytes (mainly neutrophils and eosinophils in the context of cancer) possess a relatively short lifespan, so that their tumor-infiltrating population fluctuates, unless they are constantly replenished from the circulation. Their role, however presently less well-described, appears to be in the beginning of cancer establishment [41]. NK cells, while potentially very effective at eliminating tumor cells, are suppressed by soluble factors and surface ligands expressed by cancer cells [42, 43]. With regards to cells of the adaptive arm of the immune system, the main population that has been extensively studied are T cells and their various subtypes. Early on, CD4⁺ helper T cells and CD8⁺ cytotoxic T cells, primed by tumor antigen-bearing APCs, infiltrate the tumor site [44]. Subsequently, though, cancer cells reduce the expression of neoantigens recognized by these T cells and upregulate surface ligands that suppress T cell functions and induce their apoptosis [45]. All the above-mentioned cell types, however, together constitute a minority of tumor-infiltrating leukocytes.

Ultimately, in the case of malignant tumors, cancer cells evade immune elimination and enter the escape phase, where physiologically abnormal immune cell types predominate in the TME. Myeloid-derived suppressor cells (MDSCs) and tumor-associated macrophages (TAMs) predominate in several tumor types [46, 47]. The exact origin of MDSCs cells is debated but they appear to be myeloid precursors arrested in their differentiation by circulating tumor products. Parts of the population remain in the bone marrow, while others populate lymph nodes and the tumor tissue where they contribute to suppression of effector immune cells, and tumor progression [46].

TAMs can, in principle, act as either tumor suppressors or tumor promoters through their cytokine secretion and phagocytosis [48]. Nevertheless, the majority of TAMs display features of the M2 macrophage differentiation with a distinct genetic signature, adapted to and sustaining chronic inflammatory conditions [49, 50]. Moreover, TAM number highly exceeds the other tumor-infiltrating immune cells and indeed even the tumor cells in some tumor types. On the grounds of well-described macrophage plasticity [51], the possibility of switching the M2-like

TAMs to a more proinflammatory phenotype warrants more investigation, including in the projects presented in this thesis.

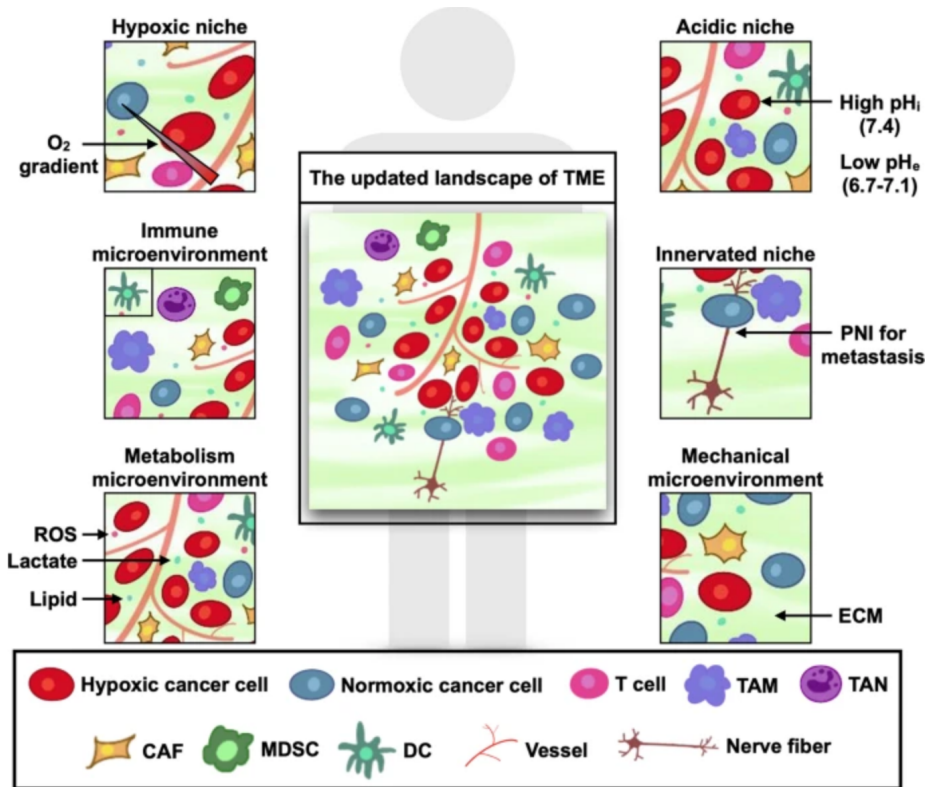


Figure 2. Overview of the various cell types present in the tumor microenvironment

According to current understanding, a single tumor contains several areas with distinct conditions. Hypoxic and acidic niches are characterized by low O₂ tension and low pH_e, respectively. Immune cell functions are inhibited under such circumstances. Other areas contain cells with high metabolic activity, turning glucose into lactate and producing ROS. The ECM is an important element of the TME, with its density facilitating tumor cell migration and metastasis. Neural axons and synapses participate in communication with tumor cells, providing tumor-supporting signals. Some cancer types also tend to metastasize along nerve fibers, a so-called perineural invasion. Adapted from Jin et al, Sig Transduct Target Ther, 2020

CAF = cancer-associated fibroblasts, MDSC = myeloid-derived suppressive cells, DC = dendritic cells, TAM = tumor-associated macrophages, TAN = tumor-associated neutrophils, PNI = perineural invasion, ECM = extracellular matrix, ROS = reactive oxygen species, TME = tumor microenvironment

Traditional brain tumor treatment

The first surgical attempts at therapy of brain tumors were done long ago in the history of medicine. In the early years, limited understanding of brain anatomy and physiology, as well as oncology, combined with insufficient neurosurgical practice led to almost universally dire patient outcomes. It wasn't until improvements in surgical technique and sterility were implemented, and especially the invention and widespread use of imaging techniques like CT and MRI that brain tumor surgery started being reasonably safe for patients. In this way, surgery in the form of maximal safe resection was the mainstay of therapy during the 20th century as the logical treatment of neoplastic growth [52, 53].

It soon became evident that surgery alone will not be enough to ensure satisfactory survival of malignant brain tumor patients. Radiotherapy has been available since the end of the 19th century, and it found its application in cancer treatment quickly after its discovery. Irradiation thus gradually became the second pillar of GBM treatment from the late 1950's, reaching mass-scale since the 1970s; however, the added benefit to overall survival was none and progression-free survival increased by single months [54-56]. The total dose, fractionation, and energy of rays were subsequently refined, albeit still with minimal added survival benefit [57-59]. Radiating the adult brain is relatively safe, as there are few areas undergoing rapid cell proliferation, which are the most sensitive to radiation damage. Nevertheless, there are adverse effects associated with whole-brain irradiation, such as seizures, coma, neurodegenerative changes and particularly, secondary tumor growth. Care must be also taken to avoid high doses to the spinal cord, an organ more prone to irreversible radiation damage.

Besides fast cell cycle, another factor that increases radiation sensitivity is high blood perfusion and O₂ saturation (due to high formation of free reactive oxygen species that attack the surrounding DNA molecules). An unforeseen advantage of radiotherapy is that irradiated tumor cells upregulate the expression of immunologically active surface molecules, such as MHC-I and FAS, leading to increased tumor cell killing by T cells [60-62]. Moreover, if the dose is high enough to kill tumor cells, certain radiation protocols trigger so-called immunogenic cell death, a type of regulated cell death that leads to adaptive immune system activation (see the specific chapter in this thesis).

Unfortunately, large areas of GBM are hypoperfused and hypoxic, which renders them less susceptible to radiation. There are limits to the cumulative dose that can be safely received by the normal brain and surrounding tissues, precluding the dose escalation until, ultimately, the GBM cells become functionally radioresistant.

First agents of chemotherapy were introduced already in the 1930's but it took some time to design substances that would readily cross the blood-brain barrier (BBB) to be even elementarily effective in brain tumors. Those used initially were intended

as radiosensitizers for hypoxic cells in the tumor that otherwise respond poorly to radiotherapy, and were therefore used neoadjuvantly, before the commencement of radiation [55]. The first agents that were used in the adjuvant sense as a distinct part of GBM treatment next to surgery and radiotherapy were nitrosurea derivatives – carbustine, lomustine and similar [63]. Despite trials investigating multi-drug combinations, e.g. with vincristine, nitrosurea-based agents were the mainstay of GBM therapy. Interestingly, no single Phase 3 RCT ever demonstrated a survival benefit of nitrosureas with radiotherapy over radiotherapy alone. It wasn't until the “small revolution of GBM treatment”, the implementation of temozolomide (TMZ) in the so-called Stupp protocol in 2004 [64] when TMZ became the standard 1st line agent in GBM treatment. It should be remarked that this significant milestone in GBM treatment reported the median OS prolongation by 2 months with TMZ addition to surgery and radiotherapy. Of note, nitrosurea derivatives are still used as second-line therapy today.

While nitrosurea derivatives and the other agents “pre-TMZ” added only several weeks to months to the OS and PFS of GBM patients, the synchronization of adjuvant TMZ and radiation showed promise in the initial trials [65, 66]. Even so, the original TMZ trial design was met with some controversies – as most clinical trials, the study population was highly selected and certain patient groups were excluded from the study. Therefore, real-life clinical outcomes suggest that even after the Stupp protocol became the accepted norm in GBM treatment, OS and progression-free survival (PFS) less than doubled since the period of 1950's-1970s where only basic surgery and radiation were available. The 5-year survival remains dismal due to rapid development of GBM cells' resistance to TMZ [67], among other factors.

It was revealed that patients who had developed MGMT-methylated GBM benefited the most from TMZ [68]. The added benefit was minimal for patients with wild-type GBM, especially when they are elderly [69, 70]. What is more, a notable percentage of patients do not receive the full treatment as defined by the Stupp protocol, either due to their overall condition being too fragile to withstand it or they cannot undergo gross total resection (GTR) of the tumor with subsequent chemoradiotherapy for other contraindications.

Since the first description of MB in 1925, the history of MB treatment followed principally a similar trajectory as that of GBM [71, 72]. The taxonomic confusion of the embryonal tumor category, however, makes interpretation of the studies somewhat unreliable. Radical surgery (lone biopsy is not recommended due to increased risk of metastasis) was largely used as monotherapy until the 1950's, where a publication demonstrated a truly large beneficial effect of radiotherapy on patient survival [73, 74]. MB tumors are more richly vascularized without the deeply hypoxic tumor core of GBM, which contributes to their high response to irradiation. Radiation protocols in MB must be adjusted, though, to address the propensity of MB to disseminate along the neuroaxis to the spinal cord and leptomeninges [18].

Consequently, the whole brain and spinal cord needs to be irradiated, often at the stage of continued CNS maturation, which leads to deep irreversible neurocognitive deficits in survivors. For this reason, radiotherapy is withheld for patients <3 years old.

Routine chemotherapy was added to the MB protocol in the 1980's and consist of a cocktail of lomustine, vincristine and cisplatin, where lomustine can be replaced by cyclophosphamide [75-77]. Patients that have optimal tumor characteristics and undergo full treatment reach the OS of about 70-80% (almost 100% in certain subtypes) [78]. Few, however, are free from long-term adverse effects. The long-term side effect most feared in children and adults is the development of malignant tumors secondary to radiation and chemotherapy exposure, including secondary GBM. Treatment-associated cognitive and neurological deficits are also commonly seen, with lifelong sequelae.

Besides the above-mentioned therapies for both GBM and MB, which are administered to patients with a curative intent, other drugs are given to alleviate symptoms of the disease. The most significant group among these are arguably corticosteroids, most commonly dexamethasone and betamethasone. They are used pre-, peri- and postoperatively to dampen cerebral edema, control ICP and decrease inflammation-mediated side effects due to their powerful anti-inflammatory action. It was proven, however, that by impeding T cell function and acute inflammation (represented by the increased levels of IFN γ , TNF α , IL12 and other proinflammatory cytokines) and promoting glioma stem cells in the TME, corticosteroids hamper the antitumoral immune response and worsen patients' prognosis [79-81].

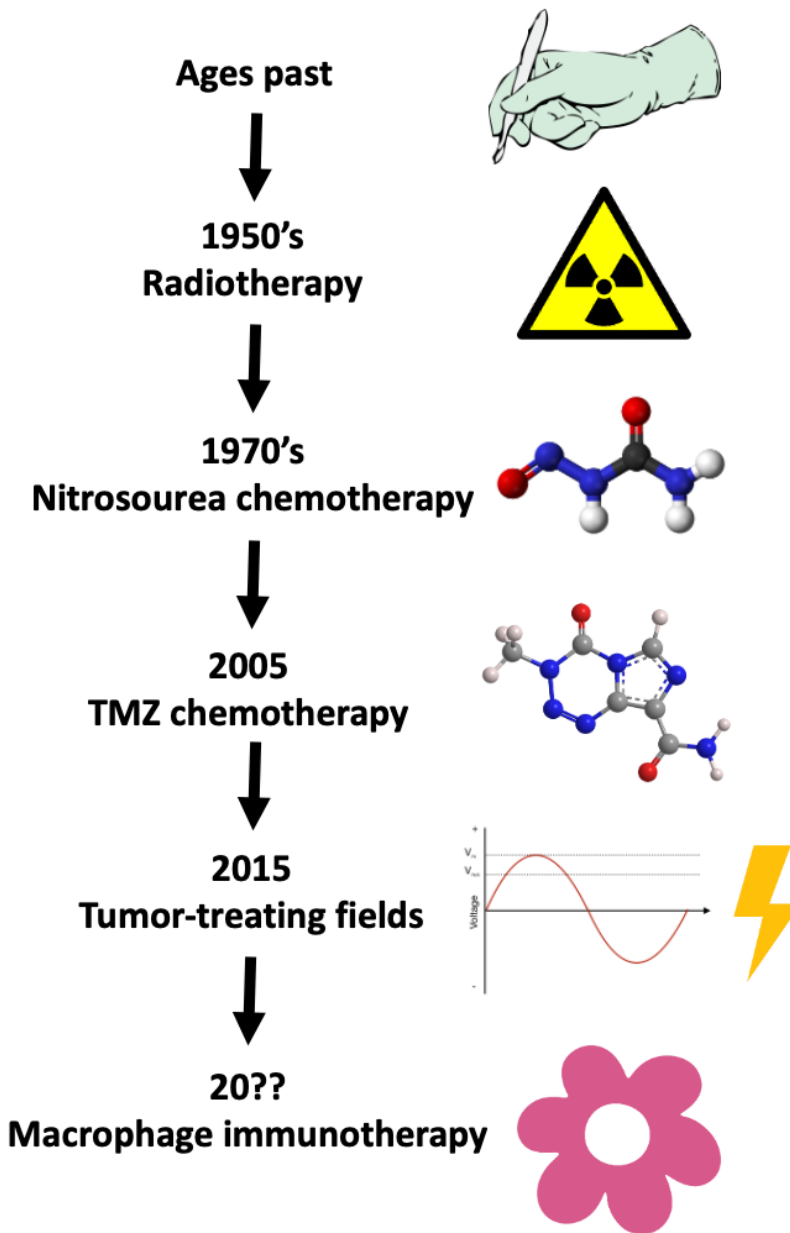


Figure 3. Graphical representation of the development of FDA-approved glioblastoma treatment

Attempts at surgical removal of brain tumors have been recorded since the ancient Egyptian era. The beginning of neurosurgery as a separate discipline date to the 1930's. Gamma-ray irradiation became a treatment modality since the 1950's. Nitrosourea derivatives, such as carmustine and lomustine were added in the 1970's. The most commonly used 1st line agent today, temozolomide was added in 2005. The newest treatment modality, tumor-treating fields (TTF), were approved by the FDA in 2015 for newly diagnosed GBM. TTF is based on alternating electrical currents that inhibit cell proliferation. Conventional immunotherapy has failed so far in GBM while macrophage-based immunotherapy is subject to intensive research at the moment.

