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Combined chemotherapy and immunotherapy against experimental malignant brain tumors

Fritzell, Sara

2013

Link to publication

Citation for published version (APA):
Fritzell, S. (2013). *Combined chemotherapy and immunotherapy against experimental malignant brain tumors*. [Doctoral Thesis (compilation), Neurosurgery]. Lund University, Faculty of Medicine, Neurosurgery.

Total number of authors:
1

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PO Box 117
221 00 Lund
+46 46-222 00 00

Combined chemotherapy and immunotherapy against experimental malignant brain tumors

Sara Fritzell

2013

AKADEMISK AVHANDLING

som med vederbörligt tillstånd från Medicinska fakulteten vid Lunds Universitet för avläggande av doktorsexamen i medicinsk vetenskap kommer att offentligen försvaras i Segerfalksalen, Wallenberg Neurocentrum, Lunds Universitet, Lund
lördag den 1 juni 2013 kl. 10.00

FAKULTETSOPPONENT

Assistant Professor Duane A. Mitchell, MD, PhD, Duke University, USA

LUNDS UNIVERSITET

Avdelningen för Neurokirurgi
Institutionen för Kliniska Vetenskaper i Lund,
Medicinska Fakulteten, Lunds Universitet

Organization

Division of Neurosurgery

Department of Clinical Sciences in Lund

Lund University

Lund, Sweden

Author(s) Sara Fritzell

Document name

DOCTORAL DISSERTATION

Date of issue

June 1, 2013

Sponsoring organization

Title and subtitle

Combined chemotherapy and immunotherapy against experimental malignant brain tumors

Abstract

Glioblastoma multiforme (GBM) is the most common and aggressive primary malignant brain tumor in adults. Despite standard treatment including surgery, radiotherapy and temozolomide (TMZ)-based chemotherapy, the prognosis for GBM patients is dismal, and there is a need for novel treatments. One possible therapeutic treatment modality presented here is immunotherapy either alone or combined with intratumoral TMZ.

In this doctoral thesis, I report enhanced cure of rats and mice with malignant brain tumors after peripheral immunizations using irradiated whole tumor cells transduced to produce different immunostimulatory cytokines such as interferon-gamma (IFNγ), interleukin-7 (IL-7) and granulocyte macrophage-colony stimulating factor (GM-CSF). In the N32 rat glioma model there is a synergistic therapeutic effect when combining immunization with IFNγ- and- IL-7-producing tumor cells and this coincides with enhanced systemic proliferation of $CD4^+$ and $CD8^+$ T cells, and an increase in the plasma levels of IFNγ, thereby strengthening the anti-tumor immune response. In addition, the synergistic therapeutic effect of immunization with irradiated GM-CSF-producing tumor cells and recombinant IFNγ in the GL261 mouse glioma model is mediated by both $CD4^+$ and $CD8^+$ T cells, and evokes a long-term memory response that protects against secondary tumors without any further treatment. Further I report that TMZ and cisplatin, two chemotherapeutic agents, could cure 45-41 % of GL261 tumor-bearing mice when delivered intratumorally using micro-osmotic pumps. Furthermore, when immunization with irradiated GM-CSF producing tumor cells is combined with intratumorally administered TMZ, the survival of tumor-bearing mice is synergistically enhanced, while systemic delivery of TMZ induces lymphopenia and abrogates the effect of the immunotherapy. Intratumoral TMZ decreases the number of immunosuppressive cells intratumorally, while sustaining the proliferation of peripherally activated T cells following immunotherapy. Intratumoral cisplatin however, does not boost the effect of the immunotherapy.

In conclusion, immunotherapy improves survival in experimental glioma models, and the therapeutic effect is enhanced by intratumoral TMZ treatment.

Key words: Cisplatin, Glioma, GM-CSF, IFNγ, IL7, Immunotherapy, Intratumoral chemotherapy, Temozolomide

Classification system and/or index terms (if any)

Supplementary bibliographical information

Language English

ISSN and key title 1652-8220

ISBN978-91-87449-34-5

Recipient's notes

Number of pages

Price

Security classification

Signature Sara Fritzell Date 2013-04-22

Combined chemotherapy and immunotherapy against experimental malignant brain tumors

Sara Fritzell

ACADEMIC DISSERTATION
Lund 2013

LUND
UNIVERSITY

Division of Neurosurgery
Department of Clinical Sciences in Lund
Faculty of Medicine, Lund University

Cover artwork by Frida Malmgren

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Lund University, Faculty of Medicine Doctoral Dissertation Series 2013:64
ISSN 1652-8220
ISBN 978-91-87449-34-5

Printed in Sweden by Media-Tryck, Lund University
Lund 2013

”I believe in God, only I spell it Nature”– architect Frank Lloyd Wright

”To be scientifically literate is to empower yourself to know when someone else is full of bullshit” – astrophysicist and science communicator Neil deGrasse Tyson

Till min stora familj

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ABSTRACT

Glioblastoma multiforme (GBM) is the most common and aggressive primary malignant brain tumor in adults. Despite standard treatment including surgery, radiotherapy and temozolomide (TMZ)-based chemotherapy, the prognosis for GBM patients is dismal, and there is a need for novel treatments. One possible therapeutic treatment modality presented here is immunotherapy, either alone or combined with intratumoral TMZ.

In this doctoral thesis, I report enhanced cure of rats and mice with malignant brain tumors after peripheral immunizations using irradiated whole tumor cells transduced to produce different immunostimulatory cytokines such as interferon-gamma (IFNγ), interleukin-7 (IL-7) and granulocyte macrophage-colony stimulating factor (GM-CSF). In the N32 rat glioma model there is a synergistic therapeutic effect when combining immunization with IFNγ- and- IL7-producing tumor cells and this coincides with enhanced systemic proliferation of $CD4^+$ and $CD8^+$ T cells, and an increase in the plasma levels of IFNγ, thereby strengthening the anti-tumor immune response. In addition the synergistic therapeutic effect of immunization with irradiated GM-CSF-producing tumor cells and recombinant IFNγ in the GL261 mouse glioma model is mediated by both CD4+ and CD8+ T cells, and evokes a long-term memory response that protects against secondary tumors without any further treatment.

Further I report that TMZ and cisplatin, two chemotherapeutic agents, could cure 41-45% of GL261 tumor-bearing mice when delivered intratumorally using micro-osmotic pumps. When immunization with irradiated GM-CSF-producing tumor cells is combined with intratumorally administered TMZ, the survival of tumor-bearing mice is synergistically enhanced, while systemic delivery of TMZ abrogates the effect of the immunotherapy. Cisplatin however, does not boost the effect of the immunotherapy.

In conclusion, immunotherapy improves survival in experimental glioma models, and the therapeutic effect is enhanced by intratumoral, but not systemic TMZ treatment.

ORIGINAL PAPERS

This thesis is based on the studies reported in the following papers:

Paper I IFNγ in combination with IL-7 enhances immunotherapy in two rat glioma models. **Sara Fritzell**, Sofia Eberstål, Emma Sandén, Edward Visse, Anna Darabi and Peter Siesjö.
Published in *Journal of Neuroimmunology*, 2013 Vol. 258 pp. 91-95.

Paper II Cure of established GL261 mouse gliomas after combined immunotherapy with GM-CSF and IFNγ is mediated by both $CD8^+$ and $CD4^+$ T-cells. Karin Enell Smith, **Sara Fritzell**, Wiaam Badn, Sofia Eberstål, Shorena Janelidze, Edward Visse, Anna Darabi and Peter Siesjö.
Published in *International Journal of Cancer*, 2009 Vol. 124 pp. 630–637.

Paper III Local intratumoral temozolomide synergizes with immunotherapy in a T cell dependent fashion. **Sara Fritzell**, Sofia Eberstål, Emma Sandén, Edward Visse, Anna Darabi and Peter Siesjö.
Manuscript resubmitted to *Cancer Immunology, Immunotherapy* (CIIM-D-13-00063).

Paper IV Intratumoral delivery of cisplatin is effective but does not boost immunotherapy in the GL261 mouse glioma model. **Sara Fritzell**, Sofia Eberstål, Emma Sandén, Edward Visse, Anna Darabi and Peter Siesjö.
Manuscript.

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PAPERS NOT INCLUDED IN THE THESIS

Inhibition of cyclooxygenase-2 enhances immunotherapy against experimental brain tumors. Sofia Eberstål, Wiaam Badn, **Sara Fritzell**, Magnus Esbjörnsson, Anna Darabi, Edward Visse and Peter Siesjö. *Cancer Immunology, Immunotherapy*, 2012 Vol. 61 (8) pp. 1191-9.

Immunizations with IFNγ secreting tumor cells can eliminate fully established and invasive rat gliomas. Shorena Janelidze, Daniel Bexell, Wiaam Badn, Anna Darabi, Karin-Enell Smith, **Sara Fritzell**, Salina Gunnarsson, Peter Milos, Johan Bengzon, Leif G Salford, Peter Siesjö and Edward Visse. *Journal of Immunotherapy*, 2009 Vol. 32 (6) pp. 593-601.

ABBREVIATIONS

APC	antigen presenting cell
APM	antigen presenting machinery
BBB	blood brain barrier
BCNU	bis-chloroethylnitrosourea
B7.H1	B7 homologue 1
CC	C-C chemokine
CD	cluster of differentiation
CED	convection enhanced delivery
CNS	central nervous system
COX	cyclooxygenase
CSF	cerebrospinal fluid
CT	chemotherapy
CTL	cytotoxic T lymphocyte
CTLA-4	CTL-associated antigen 4
DAMP	damaged associated molecular pattern
DC	dendritic cell
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
EGFRvIII	EGF receptor variant III
ENU	N-ethyl-N-nitrosourea
FoxP3	forkhead box P3
GBM	glioblastoma multiforme
GM-CSF	granulocyte macrophage-colony stimulating factor
HCMV	human cytomegalovirus
HLA	human leukocyte antigen
HMGB1	high-mobility-group box 1
IFN	interferon
IL	interleukin
i.c.	intracerebral
iNOS	inducible nitric oxide synthase
i.p.	intraperitoneal
IP-10	interferon-gamma-inducible protein of 10 KDa
i.v.	intravenous
KO	knock-out
KPS	Karnofsky Performance Scale

L	ligand
mAb	monoclonal antibody
MCA	methylcholanthrene
MDSC	myeloid derived suppressor cell
MGMT	O^6-methylguanine-DNA methyltransferase
MHC	major histocompatibility complex
MIG	monokine induced by interferon-gamma
MRI	magnetic resonance imaging
NO	nitric oxide
NK cell	natural killer cell
NKG2D	natural killer group 2 member D
NKT cell	natural killer T cell
PD-1	programmed death-1
PGE_2	prostaglandin E_2
PTEN	phosphatase and tensin homologue
R	receptor
RAG	recombination activating gene
Rb	retinoblastoma
ROS	reactive oxygen species
RT	radiotherapy
s.c.	subcutaneous
STAT	signal transducer and activator of transcription-4
TAM	tumor associated macrophage
T-bet	t-box expressed in T cells
TCR	T cell receptor
TGF	transforming growth factor
T_H cell	T helper cell
TNF	tumor necrosis factor
TMZ	temozolomide
Treg	T regulatory cell
VCAM-1	vascular cell adhesion molecule 1
VEGF	vascular endothelial growth factor
WT	wild type
WHO	World Health Organization

INTRODUCTION

Glioma

Glioma is the collective term for all brain tumors that originate from glial cells or glial precursor cells. Together they represent approximately 30% of all tumors occurring in the central nervous system (CNS) and 80% of all malignant brain tumors. They can grow throughout the brain but usually they occur in one of the four lobes of the brain, most commonly in the frontal lobe [1]. Gliomas are further divided into subcategories according to the cell type of which they originate from, or share histological phenotype with. The majority of all gliomas (75%) are astrocytic tumors (from astrocytes), but there are also oligodendroglial tumors (6%, from oligodendrocytes), mixed gliomas (3%, for example oligoastrocytic tumors of mixed glial cell origin) and ependymal tumors (7% from ependymal cells, see table 1) [1].

In order to predict the biological behavior of the tumor and to facilitate the choice of therapy regimen, the World Health Organization (WHO) has further classified all CNS tumors following a grading scheme according to their malignancy, where grade I tumors are the least aggressive and grade IV tumors are the most aggressive (table 1). Grade I tumors, such as pilocytic astrocytomas, are usually non-invasive, benign tumors with low proliferative capacity that often can be treated with complete surgical resection alone.

Neoplasms designated grade II, such as diffuse astrocytomas, are usually considered to be less aggressive although they may be transformed to more malignant tumors of grade III or IV. Even though grade II tumors have lower proliferative capacity than grade III and IV tumors, they are more infiltrative than grade I tumors, and therefore often recur. They generally display more abundance of nuclei atypia (abnormal cell nuclei appearance) than grade I tumors. Grade II tumors can be treated by surgery alone or adjuvant radiotherapy (RT) and/or chemotherapy (CT) depending on resectability and subtype.

Grade III tumors, such as anaplastic astrocytomas, are neoplasms showing nuclear atypia, high mitotic activity and anaplasia (loss of differentiation). Patients with grade III tumors typically receive adjuvant RT and/or CT.

Grade IV tumors, such as glioblastoma multiforme (GBM), are malignant tumors with nuclear atypia, high mitotic activity, anaplasia and microvascular proliferation (multi-layering of endothelium) and/or necrosis. Grade IV tumors

typically infiltrate the surrounding healthy brain parenchyma and are associated with rapid disease progression either pre- or- post-operatively with a fatal outcome [2].

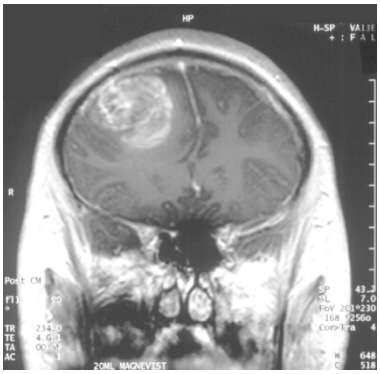
Table 1 Gliomas and subcategories of astrocytic tumors according to grade

Astrocytic tumors (classified according to malignancy) 75% of all gliomas	I	II	III	IV
	Subependymal giant cell astrocytoma <1%	Pilomyxoid astrocytoma <1%	Anaplastic astrocytoma 6%	Glioblastoma multiforme 54%
	Pilocytic astrocytoma 5%	Diffuse astrocytoma 10%		Giant cell glioblastoma <1%
		Pleomorphic xantho-astrocytoma <1%		Gliosarcoma <1%
Oligodendroglial tumors		6%		
Oligoastrocytic tumors		3%		
Ependymal tumors		7%		
Glioma malignant (not otherwise specified)		7%		
All other gliomas		2%		

Glioblastoma multiforme (GBM)

GBM, designated WHO grade IV, represents the most common and aggressive primary malignant brain tumor in adults. 16% of all primary brain tumors and 54% of all gliomas are GBMs [1]. Among the approximately 14,000 new cases of malignant gliomas that are diagnosed in the United States each year, GBMs account for around 60-70% [3]. No underlying causes have been identified for the majority of malignant gliomas including GBMs, but one established risk factor is exposure to ionizing radiation [3]. About 5% of the GBM patients have a family history of gliomas, but in most of these familial cases no common genetic aberration has been identified. GBM tumors can occur in any age group, but is more common in older adults with a median age of 64 years at diagnosis. Moreover, the incidence rate of GBM in males is 1.6 times higher than in females [1].

The prognosis for patients with GBM is dismal and largely depends on whether effective treatment is available, which generally includes extensive surgical resection, RT and concomitant and adjuvant CT. Among patients diagnosed with GBM between 1995 and 2009 in the United States, less than 36% of the patients



survived longer than 1 year following diagnosis and the 5-year survival was less than 5%. Older patients generally have a worse prognosis than younger patients, with a 1-year survival of less than 10% for patients over the age of 75 [1].

