Immunotherapy against malignant brain tumors - of mice and men

Enell Smith, Karin

2008

Immunotherapy against malignant brain tumors
–
of mice and men

Karin Enell Smith

2008

Akademisk avhandling

Som med vederbörligt tillstånd av Medicinska fakulteten vid Lunds Universitet för avläggande av doktorsexamen i ämnet Neurokirurgi kommer att offentligen försvaras i Segerfalkssalen, Wallenberg Neurocentrum, Lunds Universitet, Lund

Lördagen den 24:e maj kl 9:15

LUND
UNIVERSITY

Avdelningen för Neurokirurgi
Institutionen för Kliniska Vetenskaper
Lunds Universitet

Fakultetsopponent:
Prof. agrégé sous octroi Luc Vallières
Université Laval
Quebec, Canada

Organization
LUND UNIVERSITY

Dept. of Clinical Sciences
Div. of Neurosurgery
Rausing laboratory

Document name
DOCTORAL DISSERTATION

Date of issue

Sponsoring organization

Author(s)
Karin Enell Smith

Title and subtitle
Immunotherapy against malignant brain tumors – of mice and men

Abstract

Glioblastoma Multiforme (GBM) is the most abundant and most aggressive primary brain tumor in adults. Due to the infiltrative growth of the tumor, surgery will never be radical. Radiation and chemotherapy only marginally improve the poor prognosis due to the ability of the GBM to develop resistance towards these treatments. Therefore it is of great importance to find new efficient treatment modalities.

The aim with this thesis was to develop and evaluate a cytokine based immunotherapy as treatment for GBM in both an experimental mouse glioma model as well as in patients suffering from GBM.

In paper I we demonstrate the establishment of a GM-CSF and IFNγ based immunotherapy in the mouse glioma model, GL261. Mice with intracranial gliomas were immunized intraperitoneally with GL261 cell genetically modified to produce GM-CSF combined with recombinant IFNγ. This combination synergistically enhanced survival to 90%. In paper II-III we investigated the immune responses elicited by these immunizations both systemically as well as locally in the tumor. We found that the immunizations with GM-CSF and IFNγ were highly dependent on T-cells for mediating survival of the mice. In paper IV we monitored the immune responses elicited in GBM patients receiving IFNγ based immunotherapy using ELIspot and CBA.

Immunotherapy enhances the patient's own antitumoral immune responses otherwise suppressed by the tumor. We believe that this treatment, in combination with conventional treatments such as surgery, radiotherapy and chemotherapy, has a great promise for the future treatment of patients with GBM.

Key words:
Glioma, immunotherapy, mouse, GM-CSF, IFNγ

Classification system and/or index termes (if any):

Supplementary bibliographical information:

Language

ISSN and key title:
1652-8220

ISBN
978-91-86059-09-5

Recipient's notes

Number of pages

Price

Security classification

Immunotherapy against malignant brain tumors
–
of mice and men

Karin Enell Smith

Department of Clinical Sciences
Division of Neurosurgery
The Rausing Laboratory
Lund University

LUND
UNIVERSITY

Academic dissertation
Lund 2008

Cover artwork by Karin Enell Smith and Bengt Mattsson

ISBN 978-91-86059-09-5

Printed by Grahns Tryckeri AB, Lund, Sweden

"The best-laid plans of mice and men,
go oft awry"

"To a mouse" by Robert Burns

To Ruben

CONTENTS

ORIGINAL PAPERS

Paper I — Smith KE, Janelidze S, Visse E, Badn W, Salford L, Siesjö P, Darabi A. Synergism between GM-CSF and IFNγ: enhanced immunotherapy in mice with glioma. *Int J Cancer*. 2007 Jan 1;120(1):75-80.

Paper II — Enell Smith K, Fritzell S, Badn W, Eberstål S, Janelidze S, Visse E, Darabi A, Siesjö P. Cure of established GL261 mouse gliomas after combined immunotherapy with GM-CSF and IFNγ is mediated by both $CD8^+$ and $CD4^+$ T-cells. *Submitted to Int J Cancer*

Paper III — Enell Smith K, Fritzell S, Eberstål S, Janelidze S, Visse E, Darabi A, Siesjö P. Enhanced lymphocyte infiltration in tumor and draining lymph nodes after immunization against intracerebral GL261 glioma with GM-CSF and IFNγ. *Manuscript*

Paper IV — Visse E, Enell Smith K, Darabi A, Esbjörnsson M, Agemark Fellert M, Janelidze S, Skagerberg G, Widegren B, Salford LG and Siesjö P. Immunoreactivity is coupled to clinical responses after immunotherapy of glioblastoma multiforme. *Submitted to Neuro-Oncology*

PAPERS NOT INCLUDED IN THE THESIS

Janelidze S, Enell K, Visse E, Darabi A, Salford LG and Siesjö P. Activation of purified allogeneic CD4(+) T cells by rat bone marrow-derived dendritic cells induces concurrent secretion of IFN-gamma, IL-4, and IL-10.
Immunol Lett 2005 Nov 15;101(2):193-201

Badn W, Visse E, Darabi A, Smith KE, Salford LG and Siesjö P. Postimmunization with ifn-gamma-secreting glioma cells combined with the inducible nitric oxide synthase inhibitor mercaptoethylguanidine prolongs survival of rats with intracerebral tumors.
J Immunol 2007 Sep 15;179(6):4231-8.

Badn W, Hegardt P, Fellert MA, Darabi A, Esbjörnsson M, Smith KE, Janelidze S, Salford LG, Visse E and Siesjö, P. Inhibition of inducible nitric oxide synthase enhances anti-tumour immune responses in rats immunized with ifn-gamma-secreting glioma cells.
Scand J Immunol 2007 Mar 65(3):289-97.

ABBREVIATION LIST

APC	Antigen presenting cell
ARG1	Arginase 1
BBB	Blood-brain barrier
CBA	Cytokine bead assay
CNS	Central nervous system
CSF	Cerebrospinal fluid
CTL	Cytotoxic T lymphocyte
CTLA-4	Cytotoxic T lymphocyte antigen-4
DC	Dendritic cell
GBM	Glioblastoma Multiforme
GFAP	Glial fibrillary acidic protein
GITR	Glucocorticoid-induced TNF receptor
GL-GM	GM-CSF producing GL261 cells
GM-CSF	Granulocyte macrophage colony-stimulating factor
i.c.	Intracerebral
i.p.	Intraperitoneal
IFNγ	Interferon gamma
IFNGR	Interferon gamma receptor
iNOS	Inducible nitric oxide synthase
MCA	Methylcolanthrene
MHC	Major histocompatibility complex
NK-cell	Natural killer cell
NO	Nitric oxide
PBL	Peripheral blood lymphocyte
PEG	Polyethylene glycol
PGE2	Prostaglandin E2
ROS	Reactive oxygen species
TAM	Tumor associated macrophage
TCR	T-cell receptor
TDLN	Tumor draining lymph node
TGFβ	Transforming growth factor beta
TIL	Tumor infiltrating lymphocyte
TNFα	Tumor necrosis factor alpha
VEGF	Vascular endothelial growth factor
WHO	World health organization

SVENSK POPULÄRVETENSKAPLIG SAMMANFATTNING

Introduktion

Den vanligaste formen av maligna gliom, Glioblastoma Multiforme (GBM), är också den mest elakartade typen av hjärntumör hos vuxna. Tumörens infiltrativa växtsätt i omkringliggande, normal hjärnvävnad gör att kirurgisk behandling aldrig kan bli radikal utan enbart minska tumörbördan. GBM är dessutom till hög grad resistent mot både strålning och cytostatika. Det är mycket ovanligt att man kan bota patienter med GBM, därför är det av stort intresse att hitta nya behandlingsformer.

Experimentell gliommodell i mus, GL261

Vi har utvecklat en immunterapi mot ett intracerebralt växande gliom i mus. Musgliomceller, s.k. GL261-celler (GL-wt), inokuleras i hjärnan dag 0. Obehandlade, dör dessa möss inom 20 dagar, pga. den aggressivt växande tumören i hjärnan. Med start dag 1 och därefter varannan vecka, vaccinerar vi de tumörbärande mössen i buken med rekombinant interferon-gamma (IFNγ) tillsammans med GL261-celler genetiskt modifierade att producera granulocyt macrophage-colony stimulating factor (GM-CSF) (så kallade GL-GM celler).

Immunterapi med GM-CSF och IFNγ förstärker immunsvaret

Då mössen vaccineras attraheras de antigen presenterande cellerna (dendritiska cellerna) till vaccineringsplatsen. De dendritiska cellerna fångar upp de vaccinerade tumörcellerna. De dendritiska cellernas utmognad påskyndas ytterligare av närvaron av GM-CSF och IFNγ. Därefter migrerar de dendritiska cellerna till de närliggande sekundära lymfoida organen (mjälte, lymfkörtlar) där T-cellsaktiveringen sker. De dendritiska cellerna visar upp de uppfångade tumörcellsproteinerna för naiva T-celler. Dessa naiva T-celler kan då aktiveras till effektor T-celler, dvs. tumöravdödande $CD4^+$ T hjälpar celler och cytotoxiska $CD8^+$ T-celler, som bl.a. kan producera IFNγ. Dessa tumöravdödande T-celler migrerar sedan till hjärntumören. Effektor T-cellerna känner där igen hjärntumörcellerna och kan avdöda dessa via olika cytotoxiska mekanismer. Direkt tumöravdödande immunceller är förutom T-cellerna dessutom makrofager och NK-celler.

Immunförsvaret motverkas av tumören genom immunsuppression

Immunförsvaret motverkas däremot av att tumören utvecklar flera immunosuppressiva metoder under dess tillväxt. T.ex. kan tumören inducera så kallade myeloida suppressor-celler (MDSCs). MDSCs är en blandad omogen cellpopulation som, beroende på olika vilka faktorer som finns närvarande i dess miljö, har förmågan att mogna ut till flera olika celltyper (makrofager, dendritiska celler och granulocyter). Så länge MDSCs inte har mognat ut motverkar de immunförsvaret, bl.a. genom att stimulera bildningen av hämmande T-celler, så kallade regulatoriska T-celler. Det är välkänt att tumören utsöndrar hämmande faktorer som kan öka mängden immunohämmande celler både i tumören såväl som i de lymfoida organen. Dessa celler blockerar immunlogiska aktiviteter riktade mot tumören, vilket gynnar tumörtillväxten.

Denna immunhämning är inte konstant. Med immunterapi kan vi förändra den immunohämmande miljön genom att t.ex. mogna ut den omogna populationen av MDSCs till aktiva dendritiska celler. De aktiverade cytotoxiska T-celler kan också, förutom att vara direkt tumöravdödande, dessutom förstärka utmognaden från immunohämmande MDSCs till tumöravdödande makrofager. Denna effekt som de cytotoxiska T-celler har på MDSCs är viktig både i de lymfoida organen så väl som i och kring tumören för att förstärka det tumöravdödande immunsvaret.

Inflöde av immunceller i tumören

Ökat inflöde av immunceller i tumören efter immunterapi associeras ofta med en god prognos. Med avseende på vad de producerar för faktorer har infiltration av makrofager i tumören visats antingen kunna gynna tumörtillväxt eller verka tumöravdödande. Makrofager i tumörer med en hög produktion av proteinet tumor necrosis factor alpha (TNFα) har visat sig vara effektiva tumöravdödande immunceller För att kunna påvisa att $CD8^+$ och $CD4^+$ T-celler i tumören är cytotoxiska T-celler och inte regulatoriska T-celler måste deras funktion som tex deras förmåga att dela sig och IFNγ-produktion studeras. Genom att bara titta på utseendet och inte funktionen av immunceller vet vi inte vilken roll de spelar, dvs. om de är tumöravdödande eller immunhämmande.

Delarbeten

Arbete I

I det första arbetet etablerar vi vår immunterapi där vi kombinerar både GM-CSF producerande tumörceller och proteinet IFNγ i vårt vaccin till möss med hjärntumörer. Vi visar att möss med gliom som vaccineras med GM-CSF och IFNγ dag 1 efter att tumören satts in har en överlevnad på nästan 90 % och vi får dessutom en förstärkande effekt av överlevnaden när vi kombinerar de båda cytokinerna GM-CSF och IFNγ. När vi tittar i tumören efter vaccinering ser vi ett ökat inflöde av makrofager.

Arbete II

I arbete II vill vi undersöka immunsvaret som uppstår efter vaccineringen med GM-CSF och IFNγ. Efter första vaccineringen med GM-CSF och IFNγ ser vi primärt att de omogna MDSCs ökar i de lymfoida organen. Däremot har dessa celler mognat ut till dendritiska celler efter andra vaccineringen. T-cellerna i dessa lymfoida organ har en hög tumöravdödande aktivitet. När vi vaccinerar möss, som inte har några T-celler, med GM-CSF och IFNγ överlever ingen. Dvs. T-cellerna är av yttersta vikt för att utföra tumörcellsavdödning. Vaccinering med GM-CSF och IFNγ framkallade dessutom ett immunologiskt minne eftersom möss som blivit vaccinerade och därmed överlevt sin första tumör även överlevde sin andra tumör utan några nya vaccineringar. I detta arbete avslutar vi med att visa att vaccineringen också gav en överlevnad mot redan etablerade tumörer.

Arbete III

I arbete II vill vi studera immunsvaret i tumören och i de lymfoida organ som dränerar tumören. Vi ser att vaccinering med bara GM-CSF ökar mängden MDSCs och regulatoriska T-celler i de dränerande lymfoida organen jämfört med när vi tillsätter IFNγ till vårt vaccin. Dvs. vi minskar

den hämmande miljön runt tumören. Antalet T-celler ökar efter vaccinering med GM-CSF och IFNγ. IFNγ som produceras av aktiverade T-celler kan aktivera produktionen av TNFα från makrofager i tumören. När vi undersöker makrofagerna i tumören ser vi att fler makrofager producerar TNFα efter vaccinering med GM-CSF och IFNγ, vilket är viktigt för att mediera döda tumörceller.

Arbete IV

Baserat på tidigare studier, där råttor med malignt gliom behandlats med IFNγ baserad immunterapi, har en klinisk fas I studie startats. 8 patienter med GBM har behandlats och dessa hade en förlängd överlevnad (jämfört med en relevant kontroll grupp) efter att de vaccinerats med de egna tumörcellerna som genetiskt förändrats så att de producerar IFNγ. I arbete IV ville vi undersöka två laborativa metoder (ELIspot och CBA) för att mäta det immunsvar som uppstår hos patienternas före och under behandlingen och eventuellt kunna jämföra detta med överlevnadslängd. Med dessa tekniker kunde vi uppmäta förhöjda nivåer av IFNγ ifrån immunceller hos de patienter som hade de överlevde längst efter immunterapibehandlingen.

AIMS

The general aims with this thesis was to improve our existing IFNγ based immunotherapy as well as monitor the immune responses elicited by the immunotherapy.

Specific aims were:

- To establish GM-CSF and IFNγ based immunotherapy in the mouse glioma model GL261 (Paper I)

- To elucidate the mechanisms behind the combined therapy by monitoring the systemic as well as intratumoral immune responses elicited by the combined treatment of GM-CSF and IFNγ, leading to the synergistically enhanced survival (Manuscript II and III).

- To assess whether the *in vitro* assays ELIspot and CBA were feasible for monitoring the immune responses elicited in patients with GBM receiving IFNγ based immunotherapy (Manuscript IV).

GLIOBLASTOMA MULTIFORME

Introduction to Glioblastoma Multiforme

Gliomas comprise a group of primary brain tumors that develop from cells of a glial origin in the central nervous system (CNS) including astrocytomas, oligodendrogliomas and ependymomas. Among the gliomas, the Glioblastoma Multiforme (GBM), of an astrocytic origin, is the most aggressive brain tumor. According to the I-IV world health organization (WHO) grading system ("malignancy scale") of CNS brain tumors, GBM accounts as a grade IV neoplasm. Grade IV include the following characteristics highly malignant, aggressive growth pattern and necrosis-prone neoplasm, associated with a rapid development of the disease often followed by a fatal outcome. GBM is usually localized in the white matter of the cerebral hemispheres, most frequently in the frontal lobes. The symptoms are due to focal tissue damage, local edema and increased intracranial pressure and are therefore diverging, depending on the localization of tumor. Common symptoms are headache, nausea, focal neurological deficits (visual impairment) and epileptic seizures (1, 2).

