Katholieke Universiteit Leuven
Group Biomedical Sciences
Faculty of Medicine
Laboratory of Experimental Immunology
Department of Neurosurgery

DENDRITIC CELL-BASED TUMOR VACCINATION FOR HIGH-GRADE GLIOMAS

Hilko ARDON

Jury:

Promoter: prof. dr. S. De Vleeschouwer
Co-promoter: prof. dr. S. Van Gool
Chair: prof. dr. J. Goffin
Secretary: prof. dr. P. Clement
Jury members: prof. dr. J. de Vries
prof. dr. A. Heimberger
prof. dr. F. Lefranc
prof. dr. R. Oyen
prof. dr. P. Vandenberghe

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Doctoral thesis in Medical Sciences

Dendritic cell-based tumor vaccination for high-grade gliomas

Is all that we see or seem
But a dream within a dream?

Edgar Allan Poe

I wish I knew how it would feel to be free

Nina Simone (Billy Taylor)

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LIST OF ABBREVIATIONS

APC, allophycocyanine

ATRT, atypical teratoid-rhabdoid tumor

BBB, blood-brain barrier

BTIC, brain tumor-infiltrating cell

c.i., confidence interval

CNS, central nervous system

CTC, Common Toxicity Criteria

CTL, cytotoxic T lymphocyte

CTLA-4, cytotoxic T lymphocyte-associated antigen-4

DC, dendritic cell

DTH, delayed-type hypersensitivity

EGFRvIII, epidermal growth factor receptor class III variant

EORTC, European Organization for Research and Treatment of Cancer

FACS, fluorescence-activated cell sorting

FITC, fluorescein isothiocyanate

FMH, Fertigkeitenskala Münster-Heidelberg

Foxp3, forkhead box p3

FU, follow-up

GBM, glioblastoma multiforme

HGG, high-grade glioma

HSA, human serum albumin

IFN, interferon

IL, interleukin

ITT, intent-to-treat

KPS, Karnofsky Performance Score

LF, leukapheresis

mAb, monoclonal antibody

MB, medulloblastoma

MDSC, myeloid-derived suppressor cell

MGMT, O^6-methylguanine-DNA methyltransferase

MHC, major histocompatibility complex

MLR, mixed lymphocyte reaction

MMSE, Mini Mental State Examination

MRI, magnetic resonance imaging

NCI, National Cancer Institute

NK cell, natural killer cell

NKT cell, natural killer T cell

OS, overall survival

PBMC, peripheral blood mononuclear cell

PBS, phosphate-buffered saline

PE, phycoerythrin

PerCP, peridinin chlorophyll protein

PET, positron emission tomography

PFS, progression-free survival

PGE_2, prostaglandin E_2

PNET, primitive neuro-ectodermal tumor

QOL, quality of life

RPA, recursive partitioning analysis

RTOG, Radiation Therapy Oncology Group

TAA, tumor-associated antigen

TGF-β, transforming growth factor β

TIL, tumor-infiltrating lymphocyte

TNF-α, tumor necrosis factor α

Treg cell, regulatory T cell

TMZ, temozolomide

TMZm, maintenance temozolomide

V, vaccine

WHO, World Health Organization

6mo-PFS, progression-free survival at 6 months

CHAPTER 1. INTRODUCTION [a,b]

1.1 HIGH-GRADE GLIOMA

The term 'glioma' refers to all tumors of glial cell origin, including astrocytic tumors, oligodendrogliomas, ependymomas and mixed gliomas. Gliomas account for approximately 75% of primary malignant brain tumors. Within the astrocytic tumors, glioblastoma multiforme (GBM) is not only the most frequent brain tumor in adults, but represents also the most malignant one and is assigned grade IV by the World Health Organization (WHO). Together with WHO grade III anaplastic astrocytoma, anaplastic oligodendroglioma, anaplastic oligoastrocytoma and anaplastic ependymoma, GBM are categorized as high-grade glioma (HGG). This grading is associated with cytologically malignant, mitotically active, necrosis-prone neoplasms with rapid disease evolution and fatal outcome.[1,2]

Despite state-of-the-art oncological treatment, prognosis remains dismal for both adult and pediatric GBM patients. Median survival for adult patients is only 14.6 months with standard therapy, consisting of maximal safe resection of the tumor followed by temozolomide (TMZ) chemotherapy and radiotherapy.[3] Age, Karnofsky Performance Score (KPS) and extent of resection are the most important prognostic factors in newly diagnosed GBM.[4,5] Relapse is universal and at time of relapse, prognosis is even worse and virtually all patients are dead within 18 months.[6-8] The five-year survival rate is less than 10%.[9] The incidence of GBM is approximately 3-4 patients per 100,000 per year, which makes it an orphan disease.[10] Like for most types of cancer, the incidence of central nervous system (CNS) tumors increases with age, with a peak incidence for GBM between 50 and 60 years.[11]

1.2 CANCER AND IMMUNITY

The concept that the immune system can recognize and eliminate primary developing tumors in the absence of external therapeutic intervention has existed for nearly 100 years. Although tumor-genetics and cancer cell biology have claimed the greatest interest in cancer research

[a] Hilko Ardon, Steven De Vleeschouwer, Frank Van Calenbergh and Stefaan Van Gool: High-Grade Gliomas: Dendritic Cell Therapy, in: Hayat, M.A. (Ed.), Tumors of the Central Nervous System, Volume 2. Springer Company (in press).

[b] Van Gool S, Maes W, Ardon H, Verschuere T, Van Cauter S, De Vleeschouwer S. Dendritic cell therapy of high-grade gliomas. Brain Pathol 2009;19:694-712.

the last decades, tumor-immunology has recently gained renewed attention. Initially the question of specificity was raised, i.e. the capacity of the immune system to distinguish normal cells from tumor cells. In recent years though, many tumor-associated antigens (TAA) have been described and these antigens can be recognized by innate and adaptive components of the immune system.[12] Furthermore, innate immune cells can recognize specific molecular structures on tumor cells. The specificity of the immune system has therefore been widely accepted. However, new questions have arisen, most importantly on how a tumor can escape the immune system ('tumor immune escape'), and whether and how the immune system can be (re-)activated against the tumor.

The first immunotherapeutic approach against cancer was adapted by William Coley at the end of the nineteenth century. He used bacterial toxins ('Coley's toxins') to activate the immune system against tumors. The current immunotherapeutic approaches are mainly aimed at inducing a tumor-specific immune response. Several types of cancer (including glioma, melanoma, prostate carcinoma, renal cell carcinoma and non-small cell lung cancer) have been treated with immunotherapy and the largest experience has been gained in malignant melanoma.[13] So far, overall results seem promising and intriguing, although efficacy of immunotherapy cannot be adequately measured by classical response criteria.

Until recently, the CNS has always been looked upon as an immune-privileged organ in which the immune system could not play a role of importance due to the blood-brain barrier (BBB) and the absence of lymphatic vessels and immune cells. However, recent data do point to immune responses in the CNS and it would therefore be better to speak of an 'immune-distinct' environment.

1.3 'CANCER IMMUNOEDITING'

The growth of tumor cells is on the one hand regulated and prevented by intrinsic non-immune surveillance systems, such as DNA-repair and intracellular control mechanisms (e.g. p53 pathway). On the other hand, both innate and adaptive immunological control mechanisms exist, which form the so-called 'immunosurveillance'.[14-17] The interactions between a tumor and the immune system can be divided into 3 different phases of 'cancer immunoediting' (**Fig. 1.1**). This process is responsible for both eliminating tumors and sculpting the immunogenic phenotypes of tumors that eventually form in immune competent hosts.[14-17] In a first phase of elimination, equivalent to immune surveillance, nascent

transformed cells are eradicated by the immune system. The innate immune system is activated by inflammatory cytokines released by the tumor, macrophages and stromal cells surrounding tumor cells. This results in the activation of natural killer (NK) cells, natural killer T (NKT) cells and γδ-T cells. These cells produce other pro-inflammatory cytokines (such as interleukin (IL)-12 and interferon (IFN)-γ) that enhance the immune response. Tumor cell death by NK cells results in release of TAA that can lead to activation of the adaptive immune system. Uptake of TAA by dendritic cells (DC) will lead to tumor-specific antigen-presentation resulting in a clonal expansion of tumor-specific CD8+ cytotoxic T cells. In a second phase, there is a balance between the elimination of immune-sensitive tumor cells and the outgrowth of newly-formed immune-resistant tumor cells: 'equilibrium'. Due to continuous, genetic adaptations of the tumor cells ('mutation-drift'), tumor cell clones with a non-immunogenic phenotype will arise ('immune resistance'). The immune selection pressure will favor the growth of these tumor cell variants resulting in ongoing tumor formation. Eventually, the balance will tip in favor of the immune-resistant tumor cells and then the final phase will set in: 'immune escape'. Most of the tumor cells will now evade the immune system, as they are insensitive to immunological detection and subsequent elimination. Hence, this will result in clinically detectable malignant lesions. Three categories of 'tumor immune escape' can be recognized: 1. loss of TAA-recognition by the immune system; 2. diminished susceptibility of tumor cells to cell death; 3. induction of immune dysfunction.[18] Tumor cells can release 'tumor-derived soluble factors' that can suppress the immune response (e.g. transforming growth factor β (TGF-β), prostaglandin E_2 (PGE_2) and IL-10). The extracellular matrix can bind TAA in competition with DC leading to decreased antigen-presentation by DC and subsequently less activated T cells. Also, the tumor can induce regulatory T (Treg) cells from effector T cells, resulting in a suppression of the immune system. These peripherally 'induced' Treg cells differ from the naturally occurring Treg cells, which develop in the thymus and are essential for maintaining peripheral tolerance, preventing autoimmune diseases and limiting chronic inflammatory diseases. Moreover, the functional phenotype of intratumoral macrophages can be switched to M2-type macrophages, which can promote tumor growth and angiogenesis. Finally, myeloid-derived suppressor cells (MDSC) can further disturb the immune response.

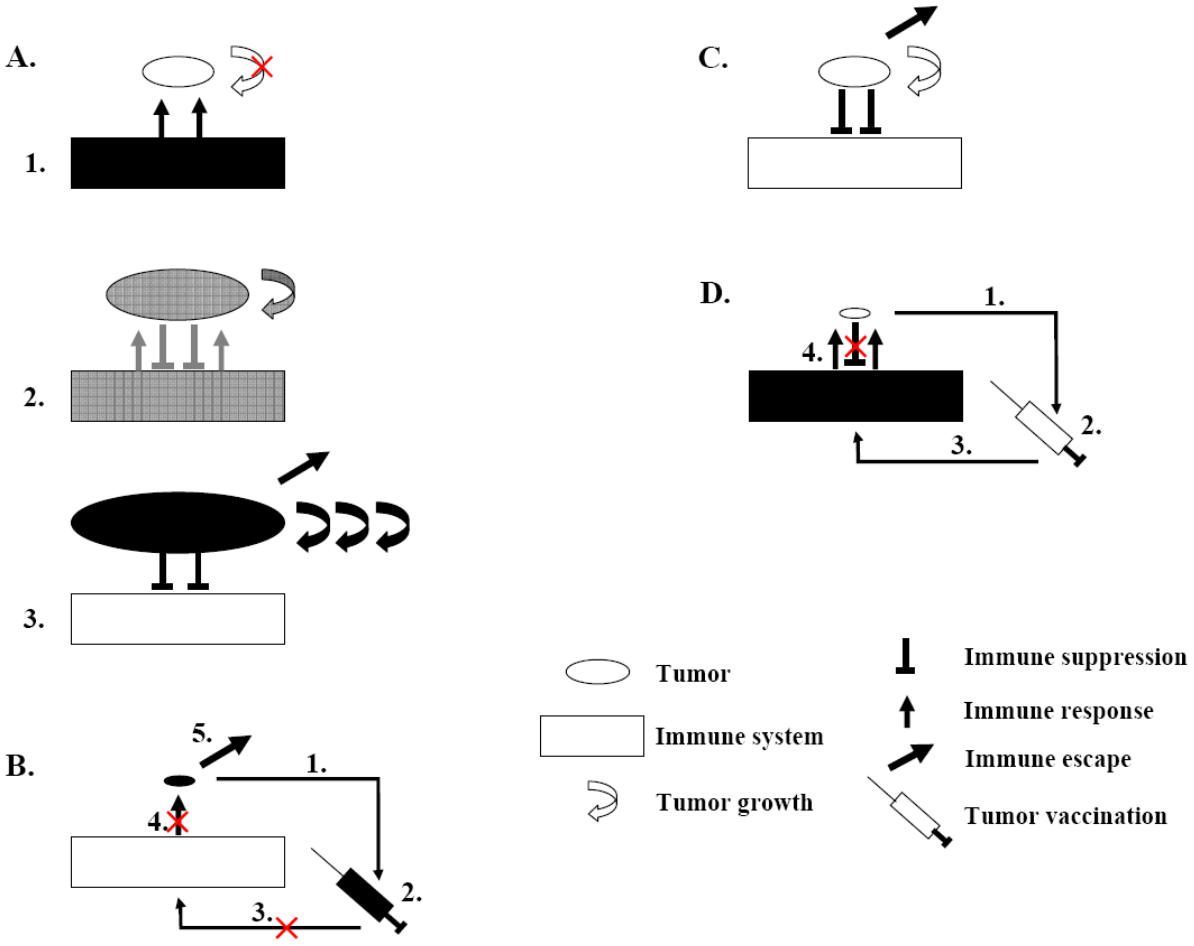


Fig. 1.1 Cancer immunoediting and relevance to tumor vaccination against malignant glioma

A. General principle of cancer immunoediting: 1. In the first phase of elimination, the immune system is capable of eliciting an effective immune response (illustrated as a black box) against an immunogenic tumor (illustrated as a white ellipse), and tumor growth is prevented. 2. In the second phase, there is an equilibrium between tumor growth and elimination of tumor cells by the immune system (illustrated by a gray ellipse and gray box). Due to immunoediting and sculpting of the tumor cells, some tumor cells become non-immunogenic and evade the immune response. Tumor-induced immune suppression counteracts the immune response as well. 3. In the third and final phase, the tumor is fully 'immunoedited' and has become non-immunogenic (illustrated as a black ellipse). This leads, in combination with the ongoing tumor-induced immune suppression, to tumor immune escape and outgrowth of the tumor. The immune system and response have become ineffective (illustrated by a white box).

B. Tumor vaccination (general): At the time of immune escape, the 'immunoedited' tumor is diagnosed and surgery is performed leading to a state of minimal residual disease (1). The 'immunoedited', non-immunogenic tumor cells are used to generate a tumor vaccine (2), but tumor vaccination does not lead to re-activation of the immune system (3), since stimulation of the immune system is done with non-

Fig. 1.1 (continued)

immunogenic cells. Thus, there is no effective immune response to eliminate the tumor (4), resulting in renewed tumor immune escape (5).

C. Glioma immunoediting: From the start of gliomagenesis tumor-induced immune suppression is very potent, leading to an ineffective immune response. Consequently, the tumor can grow and evade the immune system, without the need for editing / sculpting of the tumor cells.

D. Tumor vaccination (glioma): At the time of diagnosis, the tumor consists of a 'non-immunoedited', still immunogenic phenotype. Surgery is performed, leading to a state of minimal residual disease (1). The 'non-immunoedited', immunogenic tumor cells are used to generate a tumor vaccine (2), which is able to activate the immune system (3), in combination with inhibition of the tumor-induced immune suppression (4). Activation of the immune system leads to an effective immune response and the tumor is eliminated (4).

Based on the strong interactions between tumor cells and the immune system, one could speculate about a remarkable hypothesis; tumors that are very potent in suppressing the immune system might mainly rely on this immune suppression for their immune escape. The need for 'editing' the tumor cells to evade the immune system might therefore be less in these immune suppressive tumors. Since the tumor cells will not be attacked by the suppressed immune system, the immune selection pressure will be less and fewer genetic adaptations of the tumor cells will occur. This will result in less tumor cell clones with a non-immunogenic phenotype and more cell clones with an immunogenic phenotype. Following this line of thought, an active immunotherapeutic approach to such a tumor at time of minimal residual disease (e.g. after total resection of the tumor), in combination with a strategy to block the tumor-mediated immune suppression, might be of more benefit. This could be in contrast to a tumor with an 'immunoedited' phenotype, since the former tumor might still be immune sensitive (**Fig. 1.1**). In other words, tumors that do not lead to spontaneous immune reactions due to tumor induced immune suppression, might be best suited for immunotherapy.

1.4 CENTRAL NERVOUS SYSTEM AND IMMUNITY

In contrast to antigen-specific immune responses in peripheral tissues, the details are less clear for immune responses to antigens in the CNS. The biology of immune responses within the CNS would appear to be distinct due to the lack of secondary lymphoid tissues within the brain parenchyma, as well as the possible obstacles presented by the BBB and the relative lack of professional antigen-presenting cells. In general, TAA have to be acquired and presented by antigen-presenting cells, leading to the activation of tumor-specific lymphocytes

that will have to infiltrate the brain tumor. Research in this area is ongoing and much is still to be learned.

1.4.1 Cervical lymphatic drainage of the brain

Although lymphatic vessels cannot be identified in the brain parenchyma, several animal studies have shown that antigens can be drained to the cervical lymph nodes.[19,20] Possibly, the antigens run through the subarachnoid space along the olfactory nerve and cross the cribriform plate to the nasal mucosa where they access lymphatic drainage basins. The potential of antigens to drain to cervical lymph nodes points to possible interactions between the CNS and peripheral lymphoid tissue, highlighting a theoretical starting point for the initiation of brain tumor-specific immune responses.

1.4.2 Antigen-presenting cells in the central nervous system

Several cell types in the CNS – including vascular endothelial cells, smooth muscle cells, astrocytes, microglia, perivascular macrophages, choroid plexus epithelial cells, neurons, and DC – have been put forward as potential antigen-presenting cells. Already 3 decades ago, Hickey *et al.* found very rare Ia-positive astrocytes in an experimental allergic encephalomyelitis model in the rat, suggesting the possibility of antigen-presentation.[21] Moreover, they showed in the same model, that perivascular microglial cells are competent to present antigens to lymphocytes in the CNS.[22] Recent publications point especially to DC as most important antigen-presenting cells in the CNS,[23] although their presence in the CNS is limited. DC capture TAA from dying tumor cells in the CNS and migrate to draining lymph nodes in the cervical region. There, antigens are presented to different subtypes of T cells in a 'major histocompatibility complex' (MHC)-I and MHC-II context. Antigen-presentation in a MHC-I molecule (cross-presentation, a typical feature of DC) leads to activation of CD8+ T cells, presentation in a MHC-II molecule to activation of CD4+ T cells. Antitumoral CD4+ T cells support a strong and prolonged CD8+ T cell reaction, promote the maturation of DC and stimulate B cells to produce antitumoral antibodies.[24-26] In several animal studies, it has been shown that antigen-specific T cells are primed by immigrant, antigen-loaded DC in the cervical lymph nodes, after which the activated T cells traffic to sites of antigen challenge in the brain.[12] However, further research in brain tumor models is necessary to validate this principle.