Other ways of therapy delivery – intratumoral

Of the three components of current malignant brain tumor treatment, surgery and radiotherapy have already been optimized to a large extent. The ultimate goal of surgery is gross total resection (GTR), where all tumor tissue is resected without any detectable remnants [82]. In practice, this can sometimes be done but two issues remain – first, in certain cases, the tumor’s primary location is close to essential areas, so-called eloquent brain, and the iatrogenic destruction of which would result in catastrophic morbidity. Second, in highly aggressively growing tumors like GBM and MB, the emphasis is on the word “gross”, meaning macroscopic, because even when the resection is deemed GTR, there is still high chance of residual infiltrating tumor cells embedded in the normal tissue. It is not within the power of surgery to eliminate those cells [83, 84].

Radiotherapy has been refined since its conception so that whole-brain irradiation is used less. Dose fractionation enabled higher cumulative doses and employing high-energy particles like protons and electrons results in lethal doses in the tumor while partially sparing normal tissue. The biological limit of normal brain and spinal cord tissue firmly dictates the maximal safe tolerance for radiotherapy.

It is chemotherapy that, in my opinion, has the widest space for optimization. The major challenge of effectively delivering drugs to the CNS is the BBB, which is impenetrable to large or hydrophilic substances. This reduces the selection of available agents where the majority of highly potent chemotherapeutics cannot diffuse to the desired site of action. Moreover, it excludes many biological therapy agents, e.g. antibody-based compounds [85].

Another issue rests with the systemic administration of chemotherapy into the blood stream. Currently, this is the most common route whereby the drugs are injected into the venous blood and carried throughout the body until they reach the tumor site. This route is thus highly untargeted, affecting other highly metabolically active tissues like bone marrow cells or enterocytes in the intestines, giving rise to the serious dose-limiting side effects. Bone marrow leukocyte suppression, besides leading to life-threatening susceptibility to infection, also hampers the potential generation of anti-tumoral lymphocytes [86, 87].

An option to address both the BBB impermeability and the dose-limiting adverse effects could be to administer drugs intratumorally, i.e. locally directly into the tumor bulk. There are several ways of achieving this – intraarterial administration into a local artery that carries the drug to the site of action, implantation of biodegradable materials containing the drug into the surgical cavity, methods relying on simple diffusion (injections, subcutaneous reservoirs) or convection-enhanced delivery (CED) devices. In the majority of the projects included in this thesis, we used CED to deliver therapy agents into the tumor.

The concept of CED has been described in detail by others, including the previous PhD students in our group [88-90]. In short, this method was invented specifically with CNS applications in mind [91]. It is based on convection, which is a laminar flow of liquid containing the substance of interest from a pump/reservoir through a catheter into the tumor site. It utilizes a pressure gradient; therefore, it is less prone to system blockage and diffusion failure than simple diffusion, where intended therapy follows concentration gradients only passively [92]. CED has been extensively studied as an alternative mode of therapy delivery in neurooncology, with several chemotherapeutic agents [93-98].

Nonetheless, this method also has its downsides [99]– not all substances can be delivered this way (for instance alcohol-based or highly viscous compounds), the pump/catheter assembly can disconnect, intratumoral pressure might exceed the pumping pressure or the line can get infected.

Overall, 26 clinical trials are registered at clinicaltrials.gov under the keywords “Glioblastoma” and “CED” as of the date of writing of this thesis. One clinical trial is registered under the keywords “Medulloblastoma” and “CED” but interestingly, it is aimed at children above 12 years with Grade III and Grade IV gliomas, including GBM. MB is listed in the diagnoses included, nevertheless, limiting the inclusion age to 12 and older presents a serious MB selection bias on that study. To my knowledge, no other trials of local therapy in MB are ongoing.

In the past, several pilot studies on CED of various cytostatic agents in GBM have been published. None, however, was followed up by a Phase 3 randomized controlled trial. Ultimately, whether CED as a route of treatment administration becomes widely accepted in clinical neurosurgery remains to be seen.



Figure 4. Actual-size graphical representation of a subcutaneously implanted CED pump in a mouse
A pump with reservoir is implanted into a pocket on the mouse's neck. A catheter connected to the pump delivers the treatment agent into the tumor (not shown). Image reprinted with permission from manufacturer, © Durect Corporation

Immunotherapy – a paradigm shift

The hitherto described modes of malignant tumor treatment (surgery, radiotherapy and systemically administered chemotherapy) represent untargeted therapy, meaning that they negatively affect tumor and healthy tissue indiscriminately. All efforts are indeed made to eliminate cancer cells and protect the normal tissue, but surgery cannot always safely separate these two. As for chemoradiotherapy, it preferentially kills rapidly dividing cells with good blood and oxygen supply. In practice, there are often radiosensitive organs located in close vicinity of the tumor site and chemotherapy doses necessary for tumor elimination are limited by life-threatening side effects. What is more, some areas within the tumor tissue receive less blood supply and the cells have adapted to hypoxia and acidosis, which decreases sensitivity of chemotherapeutic drugs [32, 100]. Both chemo- and radiotherapy also increase the risk of secondary cancer growth in the future.

To address these issues, a vigorous development of more precise, tailored cancer treatment has been underway. One such method is immunotherapy, i.e., treatment that utilizes the cellular or acellular components of the host immune system to precisely target and eliminate cancer cells. The idea and concept of immunotherapy is not novel as already in the late 19th century, Coley and others elsewhere discovered that tumors could recede or even completely disappear after the host immune system was stimulated by e.g., a microbial infection [101, 102]. The early experience with active usage of immunotherapy was anecdotal with little understanding of the underlying mechanisms, unsurprisingly resulting in suboptimal treatment success. However, the theoretical benefits of less off-target adverse effects seemed appealing. What is more, immunotherapy carried a promise of an immune response adaptable to the cancer evolution, with the possibility of generating long-lasting immunological memory that would prevent tumor relapses.

The second half of the 20th century was characterized by gradually gaining preclinical knowledge about tumor signaling and immune cell interactions. During this time, it was discovered that athymic, immunocompromised mice suffer from cancer more frequently than wild-type mice [103], underlining the essential role of the immune system in tumor control. Moreover, as a first large-scale utilization of immunotherapy principles, inoculation of attenuated *Mycobacterium bovis*, the so-called Bacillus Calmette-Guérin (BCG) into the urinary bladder was established as treatment of human bladder carcinomas [104]. This procedure, which causes local macrophage-rich inflammation of the bladder mucosa with resulting tumor elimination, is still used in selected cases to this day.

Overall, considerable attention at that time was aimed at vaccine therapy, which can be either protein/peptide-based or tumor cell-based. The latter is prepared by resecting autologous tumor tissue, expanding the tumor cells *in vitro* and deactivating them, usually by lethal irradiation. The dying cell suspension is then

injected in the vaccination site, where it is phagocytosed by resident and infiltrating APCs [105].

The underlying mechanism of vaccine therapy in cancer is the introduction of tumor neoantigens or tumor-associated antigens (TAAs) to a peripheral site (commonly subcutaneously) to boost their uptake by APCs [106]. APCs then introduce the processed tumor antigens to populations of T and B cells in the regional lymph nodes, thus increasing the priming of these cells to recognize the neoantigens. These tumor-primed T cells then infiltrate the tumor site and trigger cancer cell death. This is exemplified by the first two melanoma-specific antigens to be used as vaccine antigens [107, 108].

Some challenges of this strategy include selection of specific antigens to prevent tumor cells adaptation or autoreactive T cell clone selection. The most common mechanism with which tumor cells evade tumor vaccine immunity is by decreasing the expression of the targeted antigen through selection pressure [109, 110]. It is laborious yet essential to select a target specific enough to reduce autoimmune side effects to a minimum while ensuring its potency in vaccine therapy. The antigen molecule should be fundamental in the cancer cell cycle so that it cannot be downregulated. One way to overcome the ensuing inevitable resistance is to use a combination of TAAs or combining vaccines with other cancer treatment modalities [111, 112].

Another strategy of minimizing the effectiveness of therapy based on TAA immunization employed by cancer cells is upregulation of surface molecules that inactivate the effector immune cells. They are called immune checkpoints and they are mentioned in more detail below. In the case of whole tumor cell vaccines, the time required to obtain sufficient numbers of tumor cells to re-inject may in some cases be so long as to preclude timely cancer cure [113]. Of note, ethical considerations cause some ethical review boards to reject studies where cancer cells are reimplanted into the patient's body.

Similarly, instead of relying on passive immunization with TAAs that need to be engulfed by APCs and subsequently presented to naïve T cells, injection of APCs and T cells that had been pre-primed to TAA *ex vivo* can be used. Currently, the so-called adoptive transfer of dendritic cells (DCs) as APCs, and chimeric antigen receptor T cells (CAR T cells) has been the most investigated [114, 115]. Dendritic cells can be derived *ex vivo* from autologous monocytes isolated from blood by incubation with appropriate cytokines. Afterwards, they are primed by antigens derived from the patient's tumor samples obtained by surgery and matured by further cytokine treatment [116, 117].

Besides DCs, other types of immune effector cells can be infused, as well. The cell types that have been researched most are autologous monocytes, unspecified cytokine-pulsed lymphocytes (probably poorly characterized T cells) and nascent T cells. A special category are CAR T cells, that involve introducing genetically

engineered T cell receptors into normal autologous T cells, which binds specifically to TAAs without the need of further co-stimulation (that normally occurs through e.g. CD28) [118]. This theoretically intensifies the T cell antitumoral response while eliminating the possibility of T cell silencing by cancer cells. Early experience with clinical CAR T cell therapy has recorded remarkable successes especially in the field of hematooncology [119-121]. Nonetheless the cost of such therapy makes it insofar inaccessible to most patients, while also suffering from the same principles of treatment resistance and autoimmune adverse effects as passive vaccines.

Next tactic of cancer treatment that relies on the host immune system is employing genetically engineered oncolytic viruses to infect cancer cells. The virus load can also be delivered intratumorally, into the postsurgical cavity. After administration, they home in onto cancer cells via surface receptors and both directly trigger cell death, lead to a release of TAA and increase the immune system activation [122]. Additionally, the nucleic acid of viral particles can be altered so that they force cancer cells to e.g., express enzymes that activate chemotherapy drugs [123]. Despite these preclinically-described benefits, few Phase III trials with oncolytic viruses as cancer therapy have recorded satisfactory results [124, 125]. One of the challenges that virus-based therapy faces is the elimination of therapeutic viral particles by host immune cells through pathogen-recognition receptors, thus decreasing the viral efficacy [126].

Finally, the strategy that proved to be extremely successful in activating the immune system against tumor cells and embodies a revolution in treatment of several cancer types (especially renal cell carcinoma, lung small cell carcinoma and melanoma) is immune checkpoint inhibition (ICI). Immune checkpoints are ligands such as PDL-1 and CTLA-4, which upon binding to respective receptors cause an effective suppression of immune cell responses, partly due to apoptosis of effector immune cells.

In physiologic conditions, these molecules and their associated signaling pathways prevent an exaggerated immune reaction with possible autoimmune targeting of self-tissues. Consequently, activated T cells are silenced and the inflammation-resolution balance is maintained. This evolutionary protective mechanism was hijacked by cancer cells which utilize immune checkpoint overexpression to dampen antitumor immune reactions by silencing infiltrating T- and other cells [131]. Monoclonal antibodies against PDL-1 and CTLA-4 have been developed, marketed, and used in treatment of cancer with profound results now for several years [127-130].

Besides cost, the main disadvantage of ICI is low numbers of tumor-infiltrating lymphocytes and low mutational burden in some tumors [132, 133], which renders this therapy ineffective. Serious adverse effects of ICI are mostly of an autoimmune character (colitis, dermatitis, hepatitis, encephalitis) and they are exacerbated by agent combination [134].

Originally, immune checkpoints were described based on T cell reactivity, however, molecules that inhibit other cell types, such as macrophages and NK cells are now assigned to this category, as well.

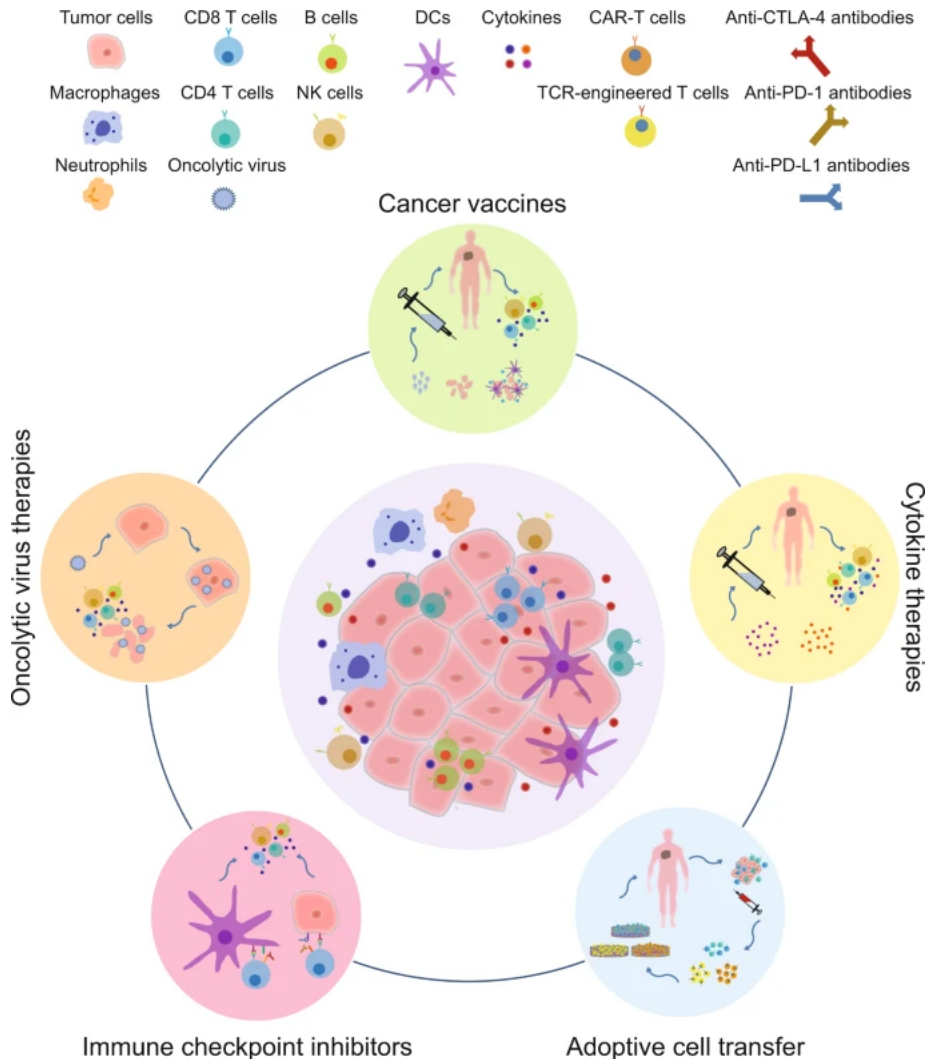


Figure 5. Depiction of various immunotherapy modalities

The concept of cancer immunotherapy has been investigated for over 100 years. In the last two decades, it has achieved remarkable success in the treatment of several tumor types. An overview depicts the different cell types present in the TME with simplified mechanisms of action of the 5 pillars of immunotherapy – cancer vaccines, cytokine therapies, adoptive cell transfer, immune checkpoint inhibitors and oncolytic virus therapies. Of these, only immune checkpoint inhibitors have so far become standard part of cancer protocols. Adapted from Zhang Y et al, Cell Mol Immunol, 2020

...not for everyone – challenges in brain tumors

Despite the above-mentioned ground-breaking discoveries in the field of ICI and immunotherapy in general, the new agents are not used clinically in malignant brain tumor protocols at the time of writing of this thesis, unlike in other tumor types [135-137]. I shall review the mechanisms of immunotherapy that are specific to malignant brain tumors below. They all stem from biological characteristics of malignant brain tumor cells and properties of brain tissue.