GBM is the most common of the primary brain tumors in adults, representing almost 15% of all intracranial neoplasms and 60-75% of all brain tumors of astrocytic origin (1). The incidence rate is 3-4 new cases per 100 000 per year (1). Despite current treatment encompassing surgery, radiotherapy and chemotherapy, the median survival rate is still very poor, between 12-15 months with a two-year survival approaching zero (3). The reasons behind this poor outcome are several. The growth pattern of GBM is rapid and invasive and the tumor infiltrates into the surrounding tissues, making it impossible for a complete surgical resection of the tumor. GBM is also known to be highly resistant towards therapy due to poor drug delivery over the partial blood-brain barrier (BBB), genomic instability, the presence of a neural stem cell-like population of cells harboring resistance mechanisms and efficient DNA-repair mechanisms abrogating the effects of chemo- and radiotherapy (4-7). Several studies have also shown that patients with GBM are immunosuppressed. The tumor itself produces factors down-modulating the immune response, leading to tumor immune escape.

Experimental mouse glioma models

Experimental animal models are a prerequisite for the testing of new treatment modalities. For this reason there are several models developed in mice. There are xenograft models available where human brain tumor cell-lines are transplanted into immunodefficient mice. However due to the lack of a functional immune system in this mouse strain, these models are not optimal for the development of new treatment modalities, including immunotherapy. Transgenic models are optimal for the study of genes involved in the development and establishment of glioma. Spontaneous tumors do occur, although very rarely in animals kept in the laboratory. However the inbred mouse strain VM/Dk spontaneously developed astrocytomas, from which two cell-lines have been established, SMA-497 and SMA-560. These cell-lines share common features of human GBM when implanted intracerebrally (i.c.) into syngeneic mice (8, 9).

GL261 mouse glioma model

The mouse glioma model, GL261, was initially induced in the 1930s by implanting the carcinogen methylcolanthrene (MCA) into the brains of mice of a C57Bl/6 background (10). The brain tumor that developed was passaged through subcutaneous implantation several times and later established as a cell-line that could be grown and maintained *in vitro* and implanted i.c. into

syngeneic mice (11). Due to the induction mode of the tumor by using MCA, it is debated whether or not this tumor has a glial origin. Probably, the serial passages performed when establishing the tumor cell line is the reason behind the lack of glial fibrillary acidic protein (GFAP) expression on the GL261 cells. GFAP expression is specific for cells of an astrocytic origin and is a common but not obligatory feature of GBM tumor cells, where GFAP expression has been shown to gradually decline with increasing malignancy (12).

The GL261 forms fatal tumors within 20 days after intracranial tumor inoculation and these tumors display similarities with human GBM, encompassing aggressive growth pattern, infiltrative growth and necrotic areas (**Fig. 1.**). Though, others have found the GL261 tumor more similar to a rare ependymoblastoma seen in children (2). Spontaneous tumor regression has never been reported for this tumor model. However, the GL261 tumor is slightly immunogenic, as demonstrated by the induction of survival after both pre- and post –vaccination of tumor bearing mice with irradiated GL261 tumor cells (13, 14). Similarly to human GBM, the GL261 cells exhibit a low expression of major histocompatiblity complex (MHC) I and II molecules and harbor *p53, K-Ras* and *c-myc* mutations (14).

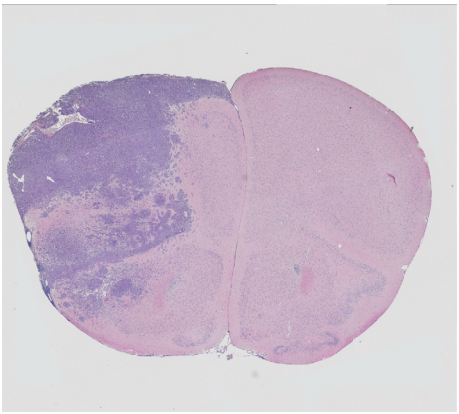

Figure 1. Coronal section of a mouse brain demonstrating the GL261 tumor day 20 after tumor inoculation (Hematoxylin and Eosin staining). The tumor (the dark area) fills up most of the hemisphere, performing a pressure on the opposite hemisphere. This picture clearly shows necrotic areas and the infiltrative growth of the tumor into the surrounding brain tissue, which are common features of GBM.

CNS IMMUNOLOGY AND IMMUNE PRIVILEGE

Certain organs constitute immune privileged sites, e.g. the eyes and gonads, where an inflammatory response would have detrimental consequences for organ homeostasis. An uncontrolled inflammatory response in the brain would raise intracranial pressure due to space limitations in the skull and induce cell death of neurons essential for motor, sensory and cognitive functions, therefore inflammatory and immune responses are downregulated in the CNS. Studies of the occurrence of both innate and adaptive immune responses have shown that the immune privilege is limited to the CNS parenchyma as opposed to the ventricles and the meningies (15) (**Fig. 2**). There are several reasons why the CNS parenchyma has been considered as immune privileged; absence of MHC I and II on parenchymal cells; the presence of the BBB, thus preventing peripheral T-cells from encountering CNS antigens; absence of lymphatic drainage to local secondary lymph nodes, significant for mediating CNS-antigen specific T-cell activation; and the absence of resident antigen presenting cells (APCs) and thereby prevention of presenting CNS derived antigens for T-cells (15, 16). However, recent studies (though, mostly studies involving the initiation and establishment of multiple sclerosis in experimental animal models) have shown that the concept of immune privilege in the CNS parenchyma should be reassessed.

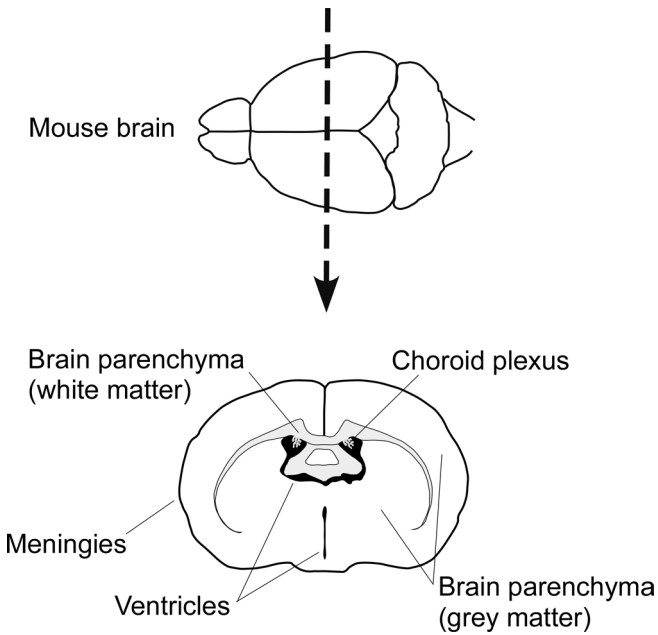

Figure 2. Coronal section of the brain demonstrating different compartments in the CNS. The inner meningies cover the brain parenchyma, the part of the brain having a BBB. The brain parenchyma is surrounded by CSF that is produced by the choroid plexus, situated in the ventricles.

Blood brain barrier

Capillaries in the CNS parenchyma are tightly controlled by the BBB, protecting the parenchyma from the systemic circulation. Tight junctions between the endothelial cells lining the vessels control the transport of antibodies and molecules, such as toxins and blood-borne metabolites, into the CNS parenchyma (16). However, the BBB is not wholly un-permeable to leucocytes and several studies have shown that activated $CD4^+$ T-cells can enter the CNS across the BBB (17, 18).

Within a glioma however, the BBB is severely disrupted. Once inflammation is established, as is the case for a growing brain tumor, the BBB is disrupted, e.g. absence of the tight junctions, hence the concept of immune privilege in the CNS parenchyma during brain tumor development is undermined (19, 20). Whether the disrupted BBB and the abnormal endothelial cells, lining the vessels, negatively or positively affect the efficient lymphocyte homing and trafficking into the CNS is disputed.

Lymphatic drainage in the CNS

For initiation of antigen specific immune responses, APCs present their phagocytosed antigens to T-cells in a secondary lymph node. The CNS parenchyma lacks a conventional lymphatic drainage and was therefore believed to be unable of mounting a CNS-specific immune response.

Studies injecting either dye tracers or albumin have shown that cerebrospinal fluid (CSF) drain to the deep cervical lymph nodes, via the perivascular and subarchnoid spaces through the cribiform plate into the lymphatics of the nasal submucosa (21). Furthermore, APCs in cervical lymph nodes have been shown to activate a CNS-specific T-cell response (22-24), confirming that CNS-antigens also drain via this route. Not only antigens drain to the cervical lymph nodes. It has been postulated by *Tsugawa et al.*, that DCs injected into intracerebral tumors also localize to the cervical lymph nodes (25). Migration of intracranial dendritic cells (DCs) to lymph nodes would facilitate the initiation of CNS-specific T-cell responses.

However, most of the previously described studies have been performed in animal models and therefore need to be confirmed for human CNS neoplasms as well.

Resident APCs in the CNS

The CNS contains several cell types of hematopoietic origin. The CNS parenchyma harbors microglia, important for neural homeostasis and tissue surveillance, while the interspace between the CNS and the blood/CSF contains bone marrow derived professional APCs (26). Several other cell types have been proposed for being potential CNS APCs as well; e.g. vascular endothelial cells, smooth muscle cells, astrocytes, choroid plexus epithelial cells and neurons (19, 27). Although both astrocytes and endothelial cells have been shown to express MHC II, they still don't have the capacity to act as professional APCs (27-29).

Microglia

Microglia are the major antigen presenting cell type in the CNS parenchyma. Microglia, however, have a hematopoietic origin, probably infiltrating the CNS during fetal development (30). Under normal conditions, protrusions from the microglia cells are constantly screening the nearby surroundings, and become activated by "danger signals" produced during tissue damage.

Microglia function as APCs, since they process antigens and have the ability to upregulate costimulatory and MHC II molecules, important for T-cell activation (31, 32). *In vitro* studies

have demonstrated microglia to be more efficient than astrocytes in antigen presentation and subsequent T-cell activation (31), however not as efficient as professional DCs.

Perivascular APCs

Perivascular APCs are localized in the perivascular spaces surrounding the vessels in the parenchyma. Unlike the microglia, perivascular APCs are constantly being replaced by new cells migrating from the bone marrow (33). This indicates that monocytic cells have the ability to be transferred over the BBB as well. The heterogenous cell population of perivascular APCs is known to comprise macrophages but probably DCs as well (26, 34).

Greter et al. have shown that especially $CD11c^+$ perivascular DCs were essential for secondary reactivation of T-cells, mediating T-cells migration into the CNS (34). This reactivation of T-cells leading to migration into the CNS was also demonstrated to be independent of the presence of secondary lymph nodes. Migration of activated T-cells over the BBB seem to be dependent on the perivascular DCs, since depletion of these cells blocked the accumulation of T-cells in the CNS parenchyma (35).

Macrophages and DCs in the meninges and choroid plexus

There is an extensive population of macrophages and DCs residing in the meningies and choroid plexus, closely related to the CSF space (36). These cells act as scavengers of pathogens, tissue debris and tumor cells.

For mounting of an efficient immune response it has been demonstrated that these cells migrate to the cervical lymph nodes. Since both DCs and macrophages accumulate in the CSF as well as perivascular spaces during neuroinflammation, the CSF probably acts as a major transport route for DCs and macrophages migrating from the CSF into the CNS parenchyma or from the CSF to the cervical lymph nodes (26, 37).

IMMUNOSURVEILLANCE AND CANCER IMMUNOEDITING

Immunosurveillance

The concept of immunosurveillance was already established in 1909 by Paul Erlich who proposed that the immune system protected the host from the development of neoplastic diseases. This concept was further fine tuned by Burnet and Thomas (38-40) who predicted that the lymphocytes must be involved in the protection against tumor development. Although, it took until the 1990s before their ideas were almost fully accepted by the scientific society. By that time, studies using knockout mice, either IFN$\gamma^{-/-}$ or perforin$^{-/-}$, showed an increased incidence of spontaneous tumor formation after implantation of the carcinogenic substance methylcolanthrene (MCA), if compared to wild type mice (41-44). Furthermore, the increased establishment of MCA-induced tumors using Rag$^{-/-}$ mice, completely lacking the T-, B- and natural killer T-cell (NKT) lymphocyte populations (45), proved that lymphocytes and IFNγ were essential for the protection of spontaneous or carcinogen-induced tumor development (46). Despite these findings, the immunosurveillance theory is still not fully accepted. *Qin et al.* strongly disagree with these recent proofs, arguing that most of these studies were performed on MCA induced experimental tumors. Therefore, they argue, that the low tumor incidence more likely corresponds to a MCA-induced inflammation and tissue repair response leading to encapsulation, rather than a T-cell mediated elimination of transformed cells (47, 48).

Immunoediting

However, if the concept of immunosurveillance is true, why do tumors exist? To explain this, *Dunn et al.* refined the concept of immunosurveillance and instead called it cancer immunoediting (46, 49, 50). The concept of immunoediting can be divided into three phases; elimination, equilibrium and escape. Initially the innate and the adoptive immune system are involved in recognizing and eliminating tumor cells, earlier explained as immunosurveillance. However, this process will lead to an equilibrium, where constant mutations and genetic instability in the tumor cells coupled with the pressure elicited by the immune system, will select for less immunogenic tumor cells. These tumor cells will acquire the ability to escape detection by the immune system, which will lead to a progressively growing tumor, yet initially developed in an immunocompetent host.

TUMOR IMMUNOLOGY

Short introduction to immunology

From an evolutionary perspective, the innate immune response is regarded as our oldest part of the immune system. Leukocytes in the innate immune system recognize distinct molecular patterns on either foreign particles invading the host (e.g. bacterias and viruses) or aberrant cells (e.g cancer cells and infected cells). The cells of the innate immune response comprise mast cells, phagocytes (including macrophages and DCs), granulocytes, natural killer cells (NK-cells), NKT-cells and γδ T-cells.

The adaptive immune system is activated by the innate immune system and has the ability to recognize and remember specific antigens expressed on pathogens or aberrant cells. APCs present phagocytosed antigens to the lymphocytes, T-cells and B-cells, thereby mediating a cellular immune response or an antibody mediated immune response, respectively. Being adaptive refers to the vast number of different antigen receptors that can be expressed on the lymphocytes, which are developed due to gene rearrangements. The lymphocytes also have the ability to acquire a long-term memory towards the antigen, leading to a quicker and stronger immune response during the second encounter with the same antigen (51).

Tumor immunology

From a simplified point of view, theoretically the concept explains the ability of the immune system to eradicate tumors. The interaction between the tumor and the immune system leading to elimination of the tumor can be divided into two parts, the innate and the adaptive immunity. The innate immunity is the primary participant at the tumor site eventually recruiting and activating the adaptive immunity to finalize the job.

Innate tumor immunity

The innate immune response preceeds the adaptive response against tumors. Due to invasiveness of the tumor and tumor-induced angiogenesis, tumor growth will induce local tissue damage, thereby initiating an inflammatory response and production of "danger signals" such as heat shock proteins, extracellular matrix breakdown products and cytokines (e.g. tumor necrosis factor α (TNFα), IL-1, IFNα, GM-CSF and IL-15) (52-54).

The cells responding to these danger signals and recruited to the tumor site comprise natural killer cells (NK-cells), macrophages, γδ T-cells and NKT-cells. Specific molecular patterns expressed on the transformed tumor cells can initiate tumor cell killing via specific receptors, e.g. NKG2D, expressed on the NK-cells, NKT-cells and γδ T-cells (55). NK-cells recognize cells with low or absent expression of MHC I molecules, which is a common feature of many tumor cells. The innate immune cells induce a cytotoxic immune response that includes secretion of IFNγ, initiation of tumor cell killing as well as recruitment of other immune cells to the tumor site. The direct effect of IFNγ on the tumor cells will be further discussed later.

Adoptive tumor immunity

The innate immune response has the possibility to initiate the adaptive immune response. The inflammatory response initiated by the innate immune response, will recruit APCs (including DCs and macrophages) to the tumor site (**Fig. 3**). The DCs phagocytosing tumor antigens from apoptotic or necrotic tumor cells and concurrently stimulated by danger signals, will mature and

subsequently migrate to nearby tumor draining lymph nodes. Maturation of DCs is characterized by upregulation of MHC and costimulatory (e.g. B7 and CD40) molecules, important for proper T-cell activation (56). Although the CNS lacks a classical lymphatic drainage, the cervical lymph nodes have been proposed for draining the CNS parenchyma (as discussed previously), and hence brain tumors as well (24), as well being important during initiation of CNS immunity (57).

In the cervical lymph nodes, the mature DCs will encounter naïve T-cells and present their phagocytosed and processed tumor antigens on MHC molecules. Tumor antigens presented on MHC II molecules will induce a tumor specific Th1 $CD4^+$ T-cells response. For the induction of $CD8^+$ cytotoxic T lymphocytes (CTLs), IFNγ produced by the Th1 cells has been shown to be essential (58). Importantly, cross-presentation of tumor antigens on MHC I molecules for $CD8^+$ T-cells has been shown to occur as well, however only when DCs were receiving sufficient activation signals (e.g. T-cell produced cytokines and CD40 ligand interactions) (59). Proper costimulatory signals are required during both $CD4^+$ and $CD8^+$ T-cell activation, including DC-bound B7 interacting with the T-cell ligand CD28.