1.4.3 Blood-brain barrier

Activated, antigen-specific T cells have to traffic from the cervical lymph nodes into the brain parenchyma to infiltrate the brain tumor. Three pathways by which immune cells can access the CNS have been proposed: 1. from blood to the cerebrospinal fluid via the choroid plexus; 2. from blood to the subarachnoid space; 3. from blood to brain parenchyma. The most important obstacle is formed by the BBB that prevents passive transit of molecules between the CNS parenchyma and the systemic circulation. The barrier is formed by tight junctions of the capillary endothelial cells, creating a tight seal which limits the paracellular transport of molecules. Moreover, more than 90% of the capillary endothelium is covered by astrocyte foot processes that form the 'glia limitans'. The capillaries of the circumventricular organs of the brain represent areas in which there is no BBB, and these are thus sites that more freely permit transport of molecules across vascular endothelial cells. Additionally, the 'blood-cerebrospinal fluid barrier' restricts free movement of cells and molecules between the cerebrospinal fluid and the capillaries of the choroid plexus.[12]

Within the glioma microenvironment, the vasculature is substantially altered and structures such as the BBB are significantly compromised. Therefore, the BBB in gliomas is more porous than the intact BBB in healthy subjects. This increased permeability might lead to increased immune cell infiltration. However, efficient lymphocyte trafficking into the tumor may be significantly reduced due the fact that tumor tissue is severely dysregulated and may contain poor target substrates to attract T cells.

1.4.4 Lymphocyte-trafficking to glioma

Although it has been shown that T cells can traffic to the CNS in autoimmune diseases, it is less clear how lymphocytes traffic to developing CNS neoplasms. One possible model is the 'multi-step' model of migration, characterized by the 'rolling, sticking and migrating' paradigm. T cells homing to the brain parenchyma slow down in a first step by gradually tethering to capillary endothelium in a 'rolling' fashion, mediated by interactions between several selectins and their ligands. Subsequently, lymphocytes adhere to the vascular endothelium in the 'sticking' step, mediated by integrins that become 'activated' by chemokines. Lymphocytes ultimately transmigrate to the brain parenchyma in the 'diapedesis' (migrating) step, which may occur via trans- or paracellular endothelial transport.

Several immune cells have been shown to infiltrate gliomas, including tumor-infiltrating lymphocytes (TIL), MDSC and M2-macrophages. Among the TIL, CD4+ T cells, CD8+ T cells and immune suppressive Treg cells have been documented.[12,27,28]

1.5 GLIOMAS AND IMMUNITY

Several TAA have been identified in human gliomas: Aim-2, Trp-1, Trp-2, Sart-1, Sart-2, Sart-3, Her2/neu, GP100, Mage-1, Galt-1, Galt-3, Sox2, Sox6, Survivin, EGFRvIII, Gage-1, WT1.[12] These antigens can be recognized by T cells and form the basis for potential spontaneous immune responses against CNS tumors. Two subgroups of glioma-specific antigens have been described: 1. antigens that are solely expressed by tumor tissue; 2. antigens that are expressed both by normal and tumor tissue. Only the antigens from the first subgroup are of immunotherapeutic interest, since antigens that are also expressed by normal tissue could lead to possible autoimmune phenomena. The ideal antigens for immunotherapy are antigens that are immunogenic and tumor-specific, i.e. not expressed by normal tissue. Moreover, these antigens should ideally be present on all tumor cells or at least on the tumor cells that are responsible for recurrence of the tumor.

There are no universal glioma-specific antigens that are expressed by all gliomas and inter-individual differences are the rule. Therefore, it seems important to give every patient an individual and glioma-specific treatment, using TAA that are expressed by the tumor of the patient. Besides, even within the tumor of one patient, expression of antigens is very heterogeneous and there are no known TAA that are universally expressed by all tumor cells. Moreover, due to the plasticity of the tumor cells, with genetic alterations and loss of expression of antigens, the immunotherapeutic use of one glioma-specific antigen would lead to a selection of tumor cells that do not express that antigen (any longer) and that are not targeted by the immunotherapy (antigen-loss variants).[29-31] Recently, antigenic loss of epidermal growth factor receptor class III variant (EGFRvIII) in GBM patients vaccinated with an EGFRvIII peptide was shown by Sampson *et al.*;[32] of 11 recurrent tumors from which pathologic material was obtained, 9 (82%) had lost EGFRvIII expression.

Furthermore, it is important to know which tumor cells are responsible for recurrence of the tumor. In other words, do all tumor cells have the same tumorigenic potential or does a subgroup of glioma stem cells exist? Several studies have shown that neurosphere-forming, CD133+ glioma cells are chemo- and radioresistant and might be seen as so-called brain

tumor initiating cells.[33-38] If this truly is the case, then immunotherapeutic approaches should focus on TAA of these tumor initiating cells.

Finally, HGG can actively suppress the immune system through different immune escape mechanisms: production of immune suppressive cytokines, negative regulatory pathways in lymphocytes, lack of glioma-specific antigen recognition by immune cells (loss of MHC-I expression on tumor cells), and induction of Treg cells.[39,40] This glioma-induced immune suppression is not only evident within the tumor, but systemically as well.[41-44]

1.6 IMMUNOTHERAPY

1.6.1 Restorative, passive and adoptive immunotherapy

Immunotherapy for cancer covers a broad field of diverse approaches, all using different aspects of the immune system repertoire. The common goal is to destroy remaining cancer cells and prevent tumor (re)growth.

Restorative immunotherapy consists of systemic or local (intratumoral) administration of cytokines (including IL-2, tumor necrosis factor α (TNF-α) and IFN-γ) to enhance non-specific immunity. As for other tumors, this type of treatment showed considerable, treatment-limiting, systemic toxicity and neurotoxicity for gliomas. No conclusive evidence of efficacy was shown and treatments had to be stopped.[31,39,45]

In case of passive immunotherapy, target-specific antitumoral monoclonal antibodies (mAb) are used with or without a conjugated ligand. Antibody-dependent cellular cytotoxicity, complement-dependent cytotoxicity and induction/blockade of intracellular signals have been described as working mechanisms of tumor cell killing. Several mAb have been shown to be active, often in combination with chemotherapy: rituximab (non-Hodgkin lymphoma and chronic lymphocytic leukemia), trastuzumab (metastasized breast carcinoma), cetuximab (colorectal carcinoma, head/neck carcinoma and non-small cell lung carcinoma) and bevacizumab (colorectal carcinoma, breast carcinoma and lung carcinoma).[46] Although some studies have shown moderate results with this type of immunotherapy for glioma, especially in patients with minimal disease burden, it is unclear whether the mAb can infiltrate sufficiently into the tumor to induce an optimal antitumoral effect.[31,39] Recently, several studies have focused on the treatment of gliomas with bevacizumab, which is an angiogenesis inhibitor targeting VEGF. Although results show that symptoms might be tempered, it seems

that furtive invasion of the disease might continue or increase, unrecognized or underestimated by standard imaging modalities. Moreover, while treatment of other tumor types is improved by combining chemotherapy with anti-angiogenic drugs, inhibiting angiogenesis in GBM might antagonize the efficacy of chemotherapeutic drugs by restoring the BBB. Several studies suggest an antitumoral effect based on improved response rates and prolonged progression-free survival (PFS). However, these data are derived from non-randomized trials with PFS as primary endpoint and the true antitumoral effect is not yet clear. Available data on overall survival (OS) are less robust (phase II) and sometimes even conflicting. Furthermore, serious side-effects of angiogenesis inhibitors (e.g. venous and arterial thrombo-embolism, arterial hypertension and hemorrhage) have been demonstrated in phase II studies.[47,48]

Adoptive immunotherapy is the infusion of *ex vivo* (antigen-specific or non-specific) stimulated lymphocytes or NK cells. This can be done locally in the brain or systemically. An important advantage of this strategy is that autoimmune reactions can be avoided. Possible drawbacks however, are the risk of contamination with Treg cells and the short survival of *ex vivo* stimulated cells. Studies have shown that adoptive transfer of autologous T cells held little or no value for leukemia, but was moderately effective for malignant melanoma. On the other hand, adoptive transfer of allogeneic NK cells could play a role in selected cases of acute myeloid leukemia.[46] For gliomas, local (intracerebral) administration of different effector immune cells (including NK cells, cytotoxic T cells and activated 'killer' cells) has been widely studied. This strategy was well-tolerated but did not yield clinical responses. Additional local administration of IL-2 as 'immuno-adjuvant' resulted in significant toxicity.[31,39]

1.6.2 Active specific immunotherapy

Finally, active specific immunotherapy, or the so-called tumor vaccination, comprises the *in vivo* induction of tumor-specific cytotoxic T lymphocytes (CTL) (and/or humoral responses) and this approach has gained considerable interest the last decade.[24] Four types of tumor vaccination can be distinguished (**Table 1.1**). 1. Autologous or allogeneic tumor cell vaccines are based on the assumption that the TAA that are expressed by the tumor can induce a cytotoxic T cell response. The vaccine consists of non-proliferating tumor cells in combination with an 'immuno-adjuvant' to enhance the immune response. 2. In case of genetically modified tumor cell vaccines, autologous or allogeneic tumor cell vaccines have

Vaccine type	Advantages	Disadvantages	Tumor type [a]	Results [b]
Autologous tumor cell vaccine	Patient specific Potential to generate immunity to any TAA Humoral and cellular antitumoral immunity	Not widely applicable Time-consuming and technically challenging preparation Requires adequate tumor tissue for manufacture	MM	Limited clinical response [49,50]
			RCC	Immunological and clinical response in selected patients - phase III study (Reniale) with significant improved PFS and OS [51]
			NSCLC	Limited data available - ongoing studies [52,53]
			HGG	Limited data available [31,39]
Allogeneic tumor cell vaccine	Widely applicable Patient's tumor tissue not needed Humoral and cellular antitumoral immunity	Not patient specific Moderately complex / demanding preparation Response dictated by similarity between TAA of vaccine and TAA of patient's tumor Contaminating 'allo-' responses	MM	Limited clinical response [49,50]
			RCC	Only used after gene modification [51]
			NSCLC	Only used after gene modification [52,53]
Gene modified cellular vaccine	May enhance efficacy of non-gene modified approaches	Requires additional *ex vivo* manipulation with issues of altered cell viability and/or functionality	MM	Limited immunological and clinical response [49,50]
			RCC	Possible limited response [51]
			NSCLC	Belagenpumatucel-L: clinical response GVAX ('granulocyte macrophage-colony stimulating factor' secreting tumor cells): no response [52,53]
			HGG	Limited data available [31,39]
Peptide vaccine	Acellular technique Widely applicable Use of known epitopes simplifies immune monitoring	TAA have to be immunogenic and tumorigenic Tumor cells not expressing TAA evade immune system ('tumor immune escape') HLA restriction	MM	Extensively studied - measurable T cell response – rarely clinical response [49,50]
			RCC	Limited number of studies – phase III study (Vitespen) showed no better PFS [51]
			NSCLC	Mucin-1 vaccine: promising results – ongoing phase III study [52,53]
			HGG	EGFRvIII: immunological and clinical (better PFS and OS) response – ongoing phase III study [31,54]
DC vaccine	Utilizes most potent antigen-presenting cells Can be loaded with TAA through a variety of techniques	Leukapheresis necessary Time-consuming and technically challenging preparation Yield can be variable and patient dependent	MM	Clinical response: 7% (other active specific immunotherapy 3-4%) [13,50] Both phase III study with positive results [55] and phase III study with negative results [56]
			RCC	Immunological and clinical response [51]
			NSCLC	Immunological response – no clinical response [52,53]
			HGG	Immunological and clinical response (some correlation) [24,31,39,57-65]

Table 1.1 Active specific immunotherapy

DC, dendritic cells; EGFRvIII, epidermal growth factor receptor class III variant; HGG, high-grade glioma; HLA, human leukocyte antigen; MM, malignant melanoma; NSCLC, non-small cell lung carcinoma; PFS, progression-free survival; OS, overall survival; RCC, renal cell carcinoma; TAA, tumor-associated antigen

a not exhaustive

b phase I/II studies and case reports, unless otherwise stated

been genetically engineered; genes encoding immunostimulatory cytokines or positive co-stimulatory molecules have been integrated into the tumor cells by transfection. This strategy is based on evidence that local cytokine secretion can activate T cells and NK cells, as well as induce inflammatory responses against tumors. 3. Peptide-based vaccines make use of one or more immunogenic TAA that are expressed by the tumor cells. 4. DC-based tumor vaccines use DC as antigen-presenting cells to activate CD4+ and CD8+ T cells. Moreover, DC can activate NK and NKT cells as well. DC are generated *ex vivo* and subsequently loaded with a source of TAA, that will be presented by the DC. Several cellular products can be used to load the DC: purified defined peptides, undefined acid-eluted peptides from autologous tumor, tumor cell lysate, viral vector containing genes, apoptotic bodies, tumor homogenate, mRNA, and necrotic tumor cells. From a theoretical point of view, the use of total tumor-derived material (lysate/homogenate) as a source of tumor antigens may be preferable because of the emergence of antigen-loss variants if the immunization is only targeted at one TAA, which might not be expressed by a subpopulation of tumor cells that will be positively selected.[29,31]

1.6.3 Active specific immunotherapy for glioma

For glioma immunotherapy, the best responses have been reported with tumor vaccination as adjuvant postoperative therapy.[39,54,60] Autologous tumor cell vaccines in combination with cytokine-secreting fibroblasts and genetically modified autologous tumor cell vaccines have been used to treat gliomas, but especially the results of peptide- and DC-based immunotherapeutic approaches seem promising. Peptide-based vaccines use EGFRvIII to induce an immune response. EGFRvIII is expressed by many, but not all malignant gliomas and can induce specific cellular and humoral immunity. Preclinical and clinical studies have shown that the use of EGFRvIII vaccines is safe and feasible. Results of an ongoing phase III study are eagerly anticipated.[54] Furthermore, much preclinical and clinical research has been done on the role of DC-based tumor vaccination in the treatment of malignant gliomas. So far, eighteen clinical phase I/II studies and case reports have been published in the literature with emphasis on feasibility and toxicity of the treatment approach (**Table 1.2**). No autoimmune phenomena were observed and the treatment was well-tolerated. As for the EGFRvIII-based vaccines, results are promising.

Author	Year of publication	No. of patients (type of trial)	Cell product	Administration	Treatment schedule	Immune response	Clinical response
Liau *et al.*[62]	2000	1 (case report)	allogeneic MHC class I-matched GBM peptides	intradermal	3 biweekly vaccines	T-cell proliferation to allogeneic acid-eluted tumor peptides	none
Yu *et al.*[63]	2001	9 (phase I)	tumor-specific MHC-I associated peptides	subcutaneous	3 vaccines at 2-wk intervals	systemic CTL cytotoxicity against tumor (n=4) (JAM assay)	prolonged survival compared to control group
Kikuchi *et al.*[66]	2001	8 (phase I)	DC fusion with autologous glioma cells	intradermal	3-7 vaccines at 3-wk intervals	increase in NK cells (n=4); increased IFNγ in supernatant (n=6)	mixed response (n=1); steroids during vaccination (n=5); 2 minor responses
Yamanaka *et al.*[67]	2003	10 (phase I-II)	autologous tumor lysate	intradermal and / or intratumoral (Ommaya)	1-10 vaccines at 3-wk intervals	increase in NK cells (n=5); positive DTH reaction to tumor lysate (n=3); increased T-cell mediated antitumoral activity (n=2); Elispot IFNγ	minor response (n=2)
Wheeler *et al.*[68]	2003	17 (phase I) 17 (phase II)	autologous tumor freeze-thaw lysate	not reported not reported	3 vaccines at 2-wk intervals 3 vaccines at 2-wk intervals – 4th vaccine 6 wks after 3rd (n=10)	not reported (study on CD8+ RTE)	not reported

Table 1.2 Overview of reported clinical trials on DC vaccination for gliomas

Yu *et al.*[64]	2004	14 (phase I-II)	autologous tumor lysate	subcutaneous	3 vaccines at 2-wk intervals	IFNγ release in PBMC (n=6); expansion of CD8+ antigen specific T cell clones (n=4); systemic T cell cytotoxicity against tumor (n=1)	significant increase in median survival
De Vleeschouwer *et al.*[69]	2004	1 (case report)	autologous tumor lysate	intradermal	wks 1, 3, and further with 4-wk interval (6 total)	positive DTH reaction to tumor lysate	long-lasting tumor-free survival (> 5 year after vaccination)
Rutkowski *et al.*[70]	2004	12 (phase I)	autologous tumor lysate	intradermal	wks 1, 3, and further with 4-wk interval (2-7 vaccines)	positive DTH reaction to tumor lysate (n=6)	partial response (n=1); tumor-free survival (n=2; 5 year after vaccination)
Kikuchi *et al.*[71]	2004	15 (phase I-II)	DC fusion with autologous glioma cells	intradermal	vaccine + rhIL-12	positive DTH reaction to tumor lysate (n=15); increased cytotoxic activity (n=2); increased intracellular IFNγ in CD8+ T cells (n=1)	partial response (n=4); mixed response (n=1)
Liau *et al.*[65]	2005	12 (multi-cohort dose escalation study – phase I)	acid-eluted tumor associated peptides (autologous)	intradermal	≤3 vaccines	CTL response (n=6)	median TTP 19.9 mo; OS 18 to >58 mo; median OS 35.8 mo

Table 1.2 (continued)

Yamanaka *et al.*[72]	2005	24 (phase I-II)	autologous tumor lysate	intradermal or intradermal + intratumoral (Ommaya)	1-10 vaccines at 3-wk intervals	positive DTH reaction to tumor lysate (n=8); positive IFNγ Elispot (n=7)	partial response (n=1); minor response (n=3); significant increase in median survival
Okada *et al.*[73]	2007	2 (A) / 5 (B)	IL-4 transfected fibroblasts + apoptotic glioma cells (A) / autologous tumor lysate (B)	intradermal	2 vaccines at 2-wk intervals	A: increased T cell reactivity; B: no response	A: clinical and radiological response in both patients; B: no response
Wheeler *et al.*[61]	2008	34 (phase II)	autologous tumor lysate	subcutaneous	3 vaccines at 2-wk intervals – 4th vaccine 6 wks after 3rd	post-vaccine antigen-directed IFNγ response (qPCR-based assay) (n=17); DTH-test resulted in cutaneous GBM in 1 patient (DTH subsequently discontinued)	significant positive correlation between post-vaccine response magnitude and TTS/TTP spanning chemotherapy
Walker *et al.*[74]	2008	13 (phase I)	single-cell suspension of autologous tumor cells	intradermal	priming phase of 6 vaccines at 2-wk intervals; further vaccines at 6-wk intervals	increased T cell infiltration in post-vaccination tumor specimens (n=3)	9-mo survival (9/13); 12-mo survival (6/13); 18-mo or longer survival (3/13)

Table 1.2 (continued)

De Vleeschouwer *et al.*[59]	2008	56 (phase I-II)	autologous tumor lysate	intradermal	cohort comparison	positive DTH reaction to tumor lysate (9/17 after 2 vaccinations)	PFS 3 mo; OS 9.6 mo; 2-year OS 14.8%; total resection and younger age are predictor for better outcome
Sampson *et al.*[75]	2009	12 (phase I)	EGFRvIII peptide	intradermal	3 vaccines at 2-wk intervals	increased antigen-specific T cell proliferation to EGFRvIII peptide (10/12); positive DTH reaction to EGFRvIII peptide (5/9)	6-mo PFS 67%; OS 22.8 mo after histological diagnosis
Ardon *et al.*[58]	2010	45 children (phase I-II) (33 HGG)	autologous tumor lysate	intradermal	cohort comparison	not reported	PFS HGG 4.4 mo; OS HGG 13.5 mo; 2-year OS 18%
Ardon *et al.*[57]	2010	8 (pilot study)	autologous tumor lysate	intradermal	incorporated in RChT - 4 induction vaccines at 1-wk interval; 3 boost vaccines with 1-month interval, followed by 3-monthly boost-vaccinations	positive DTH reaction to tumor lysate (3/7); positive IFNγ Elispot (5/8); increase CTL (6/7)	6-mo PFS 75%; PFS 18 mo; OS 24 mo

Table 1.2 (continued) CTL, cytotoxic T lymphocyte; DC, dendritic cell; DTH, delayed-type hypersensitivity; GBM, glioblastoma multiforme; HGG, high-grade glioma; IFNγ, interferon-γ; IL-4, interleukin-4; JAM, just another method; MHC, major histocompatibility complex; mo, month; NK, natural killer; no., number; OS, overall survival; PBMC, peripheral blood mononuclear cell; PFS, progression-free survival; qPCR, quantitative polymerase chain reaction; rhIL-12, recombinant human interleukin-12; RChT, radiochemotherapy; RTE, naive recent thymic emigrant T cell; TTP, time to progression; TTS, time to survival; wk, week

1.7 EXPERIMENTAL GLIOMA MODELS FOR DENDRITIC CELL-BASED THERAPY

In preparation for human studies and to better understand the immunological processes that play a role in DC-based therapy, experimental glioma models are necessary. Over the last decades, numerous reports on heterotopic and orthotopic rat and mouse GBM models have been published, and it is important to know that there are some fundamental differences between those models. For example, models exploiting spontaneous tumor formation in genetically engineered mice differ from engrafted tumor models that are established by implantation of primary tumor cells or tumor cell lines. Spontaneous tumor models mimic gliomagenesis much better than engrafted models, but the main disadvantages are the poor reproducibility, low tumor penetrance, prolonged and unpredictable latency for tumor formation, and the need for advanced *in vivo* imaging techniques. Engrafted models on the other hand lack the stepwise genetic changes occurring during tumor progression, resulting in tumors that are well circumscribed and less invasive, lack the human counterpart of microvascular proliferation and rarely recapitulate the tumor-of-origin phenotype. Nevertheless, based on greater reproducibility, engrafted models are better suited for evaluating preclinical therapies such as DC-based immunotherapy, as long as the models are studied in immune competent animals.