Figure 1. MRI (coronal view, post contrast) of a GBM tumor in a 15-year old boy
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The clinical onset of symptoms, which usually occurs abruptly, is either due to a mass effect that increases the intracranial pressure, tumor infiltration into the normal brain parenchyma or tissue destruction. Symptoms include headaches, seizures, nausea, vomiting and focal neurologic deficits (depending on the location of the tumor), and alterations in mental status [3, 4].

Diagnostic magnetic resonance imaging (MRI) and computer tomography of GBMs (see fig. 1) often show an enhancing mass displaying a necrotic central area and a surrounding edema (abnormal accumulation of extracellular fluid).

GBM is histologically characterized by morphology and includes all hallmarks of grade IV tumors including increased cellularity, nuclear atypia, mitotic activity, anaplasia, microvascular proliferation and necrotic areas within the core of the tumor [5]. The aggressive growth pattern of GBM, with a diffuse tumor border and extensions of tumor cells migrating into the surrounding brain parenchyma as well as the existence of isolated micro-satellites of tumor cells spreading throughout the brain, makes them almost impossible to surgically resect completely.

GBMs can be divided into two groups according to biological and genetic differences: 1) *primary* and 2) *secondary tumors*. The majority are primary GBMs that typically arise in patients over 50 years of age, while secondary GBMs are less frequent and usually occur in younger patients when low-grade or anaplastic astrocytomas transform into GBMs. Genetic alterations that are frequent in both primary and secondary GBMs include loss of heterozygocity of chromosome 10q, a loci containing several known tumor suppressor genes, and abnormalities in the p16 and retinoblastoma (Rb) pathways, controlling the entry into the S-phase of the cell cycle. Furthermore, primary GBMs are characterized by amplification or mutation of the gene for epidermal growth factor receptor (EGFR) leading to a constitutively activated receptor, thus activating downstream pathways promoting cell proliferation

and survival by blocking apoptosis. The deletion of the tumor suppressor gene phosphatase and tensin homologue (PTEN) also affects the downstream pathway of EGFR, and therefore further promotes cell proliferation of primary GBMs. Secondary GBMs are characterized by mutations in the TP53 suppressor gene, but both primary and secondary GBMs may have genetic alterations in genes coding for proteins controlling the cellular levels of P53, such as loss of $P14^{ARF}$ or overexpression of the Mouse double minute 2 homologue, leading to p53 breakdown. Many of these pathways and proteins are under clinical investigation for the development of targeted therapy against GBMs [5-7].

Treatment of GBM

Following diagnostic MRI, GBM patients generally undergo maximal surgical resection, to relieve symptoms and to ascertain the diagnosis. In patients who have inoperable tumors due to critical location of the tumor, an open or stereotactic biopsy is performed [8]. The benefit of extensive surgery for overall survival is debated but patients undergoing extensive surgical removal probably have a modest survival improvement [9, 10].

Besides surgery, the standard care for GBM patients comprises fractionated external beam RT and concomitant and adjuvant CT using the oral chemotherapeutic and alkylating agent temozolomide (TMZ). In 2005, The European Organization for Research and Treatment of Cancer and the National Cancer Institute of Canada Clinical Trials Group conducted a randomized phase III clinical trial, where 573 patients below the age of 70 with a high Karnofsky Performance Scale (KPS) status (a scale for quantifying general well being and activities of daily life) were assigned to TMZ treatment. Following surgical resection, patients received either RT alone (60 Gy for 6 weeks) or the addition of oral TMZ treatment (75 mg/m^2) concomitant with RT, followed by 5-days cycles of adjuvant TMZ every 28 days for 6 weeks. Stupp *et al.* reported that the TMZ regimen increased the median overall survival of the elected patients by 2.5 months to 14.6 months vs. 12.1 months for RT alone. The 2-year survival was greater for the TMZ + RT treated patients compared with RT alone (26.5% vs. 10.4%) [11]. Moreover, the follow-up of the original study also reported an increase in the 5-year survival to 9.8% vs. 1.9% with only RT [12]. Subsequently, the TMZ regimen became the new standard of care for first-line treatment of GBMs.

TMZ is an imidazotetrazine derivate of the alkylating agent dacarbazine. At physiological pH TMZ undergoes rapid spontaneous chemical conversion to the active compound 3-methyl-(triazen-1-yl)-imidazole-4-carboxamide. This active compound attaches an alkyl group to the guanine base of deoxyribonucleic acid (DNA) that stops DNA strand uncoiling and separation, thus interfering with DNA replication and inhibiting proliferation, while promoting cell death.

The efficacy of TMZ has been reported to be dependent on the methylation status of the promoter for O^6-methylguanine-DNA methyltransferase (MGMT), a DNA repairing enzyme that neutralizes the toxic effects of TMZ. If the promoter is methylated, the MGMT gene is silenced, thus the response to therapy will be greater and prolong survival [12-14].

An epidemiological study reported a gradual modest survival improvement of GBM patients in the United States between 1993 and 2004, and the authors concluded that the improvement was most likely due to the increased prevalence of TMZ use. However, the study was based on material from a database that did not include information on which patients were treated with TMZ [15].

Unfortunately, not all patients receive the golden standard of care; hence a shorter overall survival of 9.5-10 months has been reported by others [10, 16, 17]. In a study, which enrolled 788 GBM patients between 1997 and 2000, most patients (92%) underwent diagnostic MRI. However, only 75% underwent surgical resection, 87% received RT and even fewer received CT (54%) [16]. In another study with GBM patients diagnosed in 2006, it was described that approximately 65% of the patients underwent total or subtotal surgical resection and of these patients 70% received TMZ and RT. Older patients, unmarried patients, patients with no insurance or patients whose tumors were located in more than 1 lobe were less likely to receive adjuvant RT and TMZ treatment. Although not all patients underwent surgical resection followed by adjuvant RT and TMZ, the subsets of patients that did receive RT and TMZ had an overall median survival of 15 months, suggesting a therapeutic benefit of the therapy. However, it could also demonstrate a selection bias of a subgroup of patients that are younger, suitable for gross total resection and surviving longer than several months in order to be offered RT and TMZ treatment [10]. Moreover, not all patients that receive RT and TMZ are able to complete the treatment and are obligated to interrupt therapy due to general toxicity of therapy or a low KPS score. Although it has been suggested that even patients with KPS score

below 50 may benefit from tumor resection and RT, the benefit of adding TMZ treatment to this patient group is less clear [18].

Despite optimal standard treatment, with partial responses detectable on MRI shortly following therapy, tumor cells eventually become resistant to therapy and tumors recur, usually within 6 months after treatment. Reoperation may be performed as well as RT and TMZ therapy of recurrent high-grade gliomas, although the therapeutic effects on recurrent tumors are limited [17, 19-21].

The poor prognosis for this patient group has led to an extensive research field trying to find novel treatment modalities for this disease. These therapeutic strategies include; targeted molecular therapies, for example inhibitors that target receptor tyrosine kinases such as EGFR [22], administration of chemotherapeutic agents other than TMZ, such as cisplatin, carboplatin, etoposide, irinotecan and carmustine [23-28], different chemotherapeutic delivering techniques, such as intratumoral delivery of bis-chloroethyl nitrosourea (BCNU)/carmustine (Gliadel) wafers [29, 30], finding ways of overcoming drug resistance by targeting DNA repair mechanisms via MGMT suppression [24, 31], developing different gene therapies [32], or immunotherapeutic strategies [33, 34].

Experimental glioma models

In order to develop novel therapeutic strategies including immunotherapy for glioma patients, there is a need for relevant experimental glioma models that may predict the outcome of response in human trials. Such models should therefore mimic the major characteristics of human gliomas. Preferably, the experimental glioma model should be of glial cell origin, have a glioma-like infiltrative growth pattern that is predictable and reproducible, within a time frame that permits the animal to survive a sufficiently long time in order to determine the efficacy of therapy. Also, the tumor model should preferably be non- or weakly immunogenic. There are a variety of experimental rodent models being used today that mimic human gliomas and they are separated in two distinctive groups with fundamental differences; 1) *the spontaneous transgenic tumor models* that via active oncogenes or deleted tumor suppressor genes are genetically engineered to spontaneously develop tumors with varying penetrance, and 2) *the engrafted tumors models*, where either human primary tumor cells or cell lines (xenografts) are implanted into immunodeficient animals (lacking an adaptive immune response) or rodent tumor cell lines are implanted into

syngeneic immunocompetent animals. The latter models have been induced by mutagens or viruses either *in vitro* or *in vivo*. Even though the spontaneous tumor models in many more ways resemble the growth of human gliomas, with stepwise genetical changes during tumor formation, the drawback of using these models are low reproducibility with a slow and unpredictable tumor growth demanding advanced *in vivo* imaging techniques. Due to the fairly good reproducibility of engrafted tumor models, they have been widely used for evaluating new therapeutic strategies. Xenograft models cannot be used when studying immune-mediated tumor eradication; subsequently syngeneic models are frequently used for immuno-therapeutic studies.

The GL261 mouse glioma model has emerged as the golden standard engrafted model for glioma research including immunotherapy due to its high reproducibility [35]. It was induced in the 1930s by the injection of pellets of the carcinogen chemical methylcholanthrene (MCA) into the cerebral cortex of the brain of a C_3H mouse. The tumor mass that was developed was further transplanted in pieces subcutaneously (s.c.) into new recipients of the same mouse strain [36]. Since then it has further been serially transplanted and maintained in C57BL/6 mice and established as a cell line growing both intracerebrally (i.c.) *in vivo* and *in vitro* [37, 38]. When transplanted i.c. the GL261 tumor has a glioma-like slightly invasive growth pattern, displaying necrotic areas, with a rapid growth rate leading to the development of lethal tumors within 20-30 days depending on the number of injected cells. The GL261 cell line has acquired genetic alterations such as point mutations in the K-ras oncogene and in the p53 tumor suppressor gene [38]. GL261 wild type (wt) tumor cells express low levels of the antigen presenting molecules major histocompatibility complex (MHC) class I and II and co-stimulatory molecules [35, 38]. However, upon irradiation (40 Gy), exposure to recombinant interferon-gamma (IFNγ) [39] or IFNγ-transduction *in vitro* [38], MHC expression has shown to be upregulated. Furthermore, GL261 tumor-bearing mice that were pre-immunized with 1×10^6 irradiated GL261 wt-cells could eradicate tumors, while post-immunized mice did not. Therefore, the model is regarded to be moderately immunogenic [38].

Rat tumor models have been used extensively since the 1970s. Of these, the majority of the engrafted rat glioma models are induced by intravenous (i.v.) or transplacental administration of nitrosourea compounds such as N-ethyl-N-nitrosourea (ENU) inducing tumors in the offspring. Resulting tumors are implanted

into syngeneic hosts. Although most rat models are induced in a similar way, there are differences in immunogenicity between the different models [40]. The C6 glioma model, induced in outbred Wistar rats [41], the 9L gliosarcoma model in Fischer 344 rats [41, 42] and the CNS-1 glioma model, derived from an inbred Lewis rat, were all induced by injections of methylnitrosourea (MNU) to adult rats. These models have been utilized extensively, although, the C6 and the 9L models are highly immunogenic and therefore not useful models for evaluating immunotherapeutic strategies. The immunogenicity of the CNS-1 model has not been studied in detail [40]. The F98 glioma and RG2 glioma (sometimes also referred to as D74) were induced at the same time by i.v. administration of ENU to pregnant Fischer 344 rats, and their progenitors developed tumors that later have been cloned and propagated *in vitro* [43]. Both models share characteristics with human GBM with a highly invasive growth pattern, which makes them suitable models for human GBM [40]. The RG2 cells are regarded to be non-immunogenic with low expression of MHC class I, that is upregulated following exposure to recombinant IFNγ [44]. Although the F98 also is regarded to be weakly immunogenic, the RG2 glioma has been reported to be less immunogenic than the F98 model as shown by less activated effector T cells within the tumor tissue or draining lymph nodes, and the lack of prolonged survival following immunization with irradiated RG2 tumor cells [45].

The N32 rat glioma model, established in our lab in the 1990s, was derived from a male offspring of a pregnant Fischer 344 rat that was exposed to ENU [46]. The tumor cells have been passaged *in vitro* and implanted *in vivo* into the striatum of syngeneic rats to yield i.c. tumors. The tumor resembles an anaplastic astrocytoma (WHO grade III) with delineated borders that rarely spread into the normal brain parenchyma. *In vitro* the tumor cells express stem cell or progenitor markers such as cluster of differentiation-133 (CD133), nestin as well as different neural markers such as the astrocytic marker glial fibrillary acidic protein, the oligodendrocyte marker 2,3-cyclic-nucleotide 3-phosphodiesterase and the neuronal marker βIII-tubulin [47]. Though the N32 tumor cells express MHC class I, post-immunization with wt-tumor cells does not eradicate tumors, showing that the model is only weakly immunogenic [48].

The immune system

The immune system is the coordination within an organism that protects its host from infections and other diseases by recognizing non-self molecules of invading pathogens, such as viruses, bacteria, and parasites. However, it also recognizes and reacts against altered self-antigens, such as tumors or infected cells, and distinguishes them from healthy tissue. The immune system is divided into 1) *the innate* and 2) *the adaptive immune system*, where the innate immune response, found in most forms of life, is characterized by an immediate immune response, while the adaptive immune response, found only in jawed vertebrates, is slower and more specific with the ability of inducing an immunological long-term memory.

The innate immune response

The cells of the innate immune system, together with different physical and chemical barriers, function as the first-line of defense against infection by preventing pathogens from entering and infecting the host. It provides an immediate defense against infection but does not convert it to a long-lasting protection against the pathogen. The cells of the innate immune system include phagocytic cells, such as dendritic cells (DCs), monocytes (circulating in the blood that mature to macrophages in the tissue), granulocytes (including neutrophils, basophils and eosinophils), mast cells and natural killer (NK) cells (see fig. 2). The activation of innate immune cells is not dependent on specific peptide antigens, but rather recognition of common subparts of the bacteria or virus called pathogen associated

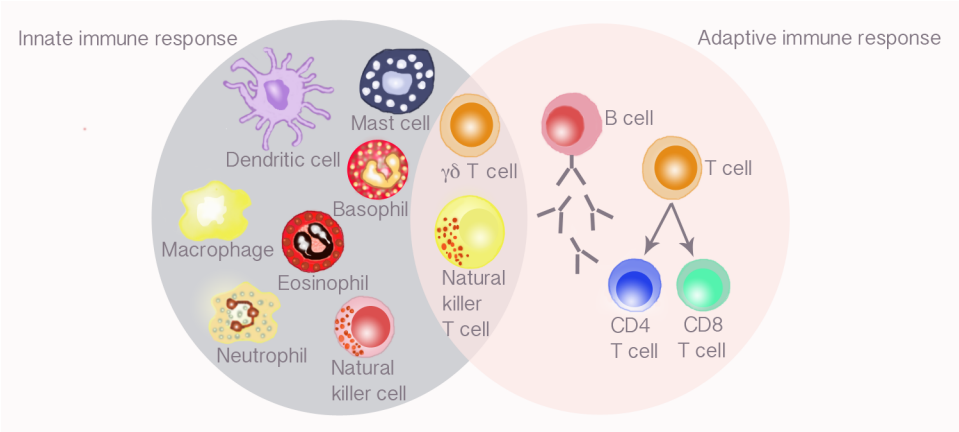

Figure 2. Cells (leukocytes) of the innate and the adaptive immune response

molecular patterns, or damaged associated molecular patterns (DAMPs), released by stressed normal cells or tumor cells, that all bind to toll-like receptors or nod-like receptors on the surface of the innate immune cells.