The activated effector T-cells, $CD4^+$ Th1 cells and $CD8^+$ CTLs, will leave the cervical lymph node and home to the brain tumor site. The CNS specific homing of the T-cells, encompassing expression of CNS homing-specific integrin- and chemokine receptors, is probably imprinted by the brain tumor antigen specific DCs (19, 60). In the CNS, re-activation by resident APCs has been shown to be important for the activated T-cells, in order to leave the circulation and to reach the tumor site. At the tumor site the $CD8^+$ T-cells will recognize the tumor cells, which will induce tumor cell killing mediated by cytotoxic effector molecules; e.g. perforin, Granzyme B and IFNγ. The tumor cell killing action of the $CD8^+$ CTLs is facilitated by the concurrent production of IL-2 by the $CD4^+$ T-cells, promoting intratumoral proliferation of the T-cells.

However, many tumors can evade recognition by the immune system and the initiation of an anti-tumor immune response is therefore inefficient for rejection of established tumors. The existence of tumor reactive T-cells during tumor growth are a proof for an active but inefficient immune response towards the tumor.

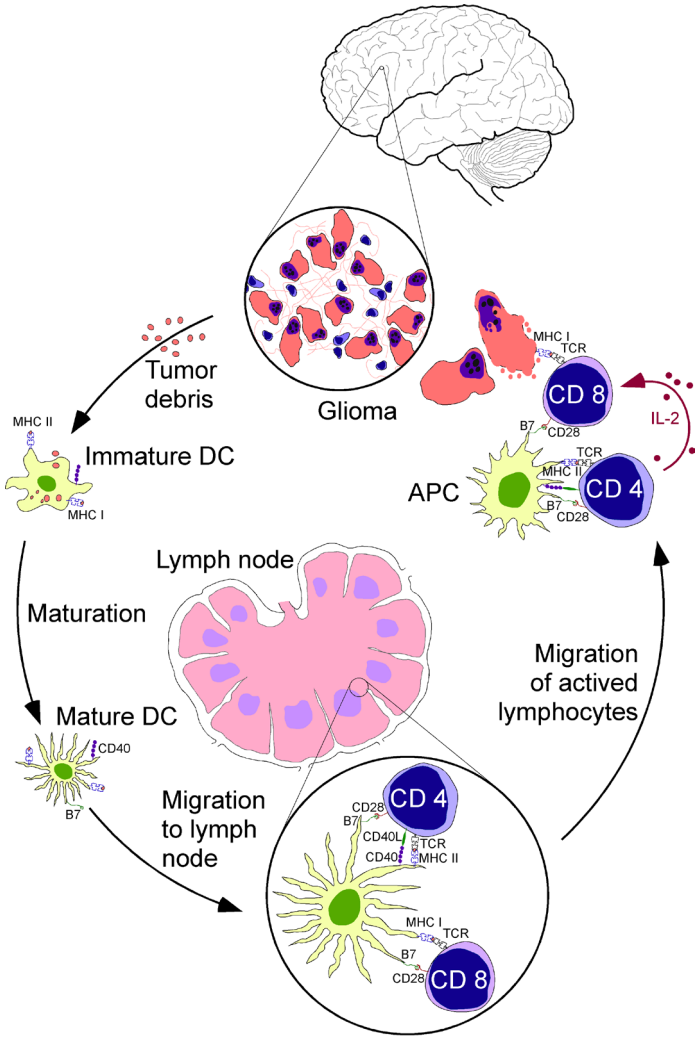

Figure 3. Simplified figure of the induction of the adaptive anti-tumor immune response. Tumor antigens are released from tumor cells dying of apoptosis or necrosis. Immature DCs attracted to the tumor site by the inflammatory response will phagocytose the tumor cell debris. When migrating to the tumor draining CLNs, the DCs will mature, expressing more MHC as well as B7 molecules on the cell surface. This maturation is further enhanced by "danger signals". In the CLNs the tumor antigens will be presented on the MHC II molecules and recognized by TCRs on the $CD4^+$ T-cells or MHC I molecules recognized by $CD8^+$ T-cells. The T-cell activation is further stimulated by binding of CD40L on the T-cells to CD40 on the DCs. The activated effector T-cells, such as CTLs and T helper cells, will migrate to the tumor site, due to expression of specific integrin- and chemokine receptors. Activated T-cells can enter the CNS despite the BBB. At the tumor site, local APCs will present tumor antigens, re-activating the activated T-cells. T-cell proliferation and the cytotoxic activities of the CTLs are further enhanced by the production of IL-2 from the T helper cells, leading to an efficient tumor cell killing.

IMMUNE EVASION

There are several ways for tumor cells to evade detection by the immune system that will be selected for during the immunoediting process. The tumor induces immunosuppression and tolerance, either directly on the tumor infiltrating lymphocytes, or systemically affecting the initiation of the immune response. Some of the evasion mechanisms elicited by the tumor will be discussed here.

Defects in tumor antigen presentation

One way to avoid recognition by the APCs in the immune system is to modify the tumor antigen presentation process. Downregulation of MHC class I or other important components of the antigen presentation machinery e.g. TAP and β2 microglobulin, are common on a variety of tumors (*7*, 61-63), thereby making them resistant against CTLs. This immune evasion is not necessarily the most efficient, since NK cells react on the reduced expression on MHC I, which will induce tumor cell killing. Tumors can also evade recognition by changing their antigenic repertoire by "antigenic drift" of surface antigens recognized by CTLs (61). It has been demonstrated that especially melanomas have downregulated specific melanoma antigens (e.g. MART-1 and gp100) (*64*, *65*).

Secretion of tumor derived suppressive factors

Several immunosuppressive factors produced by tumors have been demonstrated, but only those also secreted by brain tumors in particular will be presented in this thesis.

Transforming growth factor β (TGFβ) has a pivotal role in immune regulation, however, when produced by the tumor itself TGFβ will promote tumor progression. TGFβ is expressed by several human tumors, including GBM (*66*, *67*). TGFβ is a multifunctional cytokine with immunosuppressive properties. Some of its immunosuppressive functions are inhibition of T-cell activation, proliferation and differentiation (68, 69) as well as inhibition of antigen presentation on APCs (68). *Thomas et al*. show that TGFβ specifically inhibited the CTL mediated tumor cytotoxicity, by blocking the production of perforin, Granzyme A and B, Fas ligand and IFNγ (*70*). TGFβ has also been shown having a role in induction of T regulatory cells (*71*). The inflammatory responses of activated macrophages are inhibited by TGFβ through downregulation of nitric oxide (NO) and inducible NO synthase (iNOS) (*72*, *73*).

Prostaglandin E2 (PGE2) is important for tumor growth by promoting proliferation, invasion, angiogenesis and immunosuppression (*74*). The immunosuppressive properties consist of inhibition of T-cell activation and suppression of the anti-tumoral activites of NK cells. PGE2 has also been shown to skew the T-cell production of Th1 cytokines (IFNγ and TNFα) into Th2 cytokines (IL-*4* and IL-10) (*74*). Human GBM cells have been shown to produce PGE2 and have also been suggested for being responsible for the suppressed T-cell function seen in these patients (*75*, *76*).

IL-10 inhibits the function of APCs, by reducing their antigen presenting capacity through downregulation of MHC II. Thereby T-cell activation and concurrent IL-2 production is perturbed (*77-79*). IL-10 has been shown to be produced by GBM cells (*79*). Furthermore *Huettner et al.* have shown that the amount of IL-10 increased with malignancy of the gliomas (80). However, to add to the complexity of this research field, several studies have shown that IL-10 in some circumstances also can act immunostimulatory and thus promote tumor rejection (81, 82).

Vascular endothelial growth factor (VEGF) is produced by several tumor cells, including GBM cells, and has also been detected in sera of patients with malignant tumors (83-85). VEGF has been shown to interfere with the differentiation of human $CD34^+$ hematopoietic stem cells, which will increase the number if immature DCs (86). VEGF is also known to stimulate angiogenesis *in vivo*, thereby promoting tumor progression (87).

GM-CSF is a critical cytokine important for the myelopoiesis and differentiation of DCs (88). A constant administration of GM-CSF has been shown to increase the amount of myeloid derived suppressor cells (MDSCs) responsible for suppressing immune responses (89). In a study by *Bronte et al*. they report that 31% of all human tumor cell lines tested produced GM-CSF albeit no CNS tumors were included (89). *Karcher et al*. demonstrated that GM-CSF obtained from supernatants of GBM tissue cultures, could be correlated with a shorter survival time and a worse prognosis and hence reflected a more aggressive phenotype (90). However, it was not fully verified whether the GM-CSF production was tumor tissue specific or due to contamination of infiltrating macrophages producing GM-CSF.

IL-6 production has been found in gliomas, but whether it is a prognostic marker for GBM aggressiveness has not been clearly evaluated (91, 92). The immunosuppressive properties of IL-6 are decreased capacity of antigen presentation and T-cell activation through inhibition of differentiation of myeloid progenitor cells into DCs (93).

Myeloid derived suppressor cells

The secretion of tumor derived suppressive factors (especially VEGF, GM-CSF, IL-10, IL-6 and TGFβ) will affect the myelopoiesis in the bone marrow and subsequent accumulation of MDSCs in spleen and lymph nodes of tumor bearing hosts will follow (94). Accumulation of MDSCs in the tumor has been shown for several tumor types (93). 2007 *Gabrilovich et al*. commonly agreed that MDSC is from now on the commonly used term for the cells formerly denoted as immature DCs or myeloid suppressor cells (95). Under normal and healthy conditions, MDSCs are present in the bone marrow and spleen and have the ability to gradually differentiate into mature myeloid cells granulocyes, DCs and macrophages. Mouse MDSCs are characterized as being $CD11b^+Gr\text{-}1^+$ without displaying any markers of mature myeloid cells and functionally associated with inhibition of T-cell activation (96, 97). The inhibition of T-cell immune responses have been explained in several studies (98) and is believed to partly be mediated through down-modulation of the T-cell receptor ζ chain (CD3ζ) being part of the T-cell receptor (TCR) complex (99), inhibition of the costimulatory signaling pathway CD3/CD28 (100), prevention of CTL activity as well as decreased IFNγ production from $CD8^+$ T-cells (93).

The suppressive effects of MDSCs are believed to be dependent on the expression of iNOS and arginase 1 (ARG1) (93). MDSCs from tumor bearing mice produce reactive oxygen species (ROS) (101), e.g H_2O_2, NO and $ONOO^-$, which have been shown to be the actual mediators of the suppression of T cell immune responses. Expression of ARG1 and iNOS has been used, although in a very simplified and generalized manner, to distinguish macrophages into either iNOS expressing M1 macrophages or ARG1 expressing M2 macrophages (102). The classically activated M1 macrophages, produce inflammatory cytokines and ROS, and can act as effector cells for killing of intracellular pathogens and even tumor cells (102). In addition, the alternatively activated M2 macrophages, which secrete anti-inflammatory cytokines, have been shown to promote tumorigenesis (102). However, *Gallina et al.* showed that a subset of $CD11b^+Gr\text{-}1^+$ MDSCs, expressing IL-4Rα, shared both M1 and M2 characteristics, as regarding to iNOS and ARG1 expression and cytokine production (103). When the MDSCs were activated by

IFNγ produced by activated T-cells, they suppressed $CD8^+$ T-cells through their own production of IFNγ as well as IL-13.

However, the MDSCs still appear to be a quite heterogenous cell population, harboring some plasticity in how they react towards different types of stimuli, which may affect whether they acquire a M1 and/or M2 phenotype. So far most studies on MDSCs have been performed in mice, hence they need to be further verified in humans. For cancer treatment, MDSCs are most certainly an attractive target, either for inhibition of their immunosuppressive activities or in order to differentiate them into well functioning, non-tolerogenic APCs, which may enhance T-cell activation and subsequent tumor cell killing.

T-regulatory cells

Sakaguchi et al. were the first to characterize the $CD4^+$ T-cell population expressing the IL-2 receptor α-chain (CD25) molecules, functionally suppressing immune responses to self-antigens, supported by the finding that elimination of the $CD4^+$ $CD25^+$ T-cells mediated severe autoimmunity (104). Several T-regulatory sub-populations have been described recently, where the thymus derived $CD4^+CD25^+FoxP3^+$ are referred to as being the naturally occurring T-regs, constituting 10% of the peripheral $CD4^+$ T-cells (104). T-regs also constitutively express glucocorticoid-induced TNFRs (GITR) and cytotoxic T lymphocyte antigen-4 (CTLA-4) (105). Tumors have been shown to harbor both naturally occurring T-regs as well as locally induced T-regulatory cells (T_R1), characterized as being $CD4^+IL\text{-}10^+FoxP3^-$.

The suppressive mechanisms of T-regs have been studied extensively in *in vitro* studies and are believed to be mediated by soluble factors (e.g. IL-10, TGFβ, perforin and GranzymeB) and direct cell-cell contact (e.g. CTLA-4 and B7-H4), suppressing the functions of T-cells, APCs and NK-cells (106-108). *Chaput et al.* demonstrated that tumor infiltration of Tregs prevented the development of T helper cells as well as $CD8^+$ effector cells, further supported by depletion of Tregs which induced highly cytotoxic tumor infiltrating $CD8^+$ T-cells (109).

Tumors actively prevent the induction of anti-tumor immune responses through directly affecting the differentiation, expansion and or recruitment of T-regs. Soluble factors, e.g TGFβ and IL-10, either produced by the tumor or by MDSCs, promote proliferation and differentiation of T-regs (107). Peripherally induced T-regs can also home to tumors under the influence of the chemokines CCL17 and CCL22 (110, 111).

An increased tumor infiltration of T-regs has been found in experimental glioma models (112, 113). There are several studies performed, inversely correlating tumor infiltration of T-regs with clinical outcome (107, 111). A direct correlation of T-reg tumor infiltration and tumor grade of human brain tumors was performed by *El Andaloussi et al.,* which support the role of T-regs for suppressing anti-tumor responses, causing increased tumor growth (114, 115). However, a direct proof for this has been performed in several studies where T-regs were depleted through administration of antibodies specific for either CD25, CTLA4 or GITR, which clearly have shown that tumors were more efficiently rejected as well as enhanced the specific anti-tumor responses induced with immunotherapies (112, 116, 117).

T-regs are crucial for the immune escape of tumors, and have also been shown to be responsible for blocking the anticipated success of many immunotherapies. Therefore, immunotherapy combined with specific blocking of T-regs, needs further investigations.

TUMOR INFILTRATING LYMPHOCYTES

Prediction of clinical outcome in the context of tumor infiltrating lymphocytes (TILs) has been performed for various cancer types including ovarian cancer, melanoma and colorectal cancer. Characterization of TILs during the development of new immunotherapeutic treatments both in patients as well as in animal studies is also a valuable tool for evaluation of new treatments.

Tumor infiltration of T-cells

Several studies have correlated a high infiltration of T-cells as well as a low infiltration of T-regs intratumorally with a favorable prognosis for the patients (19, 118-121). However, the role of tumor infiltrating T-cells is disputable. As previously described the activated cytotoxic T-cells have the capability to mediate direct tumor cell killing. Though, lymphocyte infiltration *per se* is not an indicator of increased survival, since infiltrating lymphocytes may be affected by the tumor derived suppression and become anergic or dysfunctional. *Dunn et al.* summarized all studies performed on human malignant gliomas, concerning tumor infiltration of lymphocytes. However, when comparing these studies, no conclusions could be drawn, whether lymphocyte infiltration in gliomas could be correlated with a good clinical outcome (19).

The induction of a tumor specific CTL response is a requirement for most anti-tumor responses and is also the major focus during the development of an efficient immunotherapy. Several studies in human neoplasms have shown that the induction of an efficient anti-tumor immune response towards intra cranial tumors is accompanied with an increased infiltration of $CD4^+$ Th1 cells and $CD8^+$ CTLs (120, 122-125). These studies also stress the importance of cytotoxic as well as proliferative capacity of the infiltrating lymphocytes for a correlation with a favorable clinical outcome.

Furthermore, when analyzing the function and phenotype of infiltrating T-cells, there is a correlation between increased infiltration of T-regs and a poor clinical outcome (126, 127). *El Andaloussi et al.* have demonstrated, both in preclinical as well as clinical studies, a high T-reg infiltration in gliomas (112, 114). The suppressive role of the infiltrating T-regs was further supported by the finding that infiltration of T-regs increased with the malignancy grade of the brain tumors (115).