The GL261 glioma model has been widely studied and Ni *et al.*[76] underscored the immunogenicity of GL261 tumor cells by treating mice with intracranial glioma with tumor extract-pulsed cloned DC. Cured animals showed increased delayed-type hypersensitivity (DTH) responses to GL261 cells, with long-term tumor protection. Aoki *et al.*[77] showed that pulsing DC with a complex of tumor extract and cationic liposomes induces an antitumoral immune response against intracranial glioma in which CD8+ T cells are involved. Protective immunity against intracranial glioma growth, obtained through immunization with either lysate- or RNA-loaded DC, was reported by Insug *et al.*,[78] while adding recombinant IL-12 to the vaccine regimen further improved its efficacy. Vaccination with lysate-pulsed DC combined with IFN-β gene therapy resulted in a survival benefit, as was demonstrated by Saito *et al.*.[79] Similarly, the group of Okada *et al.*[73] revealed that the sequential intratumoral delivery of an IFN-α encoding adenoviral vector and DC induces long-term survival and specific CTL activity. Furthermore, the same group showed that intratumoral administration of DC, genetically engineered to secrete IFN-α, enhances the efficacy of peripheral vaccines

with cytokine gene-transduced tumor cells. Using vaccines created through electrofusion of DC and irradiated tumor cells, Kjaergaard *et al.*[80] observed complete tumor regression of established intracranial tumors with infiltration of both CD4+ and CD8+ T cells. Ciesielski *et al.*[81] have focused on survivin, a member of the apoptosis inhibition family of proteins, as GL261 TAA. In particular, the authors exploited the xenogeneic differences between human and murine survivin sequences to develop a more immunogenic tumor vaccine. The efficacy of systemic immunotherapy with DC loaded with GL261 antigens was confirmed by Pellegatta *et al.*.[82] Additionally, these authors introduced the concept of cancer stem cells in this model and reported that DC targeting of such stem cells within the GL261 tumor cell pool provides a higher level of protection against GL261 glioma. Recently, Grauer *et al.*[83,84] illustrated the pronounced impact of Foxp3+ Treg cells in the GL261 model, and suggested that Treg cell elimination is a prerequisite for successful eradication of established glioma using tumor lysate pulsed DC. Maes *et al.*[85] showed that DC loaded with tumor antigens, in the form of RNA molecules, are capable of inducing a T cell-mediated antitumoral immune response. Furthermore, the impact of Treg cells in this model was evident and elimination of Treg cells resulted in long-term surviving mice. Strikingly, upon rechallenge of long-term surviving mice after Treg cell depletion and/or DC vaccination, only those mice that were treated with DC vaccination depicted a prolonged antitumoral protective immune response.
Other preclinical studies have used murine intracerebral melanoma models to test DC-based tumor vaccination. Using such a model, Ashley *et al.*[86] showed that immunization with DC pulsed with either unfractionated tumor extracts or with total tumor RNA, elicits potent immunity against CNS tumors in mice. Heimberger *et al.*[87] demonstrated in a murine melanoma model, that immunization with DC mixed with tumor-specific peptide results in a long-lasting immunological response that recognizes the EGFRvIII mutation and significantly increases median survival times.

1.8 DENDRITIC CELL-BASED THERAPY FOR HUMAN GLIOMAS

Eighteen clinical phase I/II studies and case reports have been published in the literature since 2000 (**Table 1.2**). The median patient number in these reports was only 12, ranging from 1 to 56. The inclusion criteria, immunotherapeutic designs and interpretations varied significantly among and even within the reports, being based entirely on single-center approaches and hypotheses. Hence, no firm conclusions can be drawn regarding the optimal

immunotherapeutic strategy, nor would it make sense to perform a meta-analysis on these data. The importance of a minimal residual disease setting for adjuvant postoperative DC vaccination in HGG patients was already stressed in the first reports. In the series published by De Vleeschouwer *et al.*,[59] the importance of a gross total resection prior to DC vaccination for GBM patients was confirmed in a multivariate analysis. General awareness and agreement on this important issue is mounting and even culminating in the assumption that therapeutic tumor vaccinations should be used for early disease states. Most of the reports describe patients treated with immunotherapy at a stage of minimal residual disease, after gross total or near-total resection. Interestingly, patients have been under maintenance corticosteroid treatment just prior and/or during immunotherapy in some trials, which one would expect to have a negative effect on the generation of an effective immune response. In order to avoid this confounding variable, other trials have been restricted to patients who could obtain a total, near-total or subtotal resection and be rapidly weaned from corticosteroids. There are multiple arguments supporting the need for these restrictions. First, because there are major immune suppressive mechanisms at play within the tumor microenvironment and even systemically, only a gross total resection results in a clinically effective recovery of normal immune system function. Second, it might be difficult to produce good-quality DC out of monocytes isolated at the time of corticosteroid treatment. Third, an overwhelming peritumoral inflammatory reaction was seen in one patient with bulky residual tumor.[70] In this patient, the vaccine-induced inflammatory immune reaction occurred with a progressively increasing delay after the vaccine injection. This is in line with the hypothesis that the growing tumor and, maybe as such, the increasingly induced immune suppression counteract the vaccine-induced immune response. This observation points to a tumor-specific reaction induced by the vaccine, but possibly also to a gradually shifting balance in favor of tumor-induced immune suppression.

1.8.1 Selection of outcome measures

Immunological responses, assessed by a wide variety of immune monitoring tools, have been demonstrated in 50% of reported cases, ranging from 20% to 100%. However, most studies did not find a consistent correlation between immune monitoring data and clinical outcome. Only recently, Wheeler *et al.*[61] found a clear correlation between immunological responses and survival in vaccinated glioma patients. However, the use of peripheral immune monitoring tools might prove inadequate in case the peripheral immune system does not

mirror local immune events in the brain tumor microenvironment. Although immunological responses are mandatory to map the way and to provide proof of the principle for this therapeutic strategy, 'proof of efficacy' can only be established in large well-designed comparative clinical trials, ideally in controlled randomized trials. Especially in HGG, an old paradigm states that pre-treatment prognostic variables might have more impact on outcome than any (new) potentially active therapy or treatment strategy. To that end, a recursive partitioning analysis (RPA) for HGG patients, as originally described and validated by the Radiation Therapy Oncology Group (RTOG) in 1993 for newly diagnosed malignant gliomas treated with radiotherapy, provides us with an excellent model of prognostic classes of pre-treatment and treatment-related patients' variables. This RPA classification was adapted by the European Organization for Research and Treatment of Cancer (EORTC) for newly diagnosed malignant gliomas treated with radiotherapy and TMZ chemotherapy. Using RPA classifications, the impact of new treatments on OS in HGG can be examined in clinically similar patient groups and compared with large databases of conventionally treated patients. To date, there is consensus that clinical responses, as defined by the Macdonald criteria,[88] do not apply for biological treatment modalities such as tumor vaccination. The paradigm of a therapeutic vaccine leading to (detectable) immunological responses and hence, to (detectable) clinical responses with increased OS, is at least incomplete. Even immune monitoring tools directed toward the affected organ, that is, the brain, will have to take into account that the presence of TIL and immune effector cells does not unequivocally correlate with a good or a bad outcome. Proof of efficacy of immunotherapy implies an impact on OS in patients with HGG, of whom the different prognostic risk factors are clearly categorized.

1.8.2 Practical use of dendritic cells

All clinical reports have used monocyte-derived DC for their trials. The methodology for DC preparation is now fairly well established, and gives a sufficient yield of mature DC for injection into patients. Most of the older trials have used immature DC, while others have used maturation stimuli like TNF-α, penicillin-killed *Streptococcus pyogenes* (OK-432), monocyte-conditioned medium, IFN-γ and TNF-α in combination with IL-4-secreting fibroblasts.[60] Only one phase I study focused on the dose of DC and could not find any dose-limiting toxicity.[60,65]

At the time of the design of most published clinical trials, it was not known if and which lymph nodes would be the optimal destination of injected DC. Later on, however, data

became available that priming of T cells by DC within the cervical lymph nodes induces an integrin homing pattern toward intracerebral locations. Other preclinical, *in vivo* brain tumor models clearly showed an enrichment of tumor-specific Treg cells in the blood and cervical lymph nodes. As pointed out by Tuyaerts *et al.*,[89] the question whether DC should be injected intradermally, intravenously or in the lymph nodes is not yet resolved. This becomes particularly important as only a small amount of the intradermally injected DC ultimately reaches the T cell area of lymph nodes.[90] However, taking into account the documented failure rate of even experienced radiologists injecting DC in lymph nodes under ultrasound guidance,[83] and the desire to spread DC vaccination technology to more centers in order to perform large-scale clinical trials, most researchers in the field favor the intradermal route for DC injection.

A very interesting issue is the potential role that local injection of DC directly into the tumor region might play in immunotherapeutic approaches. In their study, Yamanaka *et al.*[72] showed some benefit from intratumoral plus intradermal injections of DC, as compared to only intradermal injections, although one should note that this observation was not derived from a prospective randomized approach. Nevertheless, a local change from an immune suppressive into a more immune stimulatory microenvironment might be induced by intratumoral administration of DC.

1.8.3 Immune monitoring

Immune responses following vaccination have been monitored in most trials. These analyses have included positive DTH skin reaction, T cell reactivity and NK cell enrichment in peripheral blood, as well as measuring T cell infiltration in tumoral tissue taken after vaccination. The reported immune monitoring remains very global in these clinical trials, mainly due to the lack of specific antigens to be targeted. Although data are still preliminary, advanced magnetic resonance imaging (MRI), eventually combined with positron emission tomography (PET), may soon provide better tools to monitor the local effects of immunotherapy for HGG.

1.8.4 Results of clinical studies

Because all clinical trials reported thus far comprise case reports, phase I, phase I/II or phase II trials, the questions answered in these trials relate to feasibility, toxicity and early efficacy, both immunologically and/or oncologically (**Table 1.2**). In all trials, it seemed feasible to administer DC-based vaccines. Moreover, all reports conclude that toxicity is minimal, except for the case reported by Rutkowski *et al.*[70], where an overwhelming inflammatory reaction around a residual tumor was observed. Of major importance, none of the reports describe autoimmune phenomena induced by DC vaccination.

Besides feasibility, toxicity and clinical outcome, all published studies on DC-based immunotherapy have aimed to assess immune responses. Several immune assays have been developed for this purpose, but so far, results have been difficult to interpret. Most studies did not find a consistent correlation between immunologically measurable effects and clinical outcome. Moreover, immunological responses are not detected in all patients. However, analysis of the phase II trial by Wheeler *et al.*,[61] suggested that DC vaccination elicits antitumoral immune responses in most GBM patients. Furthermore, the data in this trial suggest that vaccine responders enjoy improved clinical outcomes and that response magnitudes in these patients correlate with time to progression and/or survival.

Only a limited number of research groups are actively performing clinical trials world-wide. All these trials are small, and include heterogeneous groups of patients with regard to histology and/or time of vaccination. Some studies included patients with all subtypes of HGG, while others focused solely on patients with GBM. Some studied patients with relapsed HGG, while others included patients with a first diagnosis of HGG, either exclusively or combined with patients treated at recurrence. Only our group reported on children with relapsed HGG (**Chapter 4**).[58] Moreover, as pointed out before, the vaccination protocol varied enormously among research groups. Due to this heterogeneity in study design and execution, sound comparative analyses on clinical outcome are difficult. Nevertheless, in most trials, some clinical effect is observed and some important clues for future clinical studies are provided, especially from trials where there are comparisons with historical controls or between internal subgroups. For example, Yu *et al.*[63] reported that 7 patients with newly diagnosed GBM, who received 3 biweekly intradermal vaccinations with peptide-pulsed DC, had median survival times of 455 days, as compared to 257 days for 42 non-vaccinated, but otherwise comparably treated patients in the same institute. Although compared with historical control patients and although no statistics are provided, the

prolongation of the median survival is an interesting observation in this phase I clinical trial. The same group reported on vaccination at the time of relapse in 8 GBM patients, and made a similar comparison to 26 historical control patients.[64] Both patient groups underwent second craniotomy for relapsed GBM. Whereas the median survival was 30 weeks in the control group, subsequent vaccination after new resection resulted in a significant change of median survival to 133 weeks, with some patients surviving for more than 250 weeks. In a recent paper, the same group demonstrated that vaccine responders exhibited more favorable clinical outcomes relative to non-responders. Moreover, the vaccine-induced responses elicited therapeutic benefits primarily by sensitizing tumors to subsequent chemotherapy.[61] In the phase I study reported by Liau *et al.*,[65] clinical outcome in the patients with stable tumors or no residual disease at time of DC vaccination, compared favorably, even when compared with historical/concurrent data for the best prognostic subgroup of GBM patients treated during the same time period at their institute. In contrast, Okada *et al.*[73] did not show any benefit in PFS in patients treated with DC loaded with autologous tumor lysate in combination with IL-4 producing fibroblasts. In the study on recurrent HGG patients reported by Yamanaka *et al.*,[67] radiological responses were observed in the group of 5 patients who received intratumoral plus intradermal vaccinations, while no response was detected in the 5 patients who were only injected intradermally.

De Vleeschouwer *et al.*[59] reported on a large group of patients with relapsed HGG, treated with DC loaded with tumor cell lysate in a stepwise process, using a cohort-comparison study concept (HGG-IMMUNO-2003), comprising of four cohorts (A-D). Following this strategy, it was possible to compare each cohort within the trial with the other cohorts. Thus far, 3 major issues in the complexity of DC immunotherapy have been addressed: the vaccination schedule, the boosting and the maturation cocktail. In this large group of patients, a complete resection improved the median PFS and OS, as compared to an incomplete resection. Interestingly, a stepwise improvement in PFS curves could be seen by shortening the interval between the induction DC vaccines, with further improvements noted by adapting the boosting intervals and technology, and finally by changing the maturation cocktail. Reported 2-year survival was 16.7% in cohort C and 27.8% in cohort D.

In this thesis, we will report on DC-based immunotherapy integrated into the primary treatment regimen for patients with newly diagnosed GBM. Based on the results in 8 pilot patients (**Chapter 5.1**),[57] we conducted a large phase I/II trial HGG-2006, in which 77 patients were included (**Chapter 5.2**).

CHAPTER 2. AIMS AND EXPERIMENTAL APPROACH

2.1 CLINICAL TRIALS

Our working hypothesis is that postoperative adjuvant DC-based immunotherapy could be beneficial for both newly diagnosed and relapsed HGG patients and offers new treatment perspectives. Our main objective is to assess the feasibility of the different vaccination approaches and to explore the hypothesis of possible clinical benefit.

2.1.1 Relapsed HGG patients

2.1.1.1 Aim

In a large group of patients with relapsed HGG, we have implemented DC vaccination in 4 consecutive cohorts, differing in vaccination characteristics (HGG-IMMUNO-2003 trial). Patients have all been treated according to the general concept of postoperative adjuvant DC vaccination, but with slight changes in the vaccination design over the cohorts, aiming to improve the vaccination strategy.
We wanted to stratify this large group of patients in prognostic classes to better detect possible advancements in the vaccination design, now and in the future. To that end, we aimed to modify an existing prognostic model and make it applicable to the group of patients with relapsed HGG, treated with postoperative DC-based therapy (**Chapter 3**).

2.1.1.2 Experimental approach

We have implemented immunotherapy for the treatment of 117 patients with relapsed HGG, as a stepwise process using a cohort-comparison study concept (HGG-IMMUNO-2003). The RTOG RPA classification initially designed to classify patients with newly diagnosed HGG in the era before the use of TMZ, was modified to implement it in the group of patients with relapsed HGG undergoing postoperative DC vaccination: the first modification comprised the exclusion of the radiation dosage, as radiotherapy takes no part anymore in the treatment of these relapsed patients. The second modification was the cancelling of a biopsy as only

surgical act, because all patients need to be re-operated before vaccination at time of recurrence.
Apart from the modification of this prognostic model, it was used to detect possible beneficial effects of the use of imiquimod in DC vaccination (cohort D).

2.1.2 Relapsed pediatric malignant brain tumor patients

2.1.2.1 Aim

Based on the already published importance of age in DC vaccination in the patients with relapsed HGG,[59] we decided to analyze in depth the results in relapsed pediatric malignant brain tumor patients (18 years and younger), and to report on feasibility, safety and indication of efficacy (**Chapter 4**).

2.1.2.2 Experimental approach

Apart from the adult patients, 45 children with relapsed malignant brain tumors were treated with adjuvant DC-based tumor vaccination. We treated 33 patients with HGG, 5 patients with medulloblastoma / primitive neuro-ectodermal tumor, 4 patients with ependymoma and 3 patients with atypical teratoid-rhabdoid tumor (ATRT).