NK cells have the ability to kill cells that have down-regulated or lost MHC class I expression. MHC class I that is expressed on normal cells, bind to an inhibitory receptor on the surface of the NK cells such as the natural killer group 2 member A (NKG2A), leading to an inhibitory signal, thus the target cell is recognized as self and no lysis occurs. However, ligation of the activating receptor NKG2D by any of its ligands, for example the MHC class I chain-related (MIC) molecules, triggers degranulation and perforin-mediated lysis and apoptosis of the target cell [49]. Natural killer T cells (NKT) and gamma delta (γδ) T cells are regarded as both innate and adaptive immune cells (fig. 2). NKT cells express CD161 that binds to non-classical MHC molecules such as CD1d that present lipid antigens. γδ T cells are a small population of T cells that express a distinct T cell receptor (TCR) composed of one γ chain and one δ chain. Their role in immune responses is complex and spans definitions of both innate and adaptive immune responses. They have the ability to rearrange TCR genes to develop a memory phenotype and they may be phagocytic while possessing antigen-presenting capacity. They also secrete cytokines and target cell lysis rapidly in response to invading pathogens [50]. On the other hand, they have also been reported to have immunosuppressive capacity [51].

The major functions of the innate immune system is to recruit immune cells to the site of infection by producing different cytokines, to remove foreign substances, to activate the complement cascade that clears away dead cells or antibody complexes and to activate the adaptive immune system via antigen presentation.

The adaptive immune response and antigen presentation

The adaptive immune response is a slower and an antigen specific response that induces a long-term memory. The adaptive immune cells are comprised of B and T lymphocytes. T lymphocytes are further subdivided into 1) *$CD4^+$ T helper (T_H)* cells and 2) *$CD8^+$ cytotoxic T lymphocytes* (CTLs, fig. 2). While the B cells produce antibodies and are involved in humoral immune responses, the T cells are involved in cell-mediated immune responses. Tumor eradication is primarily mediated by T cells and cellular immune responses.

The activation of the adaptive immune response is performed by the professional antigen presenting cells (APCs): DCs and macrophages. These cells constitutively sample their environment for antigens (peptides), which are then loaded onto MHC class I and II molecules on their cell surface. While MHC class I is expressed on all cells, MHC class II are only expressed on specific immune cells such as APCs. Direct classical antigen presentation involves the processing of endogenous antigens (synthesized within the cell) such as virus peptides that are broken down and presented on MHC class I molecules, while MHC class II presents exogenous antigens such as extracellular bacteria that have been engulfed by APCs. However, exogenous antigens can also be phagocytized, processed and presented on MHC class I molecules on DCs through a process called *cross-presentation*, which is vital for anti-tumor reactivity [52]. Once the APCs have phagocytized the antigens, they mature and leave the tissues to enter secondary lymphoid organs such as the lymph nodes.

All lymphocytes are developed in the primary lymphoid organs from a common lymphoid progenitor cell. Though B cells are developed and mature in the bone marrow, T cells leave the bone marrow for the thymus where they mature further. Naïve T cells that have not yet encountered their specific antigen exit the thymus and recirculate to the secondary lymphoid compartments such as the spleen and the lymph nodes, sampling APCs for the presence of antigens. The expression of CD62 ligand (CD62L also known as L-selectin) and the C-C chemokine receptor-7 (CCR7) enables naïve T cells to enter the lymph nodes via the high endothelial venules. Within the lymph node, naïve T cells scan the APCs for the proper antigen presented on MHC via their TCR comprised of one α-chain and one β-chain. $CD4^+$ T cells recognize peptides presented on MHC class II while $CD8^+$ T cells recognize peptides presented on MHC class I. The recognition of the TCR binding to the correct MHC/peptide complex is not enough to activate the T cell, but appropriate co-stimulatory signals are required and delivered by the binding of CD28 on the T cell to CD80 (B7.1) or CD86 (B7.2) on the APC, and by the interaction of CD40L on the T cell and CD40 on the APC. Once activated, the T cells undergo clonal expansion, and differentiate into effector cells. Upon activation they down-regulate CD62L, leave the secondary lymphoid organs and enter the blood stream from where they infiltrate peripheral tissues, specifically targeting sites of inflammation or tumor tissue.

Once the activated T cells have entered their target tissue, the $CD8^+$ CTLs recognize and directly destroy their cell target via IFNγ, perforin and granzyme B or

by Fas/FasL. The $CD4^+$ T_H cells in turn may produce immunostimulatory cytokines that help to optimize the function of other immune cells. T helper responses are classified into T_H1 or T_H2 responses. While T_H1 responses are important for cell-mediated immunity such as eradication of tumors or intracellular pathogens, T_H2 responses facilitate optimal antibody production and elimination of extracellular bacteria.

Different types of antigens or distinct subsets of DCs and cytokines that they produce dictate the differentiation of T_H cells into either T_H1 or T_H2 [53]. Interleukin-12 (IL-12) produced by DCs, together with signal transducer and activator of transcription-4 (STAT4), IFNγ and the transcription factor T-box expressed in T cells (T-bet), have been shown to promote differentiation into T_H1 cells. These in turn produce IFNγ, IL-2, tumor necrosis factor alpha (TNFα) and TNFβ [54-56]. IL-4 and STAT6 signaling induces the expression of the transcription factor GATA3 and promotes differentiation into T_H2 cells, which produce IL-4, IL-5, IL-6 and IL-13 [57]. A third subset of T helper cells, promoted by IL-6, transforming growth factor beta (TGFβ) and IL-21, have been characterized: the T_H17 cell lineage. These cells produce IL-17 and IL-22. Although the effect of T_H17 cells is not fully explained, they have been identified as key mediators for immune responses against pathogens that have not been adequately handled by T_H1 or T_H2 cells and are potent inducers of tissue inflammation [58].

There is also a subpopulation among the circulating $CD4^+$ T cells that upon strong TCR activation and TGFβ stimulation turns into T regulatory cells (Tregs) with immunosuppressive function. They are usually defined as cells expressing the IL-2 receptor α-chain (IL2Rα, also known as CD25) and the transcription factor forkhead box P3 (FoxP3). Their main function is to suppress immune responses against self-antigens to maintain self-tolerance and avoid autoimmunity [59]. Also, activated T cells up-regulate CTL-associated antigen 4 (CTLA-4), programmed death-1 (PD-1) and B and T lymphocyte attenuator, and the binding of these molecules to co-inhibitory molecules of the B7 family: CD80 (B7.1)/ CD86 (B7.2), PDL1 (B7 homologue 1, B7.H1)/ PDL2 (B7.DC) and B7.H4 expressed on DCs, inflamed tissues or tumors, induce an inhibitory signal resulting in T cell inhibition [60, 61].

Once the effector T cells have eliminated and cleared their target, most T cells undergo apoptosis. However, 5-10% of the T cells survive and become memory T

cells that upon reencountering a specific antigen immediately can kill their target without further activation by an APC.

Cytokine production

Communication between immune cells or between immune cells and their target cells can be direct via cell-to-cell contact (such as the perforin and granzyme B of a CTL) via receptors and ligands or by cytokine and chemokine production. Cytokines can either be secreted at a close distance to the target cell or travel long distances, thereby recruiting immune cells from the bone marrow and blood stream into inflamed tissues or tumors.

Interferon-γ (IFNγ)

Amongst all cytokines produced by the immune system, IFNγ is one of the most multifunctional cytokines. It is mainly produced by T_H1 cells, CTLs, NK-cells and NKT cells, but also by certain macrophages and DCs [62, 63]. IFNγ further enhances T_H1 polarization, together with CD40L by promoting IL-12 production of DCs, and by inducing T-bet [64, 65]. During virus infection IFNγ acts directly on $CD8^+$ T cells to increase their abundance [66]. IFNγ plays a key role in macrophage activation, T helper cell responses, Treg differentiation, protection against the development of cancer (cancer immunoediting, [67] discussed below) and cell-mediated anti-tumor immune reactivity. Besides its multiple and direct effects on immune function, IFNγ also affects cell growth control, apoptosis and angiogenesis, and many of its functions are mediated by cross regulation of cellular responses to other cytokines [68, 69]. IFNγ has shown to upregulate MHC class I and II on tumor cells, macrophages and microglia but also other components of the antigen presenting machinery (APM), such as the immunoproteasome complex, thus enforcing and increasing the pool of antigens available for T cell presentation [44, 70-75]. By these features, IFNγ is crucial for immune reactivity of tumors and as a consequence, knock-out mice (KO) lacking the IFNγ gene or any of its downstream targets, have an increased risk of developing tumors when exposed to the carcinogen MCA [72, 76].

Interleukin-7 (IL-7)

IL-7 is constitutively produced in the bone marrow and thymus by stromal and epithelial cells, and in secondary lymphoid organs by lymphatic endothelial cells and fibroblastic reticular cells in the T cell zone [77-79]. IL-7 is a homeostatic lymphocyte survival factor and plays a central role in the development and maturation of naïve T cells [80, 81]. Naïve T cells rely on survival signals through contact with self-antigen loaded MHC molecules and IL-7; while the survival of antigen-experienced memory T cells is MHC-independent but dependent on IL-7 and IL-15. IL-7 promotes proliferation of naïve and memory T cells during lymphopenic conditions and together with IL-15 it also promotes proliferation of memory $CD8^+$ T cells during normal physiological conditions [81-84]. The role of IL-7 for the generation and survival of $CD4^+$ memory T cells is less clear [82, 85, 86]. IL-7 mainly mediates its effects on naïve and memory T cells through a heterodimer involving the IL-7 Receptor α (IL-7Rα, CD127) and the common γ chain (CD132), shared by the receptors for IL-2, IL-4, IL9, IL-15 and IL-21 [87]. The availability of IL-7 is regulated by its production and its consumption by T cells. T cells that have received a pro-survival signal via IL-7, IL-2, IL-4, IL-6, IL-15 or through MHC-TCR interaction, down-regulate the IL-7Rα, and do not compete with remaining survival signals for naïve T-cells [88-91]. Therefore activated T cells are less responsive to IL-7. Tregs only express low levels of the IL-7Rα and therefore, administration of recombinant IL-7 to humans induces a selective increase of naïve and memory $CD4^+$ and $CD8^+$ T cells without expanding Tregs [92, 93]. γδ T cells, which express high levels of both IL7Rα and IL15R-α, are dependent on both IL-7 and IL-15, where as NK-cells are more dependent on IL-15 [94, 95].

IL-7 has been shown to down-regulate the immunosuppressive cytokine TGFβ [96].

Other immunostimulatory interleukins

Other important immunostimulatory cytokines are IL-2, IL-12 and IL-15. IL-2, mainly produced by activated T cells, promote the survival and activation of T and NK cells. However, since IL-2Rα/CD25 is highly expressed on Tregs, it also induces proliferation of these immunosuppressive cells [97]. IL-2 preferably induces differentiation of naive $CD4^+$ T cells into Tregs rather than T_H17 cells [98].

IL-12, a T_H1 cytokine secreted by macrophages, DCs and B cells, induces proliferation of T cells and stimulate IFNγ production together with IL-7 [99].

IL-15, mainly produced by activated macrophages, is an important survival signal for activated T cells, memory T cells (preferably $CD8^+$ T cells), γδ T cells and NK-cells, and crucial for a long-lasting reactivity against pathogens and tumors [100].

Granulocyte macrophage-colony stimulating factor (GM-CSF)

Granulocyte macrophage-colony stimulating factor (GM-CSF), produced by a number of cells including epithelial cells, endothelial cells, monocytes, macrophages, NK cells and activated T cells, is a hematopoietic cytokine that stimulates the differentiation of myeloid progenitor cells into granulocytes, monocytes/macrophages and DCs. During inflammation it recruits myeloid progenitor cells from the bone marrow or monocytes from the blood into the inflamed tissue and to the local draining lymph nodes [101-103]. GM-CSF endorses antigen presentation of DCs and macrophages by inducing expression of CD80 and MHC class II.

However, high constant levels of GM-CSF produced by tumor cells, may also recruit and expand a population of immature myeloid derived suppressor cells (MDSCs) with an immunosuppressive capacity [104].

CNS immunology –immune privilege and the blood-brain barrier

Certain organs, such as the CNS, are extremely sensitive to tissue damage caused by inflammation. The brain has only a restricted space within the skull, and an uncontrolled immune response within the brain would induce edema, leading to an increased brain volume and increased intracranial pressure that in turn could induce an immediate death of neurons. Consequently, the CNS has developed several mechanisms that limit immune responses, a phenomenon called *immune privilege* [105, 106]. Although, it should be noted that immune privilege of the CNS is not an absolute state but is rather relative to other organs. Moreover, the immune privilege is limited to the CNS parenchyma, whereas the immune reactivity of the ventricles, choroid plexus, meninges and circumventricular organs is comparable to the peripheral reactivity [107]. The mechanisms of immune privilege in the CNS are presented here:

1) There are no lymphatic vessels in the brain parenchyma that drain antigens or APCs from the CNS to peripheral lymph nodes; hence a non-inflamed brain does not present antigens to naive T cells. Even though the brain lacks a conventional lymphatic system, drainage of antigens is partially served by the cerebrospinal fluid (CSF) that may track along the perivascular spaces and through the subarachnoid space (between the arachnoid membrane and the pia mater) along cranial nerves to the nasal mucosa where antigens access the draining deep cervical lymph nodes [108, 109].
2) There are no resident professional APCs within the brain parenchyma that are able to maintain immune responses, although this statement has been revised recently. Resident microglia, which belong to the monocyte-macrophage lineage, represent the endogenous immune cells in the brain, and they constantly sample the brain for danger signals. Upon tissue damage, their extensions may form a protective shield surrounding the damaged tissue [105]. Their capacity to present antigens and evoke cellular immune responses has been debated [110-113]. Therefore a variety of other cells have been proposed to be the primary APCs of the CNS. Macrophages and DCs in the perivascular spaces, meninges and choroid plexus scan the CSF for the presence of antigens. Upon antigen uptake they leave the brain via the CSF and migrate to the deep cervical lymph nodes [114, 115]. In rats, DCs injected into the CSF (but not when injected into the brain parenchyma) accumulate in the cervical lymph nodes, where they may present antigens [116]. Also, it has been shown that selective traffic of antigen-specific $CD8^+$ T cells into the brain *in vivo* was dependent on luminal expression of MHC class I by cerebral endothelial cells and that this was independent of antigen presentation by perivascular macrophages [117].
3) The capillaries within the CNS parenchyma are tightly controlled by the *blood-brain barrier* (BBB, see fig. 3). The BBB consists of endothelial cells with low pinocytic activity lining the vessels, with tight junctions between the endothelial cells. These cells control the transport and restrict diffusion of most blood molecules, including leukocytes, into the brain. Under normal physiological conditions, the endothelial cells express low amount of adhesion molecules and chemokines, which are vital for all leukocytes to cross the endothelial wall, hence, the trafficking of leukocytes into the brain is generally low [106, 114]. Underneath the endothelial wall two basement membranes are situated: the endothelial membrane, with embedded stabilizing pericytes, and the parenchymal

membrane, with astrocytic foot processes (or glial limitants). They function as two barriers for leukocyte extravasation. However, the BBB is not absolutely impermeable to leukocytes since effector T cells, memory T cells, macrophages and DCs can traverse the BBB. In between the two membranes there is a perivascular space containing cerebrospinal fluid (CSF) and perivascular APCs, which may present antigens from the brain. T cells that have extravasated through the endothelial wall and recognize their cognate antigen are activated here. Without antigen-triggered activation, these cells will not persist nor traverse into the brain parenchyma [105, 106]. During inflammation and disease, the expression of integrins: E-selectin, P-selectins, vascular cell adhesion molecule 1 (VCAM-1) and chemokines: CCL2, CCL4 and CCL5, normally not expressed on the endothelial cells or astrocytes, is augmented, and leukocyte trafficking is increased. Furthermore, brain tumors lack specialized vascular structures such as the BBB, which makes the *blood-tumor barrier* more leaky [105, 114].