GBM and MB cells possess relatively few genomic mutations and resulting abnormal proteins, which makes them only weakly immunogenic [138-141]. From the perspective of antigen vaccine utility (shared also by DC-based therapy and adoptive cell transfer), one general weakness is the requirement to select an antigen that is indispensable to the functioning of tumor cells. Otherwise, cancer cells will inevitably adapt to the selection pressure by downregulating the targeted molecule so that the antigen-primed T cells become ineffective, a process called antigen escape [109, 142]. In this regard, malignant brain tumor cells contain certain molecules that could serve as specific antigens – mutated EGFR and IDH in GBM, etc. [143, 144]. Such antigens are expressed dynamically and the selection pressure on tumor cells can result in their gradual depletion in recurrences, for instance [110, 145]

Furthermore, GBM and MB possess a lymphocyte-poor immune infiltrate. The vastly predominant cell type in the TME are TAMs, regulatory T cells (Tregs) and other immunosuppressive cells. Together with other stromal cells hijacked by tumor cells, these create a milieu hostile to cytotoxic T cells [140, 146, 147]. The spatial heterogeneity of GBM and MB results in areas with poor perfusion, where T cells struggle to home into. Moreover, once past the blood vessel wall, the hypoxic acidotic conditions interfere with the cytotoxic activity of T cells [148]. This means that pockets of cancer cells remain resistant to natural or CAR T cells even despite antigen-priming and are then able to repopulate the tumor with cells unresponsive to immunotherapy.

Oncolytic viruses have been used in clinical trials in GBM with limited success [149, 150]. More favorable outcomes have been reported after the viral genome had been edited to cause the production of proinflammatory cytokines, proapoptotic factors, checkpoint inhibitors and other immune stimulants [151].

Agents from all 5 immunotherapy modalities have been tested in GBM preclinically and in pilot human trials. Nevertheless, due to the above-mentioned pitfalls, the treatment attempts did not succeed in overcoming the immunosuppressive TME.

Specificities of brain tumor immune compartment

The hitherto unsatisfactory results of immunotherapeutic agents are derived in part from the specific features of brain TME. Besides the characteristics mentioned above, such as the impermeable BBB and low mutational burden of GBM and MB, the immune component of TME in malignant brain tumors consists of cells unreceptive to conventional T cell-based immunotherapy.

The BBB is an anatomical and physiological partition between the systemic circulation and the CNS tissue, composed of capillary endothelial cells with tight junctions, basement membrane, adjacent pericytes and surrounding processes of astrocytes. Under physiologic conditions, an intact BBB regulates the transfer of molecules and immune cells entering the CNS, thus playing a key role in CNS homeostasis. This nevertheless means that access of treatment agents from the systemic circulation into the brain is severely limited. It should be noted that the BBB does not isolate the brain completely and there are areas with physiologically thin and permissive BBB [152].

As a brain tumor proliferates and grows, angiogenic growth factors released from cancer cells induce an ingrowth of new capillaries, so-called neoangiogenesis. Logically, the proper architecture of the BBB is oftentimes not established, leading to hemorrhages and fluid leaks into the extravascular space. Such a defective structure is termed the blood-tumor barrier (BTB) and its increased propensity to leakage facilitates drug and T cell transfer into the brain, as well as tumor DNA or even whole cells into the circulation [153, 154]. Moreover, BTB does not have uniform properties among different tumor types or areas within one tumor. For instance, WNT-driven MB contains a more loose BTB permissive to larger doses of chemotherapy, compared to the SHH-driven MB [155]. Continuous deposition of plasma proteins in the TME through leaky BTB may also lead to a sustained pro-tumorigenic inflammatory reaction [156, 157].

The brain has been dubbed “an immune-privileged organ” reflecting the specific characteristics of immune responses and comparatively thin lymphatic vasculature [158]. However, T cells and macrophages indeed routinely enter from the systemic circulation to certain areas of the brain, although they are normally absent from the parenchyma [159-161]. Besides these temporary “visits” by circulating immune cells, there is a large pool of resident brain macrophage-like cells called microglia. Microglia have a different embryonic origin from systemic macrophages, and they are critical to providing stimulatory signals to neurons during brain development. They are continually replenished by self-renewal under physiological conditions, without support from circulating monocytes [162-164].

During the course of malignant brain tumors, the BBB/BTB is further damaged, either by the effects of tumor cells or by treatment. This allows for increased infiltration by circulating monocytes and lymphocytes. These monocytes mature

under the influence of tumor-secreted factors into TAMs, forming the predominant TME cell population in malignant brain tumors [165, 166]. Unlike in other tumor types, large populations of TAMs have been associated with tumor progression and lower survival in GBM and MB [167-169]. Through cytokine-mediated reprogramming, tumor cells prevent the pro-inflammatory M1-like maturation of TAMs, directing them instead to the M0/M2-like phenotype [170]. Furthermore, these tumor-promoting TAMs inhibit cytotoxic T cell activation and recruit more inhibitory Treg cells [171]. The highest density of the tumor-promoting TAMs can be found in the hypoxic, glioma stem cell-rich tumor core [172, 173]. The percentage of TAMs differs across the GBM and MB subtypes, with mesenchymal GBM and SHH-driven MB containing the most [174, 175]. Of note, high levels of plasticity have been observed in the TAM populations, rendering the understanding of cells as being either M1-like or M2-like oversimplified. In reality, TAMs (and macrophages in general) display phenotypic and functional features of both maturation pathways and they can switch the functionality in time [176].

The non-tumorous brain TME consists not only of resident microglia, circulating TAMs, T cells, NK cells and other leukocytes, but also of considerable populations of cells normally present in the CNS – neurons and the supporting glia (especially astrocytes and oligodendrocytes). Their role in glioma progression and propagation stands less at the forefront of malignant brain cancer research but it is significant, nevertheless. An extensive communication between cancer cells and neurons via extracellular vesicles (EVs) that contain tumor-derived receptors, signaling molecules and genetic information has been documented [177, 178]. Neurons also support cancer cells by releasing paracrine stimulants of tumor growth [179, 180]. The crosstalk between astrocytes and microglia, among others, induces the genetic reprogramming of microglia and monocyte-derived TAMs towards the immunosuppressive milieu hostile to tumoricidal immune cells [181, 182]. Some TAM/microglia populations can nevertheless retain the tumor-killing capacity, as evidenced in SHH MB [183].

Various clinical trials of molecular targets that are fundamental to TAM function and development in malignant brain tumors showed underwhelming results, often in contrast to preclinical experiments. These include attempts at CSF1R inhibition, CCL2/CCR2 blockade, CD47 inhibition or CD40 stimulation [184-187]. The reason for this failure stems from missing knowledge on how to overcome the human brain TME challenges. A lack of faithful preclinical models of the complex TME interplay lies at the core of the problem, warranting re-evaluation of the faithfulness of tumor cell lines, immunocompetence of research animals and overall proximity to the situation in humans during preclinical trials. Highly informative reviews on the topic have been elaborated by several research groups [188-190], among others.

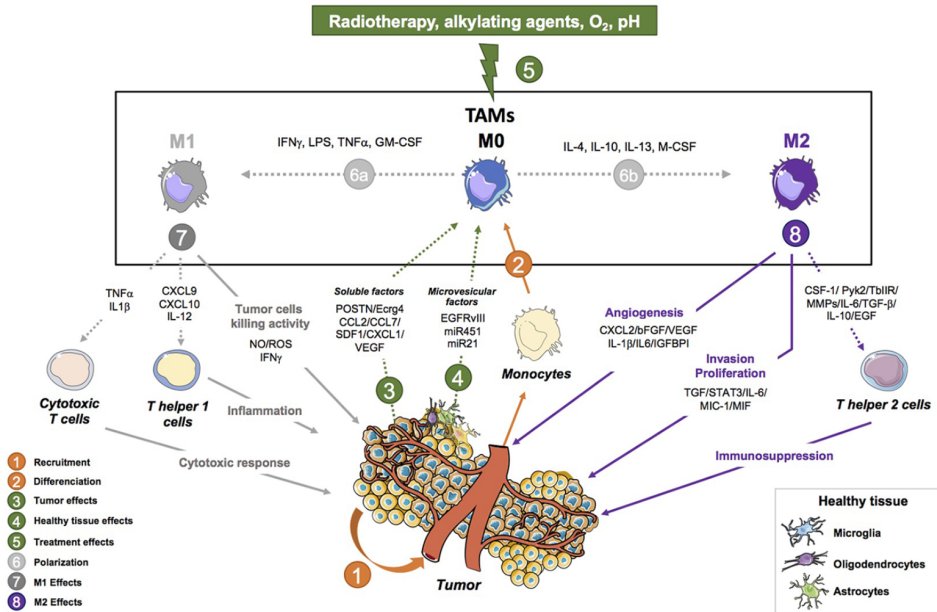


Figure 6. Complex interactions in the malignant brain tumor microenvironment.

Peripheral monocytes are recruited from the circulation by chemokines secreted by tumor cells (1). They differentiate into naïve M0 TAMs (2). This is modulated by tumor- and normal cell signalling molecules (3 and 4). Treatment modalities, such as chemo- and radiotherapy, as well as the hypoxic acidotic environment influence the macrophage homeostasis (5). According to the predominance of proinflammatory or immunosuppressive cytokines, TAMs differentiated along the M1 – M2 axis, respectively (6a, 6b). Significant macrophage phenotypic fluidity takes place *in vivo*. M1-like TAMs activate and sustain cytotoxic CD8+ T cells and proinflammatory Th1 cells, besides their own tumoricidal ability (7). M2-like TAMs promote the immunosuppressive Th2 cells, while also secreting factors involved in neoangiogenesis and tumor cell proliferation (8). M2-like TAMs and microglia are the majority population in GBM and MB microenvironment. Adapted from Grégoire et al., Front Pharmacol, 2020

Antisecretory factor and its derivatives - introduction

The antisecretory factor (AF) was described for the first time in 1984 by a group of Swedish scientists in pigs and two years later in rats. Immunohistochemical analyses revealed abundant expression of the protein in the pituitary gland, cerebral and intestinal mucosa tissue [191, 192]. During the initial mechanistic studies, it was shown that the new protein can robustly inhibit the intestinal mucosal fluid secretion triggered by cholera toxin and other substances and it was therefore named after its principal property [193, 194]. As research on AF progressed, its expression was discovered in more tissues, including cells of the immune system (macrophages, GALT, spleen and thymus), as well as more species, namely all tested mammals (including humans) [195, 196]. Moreover, the structure of the protein seemingly shares a high degree of homology between species as AF isolated from human and porcine pituitary glands inhibited toxin-induced mucosal secretion in rats [194].

After isolating the gene and describing the protein structure in detail, protein fragmentation experiments revealed several peptides were responsible for the antisecretory effect. The peptide with the highest stability and most potent effect was 16 amino acids long and was therefore dubbed AF16. Subsequent mechanistic experiments proved that besides blocking fluid secretion, AF also diminished mucosal inflammation in the toxin-stimulated colon [197]. This raised interest as a potentially fully endogenous anti-inflammatory agent without side effects would have a wide range of clinical applications. Research on the effect of AF on T cell cultures demonstrated that antibody-mediated blocking of AF caused an increase in the proinflammatory cytokines IL-18, IL-6 and decreased the anti-inflammatory IL-10, implying that AF itself had anti-inflammatory properties [196]. Moreover, *in vivo* experiments proved that such blockade of AF resulted in a longer course of autoimmune encephalitis with higher severity in rats [198]. Further studies on other immune cell types with AF16 were not done and a conclusion was made that AF has anti-inflammatory properties.

Besides immunomodulation by AF16, other beneficial effects were described in a herpes simplex virus (HSV)-encephalitis model in rats. Like in other cases of inflammation, the release of cytokines, and immune cell infiltration are accompanied by fluid extravasation, resulting in tissue edema. When brain tissue swells encased in the rigid skull, the intracranial pressure (ICP) rises. If the brain edema is severe, the ICP can rise above the blood pressure in the capillaries, which can lead to perfusion impairment and tissue damage on a microscopic level, and brain herniation resulting in death of the organism. AF16 suppressed the general increase of ICP and eliminated high pressure spikes in the rat HSV encephalitis. No effect of AF16 was found on the immune cell infiltration of the brain tissue nor on the replication of the virus [199]. Whether the ICP stabilization and survival benefit was caused by an inhibition of proinflammatory cytokine release from resident and infiltrating cells or by some other mechanism was not elaborated upon.

The physiological function of AF has not been fully elucidated yet. DNA homology studies revealed that the AF gene locus is synonymous with S5A, Rpn10, Angiocidin and Proteasome 26S Subunit Ubiquitin Receptor, non-ATPase 4 (PSMD4) [200-202]. Gene knock-out experiments showed that mouse embryos with knocked-out transcription of the AF gene die *in utero*, suggesting that AF is essential in early organism development [203].

The similarity with PSMD4 led to a hypothesis that AF may exert its action through the proteasome complex. Such theory is supported by the fact that polyubiquitin, a major target for the proteasome, binds with high affinity to AF [200]. A body of evidence describes the role of proteasomes and immunoproteasomes in the regulation of inflammation, namely by enhancing MHC-I antigen presentation and T cell activation through PSMD4 [204]. Ubiquitination is also linked to immunogenic cell death regulation through the degradation of inflammatory mediators [205]. Nevertheless, no further studies with AF or its derivatives have been published in this direction.

More recently, another hypothesis was proposed. It suggests that the effects of AF are caused by inhibition of the Na-K-Cl cotransporter 1 (NKCC-1). Upon activation by phosphorylation, this ATP-dependent electrolyte pump transfers sodium and potassium cations into the cytoplasm, and chloride anions outside. Detailed experiments have not been published by other groups, either, and our preliminary studies with the NKCC-1 inhibitor bumetanide and AF16 in cell cultures did not yield conclusive results. At the time of writing of this thesis, no-one has published results that would explain the mechanism of action of AF in detail.