Tumor infiltration of macrophages

The largest population of infiltrating immune cells in human GBM consists of the macrophages (128, 129). Tumor infiltration of macrophages is correlated with tumor progression and a poor prognosis for several neoplasms (130-132). In human gliomas there are several studies demonstrating high infiltration of macrophages (133, 134). Although *Nishie et al.* were able to correlate an increased number of tumor associated macrophages (TAMs) with the malignancy grade of the gliomas, after comparing several studies it has not been possible to draw any conclusions whether infiltration of TAMs could be correlated with a favorable prognosis (19). However, the inconsistency between the various studies probably stems from differences in analysis methods, tumor malignancy grades as well as the tissues prepared for analysis.

Tumor associated macrophages

TAMs have formerly been considered to be alternatively activated macrophages, M2 macrophages (98, 135). However, in mice, *Umemura et al.* demonstrated that MDSCs and TAMs both share phenotypic similarities. As previously discussed when describing the MDSCs, the TAMs

are pleiotropic and associated with characterstics from both M2 as well as M1 macrophages (136, 137). The M2 characteristics include production of IL-10, TGFβ and ARG1 while M1 characteristics include secretion of inflammatory mediators such as TNFα, Il-6 and iNOS (102). TAMs are recruited to the tumor site from circulating monocytes, which differentiate into TAMs by tumor-derived factors such as, VEGF, M-CSF and CCL2 (135).

There are several protumoral activities associated with TAMs, leading to increased vessel density and tumor progression. Angiogenesis and tumor growth are supported by the TAMs by their production of various tissue factors (e.g. VEGF, CCL2 and CXCL8) as well as growth factors (VEGF, PDGF, EGF) (98). *Yang et al.* also proposed that $CD11b^{+}Gr\text{-}1^{+}$ MDSCs migrating to the tumor site differentiate into either endothelial cells or TAMs, thereby contributing to both neovascularization and tumor growth (138). These processes are further sustained by the release of MMP-9 and TGFβ remodeling the tissues surrounding the tumor, which in turn promote tumor invasiveness. As previously described, several of the cytokines secreted by the tumor also have immunosuppressive properties as well as the ability to recruit T-regs. TAMs have also been shown to skew the immune response into a Th2 response by the production of several chemokines (111, 135, 139).

During tumor progression the TAMs, have no or minimal tumoricidal activities and appear to be ineffective for initiating an effective immune response towards the tumor, explaining why they often are associated with a dismal prognosis. *Mytar et al.* demonstrated that tumor cells cocultured with blood monocytes were deactivated, and developed into M2-like macrophages (140). However, it has been shown *in vitro* that both the bacterial product LPS and the cytokine IFNγ can activate the deactivated TAMs harboring a predominant M2 phenotype into a M1 phenotype with anti-tumoral activities. Furthermore, it has also been demonstrated that the deactivated TAMs can be re-activated with either anti-IL-10 antibodies or certain pro-inflammatory cytokines, such as GM-CSF combined with IFNγ (141). IFNγ produced by either $CD8^{+}$ T-cells or NK-cells infiltrating the tumor have been shown to be responsible for activating the macrophages into M1 macrophages in vivo (142, 143). The anti-tumoral activities of M1 macrophages mediating tumor cell killing, include secretion of cytotoxic mediators such as TNFα, serine protease and reactive nitrogen intermediates (e.g. NO) (132, 144-146).

The explanation for the poor prognosis associated with a high infiltration of TAMs, is due to their suppression of the induced anti-tumor immune response and ability to promote tumor growth. Thus, for development of cancer vaccines, one goal is to find immunotherapies that can activate the deactivated macrophages infiltrating the tumor to become more tumoricidal.

IMMUNOTHERAPY

A new and more efficient treatment for GBM must focus on the elimination of all the tumor cells that are still residing in the brain tissue after surgery as well as to the circumvent the obstacles with induction of tumor derived immunosuppression. Therefore, new therapeutic strategies involving modulations of the host's own immune system that targets the remaining tumor cells is under development. The aim is to develop efficient treatment modalities in experimental animal models for further translation into the clinic.

Cytokine-based immunotherapy

The concepts of immunosurveillance and immunoediting enclose that albeit a growing tumor, the host can develop an active, although dysfunctional, immune response against its own tumor. The principle with immunotherapy is to vaccinate the tumor bearer, and thereby boost the host's own anti-tumor immune response in order to reject the tumor and induce a long-term immunological memory.

The cancer vaccine consists of a tumor antigen source together with an adjuvant that can stimulate the immune response. The tumor antigen source consists of a small peptide, protein, tumor cell lysate or whole tumor cells. There are conceptual differences between using tumor peptides compared with whole tumor cells. Single tumor antigens may induce a more directed and tumor cell specific immune response, however the tumor cells have the possibility to eventually become resistant and evade the immune response by downregulating the targeted tumor antigens. By using whole tumor cells the induced immune response will become broader and directed towards several antigens expressed on the tumor cells. Therefore, the risk of immune evasion via tumor antigen downregulation is minimal, however there is a greater risk of inducing autoimmunity by targeting non-cancerous cells.

Several cytokines are used as adjuvants in cancer vaccines in order to stimulate the induction of an immune response. DCs play a pivotal role in the initiation and priming of anti-tumor immune responses, therefore the purpose of several immunotherapies is to manipulate these cells. DCs can be grown and pulsed with tumor antigens *in vitro*, and have been shown to elicit potent immune responses after immunization of tumor-bearing animals (147). However, another well-used technique is to manipulate the DCs *ex vivo*, by injecting substances that stimulate their expansion and differentiation from bone marrow precursor cells.

GM-CSF based immunotherapy

GM-CSF

GM-CSF belongs to the family of hematopoietic cytokines, also including G-CSF, M-CSF, IL-5 and IL-3. GM-CSF is produced and secreted by a variety of cells e.g. monocytes, endothelial cells and activated T-cells. The receptor for GM-CSF is expressed on hematopopietic progenitor cells, where GM-CSF promotes their differentiation into granulocytes and monocytes (148).

GM-CSF based immunotherapy

Dranoff et al. used the mouse melanoma model B16 to monitor the immune response elicited after vaccinating tumor-bearing mice with irradiated tumor cells transduced with cytokines, adhesion molecules or costimulatory molecules (149). Among all the cytokines tested, they found that the

cytokine GM-CSF stimulated the most potent systemic anti-tumor response. Several studies using a GM-CSF based immunotherapy have been performed in the GL261 mouse glioma and in several rat glioma models, all demonstrating increased survival (150-155).

The anti-tumor immune response elicited by GM-CSF has been shown to be highly dependent on the local concentration and duration of the secreted cytokine (148). A too high and protracted concentration of GM-CSF has been shown to be immunosuppressive by inducing MDSCs (as described earlier) (89). *Serafini et al.* compared a high-dose GM-CSF secreting vaccine with a low-dose secreting vaccine in a mouse tumor model, and discovered that there was an upper and a lower therapeutic limit for GM-CSF (156). Immunization using cytokine-producing tumor cells is the most common way to administer GM-CSF, though for clinical purpose it would be more convenient to administer recombinant GM-CSF. Since GM-CSF has a relatively short half-life *in vivo* (0,92 ± 0,04 min) (157), injection of recombinant GM-CSF is not feasible even though it would facilitate the immunization procedure (158). Though, this could be circumvented using tumor cells expressing membrane-bound GM-CSF on the surface as tumor cell vaccine (159, 160). One approach to enhance the half-life of GM-CSF is by using polyethylene glycol-modified (PEG) GM-CSF (157). To mimic the GM-CSF transduced tumor cells, GM-CSF can be administered via repeated injections of recombinant GM-CSF or via mini-osmotic pumps, however these methods have not yet proven to be superior to GM-CSF transduced tumor cells (147, 161).

In order to enhance the effect of GM-CSF based immunotherapies, there are several studies ongoing, where GM-CSF is combined with other immunostimulatory factors. One approach is to enhance the DC-activation with the addition of CD40L (162) or TLR-ligands like CpG (163). Other studies have focused on simultaneously inhibiting the immunosuppression elicited by the tumor e.g. injection of anti-CTLA4 or antibodies (164) or blocking of VEGF (165). The use of interferons, especially IFNα, has also been shown to significantly enhance the anti-tumor immunity elicited by GM-CSF (153).

GM-CSF and mechanisms of anti-tumor immunity

The role of GM-CSF in eliciting a potent anti-tumor response including a long-lasting anti-tumor immunity has been studied extensively in a variety of animal tumor models as well as human neoplasms (149, 166). GM-CSF is a critical cytokine required for eliciting differentiation and maturation of DCs (88). The effect of GM-CSF secreting vaccines on the increased anti-tumor immunity has been suggested to be enhanced recruitment of APCs from the bone marrow to the immunization site as well as enhanced maturation of the APCs, leading to an increased antigen presenting capacity and subsequent T-cell activation in the draining lymph nodes (167). There are several findings supporting this hypothesis. After subcutaneous or intra dermal immunizations with GM-CSF producing tumor cells, the injection site was highly infiltrated by DCs, macrophages and granulocytes (168, 169). The importance for the maturation of the infiltrating DCs, expressing high levels of costimulatory molecules; MHC II, CD80 and CD86 (B7-1 and B7-2), was highlighted after comparing GM-CSF with Flt3-ligand (Flt3l). Both Flt3l as well as GM-CSF expanded the number of DCs after injection *in vivo* (157, 170). However, compared to Flt3l, GM-CSF preferentially expanded the $CD11b^{+}CD8^{-}$ population of DCs, shown to have a superior phagocytosing and subsequent T-cell activating capacity. These DCs also had a higher expression of costimulatory molecules, which in turn led to a more efficient antigen uptake and subsequent T-cell activation (170). By depletion of either $CD4^{+}$ or $CD8^{+}$ T-cells, GM-CSF based cancer vaccines have been shown to be dependent on T-cells for eliciting efficient anti-tumor immune responses (149). GM-CSF based vaccines also seem to have an

effect on NKT-cells, as seen by increased expression of CD1 on the expanded population of DCs (171).

IFNγ based immunotherapy

IFNγ

IFNγ is a type II interferon, having distinct features compared to the type I interferons (IFNα and IFNβ). IFNγ is produced by NK-cells, activated T-cells and DCs, B-cells and NKT-cells (172). IFNγ receptors (IFNGR) are distributed and expressed on almost all cell types (173). GM-CSF has been shown to increase the expression of IFNGR1 on human peripheral blood monocytes (174).

IFNγ based immunotherapy

As described previously, IFNγ has a pivotal role in promoting tumor immunogenicity. Blocking of IFNγ by using neutralizing antibodies increased the tumor growth. Similarly, IFNγ$^{-/-}$ mice on either a C57BL/6 or BALB/c background showed an increased incidence of spontaneous tumor formation (42, 44). Also, over-expression of IFNGR1 reduced the amount of soluble IFNγ, and induced a higher tumorigenicity as well as reduced immunogenicty.

The importance for the induction of an anti-tumor response after peripheral immunizations with IFNγ-transfected tumor cells has earlier been demonstrated in preclinical studies (175, 176). Intracerebral injections of retroviral or non-viral vectors encoding for IFNγ will cause a high tumor localized concentration of IFNγ, resulting in tumor regression in the rat C6 or mouse GL261 glioma model respectively (177, 178). Clinical studies have so far been inconclusive whether IFNγ has an effect on survival or not (44). This is probably due to inconsistent translation of timing and dosing schedules from animal studies into clinical studies.

IFNγ has a relatively short half-life, 25-30 min, after i.v. administration (173, 179). Hence, in order to reach a high systemic concentration leading to a high local concentration at the tumor site, IFNγ needs to be repeatedly administered. However, this procedure induced toxic side effects (such as fever, nausea, vomiting, neurotoxicity, and leucopenia) (173, 180). To overcome these IFNγ-associated toxicities studies with local delivery of recombinant IFNγ at the tumor site have been performed, thus minimizing the systemic toxic side effects as well as increasing the local effect at the tumor site (173).

However, it should be stressed that several studies on IFNγ-based immunotherapy previously mentioned, are using IFNγ as a monotherapy. The intention is to generate a high enough systemic concentration of IFNγ in order for IFNγ to reach the tumor site where it can induce an efficient anti-tumor response as well as affect the growth of the tumor. However when interferons are used as adjuvants to e.g. a GM-CSF based immunotherapy, high concentrations might only be needed at the immunization site, where GM-CSF-attracted DCs may be further activated by the co-administered interferons (153). Thereby, systemic toxicities caused by interferons may be less severe, due to a much lower systemic concentration exerted by the therapy.

IFNγ and mechanisms of anti-tumor immunity

IFNγ is a pleiotrophic cytokine, with several immunomodulatory functions essential for enhancing tumor immunogenicity. IFNγ induces growth arrest, although this effect has been shown to be ameliorated by co-administration of GM-CSF (181). Other anti-tumor effects of IFNγ have been shown to be inhibition of angiogenesis and control of apoptosis by promoting expression of

FAS, TRAIL and caspases (44, 172, 182, 183). Both MHC I and II expression is upregulated in response to IFNγ (172, 184). MHC I upregulation on tumor cells efficiently promotes detection by tumor specific CTLs, an important consequence of tumor cells that otherwise evade the immune system by downregulating MHC molecules. Indirectly, IFNγ promotes $CD4^+$ T-cell activation, through MHC II as well as B7 upregulation on APCs (185, 186). IFNγ is also important for skewing the immune response into a Th1 response. This is mediated through increased NK-cell mediated effector functions, such as; promotion of the activation and antigen-presenting capacity of APCs, which in turn enhances their production of IL-12 leading to a further increase in IFNγ production; as well as activation of cytotoxic functions of macrophages (172, 187, 188). IFNγ-activated macrophages have the capacity to mediate cell killing via TNFα (189) and ROS as well as reactive nitrogen intermediates (particularly NO) (135, 187). IFNγ has also been shown to be involved in inhibiting the generation and activation of Tregs. *Nishikawa et al.* demonstrated that IFNγ produced by $CD8^+$ T-cells blocked the development of Tregs (190)

RESULTS AND DISCUSSION

Establishment of the GM-CSF and IFNγ based immunotherapy in the mouse glioma model, GL261

Paper I

GM-CSF based immunotherapy has been widely investigated in several experimental tumor models with promising results. For a more efficient treatment of tumors, GM-CSF-based immunotherapies combined with other immunostimulatory adjuvants are under development. With one exception, the combination of GM-CSF and IFNγ has not been explored before. However, in this study *Nishisaka et al.* were not able to demonstrate any synergistic relationship on survival or tumor growth after combining both GM-CSF and IFNγ for the treatment of lung cancer in mice (191). The existence of a relationship between GM-CSF and IFNγ for the protection against tumor development was demonstrated by *Enzler et al.* using GM-CSF/IFNγ double knock-out mice. These mice had an increased incidence of spontaneous tumor formation as well as a higher susceptibility to infection and inflammation (192).

We established a GM-CSF producing tumor cell line (GL-GM), after transduction of the GL261 glioma cells with a plasmid encoding for GM-CSF. The GL-GM cells were stable producers of GM-CSF, even after treatment with IFNγ and/or irradiation. *Siesjö et al.* previously demonstrated the importance of IFNγ treatment of the tumor cells used for immunization both *in vitro* and *in vivo* for the efficient treatment of experimental rat gliomas (193). IFNγ is a multifunctional cytokine known to affect the expression of MHC I and II molecules, among others (172). MHC upregulation on the tumor cells will increase their ability to present antigens for the immune system and thereby increase their immunogenicity. Therefore the effect demonstrated by *Siesjö et al.* on survival could have been coupled to the induction of MHC class I and II upregulation of the tumor cells used for immunization. When we analyzed MHC expression on the GL261 tumor cell lines, both MHC I and II were upregulated after treatment of IFNγ *in vitro* on both GL261 as well as on GL-GM cells.

Mice were inoculated with GL261 glioma cells i.c. and thereafter therapeutically immunized with irradiated tumor cells intra peritoneally (i.p.) at day 1, 14 and 28 after tumor inoculation. Immunization with GM-CSF producing GL261 cells induced 44% survival of the tumor bearing mice. However, when co-injecting recombinant IFNγ together with the IFNγ pretreated GL-GM cells, survival was synergistically enhanced to 88%. GM-CSF based immunotherapies have been described to enhance recruitment and maturation of DCs from bone marrow precursor cells to the immunization site, thereby antigen presentation and subsequent T-cell activation is further enhanced (167). However, survival induced after immunization with GL-GM cells pre-treated with IFNγ *in vitro* was not superior to GL-GM cells only. Therefore, in this mouse glioma model upregulation of MHC was not mediating an increased anti-tumor immune response during immunization. GL261 cells alone or combined with IFNγ induced 10% survival each, demonstrating that this experimental glioma model is slightly immunogenic (14).