4) Cells within the brain parenchyma: neurons and glial cells such as astrocytes, microglia and oligodendrocytes produce TGFβ, which among its multiple functions has been shown to block IFNγ-induced upregulation of MHC class II expression on astrocytes and suppress activated microglial function [118-120]. They also express the pro-apoptotic factor FasL, leading to apoptosis of Fas^+ T cells [121].
5) The parenchymal cells express low or undetectable levels of MHC class I and II. However the expression may be upregulated following IFNγ exposure [122, 123].

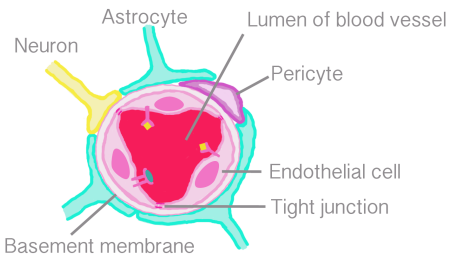

Figure 3. A schematic view of the blood brain barrier protecting the brain from toxins and inflammation. It consists of endothelial cells with tight junctions and two basement membranes: the endothelial membrane with pericytes and the parenchymal membrane with astrocytic foot processes.

Tumor immunology

The dual role of the immune system in tumor development, having both anti- and pro-tumoral function, has been known for a long time. Immune cells have the ability to recognize and attack tumors, however pro-angiogenic factors, cytokines and growth factors may also promote tumor progression by certain means. The immune system reacts against tumor cells by recognizing either tumor-specific antigens that are exclusively expressed on tumor cells, or tumor-associated antigens, normal self-antigens with an altered expression pattern.

Immunosurveillance

Already in 1909, Paul Erlich had the idea that the immune system could protect the host from potentially overwhelming occurrence of neoplasms. In the end of the 1950s and the 1970s, the hypothesis of cancer immunosurveillance was formulated by Burnet and Thomas, who proposed that lymphocytes recognize antigens of continuously arising transformed cells and eliminate tumors before they become clinically evident [124, 125].

Although, the immunosurveillance hypothesis has been debated over the years. A further proof of concept, which supported the crucial role of the immune system in preventing tumor formation, was the demonstration that recombination activating gene (RAG) KO mice (lacking lymphocytes and NKT cells) as well as $STAT1^{-/-}$ KO mice (lacking interferon-mediated pathways) displayed a higher incident of tumor formation induced by chemical carcinogens, but also of spontaneously arising tumors [72].

It has also been reported that transplant patients who receive immuno-suppressive treatment or individuals with primary immunodeficiencies exhibit a higher risk of cancer development. When analyzing data from a transplant registry of Scandinavian countries, renal transplant patients showed higher incidences of cancers of the colon, larynx, lung, bladder, lip, skin, kidney, endocrine glands and non-Hodgkin´s lymphoma, as well as prostate and testis cancer in men, and cancers of the cervix and vulva-vagina in women, as compared with the general population [126].

Immunoediting

The immune system does not eradicate all tumors, leading to a Darwinian selection of tumors with low immunogenicity. This selects for emerging tumors with escape mechanisms of immunological recognition and eradication. Consequently, the concept of tumor immunosurveillance has instead been considered to be a part of a refined and broader hypothesis of cancer immunoediting, describing the dual opposing functions of the immune system in both protecting the host from tumor formation and promoting tumors and the formation of less immunogenic tumor types [127]. Immunoediting is defined as a process with three different phases: elimination, equilibrium and escape (see fig. 4).

Elimination

Elimination corresponds to the original concept of immunosurveillance. In the elimination phase, transformed cells begin to grow invasively and they demand enhanced blood supply. The invasive tumor growth causes damage in the surrounding tissue. Cells undergoing stress start to produce endogenous danger signals that initiate an inflammatory response. These signals include heat-shock proteins, intracellular nucleotides, reactive oxygen species (ROS), extracellular matrix breakdown products, CD40L and cytokines such as TNFα, IL-1β, IFNs, GM-CSF and IL-15 [128]. The release of danger signals leads to the recruitment and activation of innate immune cells such as macrophages, dendritic cells, NK cells, NKT cells and γδ T cells that produce cytokines such as IFNγ, TNFα and nitric oxide (NO). The cytokine production of the innate immune cells will induce either direct tumor killing or production of chemokines that recruit more immune cells trans-activating each other. Also the innate immune system manifests immunoediting activity in the absence of an adaptive immunity [71]. Cell debris of dying tumor cells is phagocytized by professional APCs that home to draining lymph nodes. Within the lymph node, tumor-specific $CD4^+$ T cells become activated and start to produce IFNγ that further facilitate the development of tumor-specific cytotoxic $CD8^+$ T cells. The newly activated T cells home to the tumor site, where they attack and destroy the remaining tumor cells [127].

Equilibrium

During the equilibrium phase, the immune system and tumor cell variants that have survived the elimination phase, enter a dynamic equilibrium. The IFNγ-

producing lymphocytes exert pressure on the tumor but no longer have the ability to fully extinguish the fast growing and mutating tumor cells. During this phase tumor cells are attacked and destroyed, but new mutations provide the tumor cells with increased resistance and capacity of surviving an immune attack. This process selectively promotes the generation of less immunogenic tumor variants [127].

Escape

In the escape phase, surviving tumor cells that have acquired resistance to immunologic detection and elimination through genetic or epigenetic alterations, expand in an uncontrolled manner resulting in clinical disease [127].

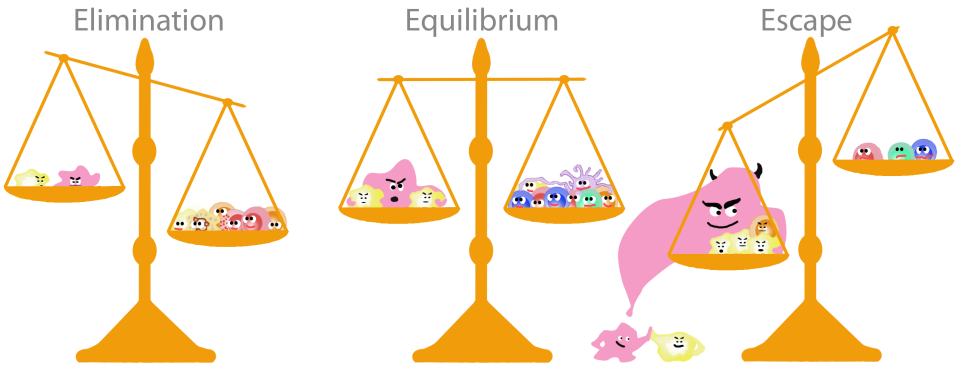

Figure 4. The three phases of immunoediting: elimination, equilibrium and escape

Immunosuppression

Tumors, including gliomas, have several ways of evading elimination of the immune system. These mechanisms include down-regulation of MHC molecule expression [129], secretion of immunosuppressive factors such as TGFβ [130], IL-10 [131] and prostaglandin E_2 (PGE_2) [132], or expression of apoptosis-inducing molecules such as FasL [133]. They may also recruit immune cells with immunosuppressive capacity such as Tregs [134], MDSCs [135] or tumor associated macrophages (TAMs) [136] to the tumor. Also, infection of human cytomegalovirus (HCMV) may contribute to the immunosuppressive environment within the tumor tissue [137].

Downregulation of MHC expression and co-stimulatory signals

One mechanism by which gliomas evade the immune system is by preventing normal antigen recognition, via downregulation or genetic mutations/deletions of human leukocyte antigen, HLA class I molecules (MHC molecule of humans) or other defects in components of the APM, such as tapasin [129]. Loss of HLA class I antigen presentation has been shown to be correlated with tumor grade [129]. Another mechanism of immune escape is lack of co-stimulation. Absence of expression of co-stimulatory molecules such as CD80 or CD86 on glioma cells has been reported, which may lead to T cell anergy, deletion or tolerance. Instead, the inhibitory molecule B7-H1 is expressed on glioma cells, thus suppressing T cell activity [138]. In addition, microglia and macrophages from glioma tissue have shown to be less efficient APCs than cells isolated from normal brain [139, 140].

Immunosuppressive factors

Cells of the glioma microenvironment, consisting of both tumor cells and infiltrating leukocytes, produce cytokines and chemokines, including high levels of immunosuppressive factors that contribute to the immunosuppression seen in glioma patients.

Human GBMs abundantly produce TGFβ [130]. TGFβ is a multifunctional regulator of inflammation, angiogenesis and proliferation with immunosuppressive function. This is achieved by inhibiting IL2-dependent proliferation and activation of T cells, inhibiting T cell effector mechanisms by blocking IFNγ-production [141-143], and regulating the maturation and function of APCs [144].

IL-10, secreted by microglia, glioma cells and Tregs [131, 145-147] has been shown to inhibit the production of IFNγ and TNFα, down-regulate MHC class II expression on TAMs [148] and promote proliferation of glioma cells *in vitro* [131].

Another immunosuppressive factor is PGE_2, a general pro-inflammatory mediator that induces pain and fever. PGE_2 and its rate-limiting converting enzyme cyclooxygenase (COX) are usually overexpressed in gliomas [132, 149, 150]. PGE_2 promotes tumor invasiveness and angiogenesis. PGE_2 down-regulates anti-tumoral T_H1 cytokine production and skews the immune response towards a T_H2 immune response and promotes Treg proliferation [151, 152].

Other immunosuppressive factors produced by gliomas include:

1) Vascular endothelial growth factor (VEGF), important for endothelial proliferation and angiogenesis, but also have immunosuppressive capacities [153].
2) Arginase, which down-regulates L-arginine that is crucial for T cell function.
3) Indoleamine 2,3-dioxygenase (IDO), an enzyme that catabolizes tryptophan, leading to tryptophan depletion and inhibition of T cell proliferation [154].
4) Inducible NO synthase (iNOS) [150, 155] that synthesizes NO, a factor with dual and antagonizing effects on tumor proliferation, vascularization, invasiveness and immune reactivity [156].

Regulatory T cells (Tregs)

The immune system has developed a regulatory mechanism that restricts self-tissue destruction by auto-reactive immune cells. Naturally occurring Tregs are important for the avoidance of autoimmune diseases. However, Tregs may also be induced by tumors that take advantage of their normal immunosuppressive mechanisms to impair anti-tumor immunity. The fraction of Tregs among the total $CD4^+$ T cell-compartment has been shown to be increased in the circulation and tumor tissue in both experimental [134] and human gliomas [157-159]. Tumor cells secrete factors that preferentially recruit Tregs to the tumor tissue, and promote their survival and expansion [159]. Tregs accumulating within the tumor tissue limit the function of tumor-specific effector T cells, by direct cell-to-cell contact via the binding of CTLA-4 or inhibit T cell production of IL-2 and IFNγ [160, 161]. Tregs also produce IL-10 and TGFβ [147, 161].

MDSCs and TAMs

In addition to Tregs, MDSCs and TAMs accumulate at the tumor site. MDSC is a heterogeneous population of immature cells with myeloid origin with the ability of promoting tumor growth. In mice, they are usually defined as $Gr\text{-}1^+CD11b^+$ cells, and can be either granulocytic or monocytic depending on their Ly6G, Ly6C and F4/80 expression [136]. They have the possibility to differentiate into granulocytes, macrophages and dendritic cells. In tumors they may suppress immune responses by secreting ROS, NO, arginase or TGFβ and induce the development of Tregs [135, 162, 163]. They are induced by GM-CSF, VEGF and PGE_2 [104, 164, 165].

TAMs, macrophages that infiltrate tumors, are divided into two different subtypes according to their function: 1) *the classical M1* or 2) *the alternative or regulatory M2* phenotype. The M1 phenotype has shown to be pro-inflammatory and

promotes anti-tumoral immunity and T_H1 responses by producing IL-12, IL-23, TNF, IL-6 and Type I IFN, while the M2 is anti-inflammatory and promotes T_H2 responses and tumor growth by IL-10 and TGFβ production. During tumor progression, the tumor microenvironment affects the TAMs to change their phenotype to more resemble the M2 regulatory cells [136, 166].

Human cytomegalovirus (HCMV)

Infection by the oncolytic human papilloma virus, has shown to increase the risk of developing cervix cancer [167]. Although still very controversial in the brain tumor field, there are viruses, such as the HCMV, with oncomodulatory capacities [168]. During a lifetime, the majority of the human population becomes infected with HCMV. In healthy individuals, following the first virus infection, the virus remains in an asymptomatic latent state throughout life in $CD34^+$ bone marrow-derived myeloid progenitor cells, $CD33^+$ myeloid granulocyte/monocyte precursors or in $CD14^+$ monocytes. However, in immunocompromised individuals such as HIV or cancer patients, the virus can be reactivated. The reactivation occurs only in differentiated cells such as macrophages or DCs [169].

Proteins of HCMV have been detected in tumor cells and tumor-infiltrating immune cells in gliomas [137, 170-172]. The role of HCMV infection in glioma development and progression is still controversial. It is unclear whether the presence of HCMV in gliomas represents an epiphenomenon of tumor-mediated immunosuppression, which enables HCMV to become reactivated within the tumor tissue, or whether the virus infects premalignant cells and contributes to the oncogenic phenotype of the cell.

HCMV has acquired certain immunosuppressive abilities, which further enhance tumor immune evasion and escape, for example by inducing COX-2 expression [173]. The viral protein UL83 (pp65) inhibits MHC presentation of viral peptides via degradation of the HLA-DRα chain [174]. UL83 also helps infected cells to escape NK cell recognition [175]. Moreover, HCMV infected glioma cancer stem cells produce cmvIL-10, which induces the immunosuppressive and tumor-supporting M2 TAM phenotype that enhances migration of tumor cells [170, 176].

Immunotherapy against gliomas

Immunotherapy is an attractive treatment strategy for GBMs. It has the ability to induce a specific anti-tumor response that is less toxic for the normal brain parenchyma than chemotherapeutic agents, and that could eradicate recurrent tumors. It also targets both dividing and non-dividing tumor cells. Today, knowing the complexity of tumor immunology, most immunotherapies are advanced and combine various strategies. Despite the progresses in preclinical studies, the results in immunotherapeutic clinical trials have been rather disappointing, and there is a demand for improvements. Although the most obvious way to validate immunotherapeutic strategies is by clinical outcome, there is also a need for intermediate markers that are able to monitor the immune response during therapy. There are both *in vitro* and *in vivo* methods for assessing T-cell number and function, however there is no consensus on which tests to use [177].

Different immunotherapeutic strategies broadly include cytokine therapy, passive immunotherapy and active immunotherapy (see fig. 5).

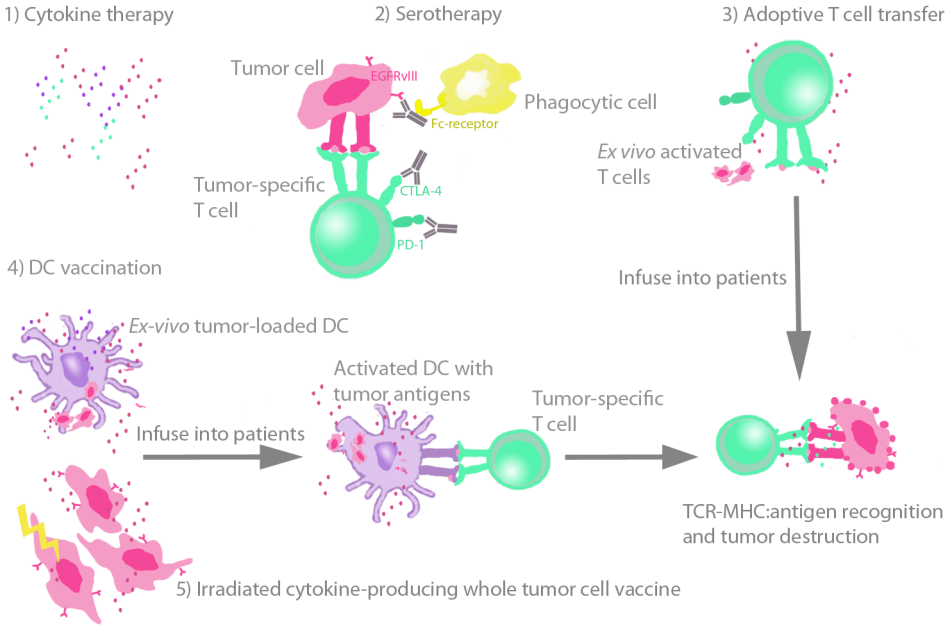

Figure 5. Different immunotherapeutic strategies including cytokine therapy, serotherapy, using antibodies, adoptive T cell transfer, DC vaccination and whole tumor cell vaccines

Cytokine therapy

Cytokine therapy is based on the administration of immunostimulatory cytokines that activate the immune system. A diverse range of cytokines have been used as immunostimulants in both experimental brain models as well as in clinical trials and include IL-2 [178-180], IL-4 [181], IL-7 [93, 182, 183], IL-12 [184], IFNα [185, 186], IFNβ [187], IFNγ [179, 188-190] and GM-CSF. Different strategies of delivering recombinant cytokines to the CNS have been explored and include either systemic or local administration. However, the use of recombinant cytokines has often shown to be highly toxic and non-effective due to a rapid release of high levels of the cytokines. More promising results have been maintained by other delivery methods utilizing gene transfer techniques that enable prolonged cytokine production by the incorporation of genes into cellular or viral carriers [178-181, 191-193]. Cytokines may also be delivered using biodegradable polymers [194, 195].