2.1.3 Newly diagnosed GBM patients

2.1.3.1 Aim

Based on the suggested synergism of DC vaccination with radio- and chemotherapy, and recognizing the importance of a minimal residual disease state as important prerequisite for immunotherapy, we have integrated DC vaccination in the state-of-the-art postoperative therapy of patients with newly diagnosed GBM, and report on feasibility, toxicity and PFS at 6 months (6mo-PFS) as primary endpoints (**Chapter 5**). OS and immune monitoring are stated as secondary endpoints.

2.1.3.2 Experimental approach

DC-based tumor vaccination was integrated in the standard primary treatment of newly diagnosed GBM, consisting of maximal safe surgical resection, radiotherapy and TMZ chemotherapy.

- Pilot trial: We started with a pilot trial in 8 newly diagnosed GBM patients to assess safety and feasibility of integrating DC vaccination within the standard postoperative therapy (**Chapter 5.1**).
- HGG-2006 trial: The survival data of the pilot study were used to power the phase I/II HGG-2006 trial (**Chapter 5.2**).

2.2 IMMUNE MONITORING

Although pronounced immunological responses are often observed in reports on immunotherapy for HGG, there is rarely a strong correlation with the clinical outcome.[60] However, the conventional response criteria used in oncology, originally developed to monitor effects of radio- and chemotherapy, might not be fully appropriate to measure the beneficial effect of active immunotherapy. Monitoring of immunological events in patients that are being treated with DC-based therapy is of crucial importance and hence, we wanted to establish clinically relevant immune monitoring tools and pre-vaccine baseline data for vaccinated HGG patients, both in the blood and the tumor, thereby considering both the effector and suppressor compartment (**Chapter 6**).

2.2.1 Establishment and validation of a robust and easy-to-use assay to monitor regulatory T (Treg) cells in glioma patients

2.2.1.1 Aim

In tumor immunology, Treg cells have claimed a very prominent role as potential suppressors of immune responses and therefore monitoring of Treg cells is essential in patients treated with immunotherapy.[12,43] Commonly, Treg cells are identified by the expression of a nearly unique transcription factor, called Foxp3. However, to monitor Treg cells, intracellular flow

cytometric detection of Foxp3 expression is both a labor-intensive and costly process. Therefore, we wanted to establish a less laborious assay for routine use in tumor vaccination trials.

2.2.1.2 Experimental approach

Intracellular flow cytometric detection of Foxp3 expression and IL-7 receptor alpha subunit (CD127)dim surface staining were correlated on CD4+(CD25+) cells in 11 HGG patients and 4 healthy volunteers. Next, suppressive function of CD4+CD127dim cells was evaluated in an allogeneic mixed lymphocyte reaction (MLR).

2.2.2 Immune monitoring in the peripheral blood

2.2.2.1 Aim

Both in the pilot study with newly diagnosed GBM patients and in the HGG-2006 trial, flow cytometric analyses on patients' blood samples were performed at pre-set points in time during the vaccination schedule. Based on these results, we hoped to find immunological patterns, both in the effector and suppressor compartment, that would correlate with or predict clinical outcome.

2.2.2.2 Experimental approach

Flow cytometry is the most routinely used tool in the monitoring of cellular immune responses and was also applied by us to monitor immune responses in vaccinated GBM patients in the pilot and HGG-2006 trial. Monitoring results were compared with clinical data.

- Pilot trial: Elispot, flow cytometry and delayed type hypersensitivity reaction
  - Blood samples were obtained at times of leukapheresis, vaccine 1, vaccine 4 and vaccine 7. In each whole blood sample, the phenotype of circulating T cell populations was determined by flow cytometry: total CD3+ population, and the CD4+ and CD8+ subpopulations, as well as the activation markers HLA-DR on CD3+ cells and CD25 on both subpopulations.

- Peripheral blood mononuclear cells (PBMC) from each blood sample were cryopreserved and thawed together at the end of the immunotherapy for use in an Elispot assay.
- DTH was tested at the first and the fourth vaccination.

- ***HGG-2006 trial: flow cytometry***
  - Blood samples were obtained at the same points in time as in the pilot trial, and flow cytometric analysis was done for the following cell populations, relevant to the effector and suppressor arm of the immune system: CD4+ and CD8+ subpopulations, as well as Treg cells based on CD4+CD127dim expression, and NK cells based on CD3-CD56+ expression.

2.2.3 Immune monitoring in the tumor

2.2.3.1 Aim

We wanted to establish a baseline (i.e. pre-vaccine) phenotypical characterization of tumor-infiltrating cells, which we could then compare to the immune status of the patient as measured in the peripheral blood. Use of peripheral immune monitoring tools might prove inadequate in case the peripheral immune system does not mirror local events in the brain tumor micro-environment.

2.2.3.2 Experimental approach

Brain tumor infiltrating cells (BTIC) were isolated out of fresh resection specimens of 10 HGG patients that were not previously treated with immunotherapy. Isolated BTIC were used for phenotypical characterization by flow cytometric analysis and comparison was made with peripheral blood samples, which were obtained at time of surgery as well. Flow cytometric analysis was done for the following cell populations, relevant to the effector and suppressor arm of the immune system: CD4+ T cells, CD8+ cytotoxic lymphocytes, NK cells, NKT cells, MDSC and Treg cells.

CHAPTER 3. RPA CLASSIFICATION IN VACCINATED PATIENTS WITH RELAPSED HGG

Recursive Partitioning Analysis (RPA) is known as a statistical model resulting in prognostic classes based on treatment and pre-treatment prognostic variables.[91] For newly diagnosed HGG, the RTOG first described 6 prognostic classes in a recursive tree analysis in 1993.[92] Thus far, the RTOG RPA model has been validated in groups of patients with newly diagnosed malignant gliomas treated in several ways. Validation has been performed for external beam radiation therapy,[93] brachytherapy,[94] chemotherapy [95] and radiosurgery.[96] More recently, the EORTC adapted the RPA model to the current state-of-the-art treatment strategy of radiotherapy and concomitant chemotherapy with TMZ in patients with newly diagnosed GBM, considering the importance of the dose of radiation, the concomitant and the adjuvant TMZ therapy.[97] However, a comparable validation in adult patients with relapsed HGG treated in a uniform way, other than with chemotherapy, has not been done. Nevertheless, especially in this population, there is a high need for patient stratification according to validated prognostic parameters, given the high number of phase I/II trials of experimental and purely innovative treatment strategies, of which the real or possible clinical value is often hard to estimate due to the usually very heterogeneous nature of the patient population. Especially the importance of pre-treatment patient-related variables should be stressed in these models, given the wide-spread belief that in HGG, pre-treatment prognostic factors have more impact on outcome than any (new) potentially active therapy or treatment strategy.[10]

Carson *et al.*[98] published a RPA model, built upon data of 10 large phase I and II chemotherapy trials in patients with relapsed GBM. As such, within this heterogeneous group of patients, the diverse treatment regimens, pooled in the model of Carson *et al.*, potentially further confound the outcome data. Moreover, it is impossible to use the Carson classification in patients treated with DC vaccination, as administration of steroids to the patient (in up to almost 1/3 of patients in the pooled trials building the Carson classes) is an important third-line question in the recursive tree. For postoperative adjuvant DC vaccination, however, steroids are to be avoided because of their immunosuppressive properties.

We modified the RTOG RPA classification in order to use it in a large group of adult patients with relapsed HGG, treated at time of relapse by re-operation and postoperative adjuvant DC vaccination in the HGG-IMMUNO-2003 cohort comparison trial.[59] The main objective is to

define and compare survival categories within this heterogeneous patient population by obtaining simple, homogenous subsets of patients with comparable outcome. This refined stratification of patients will allow a better interpretation of the results of this ongoing phase I/II cohort comparison trial and might possibly lead to a better design of future phase III studies in HGG patients using innovative, non-chemotherapy based treatment regimens.

3.1 PATIENTS AND METHODS

117 Adult patients (>18 years) with relapsed HGG, included in the HGG-IMMUNO-2003 trial to undergo re-operation at time of recurrence, followed by vaccination with autologous DC loaded with autologous tumor lysate, were analyzed in an intent-to-treat (ITT) analysis. In this cohort comparison trial, which has been approved by the local ethics committee, patients are included in consecutive cohorts, in which treatment follows the general concept of postoperative adjuvant DC vaccination, but with slight changes in the vaccination design over the cohorts, as outlined in **Table 3.1** and described in previous publications of our group.[59,60,70] Patients could only be included in the trial after written informed consent.

	induction vaccines	boost vaccines	DC maturation	adjuvant
Cohort A	DC week 1 and 3	DC monthly	TNF-α, IL-1β, PGE_2	none
Cohort B	DC week 1,3,5,7and 9	DC monthly	TNF-α, IL-1β, PGE_2	none
Cohort C	**DC week 1,2,3,4**	Lysate monthly	TNF-α, IL-1β, PGE_2	none
Cohort D	**DC week 1,2,3,4**	Lysate monthly	TNF-α, IL-1β	**imiquimod**

Table 3.1 Design and technical details of the vaccines and schedules used in each cohort

DC, dendritic cell; IL-1β, interleukin-1β; imiquimod, Toll-like receptor 7 agonist, administered in a local cream (Aldara®) at the injection site for both induction and boost vaccinations; PGE_2, prostaglandin E_2; TNF-α, tumor necrosis factor α

All patients had previously been treated with surgery, external beam radiotherapy and (sometimes multiple) chemotherapy regimens. As such, the first modification to the original RTOG RPA classification comprised the exclusion of the radiation dosage, as radiotherapy took no part anymore in the treatment of these patients (**Table 3.2**). The second modification was the cancelling of a biopsy as only surgical act, because all patients needed to be re-operated before vaccination at time of recurrence (**Table 3.2**).

RPA Class	Age	Pathology	KPS	Mental status	Surgery	Remark
I	age<50	grade III		normal		
II	age≥50	grade III				symptoms ≥ 3 months
III	age<50	grade III		abnormal (MMSE<27)		
	age<50	grade IV	90-100			
IV	age<50	grade IV	<90			
	age≥50	grade III	70-100			symptoms < 3 months
	age≥50	grade IV			resection	good neurological function
V	age≥50	grade IV	70-100		resection	bad neurological function
	age≥50	grade IV	70-100		~~biopsy~~	~~RT ≥ 54,4 Gy~~
	age≥50		<70	normal		
VI	age≥50		<70	abnormal (MMSE<27)		
	age≥50	grade IV			~~biopsy~~	~~RT < 54,4 Gy~~

Table 3.2 Outline of the modified RPA classes

KPS, Karnofsky Performance Score; MMSE, Mini Mental State Examination; RPA, recursive partitioning analysis; RT, radiotherapy

~~ exclusion of factor (as described in text), resulting in 2 subpopulations of RPA classes V and VI respectively, that are not present in this study (depicted by light grey bar)

The extent of resection could be partial, subtotal or total, but was not included in the model to try to stick as close as possible to the original RTOG class descriptions. Extent of resection was assessed by the neurosurgical report and on postoperative MRI (T1 weighted spin-echo images before and after gadolinium enhancement) within 72 hours after surgery. Gross total resection was defined as the absence of any nodular postoperative contrast enhancement. Reference pathology was classified according to the WHO classification.[99] Performance status was scored using KPS,[100] ranging from 0 to 100, with cut-offs at 90 and 70 depending on the RPA class (**Table 3.2**). Mental status was assessed using the Mini Mental State Examination (MMSE),[101] with a cut-off value of 27 (**Table 3.2**). Good neurological status, as opposed to poor neurological status, was defined as a neurological status resulting in functional independence in activity of daily living.

Treatment at the time of new recurrence after/during vaccination was at the physician's discretion and not included in the analysis.

3.2 RESULTS

3.2.1 Modified RPA classification

In **Table 3.3**, the relative distribution of the prognostic variables is depicted for the 117 patients included in the 6 classes. Remarkably, 80% of patients belong to class III to V, reflecting the classical majority of patients in this setting of (multi-)relapsed HGG. Only 9 patients belong to class VI. For obvious reasons, patients in class VI are only rarely eligible for inclusion in trials. The vast majority of patients in class III to VI had a GBM. GBM patients are, as by definition, absent in class I and II: these classes only harbor patients with WHO grade III lesions. From classes I to VI, median age of patients seems to increase, except for the inversed age relation between class II and class III, the latter having a relatively younger population than the former. KPS, expressed as the postoperative performance state before vaccination, declined from class IV to VI. Patients with an abnormal mental status, defined as a MMSE score less than 27, were only encountered in class V and VI. Although not mandatory for this modified RPA classification, extent of resection was routinely assessed using an early postoperative MRI. The percentage of patients with a gross total resection before vaccination seems to be quite constant over the different classes, with only a clear discrepancy between the patients from class II and class III. In the latter class, 60% of patients

had a total resection compared to only 30% in class II. However, in case of GBM, total resection is based on the degree of resection of contrast enhancing tumor. WHO grade III tumors, which fall into the RPA classifications I through IV, may possess minimal contrast enhancement, at which point extent of resection is based on a usually more difficult quantifiable resection on T2 weighted images. This confounding factor might have influenced the percentage of patients that achieve a total resection.

RPA Class	Number of patients	Median age (y) (range)	Pathology (% GBM)	Median KPS (range)	Mental status (% abnormal)	Total resection (%)
I	4	27.6 (19.4-48.5)	0	90 (70-100)	0	50
II	10	43.4 (25.6-55.4)	0	100 (80-100)	0	30
III	28	37.1 (18.6-49.9)	82.1	90 (40-100)	0	60.7
IV	51	50.1 (19.9-68.0)	98	70 (40-100)	0	43.1
V	15	59.0 (50.0-77.8)	93.3	60 (50-80)	6.7	40
VI	9	63.8 (56.4-76.1)	88.9	40 (40-60)	55.6	33.3

Table 3.3 Relative distribution of prognostic variables in the different RPA classes

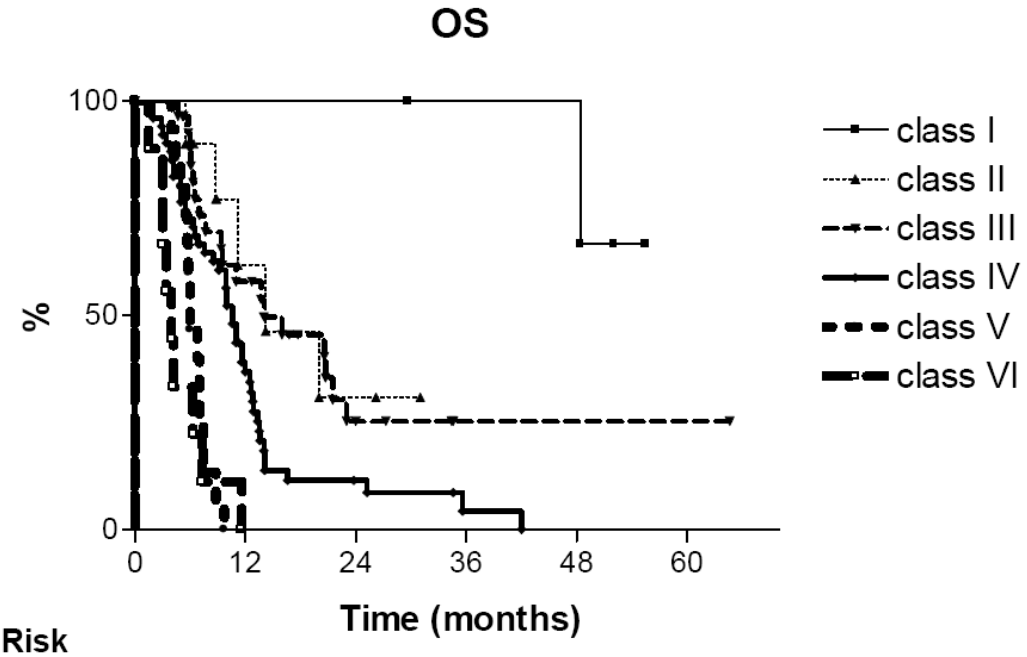
Abnormal mental status, MMSE < 27; GBM, glioblastoma multiforme; KPS, Karnofsky Performance Score; MMSE, Mini Mental State Examination; Pathology (% GBM), percentage of patients with GBM; RPA, recursive partitioning analysis; Total resection (%), percentage of patients with total resection

3.2.2 Kaplan Meier survival estimates

Log rank analysis of the Kaplan Meier survival estimates for OS are depicted in **Fig. 3.1** for each modified RPA class. The global difference in OS between the classes, reflecting different survival categories, was statistically significant ($p<0.0001$). The small differences between class II and III can be explained by the relatively good prognostic variables in class III as compared to those in class II (**Table 3.3**). In **Table 3.4**, median OS, ranges of OS, 2-year survival rates calculated from the moment of pre-vaccine re-operation and the numbers of patients still alive at latest FU are depicted. The decreasing median OS from class I to VI, but especially the differences in long-term survivors (>24 months after pre-vaccine re-operation) reflect clinically relevant prognostic differences. With a median FU of 20.8 months (range: 5.2 – 64.6 months), 24 (21%) patients with relapsed HGG are still alive after re-

operation and vaccination for recurrent HGG. No long-term survivors, however, are found in class V and VI.

Kaplan Meier survival estimates for OS in patients treated in cohort A, B and C, as compared to patients treated in cohort D, were not significantly different if all the classes or if the classes III to VI were considered. However, in a homogenous subgroup of patients from class III (*n* = 28), being the young patients with relapsed HGG in a good clinical condition, the use of imiquimod resulted in a significant survival benefit (p=0.035) (**Fig. 3.2**).



No. at Risk						
class I	4	4	4	4	3	1
class II	10	5	3	1	1	1
class III	28	16	5	2	2	2
class IV	51	17	5	2	1	1
class V	15	1	1	1	1	1
class VI	9	1	1	1	1	1

Fig. 3.1 Kaplan Meier survival estimates for overall survival (OS) according to modified RPA classes

Survival estimates are depicted in 117 adult patients with a (multi-)relapsed malignant glioma, treated with re-operation and adjuvant postoperative dendritic cell vaccination. Survival data are calculated from the time of the pre-vaccine re-operation. Log rank analysis, p<0.0001 (RPA, recursive partitioning analysis).