One step taken in this direction was a study describing one possible way of how cells can take up AF16. The authors showed that the uptake is dependent on endocytosis, which was enhanced in the presence of cell surface proteoglycans, i.e., heparin. AF16 bound to heparin with higher affinity in conditions of low pH and ionic strength, such as those that often arise in the inflammatory microenvironment and conditions with electrolyte disturbances, e.g., diarrhea. Still, AF16 was also taken up by cells that lack surface proteoglycans, albeit to a lesser degree. Glycosaminoglycans are therefore proposed as important co-factors in the antisecretory and anti-inflammatory effects of AF16 [206].

Antisecretory factor in treatment of various diseases

The unknown specificities of the mechanism of action of AF did not prevent applied research of the protein and its derivatives in clinical medicine. Importantly, it was established that gut tissue and plasma levels of AF rise after feeding animals with specially processed cereals (SPCs) [207, 208]. SPCs, also referred to as hydrothermally processed cereals exert this effect on the intestinal mucosa through a patented modulation of specific amino acids and oligosaccharide content in the grain [195]. Nevertheless, the exact mechanism is also unknown. A formulation of egg yolk enriched for AF (due to the hens having been fed SPCs), trademarked under the names Salovum or B221, enabled convenient oral administration [209].

Expression of AF has been induced in the intestinal mucosa of rats and pigs in response to cholera and clostridium toxin stimulation [197]. Such findings prompted human proof-of-concept trials, which revealed that patients with inflammatory bowel disease (IBD; ulcerative colitis or Crohn's disease) who consumed SPCs reported improved IBD symptoms compared to the placebo group. Moreover, the SPC-diet patients showed significantly higher levels of AF in their plasma and a significant decrease of histological signs of acute inflammation in their rectal biopsy samples than the placebo group. In the same study, researchers preliminarily characterized the cells most AF-positive as CD4+ and CD8+ T cells. No difference in plasma lipid levels was observed [207].

In a follow-up trial on 20 patients with ulcerative colitis, similar results were reported – a decrease in histological severity of inflammation, reduction of plasma CRP and decreased ESR after oral AF administration [210]. Despite these relative successes in the early pilot studies and case studies [211, 212], no larger Phase II trials were initiated on IBD patients. As for other diarrheal conditions, no difference between SPC diet and placebo was reported in patients with irritable bowel syndrome (IBS), who self-reported the development of their IBS symptoms [213]. In this study, only patients with the most severe diarrhea described symptom relief after a diet containing SPCs.

In a pilot trial of SPC treatment of 8 patients with post-surgical short bowel syndrome (SBS), length of the remaining small intestine was correlated with the SPC-mediated induction of AF in patients' blood. Frequency of bowel movements did not change after SPC diet [211]. A more recent study from another center recorded no benefit of SPC diet, oral Salovum or the combination thereof in 7 SBS patients. The administration of Salovum was meant to circumvent the short length of small intestine, insufficient to generate endogenous AF upon SPC stimulation. Interestingly, the high intake of osmotically active Salovum seemed to worsen fluid loss and the symptoms of SBS perceived as unpleasant [214].

Despite this finding, the interest in AF treatment in gastrointestinal applications continued. A larger randomized blinded placebo-controlled trial was done with

Salovum in 240 Pakistani children and the treatment resulted in improved stool frequency and consistency for acute, as well as chronic diarrhea. The article concludes by stating that AF-containing food products could be useful in treating diarrhea in the vulnerable children population [215]. More trials with Salovum in the pediatric population with diarrhea were conducted by the same group, with the same encouraging results [216-218]. The most recent of these studies found that none of the Salovum-treated children developed a relapse of diarrhea during 6 weeks of post-treatment follow-up [216].

The causative agent of pediatric diarrhea in this series of studies was not established. One open-label RCT focused specifically on treating cholera-associated diarrhea with Salovum in adult males. Here, no benefit of peroral Salovum was observed in relation to stool volume or duration of diarrhea. The authors planned to address this by increasing the dose of Salovum given to patients [219].

Other diseases, where tissue edema and hypersecretion play a major role, have been studied as potential indications for AF treatment. One study investigated the effects of intravenous AF-16 infusion on a porcine acute respiratory distress syndrome (ARDS) model. Generally, lung edema plays a major role in the pathology of ARDS, which is a potentially life-threatening lung condition leading to the inability of the affected lung tissue to perform effective gas exchange. It was shown that intravenous AF16 significantly decreased the volume of extravascular lung water, a parameter that is also used in assessing human ARDS patients. Other measured parameters in the study, such as wet-to-dry ratio, pressure-volume curve development, PaCO₂, PaO₂, lung tissue cytokines or histological markers of inflammation were not changed between treatment and placebo [220].

One recent study by the same group tested an intravenous infusion of AF16 as treatment of peritoneal sepsis in pigs. Control treatment was administered in the form of saline. The authors did not observe any improvement in the shock symptoms, namely hemodynamic parameters, respiratory parameters (including extravascular lung water), need for norepinephrine therapy, nor plasma TNF α /IL6 levels after AF16 infusion. However, a significant reduction in wet-to-dry ratio was described in the livers of pigs treated by AF16 compared to saline. The study authors recognize that the sample size was low (8 in each group) and inter-individual variation high. It can be argued that higher AF16 doses or continuous infusion could be necessary, as well as initiating therapy earlier, before severe hemodynamic compromise appeared [221].

Ménière's disease (MD), a chronic inner ear condition characterized by recurrent attacks of vertigo, tinnitus, and aural fullness, is caused by a periodic disbalance in production and resorption of endolymph (the fluid that fills the ducts in the inner ear). The resulting high pressure in the endolymph duct system causes the above symptoms. Histological evaluation proved that AF was expressed in human and rat cochlea and vestibule [222]. Two clinical trials (one pilot and one follow-up) of SPC

diet in patients with MD have been conducted. They reported increased plasma AF levels after SPC diet which correlated with improved symptom control in the SPC-treated group [222, 223].

Interestingly, no change was seen between groups in the objective parameters that were followed, i.e. pure-tone audiometry and an otoneurological exam. This led the authors to conclude that the SPC diet affected mainly the vertigo component of MD. Two later validation studies by different research groups reported the same results where the SPC diet improved the subjective symptom reported by MD patients [224, 225]. Contrastingly, one RCT where MD patients received the SPC treatment first with subsequent switch to placebo, failed to note any improvement of functional status or frequency of vertigo attacks [226].

An excellent review by Ulgheri *et al.* published in 2010 summarizes the hitherto known characteristics of AF, elaborates on the theories of its mechanism of action, and summarizes the clinical trials on diarrheal conditions and MD [227].

Results from studies of rat autoimmune encephalitis, herpes simplex encephalitis and brain injury-related intracranial hypertension (IH), all treated with AF16, provided ample evidence that AF16 counteracted the ICP increase and improved outcomes [198, 199, 228, 229]. In these experiments, AF16 was administered either intranasally or intravenously to the rats and its penetration through the BBB was documented [228]. Subsequently, a series of human trials on edematous conditions of the brain treated with AF derivatives was launched. In the first study of normal pressure hydrocephalus (NPH) and idiopathic IH (IIH), 10 NPH and 8 IIH patients received daily oral Salovum and had their ICP continuously monitored. ICP wave readings were recorded before and after Salovum treatment. The authors found no change of ICP waves after Salovum administration [230]. Unlike in the animal studies, however, plasma AF was not monitored in the patients, thus it is difficult to argue whether the Salovum dose was sufficient to raise systemic and brain AF levels. Also, no other disease variable, e.g. subjective symptom evaluation, was recorded. Overall, the authors concluded that neuronal cell edema does not contribute to the pathological change of ICP waves in NPH and IIH [230].

It should be noted, however, that the etiology of IH is different from the conditions where AF treatment recorded successes. While the precise pathophysiology remains unclear, the clinical signs and symptoms of NPH are thought to arise from abnormalities of CSF flow and absorption. The ICP generally remains mostly normal [231]. In contrast, in autoimmune or viral encephalitis, as well as posttraumatic brain edema, brain cell volume increases, leading to cell edema and spikes in ICP.

So far, two pilot case series have been published on the AF treatment of traumatic brain injury (TBI) in humans. Cedeberg *et al.* administered Salovum to unconscious patients with severe TBI via a nasogastric tube, together with standard treatment to reduce ICP. They concluded that in at least 3/5 patients, Salovum favorably affected

the ICP and demonstrated signs of clinical benefit. In those patients, ICP could be effectively decreased to the acceptable range solely by Salovum. No analyses were done to follow the AF levels in the plasma of the patients and the authors acknowledged that the oral administration of Salovum presented a challenge in patients suffering from periods of gastroparesis (when absorption of Salovum through the GIT is impaired) [232]. The generally encouraging findings motivated the initiation of two randomized prospective, double-blinded, placebo-controlled trials in South Africa and Sweden, which are recruiting patients at the time of writing of this thesis (NCT03339505 and NCT04117672).

A contemporary case series of TBI patients treated with Salovum attempted to address the limitations of the previous study. Four patients with severe TBI were treated with Salovum orally and rectally in this publication. The rectal route was chosen after the first patient experienced periods of gastroparesis, which resulted in suboptimal blood AF levels with failed ICP control. In the remaining patients, the route of administration was switched to rectal, which caused statistically significant reductions of ICP even with Salovum monotherapy. The authors stated that oral administration of Salovum could result in gastric overload and stress, with poor AF absorption and therefore, rectal route is preferred in unconscious patients. Moreover, intravenous infusion of AF16 could provide better treatment control and precise pharmacokinetic data in the future [233].

It is worth noting that in none of the above-mentioned animal or human studies, AF treatment-related adverse effects were observed, even at large doses. This implies a remarkable advantage of a treatment agent for indications where the currently used drugs have a much narrower therapeutic window.

Table 2. Overview of human studies where AF derivatives were used in treatment of various conditions (next page)

TBI = traumatic brain injury; MD = Ménière's disease; SBS = short bowel syndrome; IBS = irritable bowel syndrome; UC = ulcerative colitis; IDB = inflammatory bowel disease; RCT = randomized controlled trial; p.o. = orally; p.r. = rectally; * exact number of patients who received treatment was not possible to discern

Author	Year	Country	Study type	Disease	Treatment	# Patients	Endpoint	Outcome
Gatzinsky K et al.	2020	Sweden	Case series	severe TBI	Salovum p.o./p.r.	4	ICP decrease	4/4 patients decreased ICP upon Salovum, rectal route leads to more reliable absorption
Cedeberg D et al.	2020	South Africa	Case series	severe TBI	Salovum p.o.	5	30-day mortality	good response, 1 death of unrelated causes, unclear proof of Salovum related ICP decrease
Scarpa A et al.	2019	Italy	Open-label study	MD	SPC p.o.	13	functional questionnaire	no hearing improvement, augmented subjective tinnitus intensity
Viggiani M T et al.	2019	Italy	Open-label study	SBS	SPC ± Salovum p.o.	4	biometric parameters	no significant improvement
Zaman S et al.	2018	Pakistan	Open-label study	infant acute diarrhea	Salovum p.o.	40	stool frequency and consistency	80% improvement after high dose Salovum
Ingvarsdén C et al.	2016	Denmark	placebo-controlled RCT	MD	SPC p.o.	19	self-assessment questionnaire	no significant improvement
Zaman S et al.	2014	Pakistan	RCT	infant acute diarrhea	Salovum p.o.	18	stool frequency and consistency	halving of diarrhea duration
Leong C S et al.	2013	UK	placebo-controlled RCT	MD	SPC p.o.	39	self-assessment questionnaire	59% reported improvement
Alam N et al.	2011	Bangladesh	Open RCT	adult cholera	Salovum p.o.	20	stool weight and diarrhea duration	no difference between Salovum and placebo
Hanner P et al.	2010	Sweden	placebo-controlled RCT	MD	SPC p.o.	27	self-assessment questionnaire	52% reported improvement
Ekesbo R et al.	2008	Sweden	RCT	IBS	SPC p.o.	61*	self-assessment questionnaire	no difference between SPC and placebo
Zaman S et al.	2007	Pakistan	RCT	infant acute/chronic diarrhea	Salovum p.o.	76	stool frequency and consistency	83% and 91% improvement of acute and chronic diarrhea, respectively
Hanner P et al.	2004	Sweden	Open-label study	MD	SPC p.o.	24	self-assessment questionnaire	50% reported improvement
Svensson K et al.	2004	Sweden	RCT	mastitis	SPC p.o.	12	frequency of mastitis, AF blood levels	significant reduction of mastitis incidence, increased plasma levels of AF after SPC
Eriksson A et al.	2003	Sweden	RCT	UC	Salovum p.o.	16	histological changes in intestinal mucosa	significant improvement of inflammatory parameters and histological picture after Salovum
Björck S et al.	2000	Sweden	RCT	IBD	SPC p.o.	53	self-assessment questionnaire, intestinal biopsy of histology	35% reported improvement of symptoms, unclear benefit of histology

Treating cancer with AF16 and Salovum

Following the steady volume of published results that show the anti-inflammatory and anti-edematous effect of AF in various pathological conditions, the first explorations into the field of cancer research were made. There have, however, been few studies published on cancer so far, compared to the relatively abundant trials in the field of general immunology and gastroenterology.

The first study published on AF16 in cancer revealed that the peptide significantly but transiently lowered the interstitial fluid pressure (IFP) in two types of rat mammary malignant tumors [234]. As described previously, IFP is regularly increased in the tumor tissue due to abnormal vessel growth in neoangiogenesis, which causes fluid leaks [235, 236]. This interstitial fluid hypertension contributes to decreased blood perfusion in the tumor tissue, with the resulting hypoxia and acidosis. Moreover, high IFP exerts resistance to the capillary filtration pressure, therefore decreasing the amount of cytostatic agents and other drugs to be delivered into the tumor. It is therefore of interest that AF16 could in this way improve the delivery of e.g. chemotherapy to the tumor tissue.

A more recently published follow-up article used rats with chemically induced mammary tumors that were treated by intranasal AF16. The authors demonstrated that AF16 treatment increased the intravascular volume in the tumor, liver and kidney tissue (as measured by Evans blue quantification), compared to sham-treated animals. They concluded that AF16 enhanced blood supply to the tumor tissue, likely by opening capillaries otherwise collapsed due to high IFP [237].

Facilitating blood supply to cancer cells could bring more oxygen and nutrients for their proliferation. On the other hand, cancer cells deactivate the metabolically demanding processes of oxidative breakdown of glucose and fatty acids, relying instead on simple anaerobic glycolysis [238]. This is tentatively because cancer cells divide too rapidly, and their metabolic apparatus is defective, therefore access to more oxygen would not benefit them. Instead, a more mature vasculature inside the tumor core could carry more immune cells that would not be inhibited by the acidotic and ischemic conditions normally present in the TME. This could result in decreased HIF induction and subsequent modulation of transcription of various tumor-promoting genes. Moreover, higher levels of treatment agents could be delivered into the tumor.