All together, cytokines may be effective when delivered optimally, but the lack of specificity of most cytokine therapies, suggests that they may be more useful as complements or adjuvants to other types of immunotherapies [196].

Passive immunotherapy

Passive immunotherapies include serotherapy, where antibodies are administered to help immune recognition of tumor cells or to deliver toxins to the tumor cells, and adoptive transfer, where tumor-specific T cells are expanded *ex vivo* and then reinjected into the patient.

Serotherapy

Serotherapy uses monoclonal antibodies (mAbs) to activate an antibody-dependent cell-mediated immune response or to specifically deliver toxic compounds to the tumor cells. In order to achieve an optimal therapy, there is a need of identifying specific tumor antigens only expressed on tumor cells, thus limiting toxicity to the normal brain parenchyma. Glioma antigens that have been selected for therapy include EGFR, often overexpressed in gliomas, and its constitutively active mutated form, EGFR variant III (EGFRvIII) [34, 197-199]. Although these therapies have shown promising results with specificity of delivery, low toxicity and clinical responses, there is a risk of tumors down-regulating the targeted tumor antigen and thereby developing resistance to therapy.

In addition to tumor cell killing, mAbs that deplete specific immune cells, for example mAbs targeting CD25 expressed by Tregs, have been shown both to enhance immunotherapies in preclinical and clinical trials, but also to inhibit clonal expansion of tumor-specific T cells [200-202].

Immunostimulatory mAbs that bind to and activate receptors such as the co-stimulatory molecule CD28, or mAbs that blocks inhibitory receptors, such as the T-cell receptor CTLA-4, have been clinically tested [203]. Anti-CTLA-4 mAbs have been shown to prolong survival of patients with malignant melanoma in a phase III clinical trial [204]. Though these therapies are potentially very useful agents, an initial clinical trial with super agonist antibodies against CD28 ended in serious toxicity of healthy volunteers, which is a reminder of the need for careful preclinical and clinical testing, starting with very low doses to avoid further incidences [205].

Adoptive transfer

Adoptive immunotherapy uses immune cells that have been isolated from peripheral blood, lymph nodes or tumor tissue of patients, expanded *ex vivo* with cytokines and/or tumor antigens, and reintroduced into the patient. Most adoptive therapies utilize harvested lymphocytes that have been stimulated with IL-2, to yield lymphokine-activated killer cells. GBM patients that were treated with lymphokine-activated killer cells placed in the resection cavity displayed a prolonged median overall survival of 17.5 months compared with 13.6 months in a control group [206]. Others have used T cells from blood, activated *ex vivo* by IL-2 and autologous tumor cells to generate CTLs, and then reinjected intracranially. One patient responded to therapy [207]. In another study, autologous CTLs derived from draining lymph nodes following GM-CSF injection were reintroduced to the patients; resulting in 3 out of 10 patients showing tumor regression [208]. Tumor-infiltrating lymphocytes have also been isolated and expanded *ex vivo*, and together with IL-2 injected back into the tumor resection cavity, with one out of six patients showing tumor regression for 45 months [209]. In order to optimize this treatment modality, depletion of intratumoral suppressive Tregs could be considered. Although adoptive transfer is not optimal by itself, it may be combined with standard treatment or other immunotherapies.

Active immunotherapy

Active immunotherapy involves the immune system to respond to tumor antigens, by administering professional APCs, or by administering tumor antigens, lysates or irradiated whole tumor cells.

DC vaccination

To augment tumor antigen presentation professional APCs have been utilized as glioma vaccines. Autologous DCs are generally obtained from monocytes in the peripheral blood using leukapheresis or from myeloid precursors in the bone marrow. They are then matured *ex vivo* by exposure to a cocktail of cytokines (for example GM-CSF, IFNα, IFNγ, IL-1β, IL-4, IL-6, PGE_2, TNFα), loaded with antigens via exposure to autologous whole tumor cells, isolated peptides, tumor lysates or RNA, and reinfused intradermally or s.c. into the patient where they activate T cells [210-216]. Encouraging results with clinical responses, including a reduction in tumor size and improved survival of patients, have been reported [210, 211, 216, 217]. However, the non-specific selection of tumor extracts for DC priming increases the risks of autoimmunity towards normal brain antigens [218], which has to be weighed against the risk of developing therapy-resistance when using specific tumor antigens [198]. The best protocol for antigen preparation has been evaluated; DCs were either fused with tumor cells, pulsed with apoptotic tumor cells, peptides, tumor lysate or RNA lysate. DCs pulsed with apoptotic tumor cells were superior at activating CTLs and NKT cells and inducing anti-tumor immunity [219, 220]. Other matters that need to be evaluated are the best source of DCs, the right combination of maturation cytokines, and the best administration route.

Whole tumor cell vaccine

Autologous whole tumor cells as a source of tumor antigens have been used for several decades in glioma research [196, 221, 222]. Autologous resected tumor cells are expanded *ex vivo*, and usually genetically modified to produce various cytokines for further immunostimulation. To avoid tumor growth at the injection site, tumor cells are inactivated by irradiation before they are reinjected into the patient. Irradiation may also enhance the immunogenicity of the injected tumor cells, as discussed below. At the immunization site, the tumor cells are phagocytized by infiltrating APCs, recruited to the immunization site and further stimulated by the cytokine production of the tumor cells. The APCs mature and migrate to the

secondary lymphoid organs, where they will present tumor antigens and activate T cells. By using whole tumor cells, the immune response will be directed towards several tumor antigens, thereby diminishing the risk of therapy resistance (as previously described). However, as with most DC vaccines, there is a risk of developing autoimmunity. The limiting factor of whole cell tumor vaccines is the time-consuming step of culturing and expanding the tumor cells *in vitro*.

IFNγ immunotherapy

The previously described key role of IFNγ as an important regulator of cancer immunoediting and anti-tumoral responses makes it an attractive candidate for immunotherapeutic strategies. When utilized together with whole tumor cellular vaccine it has the potential of increasing the immunogenicity of tumor cells, thus promoting T cell activation and tumor eradication. Indeed, we and others have shown that following IFNγ-based immunotherapy there is an increased T cell tumor infiltration and T cell mediated killing [191, 223, 224], a decrease of tumor size [224, 225] and prolonged survival [48, 191, 223-226]. The promising experimental data from our group lead to the initiation of a clinical trial where eight patients diagnosed with GBM were, following surgical resection and RT, repetitively immunized intradermally with autologous tumor cells genetically engineered to produce IFNγ. The immunization resulted in a median overall survival of 17.2 months, which was significantly longer compared with a historical age-matched control group who survived for 10.7 months ([227], *manuscript in preparation*). It should be noted that the study was performed before the introduction of TMZ; hence the patients did not receive any TMZ or other chemotherapeutic agents.

IL-7 immunotherapy

There are several potential features of IL-7 that could be exploited for immunotherapy. For example, IL-7 has the potential of enhancing immune reconstitution in cancer patients following CT-induced lymphopenia [228], and expanding the number of naïve and memory T cells that are activated by immunotherapeutic strategies, such as whole tumor cell vaccine, adoptive T cell transfer or DC vaccination, with the advantage of not expanding Tregs [92, 93]. As we age, the thymic emigrants of the peripheral naïve T-cell pool decreases substantially, resulting in a highly limited repertoire of T cells available to respond to new antigens, leading to less immunocompetence in the elderly [229]. IL-7 increases

the TCR repertoire diversity via the expansion of naïve T cells and enhances the overall immune competence including improved response to immunotherapy.

IL-7 has been shown to prolong survival of tumor-bearing animals, when administered as an IL-7-based whole tumor cell vaccine, when administered as a recombinant cytokine in combination with GM-CSF-based vaccine, or when produced by intratumoral mesenchymal stem cells in combination with an IFNγ-whole tumor cell vaccine [226, 230-232]. Moreover, IL-7 has shown to augment the anti-tumor response induced by a viral vaccine, and increase the survival of tumor-bearing mice by promoting the expansion of anti-tumoral T cells, enhancing the production of proinflammatory cytokines and antagonizing TGFβ signaling [233].

In a clinical trial, melanoma cells were genetically modified to produce IL-7 and then reinjected back to the patients. The therapy induced reactive T cells in three out of six patients, and a minor anti-tumor response was seen in two patients [234].

All together, adjuvant IL-7 has the potential of expanding T cells activated by immunotherapy.

GM-CSF immunotherapy

GM-CSF has been widely used as an adjuvant for immunotherapy due to its ability to recruit and maturate APCs [102]. When produced by genetically modified tumor cells, GM-CSF has been described as the most potent cytokine to promote a long-lasting systemic anti-tumor response in a mouse melanoma model [235]. Several studies in experimental tumor models, including the GL261 model, utilizing GM-CSF transduced tumor cells has confirmed these results, by displaying an increase in the number of tumor infiltrating T cells and increase in survival [39, 236-241]. Irradiated GM-CSF-transduced tumor cells have also shown therapeutic effect in clinical trials against melanoma, prostate, lung, renal and pancreatic cancer [242-247].

Even though the results from the studies of GM-CSF-based vaccines have been encouraging, the therapeutic window for GM-CSF has been shown to be narrow, as high doses of GM-CSF may recruit immunosuppressive MDSCs [248].

Combination therapies

All immunotherapeutic strategies have their pros and cons, and in order to achieve the optimal therapy it might be ideal to combine these immunotherapeutic strategies with each other [39, 222, 230, 232], with targeted

therapies, for example by inhibiting production of immunosuppressive factors such as PGE_2, NO or PD-1 via the administration of COX-2 [249], iNOS [250, 251] and PD-1 inhibitors [252], or with conventional treatment such as CT and RT [194, 238, 253-255], which may have immunostimulatory potential.

Chemotherapy against gliomas

The use of various chemotherapeutic drugs including cisplatin, carboplatin, etoposide, irinotecan, carmustine, and TMZ has been developed and tested in preclinical and clinical research on gliomas [23-28, 256, 257]. Systemic administration of chemotherapeutic drugs has shown to diminish tumor growth [258], increase apoptosis, reduce angiogenesis [256] and increase survival [257, 258] in experimental glioma models, which is a proof of concept of the efficiency of chemotherapeutic drugs.

More and more evidence imply that the effect of certain chemotherapeutic drugs is not only a direct cytotoxic effect on tumor cells but also an indirect effect on the immune system in the periphery or within the tumor microenvironment.

Immunosuppressive effects of chemotherapy

Conventional therapy such as CT and RT are usually considered to be cytotoxic to hematopoietic cells and regarded as being immunosuppressive. Indeed, many chemotherapeutic drugs exhibit direct cytotoxic effects on immune cells including T cells, which is typically recognized by a clear drop in leukocyte count following systemic delivery of the drug. The majority of GBM patients that receive radiation, TMZ and glucocorticoid therapy develop lymphopenia [259, 260]. In melanoma patients it has been reported that TMZ-induced lymphopenia particularly affects the $CD4^+$ T cell compartment [261]. The nadir of $CD4^+$ T cell count occurs approximately 2 months following initiating therapy, but the lymphopenia is usually sustained throughout the year following therapy. Severe lymphopenia (CD4 count below 200 cells/mm^3) has been associated with a worse prognosis [259]. The high incidence of lymphopenia is the most dose-limiting factor for TMZ treatment, as lymphopenic patients are at higher risk of developing pneumonia or other opportunistic infections [262]. The immunotoxicity could be of concern when combining immunotherapeutic strategies with systemically delivered TMZ.

Immunostimulatory effects of chemotherapy

Recent evidence suggests that the therapeutic efficacy of radiation and certain chemotherapeutics is in part dependent on the interaction with the immune system [263, 264], and could therefore work in synergy with immunotherapy. Several immunomodulatory abilities of RT and CT have been proposed (fig. 6). They can:

1) Induce immunogenic cell death [265].
2) Increase the number of tumor antigens released and presented intratumorally, thus promoting CTL killing [266].
3) Render the tumor cells more susceptible to T cell-mediated killing [267-269] or vice versa [270].
4) Induce lymphodepletion of poorly functional T lymphocytes, which induces a relative cytokine increase and provides more space for vaccine-induced tumor specific CTLs [271].
5) Increase tumor infiltration of effector cells via the upregulation of adhesion molecules such as VCAM-1 and chemoattractants such as monokine induced by interferon-gamma (MIG) and interferon-gamma-inducible protein of 10 KDa (IP-10) [272-274].
6) Deplete subpopulations of cells with immunosuppressive capacity [275, 276].

By working with these different parameters, one can design combination therapies sometimes referred to as *chemoimmunotherapies*.

Immunogenic cell death

The immunogenicity of tumor cells is defined by its MHC/peptide expression, expression of co-stimulatory molecules such as CD80 and CD86, but also by the presentation of DAMPs or "eat me" signals on the cell surface. These signals are affected by exposure to radiation [273, 277], thus enhancing phagocytosis of irradiated tumor cells, but also by certain chemotherapeutic drugs.

One DAMP signal secreted by CT-treated dying tumor cells is the high-mobility-group box 1 (HMGB1). The recognition of HMGB1 via the TLR-4 on DCs leads to efficient antigen processing and cross-presentation of antigens from dying tumor cells. HMGB1 release was shown to be crucial for successful pre-vaccination with dying tumor cells, as inhibition of HMGB1 prevented the therapeutic effect of the vaccine [278]. Other DAMP signals are the activation of chaperones such as heat-shock proteins that bind to scavenger receptors CD91 on

DCs, which induces DC maturation [279], or accumulation of high levels of extracellular ATP recognized by the purinergic receptor P2RX7 on DCs [280].

Another key molecule for immunogenicity and a pro-phagocytic signal is the pre-apoptotic translocation of calreticulin (CRT), a calcium binding protein, to the cell surface [281]. Blocking of CRT inhibits the phagocytosis of dying tumor cells by DCs and abolishes their immunogenicity *in vivo*. The pro-phagocytic signal of calreticulin surface expression is counterbalanced by CD47, an anti-phagocytic signal that functions as a ”don´t eat me” signal [282].

The CT-mediated immunogenic cell death can be advantageous when optimizing loading of tumor antigens *in vitro* during DC vaccine development [283], or at the immunization site *in vivo* when administering whole tumor cell vaccines, but it can also increase the immunogenicity of tumor cells in the tumor bulk.

However certain chemotherapeutics such as cisplatin fail to induce immunogenic cell death due to incapacity of translocating CRT to the cell surface, although this could be rescued by inducing ER-stress [284].