We speculated that recombinant IFNγ might have endogenous effects on the immune cells recruited to the immunization site by GM-CSF. IFNγ could be affecting maturation and activation of DCs, thereby skewing the induction of an anti-tumor immune response into a Th1 response, which is more efficient in tumor cell killing. It is not a realistic scenario that a single injection of recombinant IFNγ could have any effects on the tumor itself, by inhibiting tumor cell proliferation or inducing tumor cell apoptosis. This hypothesis is supported by *Prell et al.,* who

previously demonstrated that IFNα combined with a GM-CSF-based immunotherapy increased the anti-tumor T-cell responses (153).

To further investigate the immune response elicited after immunization, we analyzed the proportion of tumor infiltrating lymphocytes. The combined treatment of GL-GM and IFNγ induced a higher infiltration of macrophages, but no increase in $CD8^+$ T-cells, when compared to mice immunized with GL261 cells only. Immunizations with GL-GM and IFNγ were also correlated with a decreased tumor size. During tumor growth, gliomas will induce an immune response as demonstrated by the findings of tumor infiltrating lymphocytes despite immunotherapy, although ineffective for induction of tumor regression (19). However, immunotherapy can skew the function and phenotype of the tumor infiltrating lymphocytes, thereby suppressing tumor growth leading to tumor regression. Even though we did not monitor any changes in the amount of tumor infiltrating $CD8^+$ T-cells, their anti-tumoral activities may have been affected by the immunizations. It has been shown by *Bonnotte et al.* that IFNγ produced by tumor infiltrating $CD8^+$ T-cells are responsible for shifting the immunosuppressive M2 macrophages into tumoricidal M1 macrophages (142). Our hypothesis is that the decrease in tumor size demonstrated after the combined treatment of GM-CSF and IFNγ could be explained by the concurrent increase in macrophages probably instructed to become more tumoricidal.

Investigation of the systemic immune response induced by the GM-CSF and IFNγ based immunotherapy

Manuscript II

The pharmacokinetic profile of cytokines used for immunotherapy is crucial for the induction of an efficient anti-tumor immune response. A high local dose of GM-CSF induces differentiation and maturation of DCs and enhances antigen presentation and subsequent T-cell activation (88, 149). On the other hand, GM-CSF has been shown to be immunosuppressive if administered during a protracted time period (156). The autocrine secretion of GM-CSF from the growing tumor has been demonstrated to expand the myeloid cell population expressing CD11b and Gr-1, which suppress T-cell responses (89). We measured the systemic concentrations of both IFNγ as well as GM-CSF produced by the GL-GM cells after immunization. Both cytokines were detectable *in vivo* and the data obtained described a relatively short window of action for both cytokines after immunization (GM-CSF within 48h and IFNγ within 6h after immunization).

The systemic effect on the immune response was analyzed in the spleen 48 h after immunization with GM-CSF and IFNγ. After one immunization we could measure an increase in the proportion of $CD11b^+Gr\text{-}1^+$ MDSCs. However, after two immunizations we could also see an expansion of differentiated myeloid cell populations such as $CD11c^+$ DCs and $F4/80^+$ macrophages, followed by a decline in the proportion of MDSCs. The proportions of both $CD4^+$ and $CD8^+$ T-cells were almost unaffected by the immunizations, but the $CD4^+CD25^+FoxP3^+$ T-reg population was expanded. We argue that the short window of action for GM-CSF after immunization temporarily expanded the MDSCs, which gradually differentiated into DCs and macrophages. One of the suppressive properties of MDSCs have been shown to be induction of T-regs (194), which may explain the increase in T-regs detected. However, *Alderson et al.* recently reported an increase in T-regs after successful immunotherapy of an experimental mouse tumor, which also could explain the increase of T-regs described in our system (195).

IFNγ alone did not affect the proportion of immune cell populations analyzed, but could have an effect on the maturation of the expanded population of APCs. IFNγ has been shown to

be involved in the upregulation of MHC I and II molecules as well as costimulatory molecules such as B7-1 and B7-2, which will enhance the T-cell activating capacity of the APCs (185, 186). Despite increased proportions of T-regs and MDSCs we could measure an increased cytotoxic anti-tumor response after restimulating the splenocytes *in vitro* with irradiated GL261 cells. The induction of a cytotoxic immune response elicited by the T-cells, including production of IFNγ and Granzyme B, is of great importance for tumor eradication. However, whether these cytotoxic T-cells migrated to the tumor site and mediated either direct tumor cell killing or activated the tumor infiltrating lymphocytes need to be further clarified.

Furthermore, the induction of an efficient anti-tumor immune response by GM-CSF based immunotherapies has been shown to be dependent on T-cells (149). By depletion of either $CD4^+$ or $CD8^+$ T-cells we could demonstrate that T-cells were crucial for the induction of survival after immunization with the combination of GM-CSF and IFNγ as well. T-cells are not only important for mediating a cytotoxic immune response, but for establishing an immunological memory. We clearly demonstrated that a long-lasting anti-tumor immunological memory was induced, since mice previously immunized during their first encounter with the tumor, could survive a tumor re-challenge in the opposite brain hemisphere without any additional immunizations.

Finally, the robustness of the combined immunotherapy of GM-CSF and IFNγ was demonstrated after postponing immunization until day 5 after tumor inoculation when the tumor is more established. None of the mono-therapies were able to induce survival, whereas the combined treatment of GM-CSF and IFNγ induced a 44% survival.

Investigation of the local immune response at the tumor site induced by the GM-CSF and IFNγ based immunotherapy

Manuscript III

The CNS has long been regarded as an immune privileged site, incapable of mounting a CNS-specific immune response. However, despite the BBB, activated lymphocytes have been shown to migrate into the CNS. Studies have also demonstrated that local APCs from the CNS drain to the cervical lymph nodes, where they can activate a CNS-antigen specific immune response (16, 17). In this manuscript, the aim was to analyze the local immune response elicited in tumor draining lymph nodes (TDLNs) as well as in the tumor, in order to explain the improved survival after the combined treatment of GM-CSF and IFNγ.

In the TDLNs activated lymphocytes migrating to the tumor site will be reactivated by APCs presenting antigens from the tumor. Due to the immunosuppression elicited by the tumor, APCs in the TDLNs have the capability to induce tolerance and anergy instead of boosting the induced anti-tumor immune response. Several studies have shown an increased population of MDSCs as well a decreased population of functional DCs in the TDLNs, induced by factors produced by the tumor (94, 196). The lymph nodes responsible for draining the CNS are suggested to be the cervical lymph nodes, CLNs (24). After immunizing with either GM-CSF or GM-CSF combined with IFNγ, we found an increased number of $CD4^+$ and $CD8^+$ T-cells as well as T-regs in the CLNs. Though, by the addition of IFNγ, this increase in T-reg numbers was not statistically significant. The numbers of MDSCs as well as of differentiated myeloid cells (macrophages and DCs) were also increased in the CLNs after immunizing with GM-CSF, but by the addition of IFNγ, the increase in MDSCs was not statistically significant. MDSCs have been shown to be responsible for inducing T-regs (103). Hence, the reduced amount of T-regs in the CLNs could therefore be explained by the concurrent reduction in MDSCs after immunizing with

the combined treatment of GM-CSF and IFNγ. Without analyzing the functional properties of the lymphocytes in the CLNs, these results indicate that GM-CSF enhances the anti-tumor response, while the subsequent immunosuppression is reduced (directly or indirectly) by the addition of IFNγ.

El Andaloussi et al. have shown infiltration of T-regs in mouse gliomas, as well as correlated T-reg infiltration in human gliomas with malignancy grade (112, 115). Intratumorally we found a relative reduction of T-regs after immunizing with GM-CSF and IFNγ. Although the total number of T-regs was increased, this only followed the overall increase in T-cells after immunizing with GM-CSF and IFNγ, most likely counteracting the induction of intratumoral T-regs. Brain tumors are associated with infiltration of macrophages, but whether tumor associated macrophages promote or prevent tumor growth is not fully clarified (128, 132). However, it has been shown that TAMs harboring a M2 phenotype can be reactivated into M1 macrophages with either anti-IL-10 antibodies or certain pro-inflammatory cytokines, such as GM-CSF combined with IFNγ (141). *Sinha et al.* further support the importance for the induction of tumoricidal M1 macrophages for the rejection of established tumors (197). The number of intratumoral $CD11b^+$ monocytes had a slight tendency to increase after immunization with GM-CSF and IFNγ. When analyzing their function, we could detect a higher number as well as proportion of TNFα producing monocytes. These results imply that the combined treatment of GM-CSF and IFNγ affected the phenotype of the monocytes infiltrating the tumor. We believe that the most obvious explanation for the effects seen after immunization with GM-CSF and IFNγ, is mediated indirectly via IFNγ produced by the activated T-cells infiltrating the tumor (142).

In conclusion, analysis of TILs showed that the combined treatment of GM-CSF and IFNγ was coupled to an increase in both intratumoral $CD4^+$ and $CD8^+$ T-cells but a relative decrease in T-reg cells. Furthermore the combined immunizations displayed an increase of putative tumoricidal $CD11b^+TNF\alpha^+$ intratumoral macrophages. Further studies of the function of the tumor infiltrating T-cells are required in order to conclude if the production of TNFα of the infiltrating macrophages is dependent on the activation through IFNγ produced by the infiltrating T-cells, as well as if we have shifted the phenotype from M2 into M1 macrophages.

Figure 4, summarizing paper I and manuscript II-III

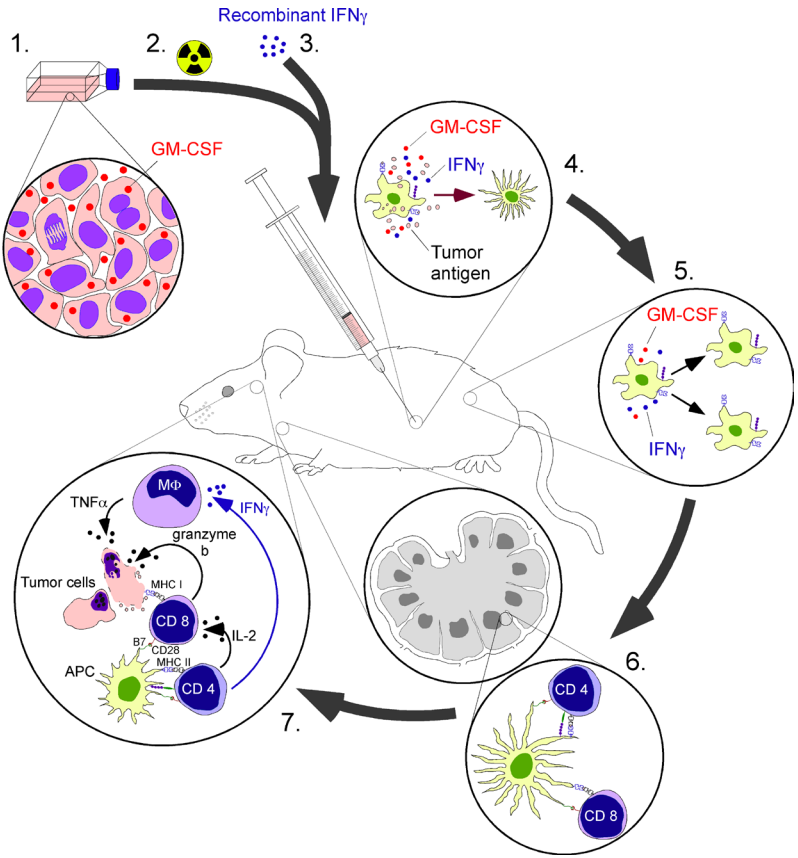

Figure 4. Suggested pathway from vaccination to induction of survival using the combined GM-CSF and IFNγ based immunotherapy. 1. GM-CSF transduced GL261 cells (GL-GM) are cultured *in vitro*. 2. Before immunization the GL-GM cells are irradiated to inhibit further proliferation *in vivo* after injection. Irradiation also increases the immunogenicity of the tumor cells, promoting phagocytosis at the immunization site. 3. Recombinant IFNγ is added to the GL-GM cell solution prior to immunization. 4. After injection i.p. GM-CSF produced by the GL-GM cells and recombinant IFNγ will affect the maturation and differentiation of the APCs migrating to the immunization site. The recruited APCs will phagocytose tumor cells and tumor antigens from the dying tumor cells at the immunization site. 5. Systemically GM-CSF enhances the recruitment and maturation of APCs from bone marrow progenitor cells to the immunization site, which are further activated by the addition of IFNγ. 6. After phagocytosis the APCs migrate to a secondary lymph node where they will present the phogocytosed tumor antigens on MHC molecules for T-cells. T-cells, properly activated with all costimulatory signals, will migrate to the tumor site, receiving additional re-activation by APCs locally in tumor draining lymph nodes and/or at the tumor site. 7. At the tumor site, cytotoxic T-cells recognizing the tumor cells will secrete cytokines and cytotoxic mediators such as IFNγ and Granzyme B. Despite having direct effects on the tumor cells, IFNγ produced by the activated T-cells will also convert tumor infiltrating macrophages into M1 macrophages, enhancing their tumoricidal activities by secretion of TNFα leading to enhanced tumor cell killing.

Monitoring the immune response induced in patients with GBM receiving IFNγ based immunotherapy

Manuscript IV

For the evaluation of clinical trials using immunotherapy, analyses of the induced immune responses are of great importance. Clinical evaluation of immune responses elicited by the lymphocytes mainly consist of three commonly used techniques; ELISPOT, tetramer analysis and cytokine flow cytometry (198). However, each laboratory and each assay have their own protocol, making comparisons between clinical studies complex (199).

A phase I trial, BRIGTT (Brain Immuno Gene Tumor Therapy), was initiated based on our previous pre-clinical studies on IFNγ-based immunotherapy (*Salford et al.,* manuscript in preparation*)*. Patients with GBM were vaccinated intradermally every third week with autologous tumor cells adenovirally transduced with IFNγ (200). The aim with our study was to choose and evaluate appropriate *in vitro* tests to monitor the immune activation against autologous tumor cells from patients with glioblastoma multiforme undergoing this immunotherapeutic phase I trial. Peripheral blood lymphocytes (PBLs) were prepared from the patients and restimulated with tumor cells *in vitro* before analyzes. The tumor cells used for restimulation were cultured *in vitro*, thus they were the same cells as were used for immunization. However, *in vitro* culture may select for certain tumor cells and therefore the tumor antigens presented *in vitro* may not reflect the tumor antigens presented *in vivo*.

When comparing IFNγ produced by the transduced tumor cells used for immunization we could correlate an IFNγ production of the tumor cells higher than 2500 pg/ml with those patients also responding to the treatment. These results also questioned if the timing and number of immunizations were optimal for eliciting the most efficient immune response in the patients.

A total of 8 patients were included in the clinical trial and a prolonged survival was recorded when compared with a relevant control group (*Salford et al.,* manuscript in preparation). Since patients with GBM have a better prognosis the younger they are, it should be emphasized that the two patients with the longest survival also were the youngest patients included in this study (1, 2). Using ELISpot and cytokine bead assay (CBA) we were able to correlate a more potent IFNγ production from those patients having a significantly prolonged survival. However, this correlation was more pronounced using ELISpot than CBA. ELISpot assays on lymphocytes have been shown by others to correlate well with survival in patients with GBM (201-203). The explanation for the differences monitored between CBA and ELISpot could be explained by the fact that in the ELISpot assay, the cytokine production was measured from non-adherent lymphocytes only, while in the CBA both adherent cells (monocytes) as well as non-adherent cells were included. Tumor derived factors have been shown to induce monocytic cell populations with immunosuppressive properties. Cancer patients have been shown to contain an increased proportion of MDSCs as well as immature monocytes, capable of suppressing the induction of immune responses (197, 204).

We were not able to see a clear correlation of an elevated IL-10 production of the lymphocytes after restimulation *in vitro* with patients having a less prolonged survival. In most patients IL-10, but not IFNγ, was detectable before surgery and initiation of immunotherapy, which could reflect steroid treatment of the patients. IL-10 is believed to be an indicator for a less efficient immune response, suppressing the production of IFNγ (186). However, as reviewed by *Mocellin et al.* several studies have shown that IL-10 also can act as an immunostimulatory cytokine, making the role for IL-10 in the context of anti-tumor responses more complex (81).

The non-immunized patient showed a predominant IL-10 production compared to IFNγ, comparing both CBA and ELISPOT assays, reflecting the efficiency of the immunotherapy. The specificity of the anti-tumor response was demonstrated by stimulating lymphocytes from a healthy control with tumor cells, demonstrating an overall low cytokine production. This was further underscored in one patient after re-operation, when comparing restimulation of the lymphocytes with tumor cells from either the recurring or the primary tumor.