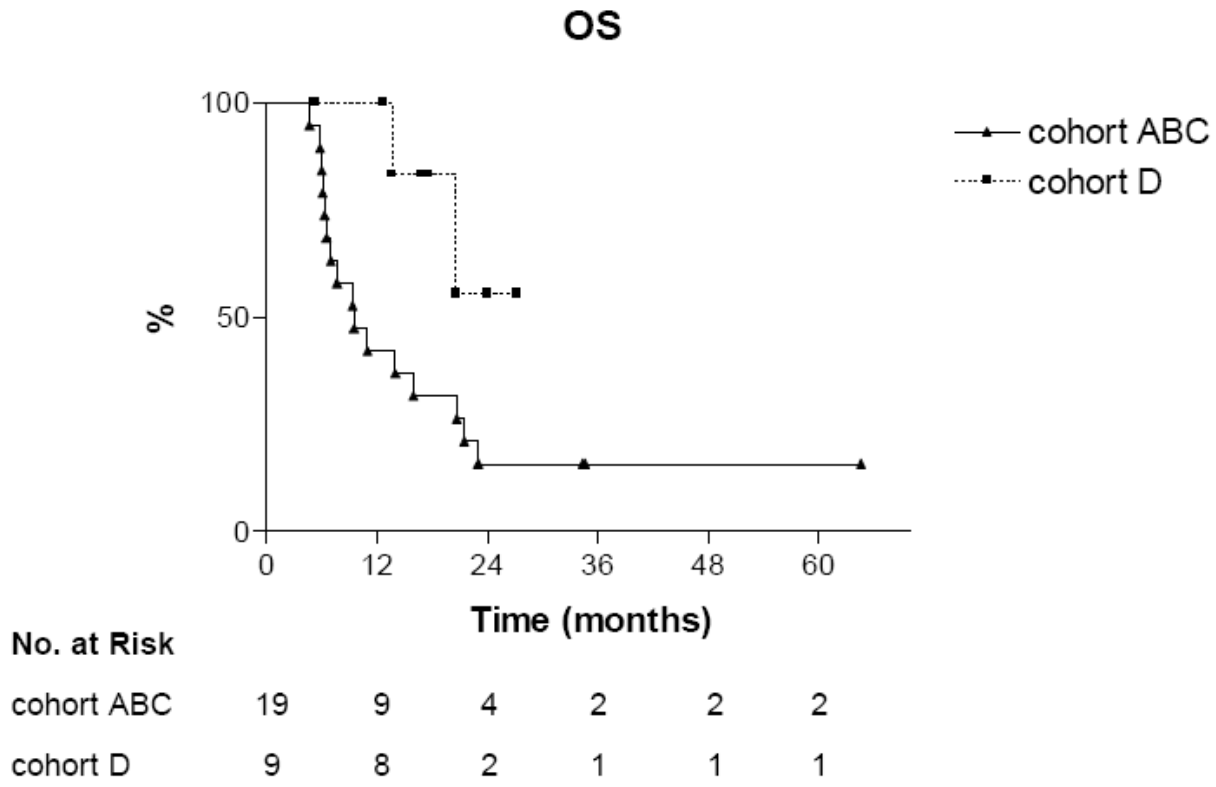


Fig. 3.2 Kaplan Meier survival estimates for overall survival (OS) for patients from RPA class III
Survival estimates for patients treated in cohorts A, B or C, versus patients treated in cohort D, in which imiquimod was used as an adjuvant. Log rank analysis, p=0.035 (RPA, recursive partitioning analysis).

RPA Class	Number of patients	OS (months)	2-year survival (%)	Number of patients still alive	FU survivors (months)
I	4	nyr (48.4-55.5)	100	3 (75%)	29.6 (52.0-55.0)
II	10	14.2 (5.5-31)	30.8	5 (50%)	8.9 (5.6-31.0)
III	28	14 (4.6-64.6)	25.3	10 (35.7%)	20.9 (5.2-64.6)
IV	51	10.6 (2.9-42)	11.5	6 (11.7%)	11.0 (7.1-34.6)
V	15	6 (4-9.7)	0	0 (0%)	-
VI	9	4 (1.5-11.6)	0	0 (0%)	-

Table 3.4 Overall survival (OS) data according to modified RPA (recursive partitioning analysis) classes
The data for OS and follow-up (FU) for survivors show the median value and the range, expressed in months (nyr, not yet reached).

3.3 DISCUSSION

We slightly modified the original RTOG RPA classification for patients with malignant glioma and made it applicable to a large group of patients with relapsed HGG who were re-operated upon and vaccinated as previously described.[24,59,70] In this heterogeneous group of patients with relapsed malignant glioma, we could define simple, clinically relevant, prognostic classes using this modified RTOG RPA model. The prognostic variables considered in this model are those from the original RTOG RPA classification, deemed relevant for this particular group of patients with (multi-)relapsed HGG: age (cut-off 50 years), WHO grading of malignancy (III or IV), KPS (cut-off at 90 and 70), mental status (MMSE score cut-off at 27), duration of symptoms before the recurrence (cut-off at 3 months) and postoperative neurological status (defined as good or poor).

This modified RPA classification resulted in 6 classes (I to VI) with a significant difference in OS in log rank analysis of Kaplan Meier survival estimates. Apart from the differences in median OS and ranges of OS, the differences in percentage long-term survivors, defined as patients with relapsed malignant glioma surviving 24 months or more after the pre-vaccine re-operation, were striking. A clear distinction in clinically relevant prognostic survival categories could be made, considering not only the median OS but especially the percentage of long-term survivors.

The long-term survivors after relapse in classes III and IV are a remarkable clinical finding, comparing favorably to almost all other trials in this population of (multi-)relapsed patients with HGG. Unfortunately, no real comparison can be made, as the classification in comparable prognostic subgroups is missing in all studies on larger series of relapsed malignant glioma patients. Compared to studies using the Carson classification,[98,102] the overall long-term survival data appear promising. Re-operation at time of recurrence, alone or in combination with several chemotherapy regimens has been extensively studied in the past, but invariably proved to be disappointing:[8] the additional survival benefit was barely half the interval between the first diagnosis and the relapse. TMZ in recurrent GBM did not result in any long-term survivors,[6] although it seemed to result in some in anaplastic astrocytoma.[103] Recently, bevacizumab (with chemotherapy) showed comparable results to TMZ in recurrent malignant glioma patients in a large single-institution trial.[104] The combination of bevacizumab and irinotecan showed promising numbers of responders with possibly some

long-term survivors in anaplastic oligodendroglioma.[105] No information, however, was given on prognostic classes in this trial.

Finally, this distinction of prognostically homogenous subgroups creates the opportunity to better assess the possible value of innovative strategies in a certain treatment schedule. As such, we investigated the changes we made to the vaccination in patients included in cohort D: PGE_2 was eliminated from the maturation cocktail of the DC because of concerns that DC maturation in the presence of PGE_2 would preferentially expand the Treg cell compartment in vaccinated cancer patients.[106] PGE_2 was replaced by the use of imiquimod cream as a topically applied 'danger signal' at the injection site of the vaccine. In this regard, we could show a clear survival benefit of the use of imiquimod as an immunological adjuvant to the vaccine in patients from class III, although this difference could not be seen in the complete group. This type of refined stratification to obtain a more homogenous study population might result in the identification of clinically meaningful modifications in therapy that might map the road for further innovation within a certain field of therapeutic research. The efforts and financial investments for randomized controlled trials could in this way be directed towards comparisons of therapeutic regimens that have already shown some benefit after stratified analysis according to validated RPA classifications.

CHAPTER 4. DC VACCINATION FOR CHILDREN WITH MALIGNANT BRAIN TUMORS [a]

Tumors of the CNS constitute the largest group of solid neoplasms in children and are second only to leukemia in their overall frequency during childhood. For all malignant pediatric brain tumors prognosis is dismal, in spite of state-of-the-art oncological therapy, including maximal safe surgical resection, external beam radiotherapy and chemotherapy. This holds especially true for atypical teratoid-rhabdoid tumor (ATRT), with a median survival of 11 months and less than 20% 2-year survival.[107-109] Although relevant therapeutic advances have been made for malignant pediatric brain tumors in recent years, the prognosis at time of relapse is clearly worse with short median survival.

Based on the already published importance of age in DC vaccination in the patients with relapsed HGG,[59] we decided to analyze in depth the results in the pediatric malignant brain tumor patients (18 years and younger). Tumor vaccination in the pediatric population was not only applied in case of malignant glioma, but in ATRT, ependymoma and medulloblastoma (MB) / primitive neuro-ectodermal tumor (PNET) as well.

4.1 PATIENTS AND METHODS

4.1.1 Patient population

45 Children (18 males and 27 females) were vaccinated after Ethics Committee approval and informed consent by the parents. We treated 33 patients with HGG (23 GBM, 5 anaplastic astrocytoma, 2 recurrent malignant pleomorphic xanthoastrocytoma, 1 oligo-astrocytoma grade III, 1 diffuse intrinsic pontine glioma and 1 anaplastic ganglioglioma), 5 patients with MB/PNET, 4 patients with ependymoma and 3 patients with ATRT. Histology at relapse was confirmed by central review in 37 patients. In the remaining patients, at least 2 independent pathological reviews in university hospitals were performed. All 3 ATRT diagnoses were performed by reference pathologists, and were based on typical immunohistochemical appearance. Patients' characteristics are described in **supplemental Table 4.1**.[b] In all patients

[a] Ardon H, De Vleeschouwer S, Van Calenbergh F, Claes L, Kramm CM, Rutkowski S, et al. Adjuvant dendritic cell-based tumour vaccination for children with malignant brain tumours. Pediatr Blood Cancer 2010;54:519-525.

[b] Available in the online version of the above-mentioned publication.

(except for one patient with primary metastasized ATRT) vaccination was performed as second or third stage treatment. All patients were operated upon and they were off corticosteroids at the time of leukapheresis/blood sampling and during vaccination.

4.1.2 Assessment of extent of tumor resection before vaccination

Total resection was defined by the neurosurgical report and the absence of any residual contrast enhancing tumoral mass on postoperative MRI (T1 weighted spin-echo images before and after gadolinium enhancement) within 72 hours after surgery. Any resection leaving a measurable tumoral mass less than 2 cm^3 was considered subtotal.

4.1.3 Tumor cell lysate

Tumor tissue was transported from the operating room into the laboratory and snap-frozen at -80°C without additives. The tissue was kept frozen at -80°C. For further preparation, the tissue was thawed and put into NaCl 0.9% with 1% human serum albumin (HSA, kindly provided by the Belgian Red Cross and Baxter), and then homogenized mechanically. Afterwards, 6 snap freeze/thaw cycles between liquid N_2 and 56°C were performed. The lysate was filtered with a 70 µm Falcon filter (BD Biosciences Europe, Erembodegem, Belgium). The amount of protein was measured using the Coomassie blue staining method and spectrophotometry at 595 nm.[110] After irradiation (60 Gy), the lysate was kept frozen in liquid N_2 until use. Tumor cell death was verified using the Trypan Blue Exclusion assay.

4.1.4 Preparation of autologous DC

In the first 7 patients, before a leukapheresis procedure was validated, PBMC were isolated from fresh blood samples to prepare each vaccination. In the following 38 patients, PBMC were obtained from leukapheresis and kept frozen in liquid N_2 until use. In 29 of these patients, it was necessary to place a double lumen deep venous access in the femoral vein, under short anesthesia, to perform the leukapheresis. For each vaccination, part of the PBMC was thawed, and adherent cells were differentiated to immature DC as described.[70] Immature DC were loaded with 200 µg of tumor proteins per 10^6 DC as described.[111] For the loading procedure, 0.01% autologous plasma was used during the first 2 h, 0.1% for the next 4 h, and finally 1% for the last 20 h. At time of loading, rTNF-α (Strathmann Biotec AG, Dengelsberg,

Germany), rIL-1β (Strathmann Biotec AG) and PGE_2 (Prostin®, Pfizer, Brussels, Belgium) were added in a final concentration of 120, 120 and 20 ng/mL respectively. After 24 hours, early mature loaded DC were resuspended in phosphate-buffered saline (PBS) with 0.5% HSA at a concentration of 2-6 $x10^6$/mL. Tuberculin syringes were filled with the loaded DC in suspension (400 μL), containing 1-2 $x10^6$ mature DC per syringe, and only administered when bacteriological release criteria were met.

4.1.5 Treatment schedule

Patients underwent maximal safe surgical resection of the tumor. Peri-operative corticosteroids were withdrawn within one week after resection. Leukapheresis was performed after histological diagnosis was obtained and inclusion criteria were assessed. Autologous DC loaded with tumor lysate were injected intradermally in the upper arms, according to a vaccination schedule as defined in the HGG-IMMUNO-2003 cohort comparison study (**Chapter 3; Table 3.1**).[59]

4.1.6 Vaccination

Vaccination was performed by intradermal injection of 0.25 – 11.9 $x10^6$ (median 2.8 $x10^6$) DC per lymph node region in the upper third of the arms (left and right). Boost vaccines (cohorts C and D) were given with tumor cell lysate with a median of 1,500 μg (range: 220 – 3,125 μg) proteins per vaccine injected in 2 syringes each containing a final volume of 400 μL.

4.1.7 Patient assessment

All patients were followed by clinical examination and MRI scanning (12 weeks after surgery and from then every 3 months). PFS and OS reported are calculated in months starting from the immediate pre-vaccination re-operation. Progression was determined radiologically on MRI, as defined by Macdonald *et al.*.[88] Statistical analysis was done by the log rank test on Kaplan Meier survival estimates.

Analysis of the patients was done based on the first inclusion in a cohort. Patients who received more than one course of immunotherapy according to the schedule(s) of the same or different cohort(s), were still assessed in the cohort in which they were initially included. At

the time of each vaccination, quality of life (QOL) was assessed using the Fertigkeitenskala Münster-Heidelberg (FMH).[112]

The FMH is a self-report questionnaire to assess the patient's ability to carry out daily life activities. In the present study, a 56-item version of the instrument was used and a total score ranging from 0 to 56 was calculated and transformed into a percentile score based on the age of the patient. In total 29 patients filled out the FMH at least once at 4 measurement moments during treatment (i.e. at time of the 4 induction vaccinations). In the other patients language difficulties made the use of the questionnaire impossible.

To analyze the data of the FMH, we used SPSS 16. First we calculated the mean percentile FMH scores of the patients. To analyze whether the scores of the patients on the FMH remained stable during treatment, we performed a repeated measurement procedure with the percentile scores on the FMH as dependent variable, and the 4 different measurement points in time as within subject variable.

4.2 RESULTS

4.2.1 Patient population

All 45 children were included for analysis and all patients had been pretreated with surgery and radiochemotherapy, except for one patient (# 43) with primary metastasized ATRT. Nine patients were treated in cohort A (8 HGG and 1 MB/PNET), 10 patients in cohort B (4 HGG, 3 ependymoma, 2 MB/PNET and 1 ATRT), 11 patients come from cohort C (8 HGG, 2 ATRT and 1 MB/PNET) and 15 patients were treated in cohort D (13 HGG, 1 MB/PNET and 1 ependymoma). Patients' treatment characteristics are described in **supplemental Table 4.1**.

Eighteen HGG patients (55%), including 13 GBM patients (57%), underwent a total resection of the tumor. Fifteen HGG patients (46%), including 9 GBM patients (39%), received additional chemotherapy during vaccination, in contrast to patients that were solely treated with immunotherapy. The addition of chemotherapy to immunotherapy was on request of the referring physician in all cases. There was no statistical difference in the FMH percentile scores at the start of the immunotherapy between the different groups (**Table 4.2**). At the time of new disease progression, possible rescue therapy was at the physician's discretion.

	Number of patients	Mean	SD	Min	Max
IChT HGG	13	35.00	14.86	9	50
IT HGG	9	31.33	15.05	7	50
IChT GBM	8	34.38	15.68	12	50
IT GBM	7	33.00	16.83	7	50

Table 4.2 Percentile scores on the Fertigkeitenskala Münster-Heidelberg (immunotherapy versus immunochemotherapy)

Means, standard deviations (SD), minima (Min) and maxima (Max) of percentile scores of relapsed high-grade glioma (HGG) and glioblastoma multiforme (GBM) patients receiving immunotherapy (IT) versus immunochemotherapy (IChT).

4.2.2 Vaccine preparation and characterization

The median number of PBMC that were thawed for preparation of autologous DC was 612.5 $x10^6$ (range: 83 – 2735 $x10^6$; n = 210). The median yield of loaded mature DC from these thawed PBMC was 0.83% (range: 0.15 – 8.12%). In absolute numbers, there was a median of 5.65 $x10^6$ loaded mature DC per vaccination session (range: 0.5 – 23.8 $x10^6$; n = 210). The phenotype of the mature DC was determined by fluorescence-activated cell sorting (FACS) as illustrated by Rutkowski *et al.*.[70] The 45 patients received a median of 7 vaccines (range: 2 – 21 vaccines). Details according to the different cohorts are given in **Table 4.3**. One patient (#43) in cohort B received a total of 21 vaccines, consisting of 16 DC vaccines and 5 boost vaccines with autologous tumor cell lysate. Three patients (#39 - #40 - #41) in cohort B, who were treated for an ependymoma, had only a one-week interval between the first and second DC vaccination. In one patient (# 44) from cohort C, only 3 DC vaccines were administered because the results of the quality control of one vaccine culture did not meet the product release criteria.

	Cohort A	Cohort B	Cohort C	Cohort D
DC vaccines	6 (3-7)	8 (4-16)	4 (3-4)	4 (2-4)
Boost vaccines	-	0 (0-5)	5 (0-11)	3 (0-6)

Table 4.3 Median numbers (ranges) of administered dendritic cell (DC) and boost vaccines

4.2.3 Clinical assessment

The clinical results (PFS, OS, FU period, 6mo-PFS and survivors) for the relapsed HGG patients are given in **supplemental Table 4.1**, **Table 4.4**, **Fig. 4.1**, **Fig. 4.2** and **Fig. 4.3**. Further details are presented below according to the different tumor types.

Tumor	PFS (mo)	6mo-PFS (%)	OS (mo)	Alive	FU survivors (mo)
HGG (all)	4.4 (1.4-85.6)	42	13.5 (1.4-85.6)	7/33	35.7 (12.1-85.6)
GBM	4.3 (1.4 – 85.6)	44	12.2 (1.4 – 85.6)	4/22	47.5 (19.7-85.6)
AA	4.5 (2.4 – 32.6)	40	18.4 (11.4 – 41.6)	1/5	13.0
PXA	1.9 – 65.7	na	12.1 – 79.1	1/2	12.1
OA gr. III	2.4	na	4.6	0/1	-
AGG	24.4	na	47.7	1/1	47.7
DIPG	1.6	na	7	0/1	-

Table 4.4 Clinical results of relapsed HGG patients

The data for PFS, OS and FU show the median value and the range, expressed in months. (AA, anaplastic astrocytoma; AGG, anaplastic ganglioglioma; DIPG, diffuse intrinsic pontine glioma; FU, follow-up; GBM, glioblastoma multiforme; gr., grade; HGG, high-grade glioma; mo, months; na, not applicable; OA, oligo-astrocytoma; OS, overall survival; PFS, progression-free survival; PXA, pleomorphic xanthoastrocytoma; 6mo-PFS, PFS at 6 months)

There were 6 long-term survivors (OS > 24 months) in the group of relapsed HGG patients (3 GBM). Four of them (#1 - #2 - #13 - #32) are still alive at last FU, at 85.6, 59.2, 35.7 and 47.7 months respectively. Five of the long-term survivors did not receive concomitant chemotherapy to the immunotherapy. Three of them (#1 - #2 - #29) come from cohort A (#1 - # 2 GBM), one from cohort B (#32) and one from cohort C (#26). The long-term surviving (GBM) patient (#13) that did receive concomitant chemotherapy (TMZ) was vaccinated according to the protocol of cohort C (**supplemental Table 4.1** and **Fig. 4.3**).