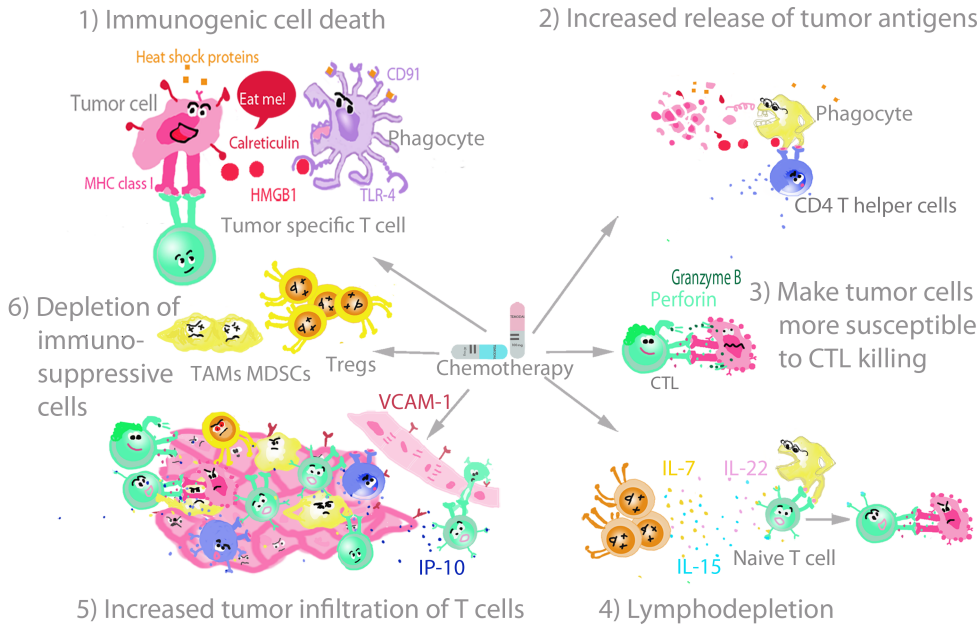

Figure 6. Immunostimulatory effects of chemotherapy

Lymphodepletion

Although lymphodepletion generally is considered to be a negative factor for activating immunotherapy, it has been described that the T cell reconstitution or peripheral homeostatic proliferation following CT-induced lymphopenia may facilitate expansion and enhance the efficacy of anti-tumor immunity [285]. One explanation is the relative increase in the availability of the cytokines IL-7, IL-15 and IL-21, which promotes the survival, expansion and function of potential tumor specific T cells that are either adoptively transferred [286-288] or host naïve T cells activated by different vaccination strategies.

Depletion of immunosuppressive cells

The administration of metronomic (repetitive low doses) regimen of certain chemotherapeutic drugs, such as cyclophosphamide and TMZ, has been reported to specifically deplete the number of Tregs in the tumor draining lymph nodes [289], the peripheral blood [276], spleen [275, 290, 291] or within the tumor [275]. Also, the suppressive function of Tregs was inhibited by cyclophosphamide via downregulation of the gene for FoxP3 [292]. The doses utilized in these experiments were substantially lower than the doses that are used in standard or dose intensified clinical regimens, which are generally suppressive for all T cells.

On the contrary, others have reported that the frequency of circulating Tregs is increased following CT or when CT is combined with immunotherapy [293-295], but that the frequency of Tregs declined intratumorally [294]. Indeed, it has been reported that gliomas express chemokines such as CCL2 that preferentially recruit Tregs to the tumor and that the production of CCL2 was diminished by TMZ treatment [296].

In another study using the chemotherapeutic agent gemcitabine, the number of MDSC found in spleens of tumor-bearing mice was dramatically reduced upon drug treatment, which enhanced the antitumor efficacy following immunotherapy [297].

Chemoimmunotherapy

A few preclinical and clinical studies have investigated the potential of combining immunotherapy with TMZ administration.

The systemic administration of IFNβ and TMZ in nude mice with human intracerebral U-87 xenografts increased survival compared with monotherapy alone [253]. In other experimental studies, systemic administration of low dose TMZ

followed by vaccination with peptide- or- RNA-pulsed DCs was reported to enhance specific T cell responses and improve the survival of GL26 glioma-bearing mice [257, 298].

A synergistic therapeutic effect and clinical responsiveness of GBM patients has been reported following DC vaccination and administration of chemotherapeutics (mainly TMZ). Patients in the combination group displayed a significantly longer survival (26 months) compared with patients either treated with CT (15.9 months) or vaccine alone (17.9 months) [299].

Despite the lymphopenia that was induced by standard or dose-intensified TMZ administration, patients that were immunized with EGFRvIII-targeted peptide vaccine had an overall median survival of 23.6 months, which was significantly longer than a TMZ-treated historical matched control (15.0 months) [295]. A case study of a patient who received the EGFRvIII-targeted peptide vaccine in combination with cycles of TMZ reported that during a single cycle, the peak of the $CD8^+$ T cells and Tregs were at different time points. The Tregs peaked at day 19, and then dropped again at day 23, when the $CD8^+$ T cell had recovered and were at their peak. Day 23, when the CD8 T cells were high and the Tregs were low, was also the time point for vaccine administration, which emphasizes the importance of finding the optimal timing when combining chemotherapy and immunotherapy [300].

Alternative administration routes of chemotherapeutics

Although there have been promising preclinical results of various chemotherapeutic drugs, the effect on survival of most chemotherapeutics tested clinically has generally been minimal [11, 301-306].

One major problem is insufficient exposure of cytotoxic drug concentrations to the tumor cells or to tumor satellites migrating into adjacent normal peritumoral brain, as many systemically delivered drugs are excluded from the CNS by the BBB [307]. In order to reach sufficient amount of drug concentrations intratumorally, the chemotherapeutic agents generally need to be administered at very high systemic concentrations. However, immunosuppressive effects of chemotherapeutics or other unwanted drug-related toxicities, such as nephrotoxicity from cisplatin, is a dose-limiting factor for systemic drug delivery [308, 309]. Therefore, novel alternative delivery techniques for local drug administration have been developed, with the

purpose of increasing therapeutic drug concentrations intratumorally, while at the same time reducing the adverse effects of the drug systemically.

Intratumoral chemotherapy

There are different localized drug delivery strategies developed in preclinical studies for gliomas that have been clinically tested [310]. They include:

1) Direct bolus injection of drugs intratumorally [311] or into the resection cavity following surgery.
2) Infusion of chemotherapeutic agents using catheters with or without implantable pumps [312].
3) Convection enhanced delivery (CED) [313, 314].
4) Various polymeric delivery systems [315, 316], such as wafers or gels [317, 318] or microparticles such as nanospheres, microspheres [319], microcapsules [320], microchips and liposomes, all containing chemotherapeutic agents.

Although the injection of nitrosourea drugs directly into the tumor has shown to be more effective and less toxic than intraperitoneal (i.p.) injection in a mouse glioma model [311], injections are generally associated with a high risk of side effects, such as infections, edema and backflow of the solution along the catheter.

Implantable pumps could offer a more constant flow of chemotherapeutic agents, and decrease the risk of toxicity. However, clinically these infusion pumps have shown to have limited efficiency with a short distribution distance from the infusion site that is dependent on the drug diffusion [310].

CED is a delivery technique where the agent is infused and distributed within the interstitial fluid from a catheter via a pressure gradient from a pump. The pressure creates a bulk fluid flow through the interstitium of the brain, which is independent on the drug diffusion, leading to longer distribution distances than by conventional infusion techniques [321]. CED has shown to achieve higher local levels of CT in rodent brain than following IV administration [322], and a better distribution volume than following simple injection [314]. The limiting factor of local CED is neurotoxicity, and there is still a risk of edema and infections. Treatment failures are usually due to high interstitial pressure, thus leading to a rapid efflux of agent from the injection site and out of the brain [323].

Polymeric implant may be biodegradable (complete erosion), such as the polyactide-co-glycolide copolymer (PLGA), or non-biodegradable. Wafers are usually implanted in the resection cavity following surgery. The use of Gliadel BCNU-loaded

polymeric wafers in combination with RT for the treatment of newly diagnosed GBM was proven to be effective and safe and became approved by the Food and Drug Administration in 2003 [29]. In one study, patients that were implanted with Gliadel wafers following surgery and then administered TMZ and RT treatment had an overall survival of 20.7 months. Patients over the age of 70 also had a greater survival in the combined Gliadel, RT and CT treatment group when compared with Gliadel and RT (21.3 vs. 12.4 months) [324].

Different types of gel formulations and microspheres have also been used to deliver TMZ intratumorally, and have shown to be safe and more effective than systemic TMZ administration in experimental models [315-320]. Although, the general drawback of both wafers, gel formulations and microspheres is the limited penetration depths of the incorporated agents into the surrounding tumor tissue [310].

Although, the pros and cons of combining systemically delivered chemotherapeutic agents with immunotherapeutic strategies have been investigated earlier, the effect of utilizing intratumoral chemotherapeutics in combination with immunotherapy has not been explored, other than in a few cytokine-based immunotherapeutic studies [194, 255]. The reduced systemic drug concentration following local delivery would be preferable when combining CT with immunotherapy. Therefore, one of my hypotheses in this thesis was that intratumoral delivery of chemotherapeutics works in synergy with immunotherapy.

AIMS OF THE THESIS

The overall aims of this thesis were to improve the development of whole tumor cellular vaccines against experimental gliomas by combining the different cytokines IFNγ, IL-7 and GM-CSF and to combine the immunotherapy with chemotherapeutic agents administered either systemically or intratumorally.

The specific aims of the different papers were:

Paper I — To improve whole tumor cell-based immunotherapy in the N32 and RG2 rat glioma models by combining IFNγ and IL-7, and to determine the IFNγ plasma levels as well as circulating proliferating T cells following therapy.

Paper II — To investigate the mechanisms behind the synergistic therapeutic effect after combined immunotherapy using GM-CSF transduced tumor cells and recombinant IFNγ in the GL261 mouse glioma model, and specifically to investigate the role of $CD8^+$ and $CD4^+$ T-cells.

Paper III — To investigate the effect of both systemic and intratumoral administration of TMZ as monotherapies or in combination with GM-CSF-cell based immunotherapy in the GL261 mouse glioma model. Also to specifically investigate the role of T cells for the therapeutic effect of TMZ treatment.

Paper IV — To assess the therapeutic effect of intratumoral delivery of cisplatin as monotherapy or in combination with GM-CSF-based immunotherapy in the GL261 mouse glioma model.

RESULTS AND DISCUSSION

Efficacy of combining cytokines for immunotherapy in rodent glioma models and the role of $CD4^+$ and $CD8^+$ T cells

Paper I: Synergistic effect of IFNγ and IL-7 immunization in glioma-bearing rats

We have earlier established two N32 tumor cell lines transduced to produce either IFNγ or IL-7. The therapeutic effect of immunization using either IFNγ or IL-7 producing tumor cells in the N32 rat glioma model has earlier been assessed. Immunization with the IFNγ- or- IL7- producing tumor cells could eradicate intracerebral N32 tumors separately [226]. However, the outcome of simultaneous immunization with the two cytokine-producing tumor cell lines has not been investigated in the N32 model. Since IFNγ and IL-7 have diverse and complementary mechanisms we hypothesized that they would synergize and enhance the effect of the immunotherapy. Combined IFNγ and IL-7 immunotherapy has never been investigated in the aggressive RG2 rat glioma model; therefore, the RG2 tumor cell line was transduced to produce either IFNγ or IL-7.

The transduced cells were first analyzed *in vitro* for their cytokine production and expression of MHC class I and II. The RG2 cells produced similar levels of IFNγ and of IL-7 as the N32 cells. Irradiation reduced the cytokine production.

The majority of the N32 cells expressed MHC class I, while the expression was much lower on the RG2 cells. Both N32 and RG2 cells were MHC class II negative. IL-7 transduction did not affect MHC expression. However, IFNγ transduction increased the expression of both MHC class I and II on the N32 cells, but induced only a minor upregulation of MHC class I and no upregulation of MHC class II on the RG2 cells. Irradiation trended towards reducing the MHC class I expression on the RG2-IFNγ cells.

The minor upregulation of MHC class I and the absence of up-regulation of MHC class II on the RG2 cells following IFNγ exposure may be due to tumor aberrations that causes alterations in components of the IFNγ signaling pathway or other components of the APM. Others have reported that RG2 cells treated with high concentrations of recombinant IFNγ increased their MHC class I expression *in vitro* [44]. However, following recombinant IFNγ exposure *in vivo* there was no increase in

MHC class I expression on the RG2 tumor cells, but an increase in $ED2^+$ cells, which presumably were perivascular APCs [44].

Next, Fischer 344 rats were inoculated with either N32 or RG2-wt tumor cells and immunized on day 1, 15 and 29 with a mix of irradiated IFNγ- and- IL7-producing tumor cells (see fig. 7).

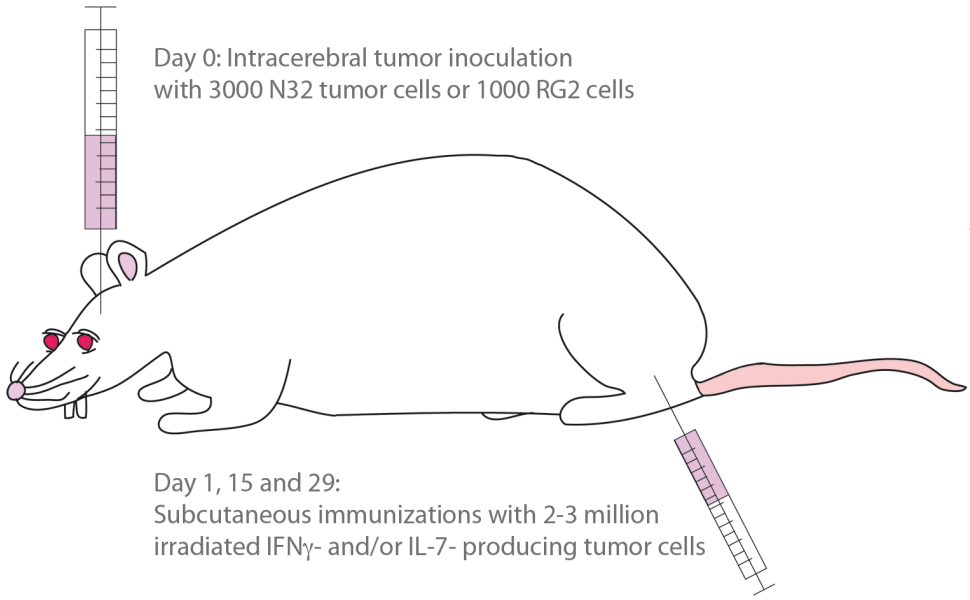

Figure 7. Experimental set-up

Immunization with both IFNγ- and- IL7-producing tumor cells synergistically increased the cure rate of N32 tumor bearing rats (75% cure), compared with both monotherapies (N32-IFNγ: 26% and N32-IL7: 19%). The therapeutic effect of the combined treatment in the RG2 model was only modest, and did not cure any rats, although rats immunized with both cytokine-producing tumor cell lines had a significantly prolonged survival when compared with non-treated rats. The low therapeutic effect seen in the RG2 model could partly be explained by the low MHC expression following IFNγ transduction and irradiation, but also by other factors such as the high proliferative rate of the RG2 cells, which limits the time for activated T cells to reach the brain and eradicate the tumor cells, or other (non-investigated) immunosuppressive factors that the RG2 cells may produce.

In order to monitor the activation of an activated immune response following IFNγ- and IL7- immunization (on day 1, 15 and 27) blood samples were analyzed on

day 1, 6, 20, and 29 and the levels of IFNγ in plasma were determined using ELISA. Before immunization and 5 days following the first immunization all rats had low levels of IFNγ. 5 days following the second immunization the levels of IFNγ increased in all rats immunized with either wt-cells or IFNγ/IL-7 cells, and continued to rise 2 days following the third immunization. The increase was more pronounced in the IFNγ/IL7-immunized group than in the wt-immunized group, suggesting that the combined treatment induced a higher activation of T cells or possibly NK-cells that produced the IFNγ. However, if the increased levels of IFNγ were produced by NK cells, we would have expected a rapid response earlier on following treatment.