The only published study so far that used AF derivatives to treat a specific tumor type was issued by Ilkhanizadeh *et al.* In this multifaceted analysis of the treatment effect of SPCs, Salovum and AF16 on GBM, the authors used immunocompromised nude mice to inject human and mouse GBM cell lines. Subsequently, the mice were treated with TMZ, doxorubicin and erlotinib combined with SPC diet, oral Salovum and intranasal AF16. The outcomes were the degree of local cytostatic diffusion, measurement of tumor IFP, GBM cell volume, proliferation assays and survival

studies, among others. The findings revealed that SPC diet induced AF expression in the brain, reduced tumor IFP and increased fluid movement in the tumor. Moreover, both SPC diet and AF16 enhanced the intratumoral gadolinium signal on MRI and raised doxorubicin and erlotinib uptake in GBM. SPC diet also induced apoptosis and decreased proliferation (as measured by Ki67 positivity) of GBM cells, resulting in slower tumor growth and better survival of treated mice [239].

Attempting to explain the mechanisms behind these effects, the authors show that AF treatment decreased GBM cell volume and prevented the normalization of cell volume in response to hypertonic conditions. GBM cells treated with AF remained shrunken in a hypertonic solution, whereas untreated cells gradually returned to normal volume. Furthermore, as untreated GBM tumor-spheres increased proliferation upon culture matrix compression, AF-treated tumor-spheres did not. Immunohistochemical analysis, gene knockout and inhibitor studies revealed that inhibition of NKCC1 phosphorylation by AF is the cause of these changes [239].

In the conclusions to this exhaustive study, its authors inferred that AF inhibited the NKCC1 transporter in GBM cells, thus resulting in impaired osmotic regulation of cancer cells, their reduced survival, and higher chemosensitivity under low IFP. The excellent survival records of GBM-bearing mice treated with SPC and TMZ are remarkable. However, certain limitations apply – the use of human GBM cells xenografted into immunocompromised mice precludes any studies of the immune system effects of AF. This fact also likely renders the GBM easier to cure as the same encouraging results could not be replicated in the immunocompetent animals. Among the abundance of data, detailed dosing of AF derivatives is sometimes lost. It is nevertheless an important breakthrough study in the AF treatment of GBM. It also suggests the emerging importance of osmotic-mediated volume mechanics of tumor cells as a factor in tumor growth and aggressiveness, with NKCC1 as a principal regulating electrolyte transporter.

In Publication II of this thesis, we characterize the effect of AF16 on the secretory repertoire of macrophages, a cell type that has not been functionally tested with AF despite its higher abundance than T cells. Moreover, in Publication III to-be-submitted, we explore the safety and feasibility of oral Salovum treatment in a pilot study of first-time GBM patients.

Interestingly, one of AF gene homologues, angiocidin has been patented for use in leukemias and solid cancers. The protein got its name after its inhibiting properties towards endothelial cells [202]. Several articles reported an induction of macrophage activation and secretion of proinflammatory cytokines upon angiocidin incubation [240, 241]. The effect of angiocidin was investigated in several tumor types [242-244], among others in breast cancer, where the protein inhibited tumor cell proliferation through activation of the NF- κ B pathway [245].

Antiphagocytic signaling – the role of CD24 and Siglec10

As was mentioned previously, TAMs are the dominant immune cell type across malignant tumors. In most tumor types, they are hijacked by cancer cell-produced signalling molecules to adopt the anti-inflammatory, immunosuppressive M2-like phenotype. Therefore, TAM count is inversely correlated with prognosis [246]. Yet, macrophages under normal conditions are effective at phagocytosis of various particles, including tumor cells, even in the M2 phenotype [247]. This process is mediated by pattern recognition receptors, scavenger receptors and antibody Fc receptors on the macrophage surface.

Tumor cells, together with TAMs, upregulate the expression of surface antiphagocytic ligand molecules. These inhibits phagocytosis of tumor cells after binding to their coupled receptors on the macrophage surface. Several so-called “don’t-eat-me” receptor-ligand couples have been discovered, e.g. CD47–SIRP α , PD1–PDL1, MHC I–LILRB1 or CD24–Siglec10, with varying abundance in different tumor types [248]. The CD47–SIRP α axis was the first to be described as a phagocytosis checkpoint. CD47 is a surface glycoprotein with a highly glycosylated extracellular domain that binds among others to the Signal Regulatory Protein α (SIRP α) on macrophages and other APCs, thereby protecting CD47-expressing cells from engulfment [249-251].

Similarly to this interaction, another phagocytosis checkpoint pair has been described more recently – CD24 binding to Siglec10. CD24 is also a glycoprotein with a small peptidic core and a strongly glycosylated extracellular domain, which grants tissue specificity [252, 253]. It was first described as a heat-stable modulatory molecule that inhibits B cells activation [254, 255]. Later, the expression of CD24 was discovered in murine hematopoietic cells, neural cells and stem cells and a wide range of functions have been assigned to it [256-258]. These functions are also dependent on the glycosylation and cellular context but include signaling via MAPK resulting in cell binding, lymphocyte development and apoptosis, as well as the oxidative burst of granulocytes [259]. CD24 also acts as a growth inhibitor of mature neurons, with high expression in the developing CNS tissue [260, 261]. Generally, CD24 is expressed more in progenitors and metabolically active cells.

The exact function of CD24 is unknown for certain cell types but it has been established quite well in the cells of the immune system thanks to knock-out studies. Those reported the essential role of CD24 in maturation of B and T cells and implicated the molecule in the development of various autoimmune diseases in mice and men [262-265]. In this context, CD24 may promote activation and proliferation of autoreactive T cells.

The effect of CD24 on the innate arm of the immune system has been shown to be more repressive. Generally, an inflammatory response is triggered in tissues by detection of pathogen-associated molecular patterns (PAMPs), foreign molecules present on invading microbes. Another way is a so-called sterile inflammation, which is not activated by invading pathogens but by so-called danger-associated molecular patterns (DAMPs) [266]. These are molecules normally present in the body, but they are sequestered under physiological condition, e.g. in the cell cytoplasm or inside organelles. After tissue injury, such as physical trauma, burns, etc., DAMPs are released from their storage into the extracellular space. Here, they are detected by tissue-resident innate immune cells that subsequently induce inflammation by secreting proinflammatory cytokines and chemokines. High mobility group box protein 1 (HMGB1), heat shock proteins (HSPs), histones and nucleolins are typical examples of DAMPs [267].

This is where CD24 comes into play. Studies showed that CD24 binds DAMPs, such as HMGB1, together with sialic acid-binding immunoglobulin-like lectin 10 (Siglec10) on DCs and macrophages [268-270]. Siglec10 is a suppressor of APC activation [271] and can together with CD24 act as a scavenger of HMGB1 to block sterile inflammation. Indeed, mice deficient in either CD24 or Siglec10 were highly susceptible to chemically induced liver necrosis. They also produced significantly greater levels of proinflammatory IL6, TNF α and MCP1 than wild-type mice [268]. Interestingly, the CD24 – Siglec10 pathway does not bind PAMPs, suggesting it discriminates sterile inflammation from pathogen-triggered inflammation [269]. In this way, the CD24/Siglec10-induced suppression of the innate immune response may protect host tissues from excessive inflammation-related damage.

This mechanism is exploited by tumor cells, which also overexpress CD24 in most tumor types investigated. Clinical studies correlated high CD24 expression with increased invasiveness, tumor size, likelihood of metastasis and overall survival [272-276]. Correspondingly, siRNA silencing of CD24 decreased tumor cell proliferation and survival *in vitro* [277]. Due to the tendency of glycosylated proteins to be sequestered in cytoplasmic vesicles, CD24 has been proposed as a marker of extracellular vesicles (EVs) released from cancer cells [278, 279]. EVs are considered an important means of cell-to-cell communication in cancer, through which tumor cells can manipulate the TME, as well as immune cells in distant lymph nodes [280].

Besides EVs, several studies have explored CD24 as a marker for cancer stem cells (CSCs). The first studies in this regard were done on breast cancer in humans, where breast CSCs were found to be CD24-low [281]. Nonetheless, the opposite was later found to be the case for prostate, colorectal and gastric cancer, among others [273, 282, 283]. In all these tumor types, a self-renewing and therapy-resistant cell population consistent with CSCs expressed high levels of CD24. This finding is in

accordance with the previously described correlation between CD24 and aggressive and invasive tumor growth.

Killing tumor cells the right way - immunogenic cell death

Broadly, two categories of how a cell can die can be described – regulated cell death (RCD) and accidental cell death (ACD). The difference lies in the character of the triggering signal and involvement of molecular cascades, among other factors. ACD is caused by a rapid, severe insult of a physical, chemical, or mechanical nature, whereas RCD is a result of an energy-consuming pathway as part of physiological processes of either tissue development, aging or elimination of undesirable cells. Moreover, RCD can be stopped by genetic or pharmacologic manipulation, while ACD cannot be prevented [284].

Whilst ACD induces cell membrane rupture and spillage of cytoplasmic and nuclear contents, eliciting a strong reaction of the surrounding cells resulting in inflammation, RCD leaves a more discreet footprint. The latter can be further subdivided into several subtypes, e.g. intrinsic and extrinsic apoptosis, autophagy, etc., which generally result in the formation of membrane-enclosed vesicles that are picked up by phagocytosing cells [285].

These cellular remnants can contain DAMPs that translocated to the membrane in order to signal to the surrounding cells and the immune system that a damaging agent is present. Other DAMPs can be released from different cellular compartments into the ECM [266]. If such release of DAMPs is sufficient to trigger an adaptive immune response in the host, then the RCD is classified as immunogenic cell death (ICD) according to the 2018 recommendations of the Nomenclature Committee on Cell Death [284]. Currently, 6 DAMPs have been linked to the induction of ICD: extracellular ATP, cell-membrane calreticulin, extracellular tumor-cell DNA, extracellular type I IFN and annexin A1 [286, 287]. Heat-shock proteins 70 and 90 (HSP70, HSP90) are counted in this category, as well [288].

These molecules are detected by pattern-recognition receptors (PRRs), purinergic receptors and toll-like receptors (TLRs) by adjacent APCs or other immune cells, which subsequently secrete proinflammatory cytokines IL6, IL12, TNF α , IFN γ and especially IL1 β and IL17. Following this, induction of a cytotoxic immune response with infiltration of CD8⁺ T cells *in situ* and creation of immunological memory ensues [288-290].

ICD can be initiated in the cells by viral infections, physical noxae (such as heat shock, high hydrostatic pressure, UV ray phototherapy) and importantly, certain dosing regimens of radiotherapy and selected chemotherapeutic agents [291-293]. The concept of ICD has been extensively studied in the cancer context and complex signaling pathways were identified, which cancer cells employ to diminish the ICD-induced immune response. For instance, calreticulin surface translocation can be antagonized by CD47 overexpression by cancer cells, where CD47 acts as a “don’t

eat me” signal described in the previous section [294]. Thus, while calreticulin exposure was associated with positive outcomes in acute myeloid leukemia patients [295], high levels of CD47 correlated with poor prognosis in several cancer types [250, 296, 297]. CD24, another “don’t eat me” signaling molecule, decreases the activity of HMGB1 and HSP70/HSP90 signaling, therefore silencing the ICD activation [268].

Other ICD-related DAMPs have their counteracting mechanisms, too. ATP can be hydrolyzed by ectonucleotidases CD39 and CD73, which are present on the surface of immunosuppressive Treg cells in the TME [298, 299]. Nucleases occurring in endosomes break down dsDNA and RNA molecules released from tumor cells, which decreases their immunogenicity [287].

It has been hypothesized that ICD could play an important role in the effect of cancer treatment, especially radiotherapy and certain chemotherapy drugs – anthracyclines (e.g. doxorubicin and mitoxantrone), bleomycin, the proteasome inhibitor bortezomib, cyclophosphamide and oxaliplatin, among others [286, 300, 301]. An agent is described as a *bona fide* ICD inducer when it elicits at least three ICD-associated DAMPs, especially calreticulin, HMGB1 and ATP [301].

We decided to employ MTX as a previously proven ICD inducer in our studies of CD24/Siglec10 modulation in GBM and MB. MTX belongs to the anthracycline family, which is derived from the *Streptomyces* sp. bacteria [302]. It is FDA-approved for treatment of a wide spectrum of human cancers, as well as being used off-label as a non-first-line agent due to its ability to induce ICD [303]. Currently, MTX is not routinely used in malignant brain tumor indications due to its poor BBB penetrance, although several trials of systemic and local MTX administration to GBM patients have been carried out, without significant improvement of OS [98, 304-306]

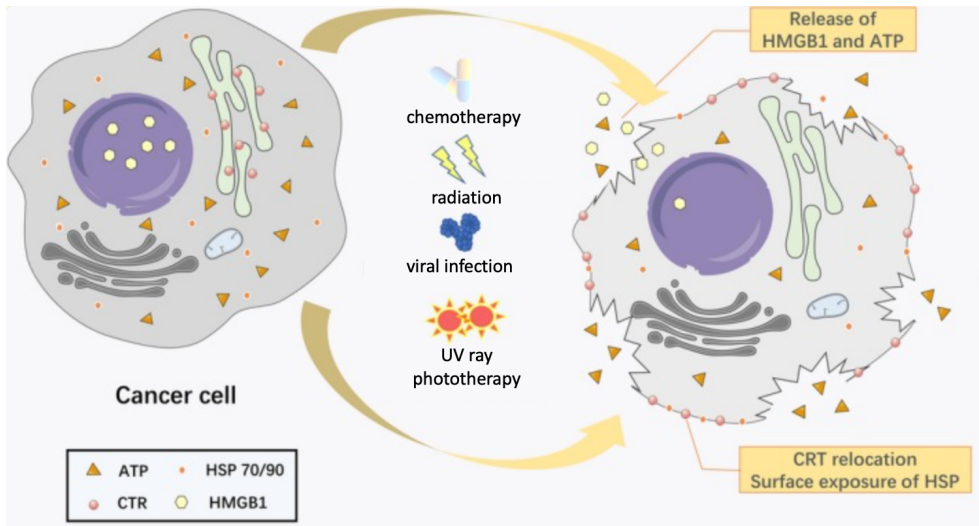


Figure 7. Activation of immunogenic cell death in a cancer cell

Following exposure to inducers of ICD, such as some alkylating chemotherapy agents, certain gamma irradiation protocols, etc., cancer cells trigger a translocation of ICD-associated molecules. Calreticulin (CRT) and ATP are released into the extracellular space from the nucleus and cytoplasm, respectively. HMGB1, originally found in the endoplasmic reticulum, together with cytoplasmic heat shock protein 70 and 90 (HSP70/90) move to the cell membrane. Adapted from Zhou et al. *J Cell Mol Med*, 2019

CD24 and Siglec10 modulation in cancer– potential for outcome improvement

The immunosuppressive pathway triggered by Siglec10 binding to CD24 and its role in protecting host tissues from excessive damage during sterile inflammation has been briefly described in the previous chapter. In recent years, several publications linked CD24 on tumor cells bound to Siglec10 on macrophages to decreased phagocytosis of tumor cells [307-309]. At the same time, identification of this tumor-promoting pathway contributed to the growing conviction that innate immune checkpoints (such as CD24) are promising new targets in cancer immunotherapy [310-312].