In the subgroup of relapsed anaplastic astrocytoma patients, 3 patients underwent a total resection of the tumor (#24 - #26 - #28) and in 2 patients the resection was subtotal (#25 –

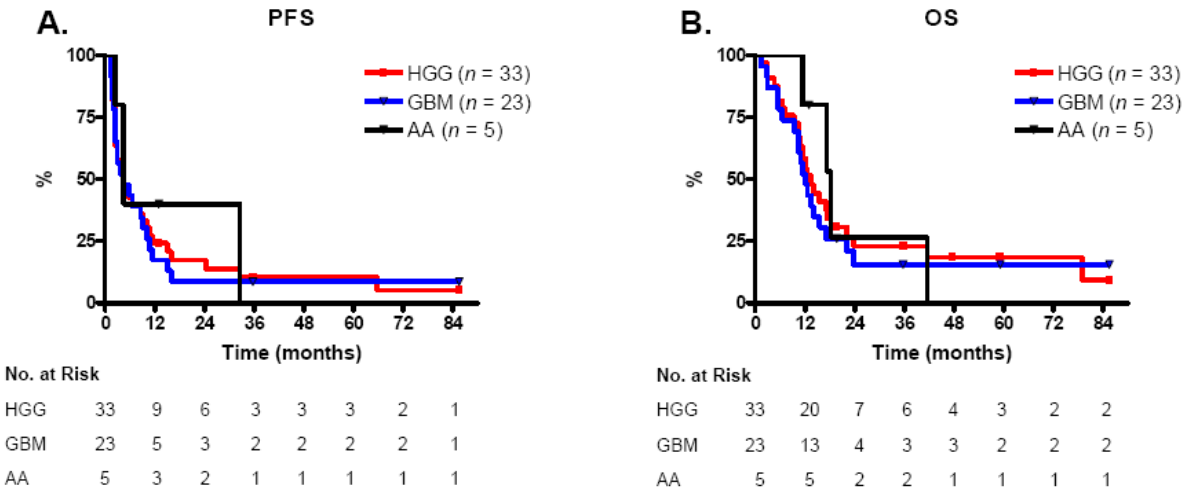


Fig. 4.1 Progression-free survival (PFS) and overall survival (OS) in relapsed high-grade glioma (HGG), glioblastoma multiforme (GBM) and anaplastic astrocytoma (AA) patients

Median PFS (A) for relapsed HGG, GBM and AA patients was 4.4, 4.3 and 4.5 months respectively. Median OS (B) for relapsed HGG, GBM and AA patients was 13.5, 12.2 and 18.4 months respectively.

#27). Patient #28 is still free of progression at last FU at 13.0 months. The other 2 patients with a total resection (#24 - #26) had PFS of respectively 4.4 and 32.6 months. Patient #24 died at 11.4 months and patient #26 at 41.6 months FU. In the patients with a subtotal resection (#25 - #27), PFS was 4.5 and 2.4 months respectively. Both patients died, at 18.4 and 17.3 months respectively (**supplemental Table 4.1**).

One of the 2 patients with a recurrent malignant pleomorphic xanthoastrocytoma (#30) is still alive at last FU at 12.1 months. The other patient (#29) died at 79.1 months FU. Tumor resection was respectively subtotal and total. PFS was respectively 1.9 and 65.7 months (**supplemental Table 4.1** and **Table 4.4**).

The patient with a diffuse intrinsic pontine glioma (#31, a radiological diagnosis) was first treated with radiotherapy and chemotherapy according to the HIT-GBM-D protocol. TMZ was given for a metastatic lesion in the right temporal lobe. Since the metastatic lesion increased in size, resection of the metastatic tumor site was performed and the resection specimen was used as source of TAA for vaccination. Histological diagnosis was GBM. During and after vaccination, TMZ and trophosphamide were given. PFS and OS in this patient were 1.6 and 7.0 months respectively (**supplemental Table 4.1** and **Table 4.4**). In the patient with an anaplastic ganglioglioma (#32), tumor resection was subtotal and PFS was 24.4 months. This patient is still alive at 47.7 months FU. The patient with an anaplastic oligoastrocytoma (#33) underwent a subtotal resection of the tumor and died at 4.6 months

FU. Tumor progression was noticed in this patient at 2.4 months (**supplemental Table 4.1** and **Table 4.4**). None of the 5 patients treated for recurrent MB/PNET (#34-38) were alive at time of analysis. The median OS was 5.7 months (range: 4.3 – 51.2 months). The median PFS ranged from 3.0 to 17.7 months with a median of 3.7 months (**supplemental Table 4.1**).

Of the 4 patients that were treated for recurrent ependymoma, one patient (#42) treated according to cohort D is still alive at 22.3 months FU. The other 3 patients (#39-41) died at respectively 31.5, 30.1 and 7.7 months. The median PFS was 4.1 months (range: 2.8 – 6.9 months) (**supplemental Table 4.1**).

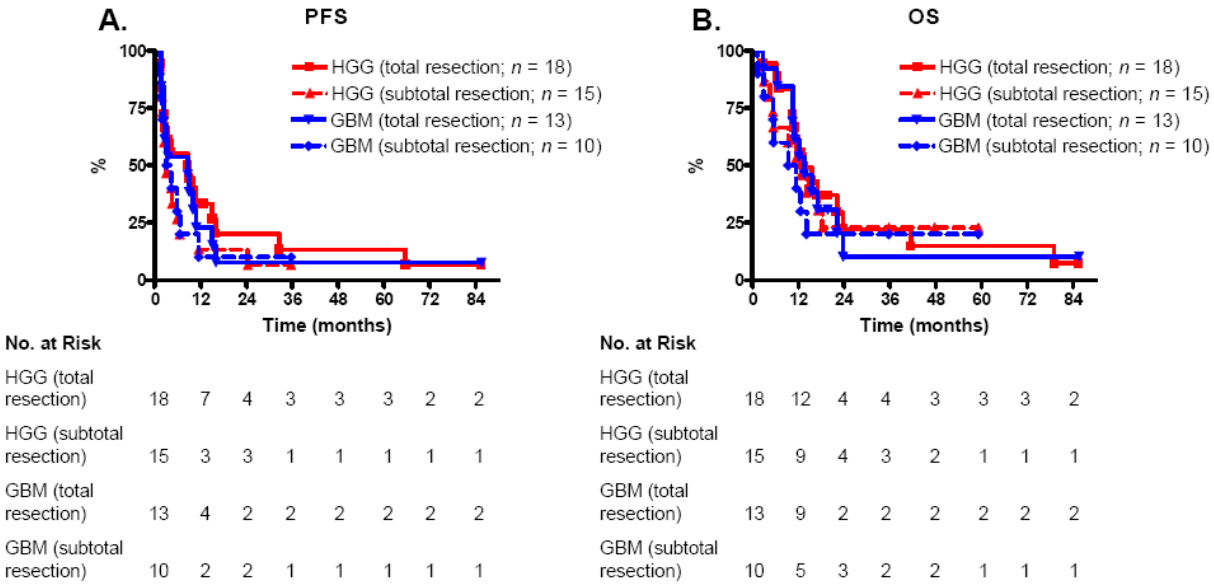


Fig. 4.2 Progression-free survival (PFS) and overall survival (OS) in relapsed high-grade glioma (HGG) and glioblastoma multiforme (GBM) patients according to grade of resection

Median PFS (A) and OS (B) for relapsed HGG patients with total resections were 8.8 and 13.5 months respectively versus 3.0 and 12.6 months for patients with incomplete resections. For relapsed GBM patients median PFS (A) and OS (B) were respectively 8.6 and 13.5 months for complete resections versus 3.7 and 10.5 months for incomplete resections.

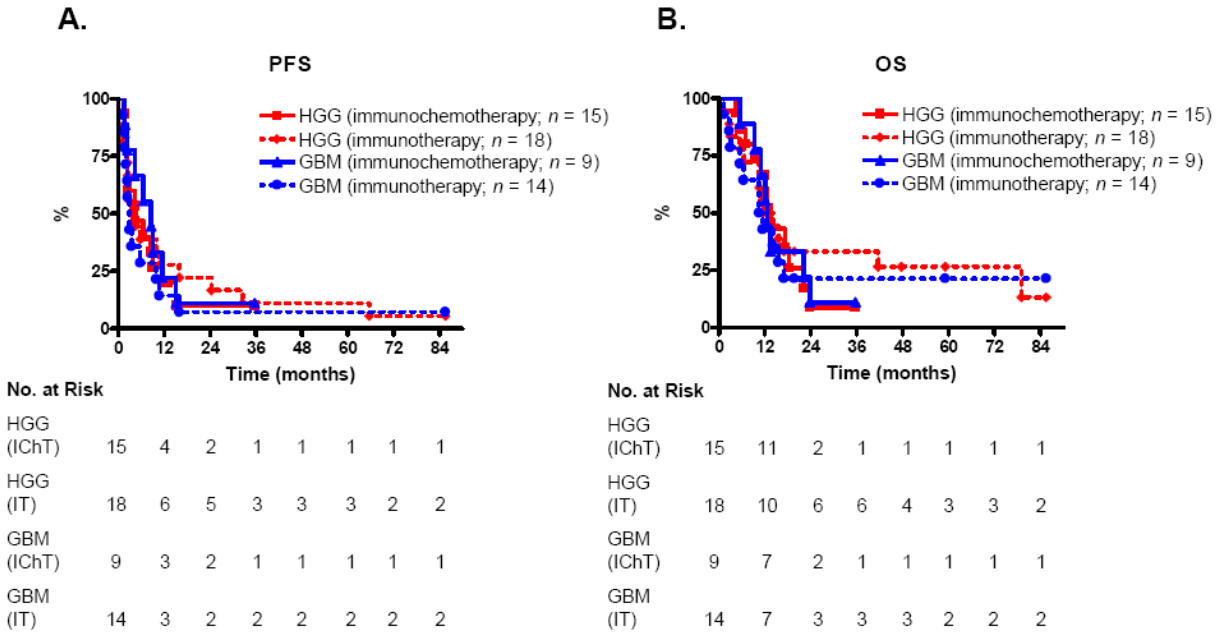


Fig. 4.3 Progression-free survival (PFS) and overall survival (OS) in relapsed high-grade glioma (HGG) and glioblastoma multiforme (GBM) patients: immunochemotherapy versus immunotherapy

Median PFS (A) and OS (B) in the relapsed HGG patients that received immunochemotherapy were 4.5 and 13.5 months respectively versus 4.0 and 12.8 months for patients that received solely immunotherapy. In the subgroup of relapsed GBM patients, median PFS was 8.6 months for patients that received concomitant chemotherapy versus 3.0 months in the patients that did not receive additional chemotherapy during vaccination. Median OS was 12.4 versus 11.0 months for patients with and without additional chemotherapy respectively, in this subgroup of relapsed GBM patients.

The 2 patients that were vaccinated for an ATRT at time of relapse (#44 - #45) are still alive at last FU, at respectively 34.1 and 52.6 months after detection of recurrence, and both patients are still free of progression. The third patient (#43), who had a primary metastatic ATRT, died at 50.5 months FU. PFS in this patient was 8.5 months. This patient had a total resection of his primary mass and of his sacral metastasis prior to vaccination (**supplemental Table 4.1**).

Five patients (#19 - #22 - #26 - #32 - #42) were re-operated upon at time of new relapse and afterwards received DC vaccines, for which the new resection specimen was used as source of TAA. Three of them (#22 - #32 - #42) are still alive at respectively 19.7, 47.7 and 22.3 months FU. The survival of the other 2 patients (#19 - #26) was 22.1 and 41.6 months respectively (**supplemental Table 4.1**).

4.2.4 Feasibility and Toxicity

For all patients that entered into the treatment protocol, it was feasible to provide the intended treatment. The vaccines were injected in an outpatient setting. Only at time of the first contact, for patients in whom a deep venous access had to be placed under short anesthesia, one overnight stay was required in order to perform the leukapheresis during the morning of the following day. The median distance between the home address and the vaccination center was 515 kilometers (range: 30 – 2,154 kilometers). Therefore, the treatment was performed in close collaboration with the referring oncological center, which remained involved for imaging studies, general support and all other necessary treatments of the patient.

Adverse Events	No. of patients	Grade according to NCI CTCAE (version 3.0)
Fatigue	8	I
Headache	5	I
General rash / itching	3	I
Post-vaccination fever	3	I
Nausea / vomitus	2	I-II
Flu-like syndrome	1	I-II

Table 4.5 Adverse events

CTCAE, Common Terminology Criteria for Adverse Events; NCI, National Cancer Institute; No., number

In **Table 4.5**, adverse events graded according to NCI (National Cancer Institute) CTC (Common Terminology Criteria) are depicted. Temporary, vaccine-induced redness with or without itching and swelling at the injection sites was the rule in all patients.

4.2.5 Quality of Life

The mean FMH percentile scores, as measured at the 4 induction vaccinations, are given in **Table 4.6**. All mean scores are situated below the 35th percentile, indicating that most patients reported problems in performing daily life activities. However, their scores did not significantly change during the treatment, as was indicated by the repeated measurement procedure.

	Number of patients	Mean	SD	Min	Max
FMH_T1	26	27.07	14.76	5	48
FMH_T2	21	28.14	16.16	6	50
FMH_T3	20	32.55	20.95	9	90
FMH_T4	21	25.95	22.51	4	100

Table 4.6 Percentile scores on the Fertigkeitenskala Münster-Heidelberg (FMH)

Means, standard deviations (SD), minima (Min) and maxima (Max) of percentile scores of patients (including all types of tumor) as measured at time of the 4 induction vaccinations (T1 = vaccine 1, T2 = vaccine 2, etc.).

4.3 DISCUSSION

In this chapter we summarized our experience in a group of 45 children with a recurrent malignant brain tumor (except for one patient with a primary metastasized ATRT), treated with autologous DC loaded with autologous tumor cell lysate. Most patients were heavily pre-treated before immunotherapy was instituted; the treatment included multiple surgeries, use of different chemotherapeutic agents and radiotherapy schedules. Therefore the group we present is very heterogeneous, which makes comparison to historical groups very difficult. Thus, the emphasis of this chapter is on the technical and clinical feasibility of DC vaccination in a large group of children with recurrent malignant brain tumors.

In previous reports from our group, we described the promising long-term survival rates in the global group of patients with relapsed HGG, treated with postoperative adjuvant DC vaccination.[59,69] In this chapter, we demonstrate the clinical feasibility of DC vaccination in children. For the relapsed HGG group of patients, median PFS was 4.4 months with a median OS of 13.5 months. For the relapsed GBM patients, median PFS and OS were 4.3 and 12.2 months respectively. This is in line with the report from Korones *et al.*[113] on TMZ and oral etoposide for children with recurrent or treatment-induced malignant gliomas, although no long-term survivors were described in this study (longest FU of 18 months). Finlay *et al.*[114] reported similar results as well, with the use of high-dose thiotepa and etoposide with autologous bone marrow rescue (median OS of 12.7 months). However, all these results compare favorably to single agent trials of TMZ and etoposide for children with recurrent brain tumors.[115-118] As could be expected, the results are better for patients with relapsed anaplastic astrocytoma, in which the median OS was 18.4 months. Median PFS was 14.5 months in these patients.

Most strikingly, and clinically relevant, there seems to be a subgroup of long-term survivors with our approach for these recurrent tumors (**Fig. 4.1**). Six out of the 33 HGG patients (18%) have an OS of more than 24 months and 4 of them are still alive at 85.6, 59.2, 35.7 and 47.7 months respectively. Five out of the 6 long-term survivors received chemotherapy, either during (n = 1) or after immunotherapy (n = 4), so one might argue that the chemotherapy had some effect as well. An increased susceptibility of GBM to chemotherapy in patients pre-exposed to DC-based vaccination has been shown by Wheeler *et al.*.[119] Other groups have reported on the possible mutual beneficial effects of immunotherapy and chemotherapy as well.[120-123] Overall, it is hypothesized that the combination of the two could potentiate the cumulative antitumoral activity, when applied in a well designed strategy.

The results in the 3 patients who were vaccinated for an ATRT are promising and compare favorably to previously published data on median survival (11 months) and 2-year survival (less than 20%).[107-109] Two patients with recurrent ATRT are still alive at last FU, at respectively 34.1 and 52.6 months, and both are still free of progression or recurrence. Both patients did receive radio- and chemotherapy prior to and/or during vaccination, and the favorable outcome might be due to possible mutual beneficial effects. The third patient died at 50.5 months FU and received radio- and chemotherapy during and after immunotherapy. However, more patients have to be treated before any definite conclusions on efficacy of immunotherapy for ATRT can be drawn, and this will be a challenge as ATRT are very rare tumors.

The results of adjuvant DC-based immunotherapy for ependymoma and MB/PNET showed no clear benefit of the vaccinations. For ependymoma, this might be explained by the fact that, as yet, no well-defined TAA have been described. Therefore, the aim of DC-based vaccination, i.e. priming and boosting of the host immune system by presenting tumor-specific antigens, might not be reached in these patients because of the lack of the necessary TAA. For MB however, Behrends *et al.*[124] showed that the humoral immune response is directed against diverse antigens that may be useful as diagnostic markers or targets for immunotherapy. Furthermore, the expression of cancer-testis antigens has been described in MB/PNET, and these might function as TAA as well. Hence, it seems that the lack of efficacy in the 5 patients treated for MB/PNET cannot be explained by a lack of necessary TAA. However, 2 of these patients were younger than 3 years of age at time of diagnosis, and infants with MB/PNET are known to have worse outcomes. Moreover, since all 5 patients had relapsed tumors that were heavily pre-treated, we can state that these tumors had proven themselves to be high-risk and therefore had poor prognosis.

Whereas our previous reports did not include patients with immunochemotherapy,[59,69,70] a substantial proportion of the children presented in this chapter were treated with combination therapies (at request of the referring physician). Both in the relapsed HGG patients and in the subgroup of relapsed GBM patients, we saw a small, but not significant difference in median PFS and OS, in favor of patients that received immunochemotherapy versus patients that were exclusively treated with immunotherapy. However, 5 of the 6 long-term survivors (OS > 24 months) in the group of relapsed HGG patients did not receive concomitant chemotherapy to the immunotherapy. In any case, it is difficult to interpret these data since the study was not designed to assess differences between immunotherapy with or without chemotherapy.

In 5 children a re-operation was performed at time of relapse, and the resection specimen was then used as new source of tumor-specific antigens. Three of these patients are still alive at respectively 19.7, 22.3 and 47.7 months FU.

The importance of minimal residual disease burden is emphasized by the finding that a total resection before vaccination seems to be associated with a better PFS/OS, in particular for PFS, although this did not reach statistical significance in this small group of patients. The same was seen in our adult patients, especially in patients under 35 years of age.[59] When we take out the HGG group as a whole, median PFS after total resection of the tumor was 8.8 months versus 3.0 months after subtotal resection. However, this beneficial effect of total resection was not as clear for median OS (13.5 months for total resection versus 12.6 months for subtotal resection). The same was seen in the subgroup of GBM patients, where a clear benefit of a total resection was evident for median PFS (8.6 months for total resection versus 3.7 months for subtotal resection), but less so for median OS (13.5 months for total resection versus 10.5 months for subtotal resection). We can conclude, as for other therapies, that a total resection is associated with a better prognosis in vaccinated children with malignant HGG.

In the first 7 patients (16%), PBMC were isolated from fresh blood samples because the leukapheresis procedure was not yet validated. In the following 38 patients (84%), it was feasible to obtain PBMC from leukapheresis, with the necessity to place a double lumen deep venous access in the femoral vein in the majority of these children (76%). For this procedure, the children were admitted to the hospital for one night without major problems. There was no difference in quality of PBMC obtained by leukapheresis versus isolation from fresh blood samples. An adequate number of early mature DC for vaccination could be generated from the PBMC in both settings, even in these heavily pretreated children. Similar findings were reported in 15 pediatric cancer patients by Jacobs *et al.*.[125]

It is important to note that there were no serious adverse events and that the therapy was well tolerated. In general, only mild adverse events were noticed for which a cessation of the treatment was not necessary.