In parallel, the percentage of circulating T cells that proliferated following each immunization was studied by flow cytometry. The percentage of proliferating $CD4^+$ and $CD8^+$ T cells following the first and second immunization was low, but increased following the third immunization, especially in the IFNγ/IL-7 group. IL-7 has previously been reported to promote the survival and expansion of $CD4^+$ and $CD8^+$ naive and memory T cells [81, 84, 86, 92, 93], and IFNγ has been shown to increase the expression of MHC class I and II on the transduced tumor cells, and facilitate antigen presentation of APCs. Therefore, we suggest that the two cytokines work in synergy to enhance an effective anti-tumor immune response evoked by whole tumor cell-based immunotherapy.

Paper II: $CD4^+$ and $CD8^+$ T cells mediate the therapeutic effect of GM-CSF and IFNγ immunotherapy of GL261 mouse gliomas

We have earlier demonstrated that immunization with irradiated GM-CSF-producing GL261 cells (GL-GM) and recombinant IFNγ on day 1, 15 and 29 could cure 90% of GL261 tumor-bearing mice [39]. GM-CSF has been reported to recruit myeloid progenitor cells from the bone marrow or monocytes from the blood and promote the maturation of antigen presenting cells such as DCs or macrophages by inducing expression of CD80 and MHC class II [101-103, 235]. However, high doses of GM-CSF may also recruit and expand MDSCs with immunosuppressive capacity [104, 248]. Therefore we analyzed the percentage of MDSCs in the spleen following the first immunization with GL-GM cells and recombinant IFNγ. Indeed, we noticed an increase in the percentage of MDSCs ($CD11b^+Gr^+$) in spleens of mice immunized with GL-GM or GL-GM + recombinant IFNγ. However following the second immunization we also detected an increase in

DCs ($CD11c^+$) and macrophages ($F4/80^+$) that coincided with a decline in the MDSCs population in both GL-GM-treated groups, suggesting that the immature MDSCs had differentiated into matured myeloid cell populations.

To further investigate the mechanisms behind the therapeutic effect, we also analyzed the proportions of T cell subsets, including Tregs, of the total number of splenocytes following the second immunization. The proportions of $CD4^+$ and $CD8^+$ T cells were not different between mice immunized with GL261-wt cells, GL261-wt + IFNγ, GL-GM or GL-GM + IFNγ. However, the fraction of Tregs was increased in the spleens of GL-GM and GL-GM + IFNγ immunized mice. MDSCs have been reported to induce Tregs [163], but the Tregs may also have been induced as a negative feedback loop following the activation of T cells induced by the therapy.

Next, we investigated the cytotoxic anti-tumor response by harvesting splenocytes of immunized mice and re-stimulating them *in vitro* with irradiated GL261 cells. The expression of Granzyme B was analyzed using ELISPOT and the production of IFNγ was measured using flow cytometry or ELISA. Splenocytes obtained from mice immunized with GL-GM or GL-GM + IFNγ had a higher production of both Granzyme B and IFNγ, implying that these cells were more efficient in tumor killing than cells from mice of the other treatment groups.

Moreover, the immunotherapy evoked a long-term memory response as all mice surviving the first tumor also eradicated a second tumor without further treatment.

Finally, we wanted to investigate the role of $CD4^+$ and $CD8^+$ T cells for the therapeutic effect of the combined GL-GM and IFNγ immunotherapy. By depleting either $CD4^+$ or $CD8^+$ T cells we could demonstrate that both $CD4^+$ and $CD8^+$ T cells were crucial for the therapeutic effect of immunization with GL-GM cells and IFNγ, as only 20% of the $CD4^+$-depleted mice and 0% of the $CD8^+$-depleted mice survived tumor challenge, while 90% of the non-depleted mice survived more than 100 days.

Efficacy of intratumoral chemotherapy as monotherapy or in combination with immunotherapy in a mouse glioma model

Paper III: Intratumoral temozolomide synergizes with immunotherapy and the effect is dependent on T cells

When Stupp *et al.* reported an increase in the median overall survival of GBM patients following administration of oral TMZ in combination with RT following surgical resection, TMZ was included in the standard of care for GBM patients [11]. As dose escalation of TMZ has been limited by systemic toxicity, intratumoral delivery of TMZ emerges as an attractive administration route to increase the efficacy of the treatment. A recent meta-analysis of TMZ in experimental glioma models reported that animals treated with local intratumoral TMZ survived longer than those treated with TMZ systemically [258].

To compare the efficacy of systemic to intratumoral administration of TMZ in the GL261 model, we treated GL261 tumor-bearing mice on day 7-9 with daily i.p. injections of 50 mg/kg TMZ (referred to as TMZ IP) or with a 3-day active micro-osmotic pump that continuously infuses TMZ intratumorally (4.2 mg/kg/day) via a catheter coupled to a brain cannula (TMZ IC, see figure 8). The pump was surgically implanted s.c. on the back of the mice on day 7 and removed after day 10 when no longer active.

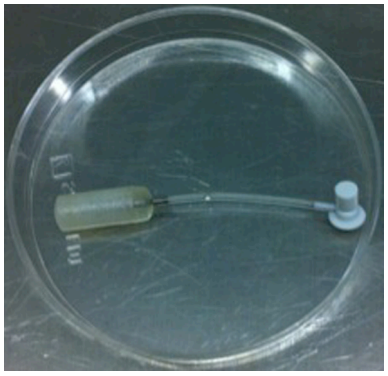

Figure 8. Micro-osmotic pump coupled to a brain infusion kit (catheter and a brain cannula)

Only 8 % of the mice treated with TMZ IP survived tumor challenge. However, TMZ IC had a superior effect over systemic administration with an increased cure rate of 45%, which is in line with previously reported results from other experimental glioma models [258, 312, 315].

When we treated the GL261 cells *in vitro* with 100 μM of TMZ we detected a significant increase in MHC class I expression, suggesting that TMZ acts as an immunomodulator on the tumor cells. However, we could not detect any upregulation of the pro-phagocytic signal calreticulin on the cell surface following TMZ treatment. In order to elucidate the immunomodulatory effect of TMZ *in vivo* we injected T cell depleting antibodies into tumor-bearing

mice and the effect of TMZ IC was monitored by survival. 33% of the TMZ IC-treated mice survived tumor challenge, whereas all T cell-depleted + TMZ IC-treated mice developed lethal tumors, implying that the effect of TMZ was dependent on T cells.

As mentioned earlier, CT can act both immunosuppressively and immunostimulatory. Since we are obligated to treat all participants of future clinical trials with TMZ, we were interested in understanding the effects of TMZ on the immune system and whether TMZ administered systemically or locally would enhance or suppress the outcomes of the immunotherapy.

Therefore, mice were immunized on day 5, 19 and 33 with irradiated GL-GM cells and then treated with systemic or intratumoral TMZ on day 7-9. Since immunization was postponed to day 5, only 25% of the GL-GM immunized mice survived tumor challenge. GL-GM + TMZ IP-treated mice all developed lethal tumors (0% cure), demonstrating that the effect of immunization was eliminated by systemic TMZ. However, GL-GM + TMZ IC cured 83% of the mice, implying that intratumorally delivered TMZ synergizes with immunotherapy.

In order to further investigate the effect of systemic or local TMZ treatment on the immune system, blood samples were collected during TMZ therapy and analyzed for different leukocyte subpopulations. 3 days following TMZ IP treatment there was a significantly lower number of macrophages, granulocytes and MDSC compared with TMZ IC. Also, the number of $CD8^+$ T cells and the percentage of proliferating $CD8^+$T cells were lower in the TMZ IP-treated mice. The TMZ-induced leukopenia was transient as all leukocytes were reconstituted to normal levels 10 days following treatment, except for the proliferating T cells that were still depressed.

The leukocyte subpopulations of immunized mice were also assessed following TMZ treatment. Following GL-GM + TMZ IP-treatment, mice had lower numbers of granulocytes, macrophages, MDSCs, $CD4^+$ and $CD8^+$ T cells, as compared with GL-GM immunized mice or GL-GM + TMZ IC-treated mice. Also, there was a lower proportion of the $CD8^+$ T cells that proliferated in GL-GM + TMZ IP treated mice (6.0%) compared with GL-GM + TMZ IC treated mice (12.3%).

We also analyzed the tumor infiltrating T cells and NK cells using immunohistochemistry of tumor sections by looking at the density of CD4, CD8 and NK1.1 stained area following immunization and/or following TMZ IP or IC treatment. GL-GM immunized mice had the highest amount of CD4 staining intratumorally, which was significantly higher than non-treated or GL-GM + TMZ

IP treated mice. The tumors with the highest amount of CD8 staining was from the GL-GM + TMZ IC treated mice, which was significantly higher than non-treated and TMZ IC-treated tumors. The treatment group with the highest NK1.1 stained area was the TMZ IC tumors, however, this was not significantly different to any of the other treatment groups.

This data suggests that systemic TMZ treatment induces immunosuppression and leukopenia, which depletes both potential APCs and effector T cells, and counteracts the effect of the immunotherapy. On the contrary, intratumoral TMZ sustains the number of potential APCs and T cells that are proliferating following immunotherapy and later infiltrate the brain and eradicate tumor cells.

Immunosuppressive Tregs may be induced by tumors or immunotherapy per se. However, certain chemotherapeutics, including TMZ have shown to specifically deplete Tregs [275, 276]. Therefore, we analyzed the fraction of Tregs among the total $CD4^+$ T cells compartment in blood. We detected a significant increase in the percentage of Tregs of GL-GM + TMZ IP-treated mice (9.2%) and in GL-GM + TMZ IC-treated mice (6.7%) when compared with GL-GM immunized mice only (5.6%). In line with our data, others have reported that the frequency of circulating Tregs is increased following CT or when CT is combined with immunotherapy [293-295], but that the frequency declined intratumorally [294]. Therefore, we investigated the Tregs intratumorally using flow cytometry. Although not significantly different, there was a trend towards lower percentage of Tregs out of all $CD4^+$ cells in GL-GM + TMZ IC-treated mice than in GL-GM + TMZ IP-treated mice.

Others have reported that MDSCs were reduced upon treatment with gemcitabine [297]; hence MDSCs were examined within the tumor. The number of MDSCs was higher in GL-GM + TMZ IP than in GL-GM + TMZ IC treated mice.

This data demonstrates that intratumoral TMZ was less toxic towards peripherally activated T cells but more toxic towards intratumoral Tregs and MDSCs than systemic TMZ. Since local TMZ was more efficient as monotherapy and in combination with immunotherapy, local TMZ would be preferable in clinical trials against GBM.

Paper IV: Intratumoral cisplatin is effective but does not boost immunotherapy of GL261 mouse gliomas

Systemically delivered drugs need to cross the BBB to reach the CNS. This could be an obstacle for certain chemotherapeutic drugs such as cisplatin, which have a poor penetration through the BBB *in vivo* [307, 325]. Moreover, cisplatin is associated with hematopoietic and renal toxicity [308, 309].

Therefore, intratumoral delivery of chemotherapeutic agents such as cisplatin or the cisplatin analogue carboplatin has been explored in glioma research, where it has been shown to cure animals [326-328] and to be well tolerated and effective in clinical trials [329]. However, the effect of combining local cisplatin treatment with immunotherapy has never been investigated.

Since cisplatin has been shown to be highly toxic, we first treated the GL261 cells with cisplatin *in vitro* before continuing with the *in vivo* studies. As suspected, very low concentrations of cisplatin were highly toxic for the tumor cells *in vitro*, with 37% cell viability after exposure to 1 μM of cisplatin.

We then investigated the therapeutic effect of intratumoral delivery of cisplatin as a monotherapy in the GL261 model, starting with a total dose of 64.8 μg of cisplatin, which was delivered using a 3-day active micro-osmotic pump (0.9 μg/μl/h). Unfortunately, the dose was lethally toxic; hence a 9-fold lower dose, 7.2 μg, was tested (0.1 μg/μl/h), but cisplatin-treated mice still displayed drug-related toxicity symptoms. Therefore the dose was further decreased 10 times to a total dose of 0.72 μg (0.01 μg/μl/h). 2 out of 24 mice still showed signs of toxicity (8.3%), but of the remaining 22 mice, 41% were cured, which was significantly different compared with non-treated mice.

Next we wanted to investigate the immunomodulatory effects of cisplatin, as many chemotherapeutics have shown to promote anti-tumor immune activity by inducing immunogenic cell death [281, 330, 331]. Following cisplatin exposure *in vitro* we detected an increase in MHC class I upregulation, but the expression of MHC class II, CD80, CD86 or calreticulin was not affected by exposure to cisplatin.

Finally, we assessed whether intratumoral delivery of cisplatin worked in synergy with immunotherapy. GL261 tumor-bearing mice were immunized on day 5, 19 and 33 with GL-GM tumor cells and then 0.72 μg of cisplatin was administered i.c. on day 5-7 using a micro-osmotic pump as previously described. 22% of the mice immunized with GL-GM cells, or treated with cisplatin were cured. However, mice

in the combination treatment group (GL-GM + cisplatin) also displayed a 22% cure rate, implying that cisplatin had no synergistic or additive effect when combined with immunotherapy.

These results clearly demonstrated the differences between cisplatin and TMZ, as intratumoral TMZ synergistically enhanced the therapeutic effect of immunotherapy (shown in paper III). Also, a total dose of 250 μg of TMZ (approximately 350-times higher than the cisplatin dose tested in this paper) administered during 3 days i.c. did not lead to any signs of toxicity as cisplatin did.

All together, cisplatin was highly toxic for the GL261 tumor cells *in vitro*, and efficient *in vivo* when administered i.c. at the optimal concentration. However, cisplatin administration did not boost the GM-CSF based immunotherapy and was lethally toxic at higher doses.

CONCLUSIONS

Concluding remarks

The main conclusions of this thesis are:

I. The cytokines IL-7, GM-CSF and IFNγ work in synergy with whole tumor cellular vaccines and enhance survival of experimental rat and mouse gliomas.

II. Cytokine-based whole tumor cellular vaccines induce maturation of APCs and the therapeutic effect is dependent on both $CD8^+$ and $CD4^+$ T-cells in the GL261 mouse glioma model, and coincides with increased systemical levels of IFNγ and circulating proliferating T cells in the N32 rat glioma model.

III. TMZ delivered intratumorally is superior to systemic TMZ delivery and the therapeutic effect is dependent on T cells.

IV. Intratumoral delivery of any of the chemotherapeutic agents TMZ and cisplatin increases the survival of GL261 mouse gliomas as monotherapies, but cisplatin induces more toxicity than TMZ and does not enhance GM-CSF-cell based immunotherapy.

V. Intratumoral TMZ, but not systemic TMZ, synergizes with GM-CSF-cell based immunotherapy in the GL261 mouse glioma model. Intratumoral TMZ sustains proliferation of activated T cells following immunization and depletes immunosuppressive cells intratumorally, whereas systemic TMZ induces lymphopenia.

Future perspectives

In this thesis I have studied immunotherapy in three experimental glioma models. As TMZ is included in the standard treatment for GBM patients and the future goal is to develop novel immunotherapeutic strategies for GBM patients, it is important to investigate the effect of TMZ and how it interacts with the immune

system both in preclinical studies but also in a clinical setting. Many immunotherapeutic strategies fail in the clinic due to shortcomings and lack of optimization of the experimental setup. The optimal dose, adjuvants, administration route and optimal timing of the vaccine with standard therapies, need to be evaluated.

In order to further clarify the mechanisms behind the therapeutic effect following immunotherapy and CT a few questions need to be addressed in our preclinical models that may help us understand the clinical situation:

Why do rats with RG2 tumors not respond to therapy, while rats with N32 tumors do? We have suggested that this may be due to unresponsiveness of the RG2 cells to IFNγ, which is common also in GBM patients, but other mechanisms may also be responsible for the minor treatment effect in this model. By inhibiting immunosuppressive factors or regulatory immune cells one may overcome the ability of tumor cells to escape recognition and elimination of the immune system.