The first study that described CD24/Siglec10 signaling in cancer was published by Barkal *et al.* They investigated the failure of other antagonists of antiphagocytic “don’t eat me” signals, like CD47 in several tumor types, hypothesizing that other molecules must be responsible for the persistent suppression of macrophage phagocytosis. Through extensive large database analyses, they discovered high expression of CD24 in many cancer types, with especially high levels in ovarian and triple-negative breast carcinomas (OC and TNBC, respectively). Moreover, a correlation could be drawn between high CD24 expression in the tumors and lower PFS and OS. A large percentage of TAMs in TNBC were found to be Siglec10-positive, unlike peritoneal macrophages from healthy patients. Interestingly, human macrophages expressing low levels of Siglec10 robustly increased its expression upon stimulation with immunosuppressive cytokines IL10 and TGF β 1; they were subsequently less phagocytic [307].

In the *in vitro* experiments, coculturing CD24-deficient tumor cells with M2-like macrophages resulted in enhanced phagocytosis, as did antibody blockade or deletion of Siglec10 on macrophages. This was observed both in an experimental tumor cell line and human OC and TNBC cells. Of note, CD47 blockade added to the effect of CD24 suppression, suggesting possible benefit of dual antiphagocytic antagonist therapy [307, 313, 314]. The authors were able to validate the findings in animal experiments, where NSG mice were engrafted with CD24-wild type and CD24-deficient MCF-7 human breast cancer cells. CD24-deficient tumors exhibited higher rate of phagocytosis, more proinflammatory TAMs, slower growth, and eventual selection of CD24+ clones. Importantly, CD24-wild type mice treated with monoclonal antibodies against CD24 displayed significant reduction of tumor growth [307].

In summary of this pioneer publication, disrupting the CD24/Siglec10 bond was described as a new approach to overcoming the “don’t eat me” checkpoints and restore the phagocytic capacity of TAMs. Moreover, neither OC nor TNBC respond satisfactorily to conventional T cell-based checkpoint inhibitors, a characteristic shared with malignant brain tumors [315-317].

The detailed molecular mechanisms behind CD24/Siglec10 binding and the enzymatic cascades triggered by it were summarized by Yin *et al.* According to this review, Siglec10 binds to CD24 based on the tissue-specific glycosylation motif. HIF1 α secreted by tumor cells in hypoxic conditions as a transcription factor induced CD24 expression in bladder, prostate, and gastric carcinomas [273, 308, 318]. Besides HIFs, noncoding RNA-rich EVs secreted by tumor cells can also exert negative feedback on CD24 transcription and translation [319].

Several preclinical studies using monoclonal antibodies against CD24 in several cancer types have been published [320-323]. The internalization of CD24 upon antibody binding is responsible for enhanced phagocytotic tumor clearance and retarded tumor growth. However, such antibody blockade is complicated by off-target effects, such as B cell depletion resulting from high CD24 levels normally found in those cells in humans, or murine erythrocyte antibody binding (human erythrocytes are CD24negative) [307].

Separately, the role of Siglec10 has been evaluated as an inhibitory signal in human hepatocellular carcinoma samples (HCC). In this study, Siglec10 was detected on TAMs of mixed M1/M2 surface phenotype with an immunosuppressive function in the TME of HCC patients, and high Siglec10 expression was associated with poor prognosis. Moreover, a link was described between the Siglec10-rich TME and higher infiltration of immunosuppressive Treg cells, lower numbers of CD8⁺ T cells, and NK cells. To exploit this in the context of treatment, Siglec10 was blocked by inhibitory Fc fragments, which resulted in a significant decrease of immunosuppressive PD-L1, TIM3, Arg1, IL10 and TGF β , and an increase of proinflammatory TNF α and IL12. The portfolio of surface receptors on CD8⁺ T cells also switched towards a more activated, antitumoral profile. To underscore this finding, combination treatment of Siglec10 inhibition and a PD-1 inhibitor pembrolizumab synergized to induce tumor cell apoptosis and reduce proliferation [324].

One caveat has been revealed by a study describing the importance of preserved nuclear CD24 expression for tumor progression when surface CD24 has been diminished. Even after surface CD24 depletion, tumor cells could retain residual cytoplasmic CD24 stores, which continue to drive growth and metastasis. Only translation blockade by siRNA resulted in an almost complete elimination of the CD24 signal and reduction of tumor proliferation. The intracellular CD24 location was pinpointed to the nucleus and this nuclear CD24 was associated with aggressive tumor phenotype *in vitro* and in animal studies, as well as poor outcome in several human cancer types [325].

One preparation of CD24 has very recently gained attention as possible treatment of a life-threatening systemic inflammatory response syndrome – soluble CD24 (CD24Fc) [326]. Originally designed to dampen graft-versus-host disease in leukemia recipients of donor bone marrow cells [327], a pilot abstract was now

published describing the use of CD24Fc in dual T cell checkpoint inhibition. When PD-1 and CTLA-4 inhibitors are used concurrently, their treatment efficacy is increased at the price of common serious autoimmune adverse effects. In the abstract, its authors described amelioration of dual ICI-related adverse effects and modest enhancement of treatment efficacy by CD24Fc. What is more, the treatment also improved the profile of tumor-infiltrating T cells. The authors mentioned no effect on TAMs in mice bearing one colon cancer and one malignant melanoma cell line [328]. This surprising pilot finding, although fraught with obvious limitations, suggests that the role of CD24 modulation in cancer is complex and establishing a fine balance will be needed in the future human cancer trials.

In none of the studies mentioned in this chapter, malignant brain tumors were given particular attention. GBM was included in the set of human cancer types analyzed for CD24 levels by Barkal *et al.* but the low CD24 level was not commented upon in the main article. Another anti-phagocytic surface protein, the beta-2 microglobulin of the MHC-I complex [329] was expressed highly in GBM, which might constitute a useful target in this disease entity [330]. Medulloblastoma was not included in any of the studies.

CD24 expression in MB has been characterized by research groups in the past. Overexpression of the protein was found in murine models of MB, especially on tumor-initiating cells in SHH MB, as well as neural progenitors and differentiating cells. Furthermore, CD24 levels were significantly higher in human SHH, Gr3 and Gr4 MB compared to the WNT subtype and normal cerebellar tissue on the gene and protein levels [331]. Similar findings have been previously reported by our group, with detailed characterization of CD24 in MB on the cellular level [332]. In this work, CD24 was significantly increased in MB compared to GBM and other brain tumors and normal brain tissue. WNT MB displayed lower CD24 levels. Human tumor cryosection analysis demonstrated CD24 staining in the majority of undifferentiated tumor cells in a unique granular/vesicular pattern. This was in contrast to gliomas with CD24 expression limited to islets of positive cells with a diffuse cytoplasmic pattern. Other stromal cell populations were found to be CD24+, namely tumor-infiltrating CD45+ granulocytes with lobulated nuclei [332].

The specific quantitative and qualitative pattern of CD24 expression could be maintained in cultures of MB cells established from patient samples. Moreover, these cells gave rise to aggressive MB tumors upon injection into the cerebella of mice and still retained the distinct CD24 expression in second-generation cultures [332]. The findings from our previous research and other supportive literature thus motivated the study of CD24 and Siglec10 modulation in malignant brain tumors, as is presented in this thesis.

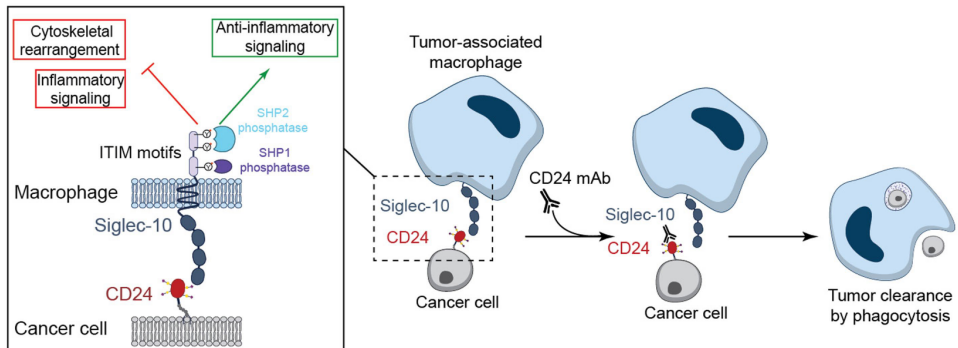


Figure 8. CD24 blocking leads to increased phagocytosis of tumor cells by TAMs

Binding of CD24 to Siglec10 leads to anti-inflammatory cytokine expression and phagocytosis blockade via inhibition of cytoskeletal rearrangement in TAMs. These changes are mediated by phosphatases SHP1/SHP2 which are linked to the intracytoplasmic domain of Siglec10. When the CD24/Siglec10 bond is disrupted, e.g. by monoclonal antibodies as illustrated here, TAMs trigger an inflammatory response which leads to tumor cell engulfment. Other disruptive modalities have been investigated, including CD24 and Siglec10 modulation by MTX in our experiments. Adapted by permission from Springer Nature, Nature, Barkal et al. "CD24 signalling through macrophage Siglec-10 is a target for cancer immunotherapy" © 2019

Ethical considerations of malignant brain tumor research

The final chapter aims to reflect on the various ethical aspects of doing research on malignant brain tumors, namely in the projects listed in the next section of this thesis. We use cell cultures and animal studies as the backbone of our research, together with patient-derived cells. Without using material from patient biopsies, we risk deriving results not applicable to actual human cancer cells as standardized cell lines don't represent primary cancer cells in many characteristics. After prolonged propagation *in vitro* (exact passage counts are unknown), cancer cells change the gene expression [333]. A concrete example is the disappearance of the distinct granular pattern of CD24 expression in 2D cultures of MB cells. With such alterations, we risk missing important targets for experimental treatment.

As for animal studies, we could expose human patients to risk if we tried new substances or routes of administration on them directly, expecting the same results extrapolated from the artificial environment of cell cultures. Once we agree that animal studies are a necessary middle step between *in vitro* research and human trials, then we must balance the ethical principle of reduction with the required number of animals to obtain an acceptable statistical power. If we reduced the number of animals too low, we again risk putting human patients in danger by assuming false positive or false negative results. Moreover, all the animals already used in such an underpowered trial would be wasted. We are required to have a strictly defined ethical permit on all our animal experiments. This is done to limit the suffering of research animals and optimize their use.

In order to use cells that we obtained directly from a human tumor sample and to be better able to validate our results, an informed consent is necessary from the patients or their guardians. Patients who donate their tissue samples to our research are in a very complicated social and private situation as they are seriously ill. This may preclude some of their rational judgements and render them vulnerable to perceived pressure to comply with the researcher's wish. The situation could be exacerbated if the person obtaining their informed consent was a member of their healthcare team.

The risk can be mitigated by explicitly stating and comprehensively explaining that non-participation in research won't in any way affect the patient's treatment or their handling by the healthcare staff. The person who obtains the informed consent must be a research nurse or another physician, but not the patient's treating physician. Efforts must also be made to clarify the purpose of the study in question. The person who administers the consent form needs to explain the benefits and possible harms caused by participation. For instance, the character of most preclinical studies is such that the tissue donors do not obtain any benefit by participating. Yet, their decision to do so provides a knowledge base used to help others in the similar situation in the future. For many patients with serious diseases, this is enough

motivation to join in. Nevertheless, it is necessary to explain this fully so as not to give rise to false hope.

Consent forms for the initial phases of clinical trials need to be structured similarly. Here, the primary endpoints are efficacy, feasibility and safety of the new treatment and its efficacy is followed secondarily. Naturally, this cannot compromise the safety of patients who might take inferior therapy. However, the exact dosing and other aspects would be fine-tuned in later stages. Patients who are offered to participate in the early phases must be made aware of this.

Needless to say, it is ethically more challenging to justify experimental treatment on children. This population is even more vulnerable than sick adults due to their age-proportionate understanding of their condition and inability to authorize their own medical decisions. Achievements of the past resulted in some survival in MB patients, so that the diagnosis no longer equals a death sentence. This situation, though, makes ethical review bodies wary of certifying more research, with concerns to compromise the safety of children. Once clinical trials are underway, obtaining informed consent must be approached carefully with the parents. Our team collects malignant brain tumor samples from children operated at the Neurosurgical Department of Skåne University Hospital in Lund. The samples are archived in our own biobank, as well as sent into the national biobank of the Children Cancer Foundation in Sweden. This unique resource has been pivotal in our research in the past and will be used in future projects to find optimal treatment of MB.

One of the main obstacles we face together with all researchers who use non-human research subjects is the limited translatability of our findings, i.e., only a minority of promising results obtained from preclinical studies is then successful in addressing the problems of humans. This is due to the artificial environment in the labs that is rarely able to sufficiently mimic the complex interactions within the human body. With that in mind, new model systems emphasizing the role of the TME and cancer cells grown in their natural milieu are developed.

Aims of the thesis

The overarching aim was to explore local and macrophage-centered therapy of malignant brain tumors. Individual studies were performed to address the following aims:

Publication I

To explore alternative ways of GBM therapy delivery, namely intratumoral CED of TMZ in order to decrease systemic dose-limiting side effects, we combined it with deactivated whole-cell tumor vaccine in mice. We wanted to study the effect on survival and the tumor immune microenvironment. We hypothesized a benefit in overall survival, immunological memory, and favorable immune cell type infiltration *in vivo* of two murine glioblastoma models

Publication II

To investigate alternative agents that could enhance the function of macrophages in the GBM microenvironment, we studied AF16, an active peptidic moiety of the AF protein, as adjuvant to TMZ in GBM treatment. We focused on the effect on survival and immune cell infiltration *in vivo*, as well as the secretome of macrophages and GBM cells *in vitro*.

Publication III

Translation of preclinical results to the clinical practice to improve treatment protocols of patients is an essential and integral part of the research process. We performed a Phase I clinical study of an AF formulation that can be taken orally, Salovum on patients with newly diagnosed GBM. The goal was to assess feasibility and safety of an intense Salovum regimen adjuvant to the traditional TMZ and radiation protocol.

Publication IV

Due to the major immune cell type infiltrating GBM and MB being macrophages, the original immunotherapy targets may not be optimally effective in this setting. Newly discovered antiphagocytic signals begin to move to the forefront of immune checkpoint inhibition. One of these signaling molecular pairs, CD24 with its receptor Siglec-10 have not been examined in brain tumors. By modulating the CD24 and Siglec-10 interaction, we aim to investigate the effect of these molecules on the GBM and MB microenvironment *in vitro* and *in vivo*.

Results

Publication I

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

In this study, we performed a series of experiments to elucidate the effects of intratumorally administered TMZ in conjunction with radiation-inactivated tumor cells injected subcutaneously in mice. This mode of therapy was investigated in two orthotopically injected murine malignant glioma cell lines, GL261 and KR158. GL261 is a widely used model of murine glioblastoma, however the cells are more immunogenic and contain a higher mutational load than primary human glioblastoma. KR158 grows more aggressively, and the cells are resistant to most means of therapy. Mice bearing the GL261 glioblastomas that were treated either with intratumoral CED of 180 μ g of TMZ, subcutaneous tumor vaccine or a combination of intratumoral CED of 180 μ g of TMZ and subcutaneous tumor vaccine had all significantly longer overall survival compared to untreated mice. The median survival of untreated mice was 39 days and 0/20 survived, compared with median survival of 49 days and 3/20 survivors after tumor vaccine, 64 days and 9/20 survivors after TMZ and undetermined median survival and 15/16 survivors after the combination treatment (median survival cannot be calculated when more than half of the subjects survive past the end of the experiment). This proves a synergistic effect of the combination treatment, which was significantly better than the effects of either monotherapy alone or combined. To show that implantation of brain catheters and pumps itself does not induce treatment effect by way of e.g., local inflammation, we implanted pumps loaded with saline into 4 mice without further therapy. All these mice succumbed to the tumor, not significantly later than mice without pumps.