We conclude that the results obtained by using CBA and ELISpot for analyzes of immune responses elicited after immunotherapy showed a trend that followed the clinical outcome of the patients. IFNγ production from PBL restimulated *in vitro* was elevated in those patients having a prolonged survival. These results may also be helpful to further improve the immunization protocol, regarding timing and total number of immunizations.

CONCLUSIONS

In paper I we described the establishment of a new treatment modality for experimental gliomas in mice. Survival after immunization with the widely used GM-CSF based immunotherapy was synergistically enhanced if combined with recombinant IFNγ at the immunization site. We could also demonstrate an increased proportion of tumor infiltrating macrophages, indicating that an enhanced anti-tumor response was initiated by the immunizations.

In manuscript II we investigated the systemic immune response elicited by the combined treatment of GM-CSF and IFNγ. We could demonstrate that despite a systemic expansion of MDSCs and T-regs, an increased cytotoxic anti-tumor T-cell response was induced and that the induction of survival was highly dependent on the T-cells. We could also demonstrate that the combined treatment of GM-CSF and IFNγ could mediate a long-lasting immunological memory. Finally we showed that immunizations with GM-CSF and IFNγ were able to treat established tumors as well.

In manuscript III we analyzed the local immune responses elicited intratumorally as well as in the tumor draining CLNs after immunization with GM-CSF and IFNγ. The addition of IFNγ reduced the amount of MDSCs as well as T-regs in the TDLNs, which were significantly increased after immunization with GM-CSF only. Intratumorally, the number of T-cells was increased and the relative proportion of T-regs was decreased after immunizing with GM-CSF and IFNγ. We also demonstrated an increased proportion of intratumoral macrophages producing TNFα after immunizing with GM-CSF and IFNγ.

In manuscript IV we monitored *in vitro* assays for the evaluation of a clinical phase I trial, where patients with GBM receive an IFNγ based immunotherapy. ELIspot and CBA assays on *in vitro* restimulated PBLs indicated that those patients having a prolonged survival also had a higher production of IFNγ. We concluded that the ELIspot and CBA assays were feasible in this context and that they also were able to demonstrate a trend in immune activities following the survival of the patients.

Concluding remarks and future perspective

Immune responses against gliomas are invoked though they are too weak to eradicate an aggressively growing glioma. Cytokine based immunotherapy will boost the immune response and shift the balance from a tumor induced immunosuppression into an efficient antitumoral immune response, hopefully leading to survival.

Before the translation of immunotherapy into the clinic, thorough evaluation has to be performed in experimental glioma models. Based on earlier studies in our rat glioma model, we are currently performing a phase I clinical trial on patients with GBM receiving IFNγ based immunotherapy. In the mouse glioma model we have now established a more efficient immunotherapy, where IFNγ is combined with GM-CSF. If feasible and proven to be safe for the patients, GM-CSF and IFNγ based immunotherapy may have a chance to be translated into the clinic in the future.

Further pre-clinical studies are still required in order to improve the treatment against GBM. Patients with GBM will be treated with both chemo- and radiotherapy and additionally with immunotherapy. Although chemo- and radiotherapy can have negative on the immune system,

they may actually act beneficially as well. There are several studies in experimental glioma models as well as human GBM demonstrating a synergistic effect on the immune responses and even survival if combining immunotherapy with either chemo- or radiotherapy in an optimal order (201, 205-207).

There is still much more to be done in order to finally cure patients with GBM. Hopefully, the results demonstrated in this thesis will bring us a small step closer to that.

ACKNOWLEDGEMENTS

Peter S, my exceptionally time optimistic supervisor, thank you for your support and endurance. Being a sometimes too pessimistic person, I really must thank you for giving me all the superlatives, since for me things never seemed to be good enough. My co-supervisor **Anna D**, your constant support during these years has of course been invaluable for where I have ended up today. Whether being on mothers leave or connected to MSN during your stay in Australia, you always had time for a chat. Probably the biggest issue between us is that we are too much alike. To my other co-supervisor, **Edward**, thank you for constantly being so open minded and enthusiastic to share ideas and thoughts with. Often there were several universes between our perspectives, luckily we could almost always agree on the basics concerning lab and science stuff and of course coffee breaks!

The GIT-group, whether it was Sorrento-trips or constant moves of our lab around the whole BMC-building, we always had a nice time. **Shorena**, your immense source of knowledge always felt like a safety and helped me to focus on the right track. **Wiaam**, thank you for all your help and support with the mice and your constant cheerfulness and fashionable lab-dresscode that made lablife much more colorful. **Sara** and **Sofia E** thank you for the nice time in the lab and for all the help and work you put into my last projects so that I finally could reach my goal (including color discussions of my thesis). Last but not the least, all of you that joined the GIT-group **Maria**, **Magnus**, **Peter M** and **Sofia H**.

A big thank you to the rest of the D14-people, for valuable science talks and support. **Anna E**, we started our PhDs at the same time and ended it all with a fantastic conference-trip to Miami together with **Linda,** that I know I will meet soon in Lund for serious discussions of our familiar projects, **Xiaolong**, **Hans-Olov**, **Bengt**, **Johan R**, **Hua**, **Nivedita, Zhongtian, Manli** and **LiuMin**.

To all the nice people from the Neurosurgery department. **Leif** for your enthusiasm and encouragement, **Daniel** and **Salina** it was always a nice time seeing you and good luck with the rest of your PhDs, **Susanne** and **Catarina** for all your help, **Gunnar,** The Mac-guru, **Seema, Oscar, Johan B** and **Petra**.

Of course I haven´t forgot all the help and the nice times with the people from or connected with the former Tumorimmunlogy department, thank you all, especially always too busy **Karin** and much more relaxed **Gabriella**, **Håkan** for the scientific DC-meetings with your group and **Eva D** who taught me how to do research.

Thank you **Bengt M** for all help with the layout and design of this thesis. The Friday breakfast Alumni-group, thank you for invaluable time-offs from the lab.

To my fantastic friends **Marie, Ulrika, Emma A** and **Emma B**, who were struggling with their own PhDs (and of course finished long before me), but actually could do and talk about other things as well.

My dearest thank you goes to my **Ruben**. Of course for helping me with the nice illustrations in my thesis, but more importantly for always being so supportive and interested in my research and for cheering me up when my confidence was fading. And of course a special little thank you goes to our little family member to come, for keeping me alert with your kicks during my endless writing sessions.

Finally, a great big hug to my beloved family.

REFERENCES

1. Louis DN, International Agency for Research on Cancer, German cancer research center, World Health Organization. (2007) WHO classification of tumours of the central nervous system / Edited by David N. Louis. 4. ed. Lyon: IARC Press.

2. Castro MG, Cowen R, Williamson IK, *et al.* (2003) Current and future strategies for the treatment of malignant brain tumors. *Pharmacol Ther* 98, 71-108.

3. Stupp R, Mason WP, van den Bent MJ, *et al.* (2005) Radiotherapy plus concomitant and adjuvant temozolomide for glioblastoma. *The New England journal of medicine* 352, 987-96.

4. Bandres E, Andion E, Escalada A, *et al.* (2005) Gene expression profile induced by BCNU in human glioma cell lines with differential MGMT expression. *J Neurooncol* 73, 189-98.

5. Shu HK, Kim MM, Chen P, *et al.* (1998) The intrinsic radioresistance of glioblastoma-derived cell lines is associated with a failure of p53 to induce p21(BAX) expression. *Proc Natl Acad Sci U S A* 95, 14453-8.

6. Doolittle ND, Abrey LE, Bleyer WA, *et al.* (2005) New frontiers in translational research in neuro-oncology and the blood-brain barrier: report of the tenth annual Blood-Brain Barrier Disruption Consortium Meeting. *Clin Cancer Res* 11, 421-8.

7. Gomez GG, Kruse CA. (2006) Mechanisms of malignant glioma immune resistance and sources of immunosuppression. *Gene therapy & molecular biology* 10, 133-46.

8. Serano RD, Pegram CN, Bigner DD. (1980) Tumorigenic cell culture lines from a spontaneous VM/Dk murine astrocytoma (SMA). *Acta neuropathologica* 51, 53-64.

9. Sampson JH, Ashley DM, Archer GE, *et al.* (1997) Characterization of a spontaneous murine astrocytoma and abrogation of its tumorigenicity by cytokine secretion. *Neurosurgery* 41, 1365-72; discussion 72-3.

10. Seligman AM, Shear, M. J. (1939) Studies in Carcinogenesis. VIII. Experimental Production of Brain Tumors in Mice with Methylcholanthrene. *Am J Cancer* 37, 364-95.

11. Ausman JI, Shapiro WR, Rall DP. (1970) Studies on the chemotherapy of experimental brain tumors: development of an experimental model. *Cancer Res* 30, 2394-400.

12. Rutka JT, Murakami M, Dirks PB, *et al.* (1997) Role of glial filaments in cells and tumors of glial origin: a review. *J Neurosurg* 87, 420-30.

13. Smith KE, Janelidze S, Visse E, *et al.* (2007) Synergism between GM-CSF and IFNgamma: enhanced immunotherapy in mice with glioma. *International journal of cancer* 120, 75-80.

14. Szatmari T, Lumniczky K, Desaknai S, *et al.* (2006) Detailed characterization of the mouse glioma 261 tumor model for experimental glioblastoma therapy. *Cancer science* 97, 546-53.

15. Galea I, Bechmann I, Perry VH. (2007) What is immune privilege (not)? *Trends in immunology* 28, 12-8.

16. Carson MJ, Doose JM, Melchior B, *et al.* (2006) CNS immune privilege: hiding in plain sight. *Immunol Rev* 213, 48-65.

17. Bechmann I, Galea I, Perry VH. (2007) What is the blood-brain barrier (not)? *Trends in immunology* 28, 5-11.

18. Hickey WF. (1999) Leukocyte traffic in the central nervous system: the participants and their roles. *Seminars in immunology* 11, 125-37.

19. Dunn GP, Dunn IF, Curry WT. (2007) Focus on TILs: Prognostic significance of tumor infiltrating lymphocytes in human glioma. *Cancer Immun* 7, 12.

20. Long DM. (1970) Capillary ultrastructure and the blood-brain barrier in human malignant brain tumors. *J Neurosurg* 32, 127-44.

21. Cserr HF, Knopf PM. (1992) Cervical lymphatics, the blood-brain barrier and the immunoreactivity of the brain: a new view. *Immunology today* 13, 507-12.

22. Karman J, Ling C, Sandor M, Fabry Z. (2004) Initiation of immune responses in brain is promoted by local dendritic cells. *J Immunol* 173, 2353-61.

23. de Vos AF, van Meurs M, Brok HP, *et al.* (2002) Transfer of central nervous system autoantigens and presentation in secondary lymphoid organs. *J Immunol* 169, 5415-23.

24. Okamoto Y, Yamashita J, Hasegawa M, *et al.* (1999) Cervical lymph nodes play the role of regional lymph nodes in brain tumour immunity in rats. *Neuropathology and applied neurobiology* 25, 113-22.

25. Tsugawa T, Kuwashima N, Sato H, *et al.* (2004) Sequential delivery of interferon-alpha gene and DCs to intracranial gliomas promotes an effective antitumor response. *Gene therapy* 11, 1551-8.

26. Mrass P, Weninger W. (2006) Immune cell migration as a means to control immune privilege: lessons from the CNS and tumors. *Immunol Rev* 213, 195-212.

27. Becher B, Bechmann I, Greter M. (2006) Antigen presentation in autoimmunity and CNS inflammation: how T lymphocytes recognize the brain. *Journal of molecular medicine (Berlin, Germany)* 84, 532-43.

28. Stuve O, Youssef S, Slavin AJ, *et al.* (2002) The role of the MHC class II transactivator in class II expression and antigen presentation by astrocytes and in susceptibility to central nervous system autoimmune disease. *J Immunol* 169, 6720-32.

29. Prat A, Biernacki K, Becher B, Antel JP. (2000) B7 expression and antigen presentation by human brain endothelial cells: requirement for proinflammatory cytokines. *Journal of neuropathology and experimental neurology* 59, 129-36.

30. Alliot F, Godin I, Pessac B. (1999) Microglia derive from progenitors, originating from the yolk sac, and which proliferate in the brain. *Brain research* 117, 145-52.

31. Aloisi F, Ria F, Penna G, Adorini L. (1998) Microglia are more efficient than astrocytes in antigen processing and in Th1 but not Th2 cell activation. *J Immunol* 160, 4671-80.

32. De Simone R, Giampaolo A, Giometto B, *et al.* (1995) The costimulatory molecule B7 is expressed on human microglia in culture and in multiple sclerosis acute lesions. *Journal of neuropathology and experimental neurology* 54, 175-87.

33. Hickey WF, Vass K, Lassmann H. (1992) Bone marrow-derived elements in the central nervous system: an immunohistochemical and ultrastructural survey of rat chimeras. *Journal of neuropathology and experimental neurology* 51, 246-56.

34. Greter M, Heppner FL, Lemos MP, *et al.* (2005) Dendritic cells permit immune invasion of the CNS in an animal model of multiple sclerosis. *Nature medicine* 11, 328-34.

35. Tran EH, Hoekstra K, van Rooijen N, *et al.* (1998) Immune invasion of the central nervous system parenchyma and experimental allergic encephalomyelitis, but not leukocyte extravasation from blood, are prevented in macrophage-depleted mice. *J Immunol* 161, 3767-75.

36. McMenamin PG, Wealthall RJ, Deverall M, *et al.* (2003) Macrophages and dendritic cells in the rat meninges and choroid plexus: three-dimensional localisation by environmental scanning electron microscopy and confocal microscopy. *Cell and tissue research* 313, 259-69.

37. Hatterer E, Davoust N, Didier-Bazes M, *et al.* (2006) How to drain without lymphatics? Dendritic cells migrate from the cerebrospinal fluid to the B-cell follicles of cervical lymph nodes. *Blood* 107, 806-12.

38. Burnet FM. (1970) The concept of immunological surveillance. *Progress in experimental tumor research Fortschritte der experimentellen Tumorforschung* 13, 1-27.

39. Burnet FM. (1971) Immunological surveillance in neoplasia. *Transplantation reviews* 7, 3-25.

40. Thomas L. (1982) On immunosurveillance in human cancer. *The Yale journal of biology and medicine* 55, 329-33.

41. Street SE, Cretney E, Smyth MJ. (2001) Perforin and interferon-gamma activities independently control tumor initiation, growth, and metastasis. *Blood* 97, 192-7.

42. Dighe AS, Richards E, Old LJ, Schreiber RD. (1994) Enhanced in vivo growth and resistance to rejection of tumor cells expressing dominant negative IFN gamma receptors. *Immunity* 1, 447-56.

43. van den Broek ME, Kagi D, Ossendorp F, *et al.* (1996) Decreased tumor surveillance in perforin-deficient mice. *J Exp Med* 184, 1781-90.

44. Dunn GP, Koebel CM, Schreiber RD. (2006) Interferons, immunity and cancer immunoediting. *Nat Rev Immunol* 6, 836-48.

45. Shinkai Y, Rathbun G, Lam KP, *et al.* (1992) RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell* 68, 855-67.

46. Shankaran V, Ikeda H, Bruce AT, *et al.* (2001) IFNgamma and lymphocytes prevent primary tumour development and shape tumour immunogenicity. *Nature* 410, 1107-11.

47. Qin Z, Kim HJ, Hemme J, Blankenstein T. (2002) Inhibition of methylcholanthrene-induced carcinogenesis by an interferon gamma receptor-dependent foreign body reaction. *J Exp Med* 195, 1479-90.

48. Qin Z, Blankenstein T. (2004) A cancer immunosurveillance controversy. *Nature immunology* 5, 3-4; author reply -5.

49. Dunn GP, Bruce AT, Ikeda H, *et al.* (2002) Cancer immunoediting: from immunosurveillance to tumor escape. *Nature immunology* 3, 991-8.

50. Dunn GP, Old LJ, Schreiber RD. (2004) The three Es of cancer immunoediting. *Annual review of immunology* 22, 329-60.

51. Kuby J. (1997) Immunology. 3. ed. New York: Freeman.

52. Gallucci S, Matzinger P. (2001) Danger signals: SOS to the immune system. *Current opinion in immunology* 13, 114-9.

53. Matzinger P. (1994) Tolerance, danger, and the extended family. *Annual review of immunology* 12, 991-1045.

54. Smyth MJ, Dunn GP, Schreiber RD. (2006) Cancer immunosurveillance and immunoediting: the roles of immunity in suppressing tumor development and shaping tumor immunogenicity. *Advances in immunology* 90, 1-50.