This study also used the Fertigkeitenskala Münster-Heidelberg as a measurement of QOL in children receiving immunotherapy. The data showed that the patients in our study reported problems in performing daily life activities from the beginning of the treatment. However, during treatment, no significant changes occurred in their QOL. We can therefore conclude that immunotherapy can be applied safely in these already affected children.

CHAPTER 5. DC VACCINATION FOR NEWLY DIAGNOSED ADULT GBM PATIENTS

Considering the promising results of surgery and adjuvant autologous DC-based tumor vaccination in the group of patients with relapsed malignant glioma,[59] the next step in the clinical development of DC-based immunotherapy for HGG is to fully integrate immunotherapy within the standard first line treatment, considering putative mutual beneficial effects of the combination of immunotherapeutic strategies with conventional therapeutic modalities, such as radiotherapy and chemotherapy.[80,119,120,126-129] In these reports, immunotherapy was added to either radio- or chemotherapy, but was never fully integrated in a comprehensive schedule of radio-chemo-immunotherapy. In this chapter we will discuss the integration of DC-based immunotherapy into the standard multimodal treatment for GBM, consisting of surgery, radiochemotherapy and maintenance temozolomide (TMZm) chemotherapy.[3]

5.1 PILOT TRIAL [a]

In a first step, we started with a pilot group of 8 patients with newly diagnosed GBM, treated with autologous DC loaded with autologous tumor cell lysate, integrated in a standardized fashion in the multimodal standard therapy. Our main interest was in the clinical and immunological feasibility and toxicity of the integration of DC vaccination in the conventional therapeutic modalities.

5.1.1 Patients and Methods

5.1.1.1 Patient population

Eight patients (5 males and 3 females) presented with a newly diagnosed primary GBM, confirmed on central review histopathology. Patients were included for DC-based therapy, if they met the inclusion criteria as summarized in **Table 5.1**. Patients' characteristics are

[a] Ardon H, Van Gool S, Lopes IS, Maes W, Sciot R, Wilms G, et al. Integration of autologous dendritic cell-based immunotherapy in the primary treatment for patients with newly diagnosed glioblastoma multiforme: a pilot study. J Neurooncol 2010;99:261-272.

described in **Table 5.2**. Their median age was 50 years (range: 31 – 62 years). All patients were operated upon and were off steroids and nonsteroidal anti-inflammatory drugs at the time of leukapheresis (as determined by the exclusion criteria) and during vaccination. Approval by the local ethics committee was obtained, as well as patients' written informed consent before the start of immunotherapy.

Inclusion criteria
Age > 18 years and < 70 years
First diagnosis of glioblastoma multiforme, histologically proved
Total or subtotal resection of tumor mass, confirmed by assessment of neurosurgeon and postoperative MRI within 72 hours
Availability of enough tumor tissue, kept dry in a sterile vial at -80°C
Perioperative administration of corticosteroids tapered within 7 days postoperatively
Life expectancy > 3 months
Histology confirmed by reference pathology
Written Informed consent by patient
Exclusion criteria
Pregnancy
Postoperative Karnofsky index < 70
Simultaneous treatment according to other clinical trials
Virus serology positive for hepatitis, syphilis or HIV
Blood counts: leukocytes < 3000/µL - lymphocytes < 500/µL - neutrophils < 1000/µL - hemoglobin < 9 g/100 mL - thrombocytes < 100000/µL two days prior to leukapheresis
Documented immune deficiency
Documented autoimmune disease
Mandatory treatment with corticosteroids or salicylates in anti-inflammatory dose
Other active malignancy

Table 5.1 Inclusion and exclusion criteria

Patient	Age at surgery (y)	Sex	Resection	Site of tumor	No. of vaccinations	RPA class
1	56	m	Subtotal	left frontal	7	IV
2	48	m	Subtotal	left frontal	13 [a]	IV
3	31	m	Subtotal	left frontal	15	IV
4	46	f	Total	left temporal	14 + 7 [b]	IV
5	60	f	Subtotal	left temporal	9	IV
6	51	f	Subtotal	left parietal	6	IV
7	49	m	Subtotal	right occipital	11	III
8	62	m	Total	right parietal	8 + 13 [b]	IV

Table 5.2 Patients' characteristics

DC, dendritic cell; f, female; m, male; RPA, recursive partitioning analysis

[a] third and fourth vaccine contaminated – not given

[b] re-operation at relapse followed by immunotherapy

5.1.1.2 Assessment of extent of tumor resection before vaccination

Total resection was defined by the neurosurgical report and the absence of any residual contrast enhancing tumoral mass on early postoperative MRI (T1 weighted spin-echo images before and after gadolinium enhancement) performed within 72 hours after surgery. Any resection leaving a measurable contrast enhancing tumoral mass less than 2 cm^3 was considered subtotal. All solid residual tumor of a measurable size $\geq$ 2 cm^3 was classified as partial resection.

5.1.1.3 Tumor cell lysate

Tumor cell lysate was generated as described in **Chapter 4.1.3**.

5.1.1.4 Preparation of autologous DC vaccines

In all patients, PBMC were obtained from a single leukapheresis. DC vaccines were prepared as described in **Chapter 4.1.4,** and only administered when bacteriological release criteria were met. The phenotype of the cells (**Table 5.3**) was determined by FACS, using fluorescein isothiocyanate (FITC) and phycoerythrin (PE)-labeled mAb purchased from BD Biosciences Pharmingen (San Jose, USA).

	Median	IQR
CD86%	81.06	14.18
HLA DR%	93.58	6.4
CD14%	2.04	1.44
CD3%	0.82	2.36
CD1a%	9.8	13.94
CD25%	5.7	5.96
CD83%	22.96	12.66
CD80%	38.42	16.78
CD19%	0	0.35

Table 5.3 DC phenotype characteristics
CD, cluster of differentiation; DC, dendritic cell; HLA-DR, human leukocyte antigen DR; IQR, interquartile range

5.1.1.5 Treatment schedule: fully integrated radio-chemo-immunotherapy

Patients underwent maximal safe surgical resection of the tumor. Peri-operative corticosteroids were withdrawn within one week after resection. Leukapheresis was performed after histological diagnosis was obtained and inclusion criteria were met (**Fig. 5.1**). After leukapheresis, patients were treated with limited field external beam radiotherapy (30 x 2 Gy) and concomitant chemotherapy with TMZ (75 mg/m²) during 6 weeks as outlined by Stupp *et al.*.[3] After radiochemotherapy, tumor lysate-loaded DC were injected weekly for 4 weeks. Following these 4 weeks, TMZm chemotherapy was started. The maintenance 28-day cycles consisted of 5 days oral intake of TMZ (150 mg/m² for the first and 200 mg/m² for the following cycles). During the first, second, third and sixth cycle, further boost vaccines with tumor cell lysate were administered at day 8 of the cycle. The decision to administer a vaccine was based on clinical and radiological findings at time of the vaccination in question. In the following conditions, vaccines were given: 1. no clinical and/or radiological progression; 2. radiological progression with a recurrent tumoral mass of less than 2 cm³ and a KPS > 70. At time of progression, possible rescue therapy was at the physician's discretion.

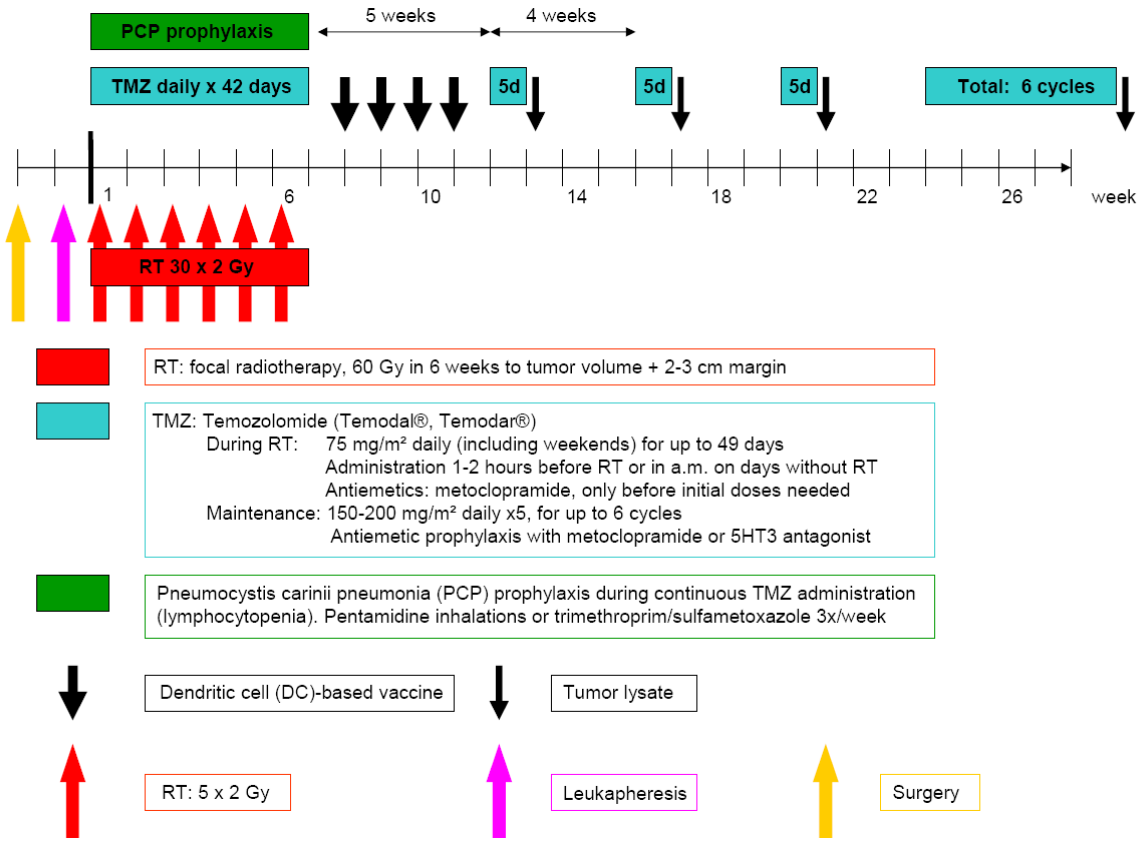


Fig. 5.1 Treatment schedule

Dendritic cell-based immunotherapy was integrated in the state-of-the-art postoperative radiochemotherapy. Leukapheresis to harvest autologous monocytes is performed once, at least 7 days after weaning of steroids and immediately before the start of the concomitant radiochemotherapy. After the radiochemotherapy, but before the maintenance chemotherapy with temozolomide (TMZ), 4 weekly induction vaccines are administered intradermally. Afterwards, maintenance chemotherapy (5/28 days) is started and 1 week after the start of the 1st, 2nd, 3rd and 6th cycle of TMZ, a boost vaccine is administered.

5.1.1.6 Vaccination

Vaccination was performed by intradermal injection of 1 – 12 x10^6 (median 4.1 x10^6) DC per lymph node region in the upper third of the arms (left and right) at weeks 1, 2, 3 and 4 (induction vaccines). Further boost vaccines were given with tumor cell lysate, with a median of 1,500 µg (range: 1,500 – 4,000 µg) proteins per vaccine injected in 2 syringes each containing a final volume of 400 µL.

5.1.1.7 Patient assessment

All patients were followed by clinical examination and MRI scanning (12 weeks after surgery and from then every 3 months). PFS and OS reported are calculated in months starting from surgery. Progression was determined radiologically on MRI, as defined by Macdonald *et al.*.[88] Upon specific indication methionine PET was performed. Statistical analysis was done by the log rank test on Kaplan Meier survival estimates.

At the time of each vaccination, QOL was assessed using the QLQ-C30 and the FMH.[112,130,131] KPS was assessed by the patients and registered at each visit.[100]

The QLQ-C30 is a 30-item questionnaire composed of multi-item scales and single items. It is designed by the EORTC for use in international clinical trials in oncology. It taps functional disability, somatic symptoms, global health and overall QOL. In this trial, only the indices for general health and overall QOL (single items 29 and 30) were used. The rating scale ranges from 1 (very bad) to 7 (excellent).

The FMH is a 56-item questionnaire, tapping the patients' ability to carry out daily life activities. In this trial, a 53-item version of the instrument was used and a total score ranging from 0 to 53 was calculated.

The KPS was used for the assessment of the patients' level of physical ability; it consists of one rating on a scale from 0 (dead) to 100 (normal functioning).

Adverse events were graded according to the NCI CTC.

5.1.2 Results

5.1.2.1 Feasibility: vaccines preparation and characterization

The patients received a median of 10 vaccines (range: 6 – 16 vaccines). The details of the vaccination for each patient are described in **Table 5.4**. Patients #4 and #8 underwent a re-operation at time of relapse with postoperative inclusion in the HGG-IMMUNO-2003 cohort comparison trial. The median yield of loaded mature DC from freshly isolated PBMC was 8.2 $x10^6$ per vaccination session (range: 2 - 24 $x10^6$; n = 30). Two vaccines (both in patient #2) could not be administered because the release criteria of the cellular product were not fulfilled.

Patient	PBMC ($x10^9$)				DC_i ($x10^6$)				loaded DC_m ($x10^6$)				tumor lysate	
	V1	V2	V3	V4	V1	V2	V3	V4	V1	V2	V3	V4	# boosts	amount/boost (µg) [1]
1	0.998	1.043	0.867	0.961	10.8	7.6	15.2	12.7	5.6	3.5	5	3.5	3	1500
2	1.088	1.075	1.024	1.034	26.4	9	30.6	na	12	4.5	na	na	11	1500
3	1.007	1.194	1.086	1.072	36	39	33	40	20.8	15.3	17	24	12	1500
4 [2]	1.099	0.92	1.186	1.191	14.7	20.6	16.8	15	7.4	9	5	8.4	10	1500
5	0.823	0.922	0.597	0.916	31	32	37	29	13	13	16	14.3	5	1500
6	1.025	0.892	1.062	1.05	16	18	17.5	26	10	8	13.5	13.5	2	1500
7 [3]	0.652	0.575	0.608	0.605	18	8.06	20.5	14	7.5	6.76	9.75	5	7	L1-2: 4000 - L3-7: 1500
8 [2,3]	0.507	0.565	0.581	0.633	8	11.5	8.3	7.25	5.75	8	2	5	4	L1-3: 1752.5 - L4: 1500

Table 5.4 Vaccination details

DCi, immature dendritic cells; DCm, mature dendritic cells; na, not available (contamination); L, lysate (boost) (L1 = V5, L2 = V6, etc.); PBMC, peripheral blood mononuclear cells; V, vaccine

[a] differences in amount/boost are irrelevant from a biological point of view

[b] vaccination details after re-operation (immunotherapy for recurrence) not included in table

[c] in patients #7 and #8 less PBMC were obtained by the single leukapheresis and therefore less PBMC were thawed for each vaccination; adequate numbers of loaded DCm could be generated in both patients

5.1.2.2 Toxicity

The clinical data during vaccination are described in **Table 5.5**, including adverse events graded according to the NCI CTC.

In patient #7, lymphopenia (650/mm^3) was diagnosed during TMZm chemotherapy, which did not require cessation or delay of treatment. In patient #2, a focal epileptic insult occurred

between the second and third vaccination. Three patients (#2, #4 and #8) experienced (transient) dysphasia postoperatively and in patient #8 there was a transient recurrence of the dysphasia after the second cycle of adjuvant TMZ chemotherapy. Four patients (#2, #6, #7 and #8) complained of fatigue during the treatment, general malaise and myalgia were each mentioned by one patient (#3 and #6 respectively).

In patient #5, a sudden onset right hemiplegia and aphasia occurred at 8 months FU (after the third boost with tumor lysate). MRI showed hyperintensities in T2 weighted images and heterogenic signals in T1 weighted images at the posteromedial side of the left temporal lobe, near the site of tumor resection (**Fig. 5.2**). These MRI data were most compatible with ischemic changes and the episode of hemiplegia and aphasia was diagnosed as an ischemic event. Inflammation, in terms of auto-immunity, could not be ruled out as no biopsy was performed. A repeat MRI 3 months after the described event showed the same changes at the posteromedial side of the left temporal lobe without any evolution (**Fig. 5.2**). We concluded that these changes most likely were ischemic, although inflammation or post-radiotherapy sequellae could not be ruled out pathologically in this patient; the clinical evolution however was favorable without administration of steroids. In the same patient the TMZm chemotherapy was stopped because of hematological toxicity (grade III) and at one year FU this same patient had a status epilepticus which was controlled with fenytoin. Tumor status at that time remained unchanged. No other serious adverse events have been reported.