The same treatment plan was repeated with mice bearing the KR158 gliomas. We confirmed the previously described radio- and chemoresistance of this cell line, where untreated mice had median survival 23 days and 0/16 mice survived. Monotherapy of tumor vaccines prolonged median survival to 38 days with 1/16 survival and monotherapy of intratumoral TMZ prolonged median survival to 25 days while curing 0/16 animals. The combination of intratumoral TMZ and tumor vaccines prolonged median survival to 39 days and cured 1/16 animals. No synergistic effect was recorded with TMZ and tumor vaccines. To summarize, the

effect of the TMZ part of the combination treatment was markedly higher in the GL261 model than in the KR158 model, where only the tumor-vaccine part of the combination was responsible for the treatment effect. TMZ prolonged survival in the KR158 model but the two parts did not synergize in their effects like in the GL261 model.

In a separate experiment, to prove that CED with subcutaneous pumps is superior to single intratumoral injection of TMZ, we administered it as a single injection bolus inside the GL261 tumor bulk to mice in decreasing doses. The bolus dose comparable to the cumulative CED dose, 175 μ g of TMZ, caused lethal toxic effects demonstrating as seizures and apnea in 2/6 mice while curing 1/6 mice. The next dose we tested, 60 μ g of TMZ, caused lethal toxicity in 1/12 mice while curing 3/12 individuals. Furthermore, 12.5 μ g of TMZ did not induce toxic effects and cured 3/12 animals while the lowest tested dose, 2.5 μ g of TMZ had neither toxic nor curative effects. Solely the 60 μ g dose showed statistically significant prolongation of overall survival, the other doses demonstrated only potential trends.

To elucidate the creation of immunological memory in GL261 survivors, we injected GL261 tumor cells into the contralateral hemisphere of mice that survived 100 days from the initial tumor inoculation (from groups CED TMZ, CED TMZ + tumor vaccines). 5/5 and 9/10 mice previously treated with the combination of CED TMZ and tumor vaccines and CED TMZ monotherapy survived the rechallenge, respectively. This suggests an important role of the immune system in eliminating GL261 tumors treated with local TMZ. To verify this hypothesis, we treated the severely immunocompromised mouse strain NOD/Scid bearing GL261 orthotopic tumors with CED pumps containing 180 μ g of TMZ. Of note, no NOD/Scid mice survived despite treatment effective in the immunocompetent strain.

A separate *in vivo* experiment was set up with the two murine cell lines and a modified treatment protocol. Now, treatment with TMZ and tumor vaccines was postponed, allowing for tumor growth in order to obtain enough tissue to analyze. The purpose of the experiment was to study the tumor size and histological characteristics from the standpoint of immune infiltration of the TME. To obtain comparable tumor samples, the whole cohort of mice was sacrificed as soon as the first individual showed symptoms of tumor growth. H&E staining was used to determine the tumor size and we observed the following: in the GL261 model, all means of therapy led to decreased tumor size compared to untreated mice. In the KR158 model, only the combination of intratumoral TMZ and tumor vaccines decreased the tumor size compared to untreated mice, TMZ or tumor vaccine monotherapies.

Using brain and tumor tissue from this experiment, we stained tumor/brain sections of all mice with antibodies against CD4 (helper T cells), CD8 (cytotoxic T cells) and F4/80 (macrophages) to describe immune cell-related changes *in situ*. We saw that, in the GL261 model, all means of therapy increased the CD4+ and CD8+ cell

infiltration in the tumors while only the combination of CED TMZ and tumor vaccines increased the tumor-infiltrating F4/80+ cells. In the KR158 model, only the tumor vaccines and combination therapy lead to an increase of CD4+ and CD8+ tumor-infiltrating T cells, not the CED TMZ monotherapy. However, intertumoral F4/80+ cells were decreased by the combination therapy and tumor vaccine monotherapy compared to the CED TMZ monotherapy. These results mirrored the survival experiment where, in the KR158 model, it was the tumor vaccine part which carried the most beneficial effect on survival.

Publication II

Intratumoral administration of the antisecretory peptide AF16 cures murine gliomas and modulates macrophage functions

In order to investigate potential new agents for non-conventional immunotherapy, we tested the 16-amino acid-long active core of the antisecretory factor protein, AF16 as part of glioblastoma treatment. Firstly, we established an *in vivo* study of OS in GL261-bearing mice treated with CED of intratumoral TMZ 180µg alone, intratumoral AF16 300µg alone, and the two agents mixed together. In the immunocompetent C57BL/6 mouse strain, we observed the survival proportions of untreated controls 0/16, AF16 monotherapy 3/16, TMZ monotherapy 7/16 and TMZ+AF16 12/16. Furthermore, the combination therapy did not give significantly higher OS than TMZ monotherapy, likely due early deaths and small sample size.

We used the same treatment setup also in the immunocompromised NOD/Scid mice, which lack T and B cells, as well as NK cells. In this model, no treatment modality produced cure. Both AF16 monotherapy and TMZ+AF16 combination significantly prolonged OS compared to untreated controls, however. The combination therapy also produced significantly higher OS than TMZ monotherapy.

Similarly to the previous project, we carried out a separate *in vivo* histological study to evaluate the tumor size and TME. The monotherapy of TMZ and combination of AF16+TMZ shrank the tumor size of mice. The addition of AF16 to TMZ also abolished the increase of tumor-infiltrating F4/80+ macrophages and T cells caused by TMZ monotherapy. AF16+TMZ also increased galectin-3 and pNKCC1 levels in the tumor. The staining for COX2, MHCII, CD11c and CD206 showed no significant differences.

We further worked *in vitro* with macrophages as our immune cell type of interest. We cultured the murine M0-like macrophage cell line RAW264.7 with AF6

2000 μ g/ml and analyzed the supernatants from cell cultures. Afterwards, we compared the treated samples with untreated naïve M0 cells, as well as differentiated M1 and M2 macrophages. AF16 induced in M0 macrophages the production of inflammatory factor profile similar to the M1 macrophages (including IL1 β , IL2, IL6, TNF α , IL12p70, IL10 and KC/GRO) at levels even higher than the M1 differentiation process. Next, we analyzed supernatants from cultures of primary human M0 macrophages and THP1-derived M0 macrophages that were exposed to AF16 1 μ g/ml and 100 μ g/ml solutions for 24 hours. We compared them to betamethasone-treated M0 macrophages, as well as differentiated M1 and M2 macrophages and we saw an upregulation of several proinflammatory factors after AF16 treatment. There were also some differences in results from the primary human macrophage and THP1-derived macrophage systems.

To elucidate the effect of AF16 on the GBM cells themselves *in vitro*, we exposed primary human GBM cells to AF16 20 μ g/ml as primary treatment and compared the supernatants with untreated cells, as well as GBM cells treated with TMZ and 20Gy radiation. AF16 increased the expression of a wide variety of chemoattractant and proinflammatory signals, while decreasing several factors associated with GBM progression. The landscape of immune modulation in GBM cells *in vitro* caused by AF16 proved to be complex with several signaling pathways involved.

Publication III

Antisecretory factor is safe to use as add-on treatment for newly diagnosed glioblastoma

Salovum is a concentrated egg yolk preparation, which contains high levels of antisecretory factor. It can be dissolved in liquids and used orally. We analyzed the safety and feasibility of this treatment adjuvant to TMZ and radiation in patients with newly diagnosed GBM in a Phase I clinical trial. Out of 10 recruited patients, 2 were excluded on the basis of withdrawn consent and death, respectively. 2/8 patients were not able to complete the full course of Salovum treatment protocol due to nausea caused by the amount of egg yolk powder consumption and pulmonary embolism, leading to TMZ and Salovum discontinuation.

Median age of the study patients was 57.5 years, and all had the preoperative ECOG score ≥ 1 . Three patients were deemed to have undergone gross total resection, while 5 had residual tumor documented. All tumors were IDH-wild type and 2/8 showed MGMT promoter methylation.

At the last follow-up, 3/8 patients were alive with one free from recurrence. Median OS was 23.0 months and median PFS was 10.2 months. The retrospectively selected, 28 matched control patients recorded the median age of 55 years, median

OS 14.8 months and median PFS 9.4 months. There was no significant difference in the OS or PFS between the control and treatment patients.

When it comes to another studied parameter – the potential for corticosteroid dose reduction after Salovum, 3/8 study patients could be weaned off betamethasone completely with further 4/8 patients could decrease the betamethasone dose in a sustained manner after an initial short dose increase. The remaining patient raised the betamethasone dose and could not taper during treatment. At the end of the trial, only 1/8 patients used > 2.5mg daily betamethasone.

Due to the potential of extra energy, and more specifically, cholesterol intake from the egg yolk-based Salovum, which could upset the serum lipid balance, study patients were followed up on serum cholesterol levels. A paired Wilcoxon signed-rank test showed no significant difference.

In summary, Salovum proved to be a safe adjuvant agent in GBM patients without any serious recorded side effects that could prevent a successful completion of the treatment regimen. The subjectively unpleasant taste could be better masked by mixing Salovum with flavored drinks or adjusting the properties of the formulation. A decrease in Salovum dose could also be considered. The positive trends in OS, PFS and betamethasone daily dose reduction were not the primary endpoints of this trial and will have to be confirmed in a larger Phase II and III trials.

Publication IV

Mitoxantrone induced immunomodulation of CD24 - implications for targeted treatment of malignant brain tumors

In the fourth project, we focused on exploring new targets for macrophage-centered immunotherapy of malignant brain tumors. We mapped the in silico gene expression of CD24 and Siglec-10 across 12 MB subgroups and 3 GBM subtypes. We show that there is CD24 gene expression heterogeneity in all MB subgroups, especially in the SHH and Gr3 MB. The SHH MB subgroups also display the highest levels of CD24, while the WNT subgroups showed the lowest. Similar, yet inverted heterogeneity exists in the expression of Siglec10, where WNT subgroups expressed the highest and Gr3 the lowest levels. As for the 3 GBM subtypes, CD24 was differentially expressed, too, with the classical subtype having the lowest and proneural the highest levels. Siglec10 was most expressed in the mesenchymal GBM subtype with the classical and proneural ones displaying no significant difference.

Next, we searched for gene expression of the proliferation marker Ki-67 in the same databases, and we plotted it with the expression of CD24. We discovered that high

expression of CD24 was associated with high Ki-67 in the SHH, Gr3 and Gr4 MB but not WNT MB. Moreover, high CD24 was linked with high Ki-67 in all unsorted GBM samples but the statistical significance was lost when divided into subtypes, tentatively due to too few samples in each subtype category.

Furthermore, we explored the *in vitro* cytotoxic potency of a known immunomodulatory cytostatic agent MTX in the murine GBM cell line SB28, as well as primary human GBM and MB cells. The results show that the IC50 dose could be reached in all three cell types after 24h, 48h and 72h, albeit with different sensitivity. At all timepoints, primary human MB cells were the most sensitive to MTX, with >90% of cells dead after MTX 0.5 μ M. Next, the SB28 cells were moderately sensitive to MTX at lower doses but even our highest tested dose of MTX 5 μ M did not cause more than 90% of the cells. This was not the case with primary human GBM cells, which died at >90% after high MTX doses.

The ability of MTX to cause cytoplasmic translocation of HMGB1 from the nucleus of the SB28, primary human GBM and MB cells *in vitro* was also investigated as a marker of immunogenic cell death. We show that this translocation is dose dependent with the clearest results after incubation with MTX 2 μ M and it was present in all three cell types. Moreover, the human cells manifested a granular or fine vesicular pattern of cytoplasmic HMGB1 expression post MTX.

Afterwards, we focused on the changes of CD24 in the same cell types after MTX exposure *in vitro*. The cells express different baseline levels of CD24 with the SB28 and human MB cells being highly positive for CD24 and human GBM cells less so. All cell types, however, displayed a decrease of CD24 with MTX 2 μ M after 72h compared to untreated cells.

In order to confirm the effect of MTX *in vivo*, we used the same setup as previously described and treated SB28-glioma-bearing mice with CED MTX in two studies. In the survival study, CED MTX cured 2/22 individuals, but the effect did not reach statistical significance. Of those 2 survivors, 1 animal survived tumor re-challenge in the opposite hemisphere, suggestive of some degree of immunological memory generation.

In the histological study, we harvested brains from the whole cohort of mice sacrificed on the same day, sectioned the material and stained for CD24 and Siglec-10. We discovered a decrease of both molecules in the tumor microenvironment after local MTX treatment.

All results described in this section are comparisons with untreated controls and they showed statistical significance unless stated otherwise.

Discussion

In this thesis, I am investigating four main projects that are linked by the overarching aim to validate a new approach to immunotherapy of malignant brain tumors – to modulate the local tumor-immune interactions so that the main immune cell type present in the TME, macrophages can better destroy tumor cells. To this end, we used local cytostatic agent delivery through mini-osmotic pumps into the tumor, investigated a novel immunomodulatory agent AF16 in preclinical models, established a Phase I clinical trial with AF-containing preparation Salovum given adjuvantly to newly diagnosed GBM patients, and investigated the modulation of CD24/Siglec10, a pathway newly described in antiphagocytic signaling.

Others have used local, intratumoral drug delivery in a range of solid tumors with promising results. Brain tumors, most often GBM, were included in several of those studies. In our projects, we utilized local drug administration in the form of CED through mini-osmotic pumps coupled to a catheter. This route circumvents the blood-brain barrier that normally excludes a wide spectrum of chemotherapeutics from being used in malignant brain tumor patients due to their impassable electrochemical characteristics. MTX, for instance, is not routinely used in brain cancer chemotherapy indications as it is deemed to reach insufficient local concentrations. *In vitro*, however, it has been shown to be highly toxic to several malignant brain tumor cell lines, even more so than TMZ.

An alternative to CED as a method to deliver chemotherapy locally could be single or repeated injections of the agent. Unlike CED, there is no equipment implanted subcutaneously, which decreases the potential for wound/pump assembly infection besides lowering costs. These advantages would be more relevant in a clinical setting, as mice in our experiments do not suffer from infectious complications. On the other hand, repeated injections carry the risks linked to multiple general anesthesia episodes and minor, yet significant surgical procedures (i.e., bleeding). According to our results, it was not possible to administer the whole CED treatment dose as a single injection safely due to treatment-related side effects, e.g., seizures and apnea. The following necessary dose reduction to safe levels resulted in a marked decrease in treatment response.

The effectiveness of local CED of cytostatic agents logically depends on the specific agent and in the case of preclinical studies, as well as on the cancer model. In the earlier projects, we used the treatment with TMZ and other agents of the orthotopic

murine GL261 GBM. This cell line is one of the oldest murine malignant gliomas and has been investigated in hundreds of experiments, however, it doesn't share the typical immune "coldness" of primary human GBM cells. On the contrary, GL261 cells express high levels of MHCI and MHCII, along other immunomodulatory receptors. This might contribute to the relative success with treating GL261 gliomas compared to human GBM.