55. Eagle RA, Trowsdale J. (2007) Promiscuity and the single receptor: NKG2D. *Nat Rev Immunol 7*, *737-44*.

56. Banchereau J, Steinman RM. (1998) Dendritic cells and the control of immunity. *Nature* 392, 245-52.

57. Prins RM, Shu CJ, Radu CG, *et al.* (2008) Anti-tumor activity and trafficking of self, tumor-specific T cells against tumors located in the brain. *Cancer Immunol Immunother*.

58. Janssen EM, Lemmens EE, Wolfe T, *et al.* (2003) CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes. *Nature* 421, 852-6.

59. Melief CJ. (2003) Mini-review: Regulation of cytotoxic T lymphocyte responses by dendritic cells: peaceful coexistence of cross-priming and direct priming? *European journal of immunology* 33, 2645-54.

60. Calzascia T, Masson F, Di Berardino-Besson W, *et al.* (2005) Homing phenotypes of tumor-specific CD8 T cells are predetermined at the tumor site by crosspresenting APCs. *Immunity* 22, 175-84.

61. Drake CG, Jaffee E, Pardoll DM. (2006) Mechanisms of immune evasion by tumors. *Advances in immunology* 90, 51-81.

62. Cresswell AC, Sisley K, Laws D, *et al.* (2001) Reduced expression of TAP-1 and TAP-2 in posterior uveal melanoma is associated with progression to metastatic disease. *Melanoma research* 11, 275-81.

63. Romero JM, Jimenez P, Cabrera T, *et al.* (2005) Coordinated downregulation of the antigen presentation machinery and HLA class I/beta2-microglobulin complex is responsible for HLA-ABC loss in bladder cancer. *International journal of cancer* 113, 605-10.

64. Croci DO, Zacarias Fluck MF, Rico MJ, *et al.* (2007) Dynamic cross-talk between tumor and immune cells in orchestrating the immunosuppressive network at the tumor microenvironment. *Cancer Immunol Immunother* 56, 1687-700.

65. Kurnick JT, Ramirez-Montagut T, Boyle LA, *et al.* (2001) A novel autocrine pathway of tumor escape from immune recognition: melanoma cell lines produce a soluble protein that diminishes

expression of the gene encoding the melanocyte lineage melan-A/MART-1 antigen through down-modulation of its promoter. *J Immunol* 167, 1204-11.

66. Bodmer S, Huber D, Heid I, Fontana A. (1991) Human glioblastoma cell derived transforming growth factor-beta 2: evidence for secretion of both high and low molecular weight biologically active forms. *Journal of neuroimmunology* 34, 33-42.

67. Kuppner MC, Hamou MF, Sawamura Y, *et al.* (1989) Inhibition of lymphocyte function by glioblastoma-derived transforming growth factor beta 2. *J Neurosurg* 71, 211-7.

68. Gorelik L, Flavell RA. (2002) Transforming growth factor-beta in T-cell biology. *Nat Rev Immunol* 2, 46-53.

69. Ranges GE, Figari IS, Espevik T, Palladino MA, Jr. (1987) Inhibition of cytotoxic T cell development by transforming growth factor beta and reversal by recombinant tumor necrosis factor alpha. *J Exp Med* 166, 991-8.

70. Thomas DA, Massague J. (2005) TGF-beta directly targets cytotoxic T cell functions during tumor evasion of immune surveillance. *Cancer cell* 8, 369-80.

71. Li MO, Wan YY, Sanjabi S, *et al.* (2006) Transforming growth factor-beta regulation of immune responses. *Annual review of immunology* 24, 99-146.

72. Vodovotz Y, Bogdan C, Paik J, *et al.* (1993) Mechanisms of suppression of macrophage nitric oxide release by transforming growth factor beta. *J Exp Med* 178, 605-13.

73. Tsunawaki S, Sporn M, Ding A, Nathan C. (1988) Deactivation of macrophages by transforming growth factor-beta. *Nature* 334, 260-2.

74. Wang D, Dubois RN. (2006) Prostaglandins and cancer. *Gut* 55, 115-22.

75. Dix AR, Brooks WH, Roszman TL, Morford LA. (1999) Immune defects observed in patients with primary malignant brain tumors. *Journal of neuroimmunology* 100, 216-32.

76. Castelli MG, Chiabrando C, Fanelli R, *et al.* (1989) Prostaglandin and thromboxane synthesis by human intracranial tumors. *Cancer Res* 49, 1505-8.

77. Fiorentino DF, Zlotnik A, Vieira P, *et al.* (1991) IL-10 acts on the antigen-presenting cell to inhibit cytokine production by Th1 cells. *J Immunol* 146, 3444-51.

78. de Waal Malefyt R, Abrams J, Bennett B, *et al.* (1991) Interleukin 10(IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J Exp Med* 174, 1209-20.

79. Hishii M, Nitta T, Ishida H, *et al.* (1995) Human glioma-derived interleukin-10 inhibits antitumor immune responses in vitro. *Neurosurgery* 37, 1160-6; discussion 6-7.

80. Huettner C, Czub S, Kerkau S, *et al.* (1997) Interleukin 10 is expressed in human gliomas in vivo and increases glioma cell proliferation and motility in vitro. *Anticancer research* 17, 3217-24.

81. Mocellin S, Marincola FM, Young HA. (2005) Interleukin-10 and the immune response against cancer: a counterpoint. *J Leukoc Biol* 78, 1043-51.

82. Book AA, Fielding KE, Kundu N, *et al.* (1998) IL-10 gene transfer to intracranial 9L glioma: tumor inhibition and cooperation with IL-2. *Journal of neuroimmunology* 92, 50-9.

83. Plate KH, Breier G, Weich HA, Risau W. (1992) Vascular endothelial growth factor is a potential tumour angiogenesis factor in human gliomas in vivo. *Nature* 359, 845-8.

84. Kondo S, Asano M, Matsuo K, *et al.* (1994) Vascular endothelial growth factor/vascular permeability factor is detectable in the sera of tumor-bearing mice and cancer patients. *Biochimica et biophysica acta* 1221, 211-4.

85. Ferrara N, Houck K, Jakeman L, Leung DW. (1992) Molecular and biological properties of the vascular endothelial growth factor family of proteins. *Endocrine reviews* 13, 18-32.

86. Gabrilovich DI, Chen HL, Girgis KR, *et al.* (1996) Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells. *Nature medicine* 2, 1096-103.

87. Connolly DT, Heuvelman DM, Nelson R, *et al.* (1989) Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis. *The Journal of clinical investigation* 84, 1470-8.

88. Inaba K, Inaba M, Romani N, *et al.* (1992) Generation of large numbers of dendritic cells from mouse bone marrow cultures supplemented with granulocyte/macrophage colony-stimulating factor. *J Exp Med* 176, 1693-702.

89. Bronte V, Chappell DB, Apolloni E, *et al.* (1999) Unopposed production of granulocyte-macrophage colony-stimulating factor by tumors inhibits CD8+ T cell responses by dysregulating antigen-presenting cell maturation. *J Immunol* 162, 5728-37.

90. Karcher S, Steiner HH, Ahmadi R, *et al.* (2006) Different angiogenic phenotypes in primary and secondary glioblastomas. *International journal of cancer* 118, 2182-9.

91. Rolhion C, Penault-Llorca F, Kemeny JL, *et al.* (2001) Interleukin-6 overexpression as a marker of malignancy in human gliomas. *J Neurosurg* 94, 97-101.

92. Chang CY, Li MC, Liao SL, *et al.* (2005) Prognostic and clinical implication of IL-6 expression in glioblastoma multiforme. *J Clin Neurosci* 12, 930-3.

93. Kusmartsev S, Gabrilovich DI. (2006) Role of immature myeloid cells in mechanisms of immune evasion in cancer. *Cancer Immunol Immunother* 55, 237-45.

94. Rabinovich GA, Gabrilovich D, Sotomayor EM. (2007) Immunosuppressive strategies that are mediated by tumor cells. *Annual review of immunology* 25, 267-96.

95. Gabrilovich DI, Bronte V, Chen SH, *et al.* (2007) The terminology issue for myeloid-derived suppressor cells. *Cancer Res* 67, 425; author reply 6.

96. Talmadge JE. (2007) Pathways mediating the expansion and immunosuppressive activity of myeloid-derived suppressor cells and their relevance to cancer therapy. *Clin Cancer Res* 13, 5243-8.

97. Yang R, Cai Z, Zhang Y, *et al.* (2006) CD80 in immune suppression by mouse ovarian carcinoma-associated Gr-1+CD11b+ myeloid cells. *Cancer Res* 66, 6807-15.

98. Sica A, Bronte V. (2007) Altered macrophage differentiation and immune dysfunction in tumor development. *The Journal of clinical investigation* 117, 1155-66.

99. Otsuji M, Kimura Y, Aoe T, *et al.* (1996) Oxidative stress by tumor-derived macrophages suppresses the expression of CD3 zeta chain of T-cell receptor complex and antigen-specific T-cell responses. *Proc Natl Acad Sci U S A* 93, 13119-24.

100. Kusmartsev SA, Li Y, Chen SH. (2000) Gr-1+ myeloid cells derived from tumor-bearing mice inhibit primary T cell activation induced through CD3/CD28 costimulation. *J Immunol* 165, 779-85.

101. Kusmartsev S, Gabrilovich DI. (2003) Inhibition of myeloid cell differentiation in cancer: the role of reactive oxygen species. *J Leukoc Biol* 74, 186-96.

102. Van Ginderachter JA, Movahedi K, Hassanzadeh Ghassabeh G, *et al.* (2006) Classical and alternative activation of mononuclear phagocytes: picking the best of both worlds for tumor promotion. *Immunobiology* 211, 487-501.

103. Gallina G, Dolcetti L, Serafini P, *et al.* (2006) Tumors induce a subset of inflammatory monocytes with immunosuppressive activity on CD8+ T cells. *The Journal of clinical investigation* 116, 2777-90.

104. Sakaguchi S, Sakaguchi N, Asano M, *et al.* (1995) Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155, 1151-64.

105. Gallimore A, Godkin A. (2008) Regulatory T cells and tumour immunity - observations in mice and men. *Immunology* 123, 157-63.

106. Grossman WJ, Verbsky JW, Barchet W, *et al.* (2004) Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* 21, 589-601.

107. Zou W. (2005) Immunosuppressive networks in the tumour environment and their therapeutic relevance. *Nat Rev Cancer* 5, 263-74.

108. Gondek DC, Lu LF, Quezada SA, *et al.* (2005) Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *J Immunol* 174, 1783-6.

109. Chaput N, Darrasse-Jeze G, Bergot AS, *et al.* (2007) Regulatory T cells prevent CD8 T cell maturation by inhibiting CD4 Th cells at tumor sites. *J Immunol* 179, 4969-78.

110. Mizukami Y, Kono K, Kawaguchi Y, *et al.* (2008) CCL17 and CCL22 chemokines within tumor microenvironment are related to accumulation of Foxp3+ regulatory T cells in gastric cancer. *International journal of cancer* 122, 2286-93.

111. Curiel TJ, Coukos G, Zou L, *et al.* (2004) Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival. *Nature medicine* 10, 942-9.

112. El Andaloussi A, Han Y, Lesniak MS. (2006) Prolongation of survival following depletion of CD4+CD25+ regulatory T cells in mice with experimental brain tumors. *J Neurosurg* 105, 430-7.

113. Grauer OM, Nierkens S, Bennink E, *et al.* (2007) CD4+FoxP3+ regulatory T cells gradually accumulate in gliomas during tumor growth and efficiently suppress antiglioma immune responses in vivo. *International journal of cancer* 121, 95-105.

114. El Andaloussi A, Lesniak MS. (2006) An increase in CD4+CD25+FOXP3+ regulatory T cells in tumor-infiltrating lymphocytes of human glioblastoma multiforme. *Neuro-oncology* 8, 234-43.

115. El Andaloussi A, Lesniak MS. (2007) CD4+ CD25+ FoxP3+ T-cell infiltration and heme oxygenase-1 expression correlate with tumor grade in human gliomas. *J Neurooncol* 83, 145-52.

116. Fecci PE, Sweeney AE, Grossi PM, *et al.* (2006) Systemic anti-CD25 monoclonal antibody administration safely enhances immunity in murine glioma without eliminating regulatory T cells. *Clin Cancer Res* 12, 4294-305.

117. Grauer OM, Sutmuller RP, van Maren W, *et al.* (2007) Elimination of regulatory T cells is essential for an effective vaccination with tumor lysate-pulsed dendritic cells in a murine glioma model. *International journal of cancer*.

118. Sato E, Olson SH, Ahn J, *et al.* (2005) Intraepithelial CD8+ tumor-infiltrating lymphocytes and a high CD8+/regulatory T cell ratio are associated with favorable prognosis in ovarian cancer. *Proc Natl Acad Sci U S A* 102, 18538-43.

119. Ohtani H. (2007) Focus on TILs: prognostic significance of tumor infiltrating lymphocytes in human colorectal cancer. *Cancer Immun* 7, 4.

120. Naito Y, Saito K, Shiiba K, *et al.* (1998) CD8+ T cells infiltrated within cancer cell nests as a prognostic factor in human colorectal cancer. *Cancer Res* 58, 3491-4.

121. Fecci PE, Mitchell DA, Whitesides JF, *et al.* (2006) Increased regulatory T-cell fraction amidst a diminished CD4 compartment explains cellular immune defects in patients with malignant glioma. *Cancer Res* 66, 3294-302.

122. Persson A, Skagerberg G, Salford LG, Englund E. (2005) Immunotreatment in patients with glioblastoma multiforme--a histopathological evaluation of reactive and inflammatory changes. *Clinical neuropathology* 24, 201-8.

123. Kondratiev S, Sabo E, Yakirevich E, *et al.* (2004) Intratumoral CD8+ T lymphocytes as a prognostic factor of survival in endometrial carcinoma. *Clin Cancer Res* 10, 4450-6.

124. Nakano O, Sato M, Naito Y, *et al.* (2001) Proliferative activity of intratumoral CD8(+) T-lymphocytes as a prognostic factor in human renal cell carcinoma: clinicopathologic demonstration of antitumor immunity. *Cancer Res* 61, 5132-6.

125. Hiraoka K, Miyamoto M, Cho Y, *et al.* (2006) Concurrent infiltration by CD8+ T cells and CD4+ T cells is a favourable prognostic factor in non-small-cell lung carcinoma. *British journal of cancer* 94, 275-80.

126. Fu J, Xu D, Liu Z, *et al.* (2007) Increased regulatory T cells correlate with CD8 T-cell impairment and poor survival in hepatocellular carcinoma patients. *Gastroenterology* 132, 2328-39.

127. Woo EY, Yeh H, Chu CS, *et al.* (2002) Cutting edge: Regulatory T cells from lung cancer patients directly inhibit autologous T cell proliferation. *J Immunol* 168, 4272-6.

128. Hussain SF, Yang D, Suki D, *et al.* (2006) The role of human glioma-infiltrating microglia/macrophages in mediating antitumor immune responses. *Neuro-oncology* 8, 261-79.

129. Morantz RA, Wood GW, Foster M, *et al.* (1979) Macrophages in experimental and human brain tumors. Part 2: studies of the macrophage content of human brain tumors. *J Neurosurg* 50, 305-11.

130. Lewis CE, Pollard JW. (2006) Distinct role of macrophages in different tumor microenvironments. *Cancer Res* 66, 605-12.

131. Dirkx AE, Oude Egbrink MG, Wagstaff J, Griffioen AW. (2006) Monocyte/macrophage infiltration in tumors: modulators of angiogenesis. *J Leukoc Biol* 80, 1183-96.

132. Bingle L, Brown NJ, Lewis CE. (2002) The role of tumour-associated macrophages in tumour progression: implications for new anticancer therapies. *J Pathol* 196, 254-65.

133. Nishie A, Ono M, Shono T, *et al.* (1999) Macrophage infiltration and heme oxygenase-1 expression correlate with angiogenesis in human gliomas. *Clin Cancer Res* 5, 1107-13.

134. Rossi ML, Hughes JT, Esiri MM, *et al.* (1987) Immunohistological study of mononuclear cell infiltrate in malignant gliomas. *Acta neuropathologica* 74, 269-77.

135. Mantovani A, Sozzani S, Locati M, *et al.* (2002) Macrophage polarization: tumor-associated macrophages as a paradigm for polarized M2 mononuclear phagocytes. *Trends in immunology* 23, 549-55.

136. Biswas SK, Gangi L, Paul S, *et al.* (2006) A distinct and unique transcriptional program expressed by tumor-associated macrophages (defective NF-kappaB and enhanced IRF-3/STAT1 activation). *Blood* 107, 2112-22.

137. Umemura N, Saio M, Suwa T, *et al.* (2008) Tumor-infiltrating myeloid-derived suppressor cells are pleiotropic-inflamed monocytes/macrophages that bear M1- and M2-type characteristics. *J Leukoc Biol*.