Why do certain chemotherapeutics such as TMZ but not others like cisplatin enhance immunotherapy? This could be further addressed by T cell depletion experiments following cisplatin treatment or investigation of different leukocyte populations following combined GL-GM and cisplatin treatment. What is the main mechanisms that induce immunogenic or tolerogenic cell death of tumor cells? Several mechanisms such as calreticulin exposure have been suggested, but since we could not detect any calreticulin exposure on the TMZ-treated tumor cells, there must be other mechanisms crucial for inducing T cell specific anti-tumor immunity.

In order to achieve high and sustainable cytotoxic concentrations intratumorally, what is the optimal delivery technique for intratumoral TMZ treatment in the clinic? How do we manage to sustain high drug concentrations when there is a high intracranial pressure in tumor patients? There may be drugs, such as the peptide known as AF16 with the ability to decrease intracranial pressure [332, 333] that may work in synergy with chemotherapeutics either administered locally or systemically.

As high doses of CT and RT may induce toxicities and disturbed cognitive functions, especially in pediatric brain tumor patients, we want to increase the specificity of treatment by lowering the doses via local CT, and to make tailored immunotherapies for each patient.

POPULÄRVETENSKAPLIG SAMMANFATTNING – SWEDISH SUMMARY

Trots ett sunt leverne med regelbunden motion och nyttig mat kan vi inte alltid förhindra risken att drabbas av vissa typer av cancer som till exempel hjärntumörer. Den vanligaste formen av elakartade hjärntumörer heter glioblastoma multiforme och är också den mest aggressiva. Symptomen varierar beroende på tumörens storlek och var i hjärnan den växer, och patienter kan få problem med andning, huvudvärk, dubbelseende, svindel, krampanfall, personlighetsförändringar samt att göra vardagliga ting som att gå eller äta.

Idag försöker man att behandla majoriteten av alla elakartade hjärntumörer med kirurgi, men tumörens förmåga att infiltrera den omkringliggande normala hjärnvävnaden gör det nästan omöjligt att ta bort hela tumören med kirurgisk hjälp. Operationen kan möjligen minska tumörens storlek och därigenom lindra många symptom. Tyvärr är sannolikheten stor att de tumörceller som finns kvar efter operation återväxer och bildar nya tumörer, vilket gör det mycket svårt att bli av med tumören. Kirurgi kombineras därför med andra behandlingsformer som strålbehandling och cellgifter. Dessa behandlingsformer är dock problematiska eftersom de ofta ger upphov till allvarliga bieffekter. Kroppens egna immunförsvar försvagas till exempel av cellgiftsbehandling vilket kan leda till allvarliga infektioner som kroppen inte kan ta hand om. Dessutom utvecklar tumörer ofta resistens mot dessa behandlingar och därför lever de patienter som drabbats av glioblastoma multiforme endast i ca 15 månader.

Jag har under mitt avhandlingsarbete arbetat med att utveckla ett vaccin mot hjärntumörer. Vi testar vårt vaccin på möss och råttor med hjärntumörer, och vi avser att sedan applicera våra resultat på patienter. Vårt vaccin består av tumörceller som vi manipulerar genetiskt så att de börjar tillverka ämnen som aktiverar immunförsvaret. Innan vi vaccinerar djuren strålar vi tumörcellerna för att förhindra celldelning och bildandet av nya tumörer vid vaccinationsstället.

I mitt första delarbete (Paper I) har jag studerat råttor med hjärntumörer. Jag har visat att om vi kombinerar vårt tumörcellsvaccin med två ämnen som aktiverar immunförsvaret: IFNγ och IL-7, börjar kroppens egna vita blodkroppar att dela sig och blir fler. Dessa vita blodkroppar börjar i sin tur tillverka mer IFNγ. Med hjälp av blodprov från djuren har vi kunnat mäta en höjning av detta ämne i blodet, vilket har

gett oss en indikation på att djuren har svarat på behandlingen. De aktiverade vita blodkropparna färdas sedan genom blodet och tar sig in i hjärnan där de känner igen tumörcellerna som främmande/farliga och dödar tumörcellerna. I min första studie blev 75% av de råttor som fick vårt tumörcellsvaccin med IFNγ och IL-7 helt botade från sina hjärntumörer. De råttor som inte fick någon behandling överlevde inte, och av de råttor som bara fick ett av de immunaktiverande ämnena i sitt vaccin, botade vi 19-25%. Detta visade att vaccinering med två immunaktiverande ämnen var mer effektivt än vaccinering med bara ett ämne.

Vi har sett att våra tumörcellsvaccin fungerar i både råttor och i möss men vi vet inte HUR det fungerar. Därför har vi i det andra delarbetet (Paper II, där jag är andra författare) undersökt möss med hjärntumörer för att ta reda på vilka vita blodkroppar som är viktiga för vårt vaccins inverkan på mössens förlängda överlevnad. Ett sätt att ta reda på detta är att ge mössen antikroppar som binder till och tar bort vissa vita blodkroppar och studera hur detta påverkar vaccinets effekt. Om de celler vi tar bort har en viktig roll för effekten av vårt vaccin förväntar vi oss att dessa möss inte överlever lika länge som de som har sina vita blodkroppar kvar. Vi tog bort två typer av vita blodkroppar som heter CD4 T celler och CD8 T celler. Resultatet blev att nästan inga möss klarade av att bekämpa tumören utan CD4 och CD8 T celler, vilket visade att de hade en vital betydelse för effekten av vaccinet.

I delarbete 3 och 4 (Paper III och IV) har jag förutom att ge mössen vårt tumörcellsvaccin också behandlat dem med två typer av cellgifter: temozolomid och cisplatin. Temozolomid ges som standardbehandling till patienter med hjärntumörer och cisplatin är ett vanligt cellgift som ges till många olika cancerformer. I delarbete 3 jämförde jag att ge mössen en hög dos temozolomid till hela kroppen (ungefär som patienter får läkemedlet idag) med att ge en mycket lägre dos direkt in i tumören. Det visade sig att när cellgiftet gavs direkt in i tumören hade det bättre effekt än när det gavs till hela kroppen (45% jämfört med 8% överlevnad). Genom blodprovstagning av mössen kunde jag också studera effekten av cellgiftet på de vita blodpropparna. De möss som fick höga nivåer av cellgifter i hela kroppen hade färre vita blodkroppar i blodet än de möss som fick lokal cellgiftsbehandling, vilket betyder att det är mindre skadligt för immunförsvaret att ge cellgifterna direkt in i tumören. Därefter testade jag att ge cellgiftet på de två olika sätten tillsammans med tumörcellsvaccinet. Av de möss som bara blev vaccinerade blev 25% botade av tumören. De möss som hade vaccinerats och sedan fick en hög dos av cellgift i hela kroppen lyckades inte överleva, vilket tyder på att cellgifterna dödade de viktiga vita blodkropparna (t.ex. T cellerna)

ansvariga för den tumördödande effekten av vaccinet. Däremot överlevde 83% av de möss som fick vaccinet och sedan lokal cellgiftsbehandling, vilket visade att T cellerna inte dog av lokal cellgiftsbehandling utan fanns kvar och kunde tillsammans med cellgiftet bekämpa tumören.

I delarbete 4 visade jag att även lokal behandling av cisplatin också kunde bota 41% av djuren, men att höga doser av cisplatin lokalt i hjärnan även hade allvarliga biverkningar vilket visar vikten av att ge rätt dos av det injicerade cellgiftet.

Vi har tidigare testat ett vaccin på patienter som bygger på resultat från våra djurstudier. Patienters tumörceller manipulerades så att de tillverkade IFNγ och sedan injicerades de in i patienterna. Vi såg en förlängd överlevnad hos vaccinerade patienter med 6-7 månader jämfört med patienter som fick standardbehandling. Förbättringarna som nu gjorts av vaccinet kommer nog i framtiden att möjliggöra en förlängning av överlevnaden hos dessa patienter. Om vi dessutom ger vaccinet med lokal cellgiftsbehandling skulle patienternas överlevnad förhoppningsvis kunna förlängas ytterligare.

ACKNOWLEDGEMENTS

Jag skulle vilja framföra mitt allra största och fetaste tack till alla ni som har hjälpt mig de senaste fyra åren och som på olika sätt gjort min doktorandtid så fantastiskt rolig, inspirerande och spännande! Detta vill jag göra i kronologisk färgordning:

För utan charmiga kompisen *Sofia H*, som föreslog att vi skulle göra ett labbprojekt under biomedicinutbildningen, hade jag aldrig börjat i GIT-gruppen. Djurförsök var inget för mig trodde jag. Men efter inte lång tid satt jag där nere i djurhuset och opererade in tumörer i mushjärnor, med vänsterhanden dessutom! (= (vänsterhänt smiley). Sedan blev det ex-jobb som handleddes av härliga *Karin*, som också är förste författare till delarbete 2. Tack för det samarbetet och för att du och Anna tog emot mig i gruppen trots att jag inte gillade sushi!

Apropå sushi, delade jag kontor ganska länge med japan-älskande *Salina*. Tack för alla gemensamma konferensresor till Barcelona, New York/Washington (då ni tvingade mig äta sushi) och Alingsås, denna metropol! Jag kommer minnas varje sekund, om inte för att du dokumenterat allt! Vi kanske ses på en annan resa, t.ex. ett pendlartåg till Danmark?

Apropå dokumentation, vill jag rikta ett speciellt tack till vår eminente isländska guru *Gunnar* för att du alltid finns till hands, speciellt när det rör sig om filmskapande eller tillagning av spanska boquerones!

Att laga god mat kan energiska *Wiaam* som i vårt labb kommer och går, men jag hoppas att vår vänskap består! Vem ska jag annars älta mina kärleksbekymmer med?

Utan vänner kommer man inte långt. Jag skulle vilja tacka alla tjejer som bott tillsammans med mig under min doktorandtid på Spole: roliga *Minh-Hang*, (vi ses väl på COS i London snart?), min alltid lika eftterlängtade hyresgäst och coola *Anna*, som också har kämpat med sin avhandling, min ständige partner in crime *Frida*, som alltid är med på galna upptåg, vare sig det är på Grand/Häktet, gåendes en power-mil till Valkärra eller i telefon Eksjö-Lund diskuterandes storlek på framsidan av en avhandling, och min allra bästa bästis *Malvina*, för att du alltid funnits där i alla stunder. Att sjunga duett med dig på en balkong vid medelhavet är grymt!

Att sjunga har utgjort en stor del av min fritid dessa år och hur skulle jag kunnat göra det utan min eminenta numera 10-åriga vän, *Carolinae Damkör*! Tack kära Caroliner för att ni förgyller mina ibland gråa tisdagar. Jag vill inte missa en enda repa! Speciellt ett stort tack till vår musikaliska dirigent *Ulrika* för att du är en så

inspirerande konstnärssjäl, och till sångfantasterna *Bella*, *Anna-Karin*, *Greta* och *Karin* för att vi blivit så goda vänner under årens lopp.

Tisdagar ja. Tråkigaste dagen i veckan tänker ni. Inte på labbet iallafall, för då är det fika! Tack kära galningar i fikagruppen: *Andreas* (kommer aldrig glömma din McGyver-sång), *Peter*, *Susanne*, *Linda*, *Johan*, *Kajsa*, *Elna*, *Camilla*, *Linda*, och *Jakob* för alla roliga konversationer om allt i från: David "Jesus pappa" till snusk och zombiemyror.

Det finns ju bara en person som kan vara så entusiastisk över något så litet som zombiemyror, kära sprudlande *Sofia*! Glädjespridaren nummer ett här på labbet som alltid ser allt på ett positivt sätt och som aldrig säger nej till en gratisfika! (= Att du orkar lyssna på allt mitt pladder! Tack för alla våra pratstunder och för ett gott samarbete under vår doktorandtid! Utan dig vet jag inte hur det skulle ha gått med alla blodprovstagningar i PET-flaskor. Tack också glada *Josefin* som fått med oss på AWs, brännbollsturneringar och Toddyspex med IKVL varje år!

En stor del av mina doktorandtidskvällar har jag tillbringat i AF-borgen tillsammans med käraste *Lundaspexarna*. Fy fan vad lövli det har varit att kunna komma dit på kvällarna efter långa labbdagar och måla fonder med konstnärssjälen *Anni* eller gå på siste april sminklunch med fantastiska *F-akademien*! Efter flertalet spexmånader har jag hittat vänner för livet.

Ett stort tack till spexige professor emeritus *Leif G Salford*, för finansiering av de första åren av min doktorandtid, och till alla övriga kollegor på *Rausing Lab*. Även ett tack går till *Immunologen* (ni på andra sidan) som har funnts väl till hands när vi behövt antikroppar! Det har varit mycket roligt att dela kök och julfester med er! Speciellt tack till f.d. kursaren och finsmakaren *Karin* som alltid har tid för en korridors-snackis.

Utvecklingen gick snabbt framåt och plötsligt skulle jag börja skriva på min avhandling. Det hade inte blivit mycket till avhandling (eller doktorandutbildning överhuvudtaget) om inte min kära bihandledare och spindeln i nätet *Anna* styrt upp allt! Tack för allt stöd du har givit mig under min tid här på labbet, med privata saker och allt som har med jobbet att göra. Du är en klippa och utan dig hade jag inte suttit här och skrivit de sista orden av min avhandling. Vi i tjejmaffian har haft så mycket kul under dessa år med trevliga middagar och mellokvällar, AWs, en massa skvaller och galet dansande på konferensresor samt på Gerdahallen. Vi ses på afron!

En till person som har haft stor betydelse för den senare delen av min doktorandtid är f.d. värstingen *Emma*. Språkgranskaren vid skrivbordet intill som

ständigt blivit avbruten i sitt arbete över någon grammatik-fråga från dysflexi-kollegan. (= Dessutom en ängel som lagt ner ofantligt mycket tid åt att hjälpa mig med immunohistofärgningar. Jag har varnat henne men hon vill stanna och avsluta sin doktorandtjänst. Och hon gillar inte sushi.. än!

Någon som kan tyda både drömmar och FACS-plots och som är mer utav en andlig vägledare är harmoniske *Edward*. Tack för alla pratstunder om allt från demokrati (eller frånvaro av det snarare) till högre energinivåer och för att du sätter världsliga saker i andra perspektiv. Vi är sällan överens och det är ju alltid kul. (=

Andra perspektiv på livet och på forskning har också en väldigt cool katt: min huvudhandledare lugne *Peter*. Tack för att du är så stresstålig, trots planerade operationer när du egentligen skulle vara på ett flyg till Sydafrika. Från en nattuggla till en annan har du delat med dig av din obekymrade laid-back stil även till mig och detta resulterade i att skriva avhandling blev för mig en fröjd!

Tillslut vill jag tacka min stora familj! Tack älskade *Mamma* och *Pappa* (farsgubben) för att ni alltid finns och bryr er om hur saker och ting går. Ni har alltid en hjälpande hand när det behövs! Tack också superenergiska syster *Hanna* för att du varit ett så stort stöd för mig dessa år! På senare tid har du fått mig på andra tankar (vilket kan vara skönt ibland), med mail om valstakter och brudklänningar. Dessutom ett stort tack för din språkgranskning av artiklarna och för att du tvingat din blivande man att korrekturläsa hela min avhandling, a BIG thank you Dr. *Jeremy*! Ett stort tack också till mina kära sångfåglar och kräftdyrkande syskon: *Martin*, *Lotta*, *Maria*, *Olle*, *Kirre* och *Ylva*, med respektive och syskonbarn, samt min stora skaldjursälskande släkt, far/morbröder, mormor, moster och kusiner (både *Perssons* och *Fritzells*). När får jag läsa era avhandlingar?

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