In our other projects, we used a newer murine GBM cell line, SB28. It was created by targeted genetic manipulation of selected oncogenes and it is therefore more similar to the low mutational burden of human GBM with its low immunogenicity. Consequently, the SB28 model proved to be more resistant to therapy with CED of MTX in our survival experiments.

Besides just survival, another outcome that helps us understand the potential of a therapeutic agent in the treatment of GBM is its modulation of tumor-immune interactions in the TME. It has been accepted as robust fact that a significant part of the effect of any given therapy on cancer is moderated by the immune system, not only by the cytotoxic properties of the treatment *per se*. This can be evidenced by the failure of most cancer treatments in severely immunocompromised human patients or animals. We observed this phenomenon when CED of TMZ at standard doses failed to cure any immunocompromised mice, despite curing 45% of GL261-bearing mice.

Adjuvant immunomodulating interventions can, by the above logic, improve the outcomes of classic therapy agents, i.e., TMZ in the case of GBM. When subcutaneous vaccinations by radiation-inactivated tumor cells were added to the CED of TMZ, the mice reached 93% survival with significantly smaller tumors. Another such modulating agent, AF16, produced 75% survival when combined in CED with TMZ while also diminishing the tumor size. Even certain conventional cytostatic agents have been described as immunogenic cell death inducers, among others MTX. According to the ICD concept, tumor cells after exposure to certain cytostatic agents are capable of revealing signaling molecules that are readily detected by cells of the immune system, thus boosting the anti-tumor response.

The immunohistological analysis of the different immune surface markers, as well as other molecules linked to tumor-associated inflammation, enabled us to study the changes caused by these novel interventions. We found that the CED of TMZ into the GL261 gliomas led to an increase in tumor-infiltrating helper and cytotoxic T cells, especially when combined with tumor vaccines. This combination also led to an increase in tumor-infiltrating F4/80+ macrophages.

The combination of CED of TMZ and AF16 caused a decrease in tumor-infiltrating F4/80+ macrophages and CD8+ T cells, possibly due to activation-induced cell death, which was described in these cells after a strong proinflammatory stimulus.

Our other staining for CD11c, CD206, MHCII or COX2 didn't show any statistically significant changes. Moreover, AF16 treatment of naïve M0 macrophages *in vitro* triggered secretion of a profile of proinflammatory cytokines similar to the M1 macrophage differentiation. We also saw an increased expression of intratumoral galectin-3 and phosphorylated NKCC-1, molecules that are linked to cell damage and macrophage activation, respectively. Taken together, AF16 treatment seems to activate tumor-associated M0 macrophages; when combined with TMZ it improves survival of GBM-bearing mice.

Another pair of cell surface molecules that have been shown to represent an emerging target in macrophage-centered immunotherapy is CD24 on tumor cells and Siglec10 on macrophages. Signaling from the CD24–Siglec10 binding results in decreased phagocytosis of tumor cells by tumor-associated macrophages and blockade of this interaction results in augmented tumor cell phagocytosis. We discovered that GBM cells treated with MTX *in vitro* decrease their expression of CD24 and while CED of MTX did not show a statistically significant benefit to survival in SB28-bearing mice, animals treated with MTX expressed less intratumoral CD24 and Siglec10. This suggests that CED of MTX could lead to a favorable modulation of antiphagocytic signals in the TME. With further research into the exact mechanisms behind this effect, optimal MTX dosing and other adjuvant agent combinations warranted, local intratumoral administration of MTX into malignant brain tumors could be a viable treatment strategy.

Ultimately, we want to shift from treating GBM-bearing mice to human patients. The project that went the farthest in this regard is the usage of antisecretory factor derivatives as adjuncts in newly diagnosed GBM. We managed to establish a Phase I clinical trial with 8 GBM patients that were treated with Salovum (an egg yolk-based preparation containing high levels of AF, currently classified as “food for special medical purposes” by Swedish and European regulators) at the Neurosurgery Department of Skåne University Hospital in Lund. Two patients had to be recalled from the study due to one severely decreased mental status and one case of pulmonary embolism precluding further therapy. The pulmonary embolism is not registered as a side effect of Salovum therapy. In the remaining 6 patients, oral Salovum mixed with a drink was added to the Stupp protocol of post-surgical TMZ and irradiation and patients were monitored with regards to feasibility and safety of Salovum treatment.

High doses of Salovum were chosen in this indication in an intensive treatment protocol. No treatment-related side effects were observed in the patient population, in agreement with previous studies. Some patients reported a somewhat unfavorable taste of the Salovum drink, an objection that should be addressed in future trials. In the treated population, overall-, median and progression-free survival were prolonged compared to historical controls, however, without statistical significance,

due to too small sample size. Corticosteroid dosing could be decreased to a large extent or fully tapered off in majority of the treated patients and blood cholesterol levels were within normal limits after the extra lipid burden. This was a pilot study utilizing Salovum as a substance completely new to cancer treatment, with all associated limitations, including the absence of blinding and placebo controls. Importantly, Salovum was deemed safe and feasible as an adjunct to standard GBM treatment protocols in humans.

Conclusions and Future perspectives

The original hypothesis supported by results from the four projects lead us to the following conclusions. Like several PhD students before me in our group, as well as other researchers have proven, intratumoral administration of cytostatic agents via CED is effective in curing GBM in a proportion of mice. Furthermore, CED of TMZ via mini-osmotic pumps is safer and more potent than repeated injections of the drug. The intratumoral route also generated favorable immunological changes in the TME that resulted in subsequent tumor rejection after a rechallenge without further treatment.

Furthermore, besides diminishing tumor size, CED of TMZ boosted by inactivated tumor cell vaccines altered the immune cell infiltration of the GBM to a more favorable composition. With more time and resources, I would like to perform detailed characterization of the immune cell types that were altered by the treatment, e.g., flow cytometry-based sorting of tumor tissue and surface markers of tumor-associated macrophages (M1, M2, MDSC panels) and lymphocytes, functional tests of T cell reactivity and antigen-presenting capacity of APCs.

Next, I would like to further investigate the TME from a genomic and proteomic perspective to elucidate which pathways are overexpressed and which are suppressed by local administration of TMZ, especially compared to the systemic route. The ultimate ambition would be to initiate pilot clinical trials with human patients; however, I am aware of the numerous practical obstacles in implementing local therapy delivery to malignant brain tumors. Implanting a sensitive assembly of a pump and brain catheter in patients carries additional risks of wound or assembly infection, and catheter misplacement or disconnection, which could impede the treatment success and increase morbidity, among others. Different means of local therapy delivery can be employed, such as slow-release wafers or coagulant foam containing cytostatic agents.

That said, I advocate for more Phase I clinical trials using local intratumoral therapy administration as part of continuous treatment protocol improvement. There are strong advantages, among others the blood-brain barrier circumvention, which enables investigators to utilize substances otherwise inaccessible to the brain. It has become clear to me that although TMZ has improved the prognosis of malignant brain tumor patients, it is not the best agent available, nor should it be used in monotherapy. CED could introduce new treatment options with significantly lower

systemic adverse effects. More about CED in GBM can be found in the dissertation of Julio Enríquez Pérez, a previous PhD student in our group.

Until suitable solutions are available, I see the implementation of other means of intratumoral cytostatic delivery as more viable than CED. A promising strategy that recently begun pilot testing in our team is admixing cytostatic agents into fibrin preparations that can then be jet-injected into surgical cavities with tumor micro-residues during the primary surgery. The initial stages of this project include investigating the stability, chemical and physical properties of the cytostatic-fibrin mixture, and basic pharmacokinetics *in situ*.

The most abundant cell type in the majority of investigated solid tumors are TAMs, in some cases even more numerous than tumor cells. It is not surprising that such a large cell population is important in the TME and the roll of TAMs in modulating tumor growth has indeed been clearly documented. Macrophages in their physiological state display a variety of powerful mechanisms that could be used in tumor elimination, e.g. tumor cells phagocytosis, recognition of tumor neo-antigens and antigen presentation to effector leukocytes. Soluble and surface factors expressed by tumor cells, however, retain the TAM in a state of continuous low-grade inflammation associated with the M2-like macrophage phenotype, which promotes tumor growth.

AF16, a 16-amino-acid-long segment of the AF protein, caused M0 macrophages in culture to secrete a range of proinflammatory cytokines similar to the M1 differentiation. It also induced GBM cells to secrete immunomodulatory factors to a much higher degree than irradiation. It cured GBM-bearing mice even in severe immunocompromise with only macrophages as a significant effector immune cell population. All these results, together with other research show that AF16 decreases intratumoral and intracranial pressure and increases uptake of cytostatic agents, make AF-based substances an interesting immunomodulatory adjuvant to current malignant brain tumor protocols.

If I could expand this project in the future, I would analyze the secretome after AF16 treatment from more primary macrophages and also investigate the effect on M1 and M2 cells, not only M0. Some such experiments have already been performed with interesting results that we didn't have time to publish. I would be interested in the specific genomic and proteomic changes that occur in the TME in response to AF16. These could be explored with the help of the laser-assisted microscopic sample extraction or intratumoral catheters connected to microdialysis.

I didn't have enough time to combine AF16 treatment with modes of therapy other than TMZ. Since one of the findings from Publication I was an increase of TAMs after CED of TMZ co-administered with tumor vaccine, it would be interesting to see whether AF16 could trigger the same proinflammatory secretion in these cells as well. Tentatively, the treatment response would be even higher. Next, a combination of CED of AF16 and another cytostatic agent, for instance MTX as a proven ICD inducer could potentiate the macrophage stimulation and ensuing T cell priming. Additionally, according to our ethical permit formulation, only 3-day CED pumps could be implanted into animals. An amendment to the permit should be submitted, where longer AF16 (and other agent) administration could be used.

Moreover, even though the scientific community has known about AF for more than 30 years, its mechanism of action is entirely unknown. The protein was found to be essential for successful embryonic development and its homologue is a component of a proteasome subunit. Whether the antisecretory and immunomodulatory properties on immune cells are mediated through proteasome activation pathways or some other, not yet described mechanisms remains to be investigated. Experiments with the effects of AF16 and proteasome inhibitors on the cell functions in e.g. macrophage cell cultures could shed more light into the matter, as would a detailed genomic analysis and characterization of the receptor through which AF16 elicits its action.

Under current circumstances, the oral administration is the most feasible in the context of AF-based adjuvants in the treatment of malignant brain tumors. AF16 cannot be administered in this way due to the destructive environment in the stomach and small intestine, therefore other AF formulations must be used. The substance that has been investigated the most in this regard is an AF-enriched egg yolk-based powder sold under the commercial name Salovum as a nutritional supplement. It is a source of AF that we used in the pilot Phase I trial on GBM patients to assess its safety and feasibility. Salovum has been used before by members of our group in clinical trials of traumatic brain injury as an agent to reduce intracranial pressure.

Perhaps without surprise, we found that Salovum was safe and feasible to add to the standard GBM treatment protocol as defined by Stupp *et al.* in 2014. These were the two primary endpoints of the study. All other results, need to be validated in the Phase II trial, which has already begun recruiting. Our ultimate goal is to prove that Salovum decreases the corticosteroid requirement in GBM patients and improves their OS and PFS. This would mean that this cheap, well-tolerated substance could be added to TMZ and radiation as current standard treatment for GBM.

The design of the Phase I trial included in my thesis counted with 10 patients being recruited. Two patients were excluded and two more could not complete the full course of therapy. The control patient population was chosen retrospectively from patients treated at the Department historically. Moreover, due to the nature of Salovum treatment, the study was not blinded. While this design was powerful enough to answer our primary objectives and endpoints for the particular study and is not exceptional with regards to other Phase I trials, I believe that the recruitment and design of the Phase II trial need to be adjusted so that a sufficient amount of patients and controls with proper blinding is introduced.

Performing advanced phases of clinical trials of new agents as a single institution is very difficult, especially when the disease in question is as rare as GBM (which, despite being the most common malignant brain tumor in adults is still comparatively infrequent). That is why the Phase II Salovum trial is designed as a multi-center study to help pool the resources and overcome the obstacles. After working with AF16 and Salovum for several years, I am aware of their somewhat irregular nature and effects. However, I remain optimistic about their future role in brain cancer treatment. They constitute a promising representative of macrophage-based immunomodulatory agents.

Another molecule that has been discovered long ago but gained awareness as a potentially new key player in the fight against cancer is CD24. After many years of being at the side line of research, close attention is now paid to the role of CD24 in tumor cell evasion of the immune-mediated destruction, coupled to the Siglec10 receptor on TAMs.

As usual due to the relative difficulty of establishing faithful models in this field, brain tumors were not included in the initial analysis of CD24 in cancer. This presented an opportunity for us to characterize the CD24 and Siglec10 landscape in GBM and MB. We strove to understand whether CD24 could be a potential candidate for targeted therapy in these brain tumor types, especially in the light of the fact that both GBM and MB have been divided into subtypes and subgroups with distinct clinical course and prognosis.

We managed to show that CD24 and Siglec-10 are expressed differently across the different subgroups of GBM and MB which lays the groundwork for further personalizing the treatment of patients suffering from these. Simply put, it was also proven that the more CD24 a malignant brain tumor expresses, the more aggressively it grows, as measured by Ki67. Moreover, CD24 can also be modulated by treatment agents, in our experiments by MTX. Such modulation by an already

approved drug means that future clinical trials could be started in an accelerated regime.

The CD24 project resulted in a manuscript that is conceptually ready for submission. Some experiments will have to be expanded in the near future. A larger dataset of primary human GBM and MB will be added to the *in vitro* analysis of CD24 expression, as well as human tumor tissue samples. More animals will be included in the survival and histological experiments with CED of MTX. The modulation of Siglec10+ macrophages will be investigated in more details, especially the exact association with CD24+ tumor cells.

More CD24-modulatory strategies will be explored, in particular monoclonal antibody blockage and genetic manipulation, e.g., gene editing to knock out CD24 and Siglec10 in GBM, MB and immune cells, respectively. AntiCD24 monoclonal antibody targeting will face the challenge of large intracellular CD24 stores present in MB and the SB28 murine GBM cell line, virtually inaccessible to the surface antibody blockade. As a research tool, gene editing offers excellent insight into the mechanisms of action of molecules and signalling pathways of interest, outweighing the relatively challenging technical aspect of the method. On the other hand, practical applicability of this methodology to a large number of patients affected by malignant brain tumors is low today and will likely remain so in the near future.

Overall, all of the above experiments (with exceptions) were performed on either GBM or MB and in the future, the complementary tumor type could be included, too.

Both GBM and MB are still extremely distressing and serious diagnoses that uproot the lives of patients and their families. Mechanisms behind cancer belong to those oldest and most evolutionarily conserved processes in living systems and therefore targeting them wholly effectively is unfortunately not possible. The brain, generally, is a fascinating organ that we shall never truly understand and conducting research on it presents its intrinsic obstacles.

Despite all these challenges, I remain optimistic regarding the enormous worldwide effort to move from heavy-handed therapeutic strategies to advantageously utilize the extraordinary potential of the immune system.

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