138. Yang L, DeBusk LM, Fukuda K, *et al.* (2004) Expansion of myeloid immune suppressor Gr+CD11b+ cells in tumor-bearing host directly promotes tumor angiogenesis. *Cancer cell* 6, 409-21.

139. van den Berg A, Visser L, Poppema S. (1999) High expression of the CC chemokine TARC in Reed-Sternberg cells. A possible explanation for the characteristic T-cell infiltratein Hodgkin's lymphoma. *The American journal of pathology* 154, 1685-91.

140. Mytar B, Woloszyn M, Szatanek R, *et al.* (2003) Tumor cell-induced deactivation of human monocytes. *J Leukoc Biol* 74, 1094-101.

141. Baj-Krzyworzeka M, Baran J, Szatanek R, *et al.* (2004) Prevention and reversal of tumor cell-induced monocyte deactivation by cytokines, purified protein derivative (PPD), and anti-IL-10 antibody. *Cancer Immun* 4, 8.

142. Bonnotte B, Larmonier N, Favre N, *et al.* (2001) Identification of tumor-infiltrating macrophages as the killers of tumor cells after immunization in a rat model system. *J Immunol* 167, 5077-83.

143. Young HA, Hardy KJ. (1995) Role of interferon-gamma in immune cell regulation. *J Leukoc Biol* 58, 373-81.

144. Urban JL, Shepard HM, Rothstein JL, *et al.* (1986) Tumor necrosis factor: a potent effector molecule for tumor cell killing by activated macrophages. *Proc Natl Acad Sci U S A* 83, 5233-7.

145. Hibbs JB, Jr., Taintor RR, Vavrin Z, Rachlin EM. (1988) Nitric oxide: a cytotoxic activated macrophage effector molecule. *Biochemical and biophysical research communications* 157, 87-94.

146. Keller R, Geiges M, Keist R. (1990) L-arginine-dependent reactive nitrogen intermediates as mediators of tumor cell killing by activated macrophages. *Cancer Res* 50, 1421-5.

147. Driessens G, Hamdane M, Cool V, *et al.* (2004) Highly successful therapeutic vaccinations combining dendritic cells and tumor cells secreting granulocyte macrophage colony-stimulating factor. *Cancer Res* 64, 8435-42.

148. Parmiani G, Castelli C, Pilla L, *et al.* (2007) Opposite immune functions of GM-CSF administered as vaccine adjuvant in cancer patients. *Ann Oncol* 18, 226-32.

149. Dranoff G, Jaffee E, Lazenby A, *et al.* (1993) Vaccination with irradiated tumor cells engineered to secrete murine granulocyte-macrophage colony-stimulating factor stimulates potent, specific, and long-lasting anti-tumor immunity. *Proc Natl Acad Sci U S A* 90, 3539-43.

150. Li B, VanRoey MJ, Jooss K. (2007) Recombinant IL-7 enhances the potency of GM-CSF-secreting tumor cell immunotherapy. *Clinical immunology (Orlando, Fla* 123, 155-65.

151. Herrlinger U, Kramm CM, Johnston KM, *et al.* (1997) Vaccination for experimental gliomas using GM-CSF-transduced glioma cells. *Cancer Gene Ther* 4, 345-52.

152. Lumniczky K, Desaknai S, Mangel L, *et al.* (2002) Local tumor irradiation augments the antitumor effect of cytokine-producing autologous cancer cell vaccines in a murine glioma model. *Cancer Gene Ther* 9, 44-52.

153. Prell RA, Li B, Lin JM, *et al.* (2005) Administration of IFN-alpha enhances the efficacy of a granulocyte macrophage colony stimulating factor-secreting tumor cell vaccine. *Cancer Res* 65, 2449-56.

154. Jean WC, Spellman SR, Wallenfriedman MA, *et al.* (2004) Effects of combined granulocyte-macrophage colony-stimulating factor (GM-CSF), interleukin-2, and interleukin-12 based immunotherapy against intracranial glioma in the rat. *J Neurooncol* 66, 39-49.

155. Chen JC, Chen Y, Wu JM, *et al.* (2005) Effects of irradiated tumor vaccine and infusion of granulocyte-macrophage colony-stimulating factor and interleukin-12 on established gliomas in rats. *Cancer Immunol Immunother*, 1-11.

156. Serafini P, Carbley R, Noonan KA, *et al.* (2004) High-dose granulocyte-macrophage colony-stimulating factor-producing vaccines impair the immune response through the recruitment of myeloid suppressor cells. *Cancer Res* 64, 6337-43.

157. Daro E, Pulendran B, Brasel K, *et al.* (2000) Polyethylene glycol-modified GM-CSF expands CD11b(high)CD11c(high) but notCD11b(low)CD11c(high) murine dendritic cells in vivo: a comparative analysis with Flt3 ligand. *J Immunol* 165, 49-58.

158. Shi FS, Weber S, Gan J, *et al.* (1999) Granulocyte-macrophage colony-stimulating factor (GM-CSF) secreted by cDNA-transfected tumor cells induces a more potent antitumor response than exogenous GM-CSF. *Cancer Gene Ther* 6, 81-8.

159. Yei S, Bartholomew RM, Pezzoli P, *et al.* (2002) Novel membrane-bound GM-CSF vaccines for the treatment of cancer: generation and evaluation of mbGM-CSF mouse B16F10 melanoma cell vaccine. *Gene therapy* 9, 1302-11.

160. Poloso NJ, Nagarajan S, Mejia-Oneta JM, Selvaraj P. (2002) GPI-anchoring of GM-CSF results in active membrane-bound and partially shed cytokine. *Molecular immunology* 38, 803-16.

161. Wallenfriedman MA, Conrad JA, DelaBarre L, *et al.* (1999) Effects of continuous localized infusion of granulocyte-macrophage colony-stimulating factor and inoculations of irradiated glioma cells on tumor regression. *J Neurosurg* 90, 1064-71.

162. Dessureault S, Alsarraj M, McCarthy S, *et al.* (2005) A GM-CSF/CD40L producing cell augments anti-tumor T cell responses. *The Journal of surgical research* 125, 173-81.

163. Sandler AD, Chihara H, Kobayashi G, *et al.* (2003) CpG oligonucleotides enhance the tumor antigen-specific immune response of a granulocyte macrophage colony-stimulating factor-based vaccine strategy in neuroblastoma. *Cancer Res* 63, 394-9.

164. van Elsas A, Hurwitz AA, Allison JP. (1999) Combination immunotherapy of B16 melanoma using anti-cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and granulocyte/macrophage colony-stimulating factor (GM-CSF)-producing vaccines induces rejection of subcutaneous and metastatic tumors accompanied by autoimmune depigmentation. *J Exp Med* 190, 355-66.

165. Li B, Lalani AS, Harding TC, *et al.* (2006) Vascular endothelial growth factor blockade reduces intratumoral regulatory T cells and enhances the efficacy of a GM-CSF-secreting cancer immunotherapy. *Clin Cancer Res* 12, 6808-16.

166. Soiffer R, Lynch T, Mihm M, *et al.* (1998) Vaccination with irradiated autologous melanoma cells engineered to secrete human granulocyte-macrophage colony-stimulating factor generates potent antitumor immunity in patients with metastatic melanoma. *Proc Natl Acad Sci U S A* 95, 13141-6.

167. Hege KM, Jooss K, Pardoll D. (2006) GM-CSF gene-modifed cancer cell immunotherapies: of mice and men. *Int Rev Immunol* 25, 321-52.

168. Dranoff G. (2002) GM-CSF-based cancer vaccines. *Immunol Rev* 188, 147-54.

169. Simmons A, Li B, Gonzalez-Edick M, *et al.* (2007) GM-CSF-secreting cancer immunotherapies: preclinical analysis of the mechanism of action. *Cancer Immunology, Immunotherapy* 56, 1653-65.

170. Mach N, Gillessen S, Wilson SB, *et al.* (2000) Differences in dendritic cells stimulated in vivo by tumors engineered to secrete granulocyte-macrophage colony-stimulating factor or Flt3-ligand. *Cancer Res* 60, 3239-46.

171. Gillessen S, Naumov YN, Nieuwenhuis EE, *et al.* (2003) CD1d-restricted T cells regulate dendritic cell function and antitumor immunity in a granulocyte-macrophage colony-stimulating factor-dependent fashion. *Proc Natl Acad Sci U S A* 100, 8874-9.

172. Schroder K, Hertzog PJ, Ravasi T, Hume DA. (2004) Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* 75, 163-89.

173. Younes HM, Amsden BG. (2002) Interferon-gamma therapy: evaluation of routes of administration and delivery systems. *Journal of pharmaceutical sciences* 91, 2-17.

174. Finbloom DS, Larner AC, Nakagawa Y, Hoover DL. (1993) Culture of human monocytes with granulocyte-macrophage colony-stimulating factor results in enhancement of IFN-gamma receptors but suppression of IFN-gamma-induced expression of the gene IP-10. *J Immunol* 150, 2383-90.

175. Abdel-Wahab Z, Dar M, Osanto S, *et al.* (1997) Eradication of melanoma pulmonary metastases by immunotherapy with tumor cells engineered to secrete interleukin-2 or gamma interferon. *Cancer Gene Ther* 4, 33-41.

176. Visse E, Siesjo P, Widegren B, Sjogren HO. (1999) Regression of intracerebral rat glioma isografts by therapeutic subcutaneous immunization with interferon-gamma, interleukin-7, or B7-1-transfected tumor cells. *Cancer Gene Ther* 6, 37-44.

177. Wu A, Oh S, Ericson K, *et al.* (2007) Transposon-based interferon gamma gene transfer overcomes limitations of episomal plasmid for immunogene therapy of glioblastoma. *Cancer Gene Ther* 14, 550-60.

178. Saleh M, Jonas NK, Wiegmans A, Stylli SS. (2000) The treatment of established intracranial tumors by in situ retroviral IFN-gamma transfer. *Gene therapy* 7, 1715-24.

179. Kurzrock R, Rosenblum MG, Sherwin SA, *et al.* (1985) Pharmacokinetics, single-dose tolerance, and biological activity of recombinant gamma-interferon in cancer patients. *Cancer Res* 45, 2866-72.

180. Quesada JR, Talpaz M, Rios A, *et al.* (1986) Clinical toxicity of interferons in cancer patients: a review. *J Clin Oncol* 4, 234-43.

181. Breen FN, Hume DA, Weidemann MJ. (1991) Interactions among granulocyte-macrophage colony-stimulating factor, macrophage colony-stimulating factor, and IFN-gamma lead to enhanced proliferation of murine macrophage progenitor cells. *J Immunol* 147, 1542-7.

182. Xu X, Fu XY, Plate J, Chong AS. (1998) IFN-gamma induces cell growth inhibition by Fas-mediated apoptosis: requirement of STAT1 protein for up-regulation of Fas and FasL expression. *Cancer Res* 58, 2832-7.

183. Park SY, Seol JW, Lee YJ, *et al.* (2004) IFN-gamma enhances TRAIL-induced apoptosis through IRF-1. *European journal of biochemistry / FEBS* 271, 4222-8.

184. Akbasak A, Oldfield EH, Saris SC. (1991) Expression and modulation of major histocompatibility antigens on murine primary brain tumor in vitro. *J Neurosurg* 75, 922-9.

185. Freedman AS, Freeman GJ, Rhynhart K, Nadler LM. (1991) Selective induction of B7/BB-1 on interferon-gamma stimulated monocytes: a potential mechanism for amplification of T cell activation through the CD28 pathway. *Cellular immunology* 137, 429-37.

186. Boehm U, Klamp T, Groot M, Howard JC. (1997) Cellular responses to interferon-gamma. *Annual review of immunology* 15, 749-95.

187. Stark GR, Kerr IM, Williams BR, *et al.* (1998) How cells respond to interferons. *Annual review of biochemistry* 67, 227-64.

188. Yoshida A, Koide Y, Uchijima M, Yoshida TO. (1994) IFN-gamma induces IL-12 mRNA expression by a murine macrophage cell line, J774. *Biochemical and biophysical research communications* 198, 857-61.

189. Philip R, Epstein LB. (1986) Tumour necrosis factor as immunomodulator and mediator of monocyte cytotoxicity induced by itself, gamma-interferon and interleukin-1. *Nature* 323, 86-9.

190. Nishikawa H, Kato T, Tawara I, *et al.* (2005) IFN-gamma controls the generation/activation of CD4+ CD25+ regulatory T cells in antitumor immune response. *J Immunol* 175, 4433-40.

191. Nishisaka N, Maini A, Kinoshita Y, *et al.* (1999) Immunotherapy for lung metastases of murine renal cell carcinoma: synergy between radiation and cytokine-producing tumor vaccines. *J Immunother* 22, 308-14.

192. Enzler T, Gillessen S, Manis JP, *et al.* (2003) Deficiencies of GM-CSF and interferon gamma link inflammation and cancer. *J Exp Med* 197, 1213-9.

193. Siesjo P, Visse E, Sjogren HO. (1996) Cure of established, intracerebral rat gliomas induced by therapeutic immunizations with tumor cells and purified APC or adjuvant IFN-gamma treatment. *J Immunother Emphasis Tumor Immunol* 19, 334-45.

194. Vasu C, Dogan RN, Holterman MJ, Prabhakar BS. (2003) Selective induction of dendritic cells using granulocyte macrophage-colony stimulating factor, but not fms-like tyrosine kinase receptor 3-ligand, activates thyroglobulin-specific CD4+/CD25+ T cells and suppresses experimental autoimmune thyroiditis. *J Immunol* 170, 5511-22.

195. Alderson KL, Zhou Q, Berner V, *et al.* (2008) Regulatory and Conventional CD4+ T Cells Show Differential Effects Correlating with PD-1 and B7-H1 Expression after Immunotherapy. *J Immunol* 180, 2981-8.

196. Ishida T, Oyama T, Carbone DP, Gabrilovich DI. (1998) Defective function of Langerhans cells in tumor-bearing animals is the result of defective maturation from hemopoietic progenitors. *J Immunol* 161, 4842-51.

197. Sinha P, Clements VK, Ostrand-Rosenberg S. (2005) Reduction of myeloid-derived suppressor cells and induction of M1 macrophages facilitate the rejection of established metastatic disease. *J Immunol* 174, 636-45.

198. Walker EB, Disis ML. (2003) Monitoring immune responses in cancer patients receiving tumor vaccines. *Int Rev Immunol* 22, 283-319.

199. Britten CM, Janetzki S, van der Burg SH, *et al.* (2008) Toward the harmonization of immune monitoring in clinical trials: Quo vadis? *Cancer Immunol Immunother* 57, 285-8.

200. Salford LG, Siesjö., P., Skagerberg, G., Persson, B. R. R., Larsson, E.-M., Lindvall, M., Visse, E., & Widegren, B. (2002) Search for effective therapy against glioblastoma multiforme - clinical immunisation with autologous glioma cells transduced with the human Interferon-gamma gene. *Developments in Neuroscience, Excerpta Medica, Elsevier 2002; Ed K Watanabe* Int. Congr. Ser. 1247, 211-20.

201. Liu G, Akasaki Y, Khong HT, *et al.* (2005) Cytotoxic T cell targeting of TRP-2 sensitizes human malignant glioma to chemotherapy. *Oncogene* 24, 5226-34.

202. Yamanaka R, Abe T, Yajima N, *et al.* (2003) Vaccination of recurrent glioma patients with tumour lysate-pulsed dendritic cells elicits immune responses: results of a clinical phase I/II trial. *British journal of cancer* 89, 1172-9.

203. Yamanaka R, Homma J, Yajima N, *et al.* (2005) Clinical evaluation of dendritic cell vaccination for patients with recurrent glioma: results of a clinical phase I/II trial. *Clin Cancer Res* 11, 4160-7.

204. Gabrilovich D. (2004) Mechanisms and functional significance of tumour-induced dendritic-cell defects. *Nat Rev Immunol* 4, 941-52.

205. Heimberger AB, Sun W, Hussain SF, *et al.* (2007) Immunological responses in a patient with glioblastoma multiforme treated with sequential courses of temozolomide and immunotherapy: Case study. *Neuro-oncol*, 15228517-2007-046.

206. Kim C-H, Woo S-J, Park J-S, *et al.* (2007) Enhanced antitumour immunity by combined use of temozolomide and TAT-survivin pulsed dendritic cells in a murine glioma. *Immunology* 122, 615-22.

207. Newcomb EW, Demaria S, Lukyanov Y, *et al.* (2006) The Combination of Ionizing Radiation and Peripheral Vaccination Produces Long-term Survival of Mice Bearing Established Invasive GL261 Gliomas. *Clin Cancer Res* 12, 4730-7.