Patient	FU (mo)	Radiological evolution	Clinical data during vaccination	NCI CTCAE (version 3.0)	PFS (mo)	OS (mo)	Alive
1	13	recurrence (MRI)	rescue chemotherapy[1]	none (grade 0)	2	13	no
2	23	recurrence (MRI/PET)	re-operation for recurrence after 11 months rescue chemotherapy[2]	focal epileptic insult (grade II) fatigue (grade I) dysphasia (grade II)	11	23	no
3	34	no recurrence (MRI)	no particular findings	general malaise after V2 (grade I)	34	34	yes
4	35	recurrence (MRI)	re-operation for recurrence after 25 months immunotherapy[3]	transient dysphasia postoperatively (grade II)	25	35	yes
5	22	inflammatory/ischemic changes after ischemic event (MRI) recurrence (MRI)	stop of TMZ because of hematotoxicity	transient confusion after V1 (grade I) ischemic event after V7 (partial recovery) (grade IV)[4] hematotoxicity (grade III)[4] status epilepticus (grade IV)[4]	18	22	no
6	17	recurrence (MRI)	re-operation for recurrence after 6 months rescue chemotherapy[1]	fatigue (grade I) myalgia after V1 + V2 (grade I)	6	17	no
7	25	recurrence (MRI/PET)	re-operation (twice)[5] rescue chemotherapy[6]	fatigue during TMZ (grade I) lymphopenia during TMZ (grade II)	17	25	no
8	44	recurrence (MRI)	re-operation for recurrence after 26 months immunotherapy[3] rescue chemotherapy[2]	fatigue with transient confusion during TMZ (grade I) discrete dysphasia (grade II)	26	44	yes

Table 5.5 Clinical data during vaccination

CTCAE, Common Terminology Criteria for Adverse Events; FU, follow-up; mo, months; MRI, magnetic resonance imaging; NCI, National Cancer Institute; PFS, progression-free survival; OS, overall survival; PET, positron emission tomography; TMZ, temozolomide; V, vaccine

[1] cetuximab

[2] TMZ

[3] immunotherapy, consisting of DC vaccinations, after re-operation

[4] as described in text

[5] second-look operation for known residual tumor - re-operation for recurrence after 17 months

[6] TMZ and Gleevec-Hydrea

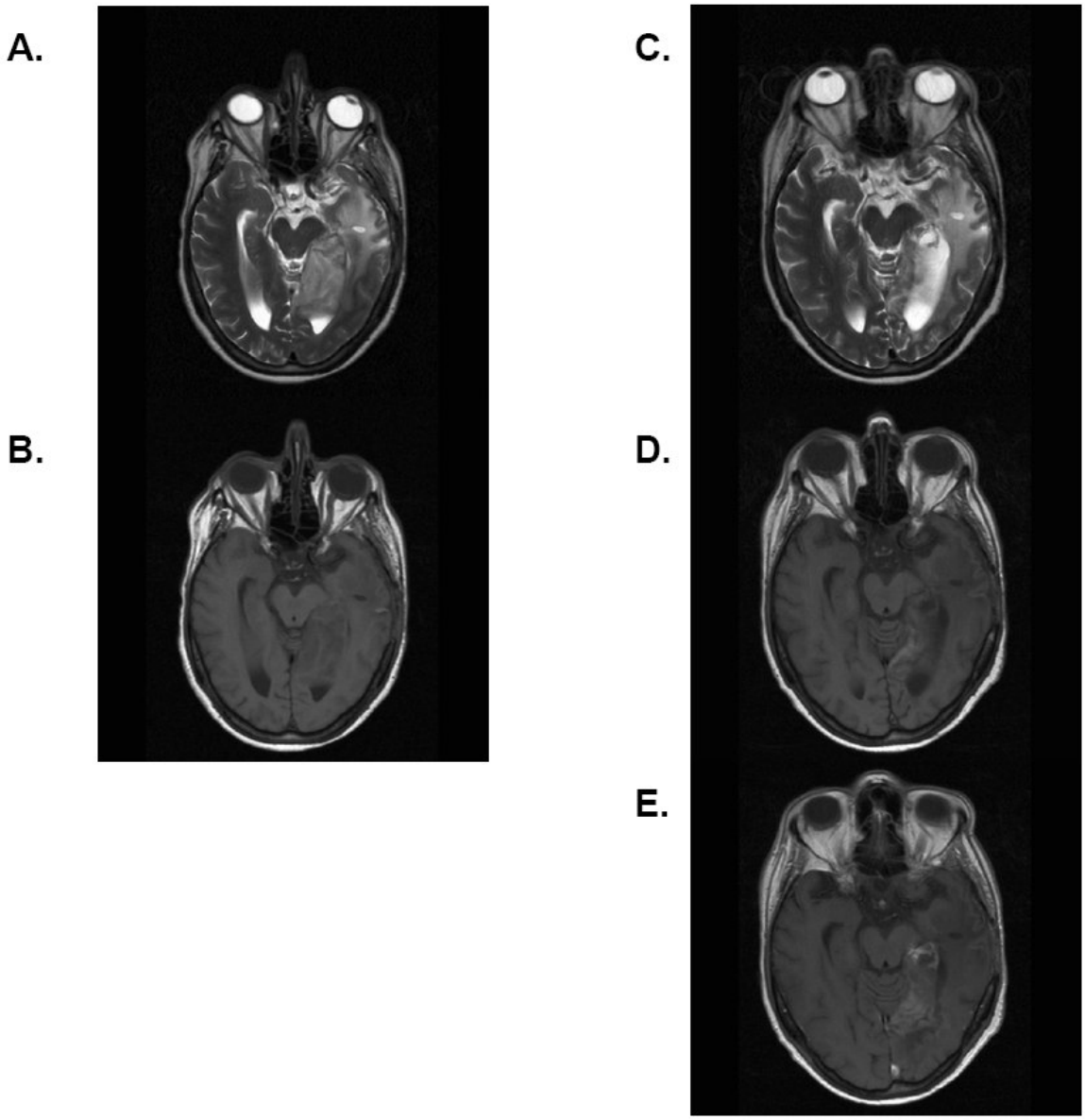


Fig. 5.2 Patient #5: MRI images at 8 and 11 months follow-up (FU)

Hyperintensities on T2 weighted images (A) and heterogenic signals on T1 weighted images (B) at the posteromedial side of the left temporal lobe, near the site of tumor resection, one day after sudden onset of right hemiplegia and aphasia (at 8 months FU). A repeat MRI 3 months after the described event shows the same changes at the posteromedial side of the left temporal lobe without any evolution; (C) T2 weighted images – (D) T1 weighted images – (E) T1 weighted images with gadolinium.

5.1.2.3 Quality of Life

The results of the QOL and disability assessments revealed that the patient-assessed KPS during the course of vaccination was equal to or exceeded 70 for 6 patients (median 85; range: 70 – 100) (reflecting a physical condition that enables the patient to care for him/herself). Patients #2 and #5 had KPS scores that varied between 50 and 60 (median 60). Moreover, for all patients the KPS remained stable or even tended to increase throughout the treatment phases.

The FMH score remained quite high as well during the course of vaccination: it exceeded 45 (median 52; range: 46 – 53) for all but one patient (#5), indicating that the patients were capable of most daily life activities and did not lose functional ability throughout the treatment phases. In patient #5 median FMH score was 36 (range 30: – 37).

Six out of 7 patients evaluated their general health (item 29) and overall QOL (item 30) on the EORTC QLQ-C30 questionnaire as good (median 5 for both items; range: 3 – 7 for both items) and these evaluations remained stable throughout the treatment phases. Only patient #2 had lower evaluations for general health (median 3; range: 2 – 4) and overall QOL (median 3; range: 3 – 4) during the course of vaccination.

5.1.2.4 Clinical assessment

The details of the clinical results are given in **Table 5.5**. Three patients (37.5%) are still alive at last FU, with median FU of 35 months (range: 34 – 44 months). The median OS for all patients is 24 months (range: 13 – 44 months). Two patients underwent a total resection of the tumor, in 6 patients the resection was subtotal. One patient is still free from progression or recurrence at the end of the FU period at 34 months. The median PFS in all patients is 18 months (range: 2 – 34 months). Using this therapeutic regimen, 6mo-PFS was demonstrated in 6 out of 8 patients (75%). In **Fig. 5.3**, Kaplan Meier curves are shown for PFS and OS.

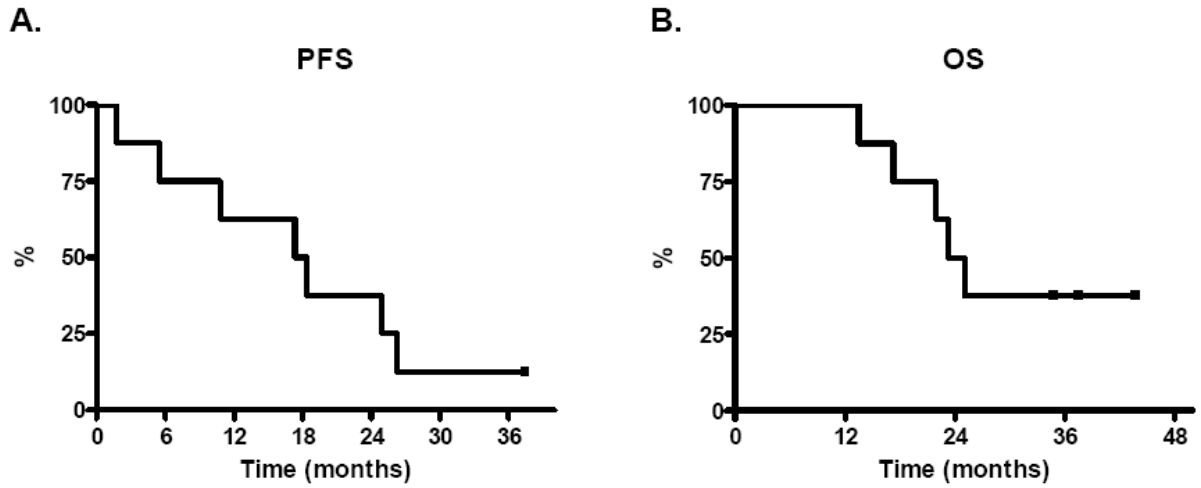


Fig. 5.3 Progression-free survival (PFS) and overall survival (OS)

Kaplan Meier curves are shown for PFS (A) and OS (B). The median OS for all patients is 24 months (range: 13 – 44 months). The median PFS for all patients is 18 months (range: 2 – 32 months). PFS at 6 months using this therapeutic regimen was demonstrated in 6 out of 8 patients (75%).

5.1.3 Discussion

We summarized our observations in 8 pilot patients with newly diagnosed GBM in whom vaccination with autologous DC loaded with autologous tumor cell lysate was fully integrated in the multimodal standard primary treatment, consisting of maximal safe neurosurgical resection, external beam radiotherapy and concomitant TMZ chemotherapy, and maintenance TMZ chemotherapy.

As shown in **Table 5.3**, DC characteristics and numbers varied considerably amongst patients. This is inherent to the nature of autologous cell therapy in an oncological patient population with highly variable baseline characteristics. Nevertheless, Liau *et al.*[65] did not find any argument for dose-related toxicity or efficacy in DC-based vaccination therapy.

It is important to note that there were no serious adverse events (NCI CTC grade IV) except for one patient in whom an ischemic event occurred during the vaccination therapy. This patient made a partial recovery and to which degree the ischemic event is related to the vaccination therapy remains a point of debate. Important though, is the fact that post-radiotherapy sequellae or inflammation could not be ruled out completely as no biopsy was performed. At one year FU the same patient had a status epilepticus which was controlled with fenytoin. Again, the relation of this event to the vaccination therapy is unclear and the status epilepticus may be linked to the possible ischemic event that the patient suffered earlier.

A favorable course of the patient's clinical symptoms was demonstrated even without the use of steroids, which makes an autoimmune reaction highly improbable. With respect to quality of life and disability assessment during treatment, one can conclude that 6 patients reported a physical condition that enabled them to take care for themselves and to perform daily life activities during treatment. Also, general health and overall quality of life were rated moderate to good by these 6 patients.

Three patients (37.5%) are still alive at last FU, with median FU of 35 months (range: 34 – 44 months). The median OS for all patients is 24 months (range: 13 – 44 months). Considering the fact that 7 patients were in RPA class IV and 1 patient in RPA class III, these preliminary data compare favorably to the RPA class-related survival estimates and RPA class-adjusted outcome in the EORTC26981/22981-NCIC CE3 Trial.[97] O^6-methylguanine-DNA methyltransferase (MGMT) promoter methylation was not assessed in these patients as it was not an in- or exclusion criterion. This warrants an even more cautious interpretation of these survival data.

5.2 HGG-2006 TRIAL

Based on our pilot trial in 8 newly diagnosed GBM patients (**Chapter 5.1**),[57] we designed the HGG-2006 phase I/II trial in which DC-based immunotherapy is integrated in a standardized fashion in the multimodal standard therapy for GBM, consisting of surgical resection, radiochemotherapy, and TMZm chemotherapy.

5.2.1 Patients and Methods

5.2.1.1 Sample size calculation

Comparison with the historical control group from the Stupp trial[3] is based on PFS. The power has been calculated to reject the null-hypothesis, using a Cox regression, of equal hazard for the historical control group and the intervention group. The alternative hypothesis has been phrased as one-sided, with alpha-level equal to 5%. The power has been calculated using a simulation study with 1,000 datasets. The power equals the percentage of the 1,000 drawn datasets for which the null-hypothesis is rejected. Note that since the data of the historical control group were not available, this dataset also needed to be simulated. It is

assumed that information for 244 patients will be available from the historical control group (excluding 15% of the patients, with partial resections or biopsy, from the 287 patients). In this control group, the recruitment period equals 21 months, with 45 months of study period. Each dataset is drawn using a set of assumptions: 1. An exponential distribution for the survival times and the drop-out times is assumed in both groups. 2. The expected dropout rate is based on the information from the historical control group (where 13% of the patients dropped out within a period of 45 months, with 21 months recruitment period). In the intervention group, 5% is assumed as expected dropout after 1 year. This dropout rate can be considered as a worst case scenario, since less dropout is expected than in the historical control group. 3. Patients enter the intervention group at a constant rate of 3.333 per month (thus 40 per year). Patients will be recruited during 18 months, with a minimal FU of 6 months. The statistical software SAS (version 9.1) has been used to program the simulation study, with PROC PHREG as procedure for the Cox regression.
With 60 recruited patients in the test group and assuming a 6mo-PFS of 54% and 70% in respectively the control and test group, the study has 91.9% power to detect a difference. Note, that assuming 68% 6mo-PFS instead of 70% in the test group, still yields a power of 86.5%.

5.2.1.2 Patient population

Seventy-seven patients (48 males and 29 females) presented with a newly diagnosed primary GBM, confirmed on central review histopathology. Patients were included for DC-based therapy, if they met the inclusion criteria (**Table 5.1**). Patients' characteristics are described in **Table 5.6**. Their median age was 57 years (range: 26 – 70 years). Patients were further subdivided according to the EORTC RPA classification for newly diagnosed GBM:[97] class III, 13 patients (median age 40 years; range: 26 – 47 years); class IV, 50 patients (median age 58 years; range: 30 – 70 years); class V, 14 patients (median age 62 years; range: 55 – 69 years). All patients were operated upon and were off steroids and nonsteroidal anti-inflammatory drugs at the time of leukapheresis (as determined by the exclusion criteria) and during vaccination. Approval by the local ethics committee was obtained as well as patients' written informed consent before the start of immunotherapy.

	All patients	RPA III	RPA IV	RPA V
Sex				
Female	29	7	20	2
Male	48	6	30	12
Age at surgery (y)	57 (26-70)	40 (26-47)	58 (30-70)	62 (55-69)
Resection				
Total	51	7	33	11
Subtotal	26	6	17	3
Site of tumor				
Right frontal	14	2	10	2
Right occipital	4	1	3	0
Right parietal	8	3	4	1
Right temporal	20	4	14	2
Right multilobular	5	0	2	3
Left frontal	5	0	5	0
Left occipital	4	0	2	2
Left parietal	5	1	3	1
Left temporal	9	1	5	3
Left multilobular	2	0	2	0
Bifrontal	1	1	0	0
Therapy				
ITT	77	13	50	14
AT	71	13	47	11
PP	39	11	25	3

Table 5.6 Patients' characteristics

AT, as-treated; ITT, intent-to-treat; PP, per protocol; RPA, recursive partitioning analysis. (Data from all the patients that were included in the trial were used for ITT analyses. Patients that received at least 3 induction vaccines and had started with radiochemotherapy were assigned as-treated. Patients that received every part of the outlined treatment, i.e. full-dose radiotherapy, concomitant and maintenance TMZ chemotherapy, 4 induction vaccines and 4 boost vaccines, were assigned treated per protocol)

5.2.1.3 Assessment of extent of tumor resection before vaccination

Extent of tumor resection was defined as described in **Chapter 5.1.1.2**.

5.2.1.4 Tumor cell lysate

Tumor cell lysate was generated as described in **Chapter 4.1.3**.

5.2.1.5 Preparation of autologous DC

In all patients PBMC were obtained from leukapheresis and kept frozen in liquid N_2 until use. DC vaccines were prepared as described in **Chapter 4.1.4**.

5.2.1.6 Treatment schedule: fully integrated radio-chemo-immunotherapy

The treatment schedule was the same as in the pilot trial, as described in **Chapter 5.1.1.5** (**Fig. 5.1**). At the time of progression, possible rescue therapy was at the physician's discretion.

5.2.1.7 Vaccination

Vaccination was performed by intradermal injection of 0.12 – 27.5 $x10^6$ (median 2.6 $x10^6$) DC per lymph node region in the upper third of the arms (left and right) at weeks 1, 2, 3 and 4. Further boost vaccines were given with tumor cell lysate with a median of 1,500 µg (range: 400 – 1,500 µg) proteins per vaccine injected in two syringes each containing a final volume of 400 µL.

5.2.1.8 Patient assessment

All patients were assessed as described in **Chapter 5.1.1.7**. Survival was calculated as time from leukapheresis to death from cancer or any other cause. Within 2 to 5 weeks after the histological diagnosis of GBM, leukapheresis took place. Radiochemotherapy had to start within one week after leukapheresis. Statistical analysis was done by the log rank test on Kaplan Meier survival estimates.

Data from all the patients that were included in the trial were used for ITT analyses. Patients that received at least 3 induction vaccines and had started with radiochemotherapy were assigned as-treated. Patients that received every part of the outlined treatment, i.e. full-dose radiotherapy, concomitant and maintenance TMZ chemotherapy, 4 induction vaccines and 4 boost vaccines, were assigned treated per protocol.

MGMT-expression of the tumor was determined for in-hospital patients. The techniques used were standard: Methylation-Specific Polymerase Chain Reaction after DNA bisulfite modification**.** MGMT promoter methylation is correlated with improved PFS and OS in

patients treated with alkylating agent chemotherapy (TMZ) and might even be a general favorable prognostic factor in GBM patients.[9,132-134]

Adverse events were graded according to the NCI CTC.

Adverse Events	No. of patients	Grade according to NCI CTCAE (version 3.0)
Fatigue	34	I
General rash/itching	4	I
Shoulder pain	2	I
Anorexia	2	I
Myalgia	1	I
Nausea / vomitus	11	I-II
Memory impairment	5	I-II
Epileptic seizure	13	II
Confusion	3	II
Humerus fracture	3	II
Lethargy	2	II
Bleeding (ectopic cerebral lesion)	1	II
Depression	1	II
Dysphasia	1	II
Oesophagitis	1	II
Otitis media serosa	1	II
Epileptic seizures	**5**	**III**
Allergic reaction on TMZ	**1**	**III**
Cerebrall abscess (surgery required)	**1**	**III**
DVT	**1**	**III**
Hydrocephalus (surgery required)	**1**	**III**
Ischemic bowel perforation	**1**	**III**
Lung- and peripheral edema	**1**	**III**
Osteoporotic D10 fracture	**1**	**III**
Dementia (Alzheimer)	**1**	**III-IV**
Focal status epilepticus	**2**	**IV**
Ischemic stroke (full recovery)	**1**	**IV**
Status epilepticus	**2**	**IV**
Overwhelming infection	**1**	**V**
Hematotoxicity		
lymphopenia	17	I
leukopenia	16	I
lymphopenia	7	II
leukopenia	5	II
lymphopenia	**12**	**III**
thrombopenia	**2**	**III**
leukopenia	**1**	**III**
thrombopenia	**3**	**IV**
lymphopenia	**1**	**IV**

Table 5.7 Adverse events

CTCAE, Common Terminology Criteria for Adverse Events; DVT, deep venous thrombosis; NCI, National Cancer Institute; No., number; TMZ, temozolomide

5.2.2 Results

5.2.2.1 Feasibility: vaccines preparation and characterization

The patients received a median of 8 vaccines (range: 0 – 17 vaccines). The median yield of loaded mature DC from freshly isolated PBMC was 5.2 x10^6 per vaccination session (range: 0.24 – 55 x10^6; n = 290).

5.2.2.2 Toxicity

Adverse events are described in **Table 5.7**. Thirty-eight serious adverse events (NCI CTC grade III, IV and V) were reported in 30 patients (39%), including 19 hematological adverse events (hematotoxicity) in 18 patients (23%). One patient died (NCI CTC grade V) due to an overwhelming infection in the postoperative period, even before radiochemotherapy or vaccination was started.

5.2.2.3 Clinical assessment

The details of the clinical results are given in **Table 5.8**: FU period, PFS, OS and 6mo-PFS. Analyses were done based on the ITT patients (n = 77), the as-treated patients (n = 71) and the patients treated per protocol (n = 39). **Table 5.9** depicts the different types of rescue therapy that were installed at time of progression.

	PFS (mo)	6mo-PFS (%)	OS (mo)	Alive / Dead	FU survivors (mo)	MGMT (methylated/unmethylated)
ITT	10.4 (1.0-37.7)	70	18.3 (1.3-42.2)	31 / 46	25.0 (10.5-42.2)	29 / 19
AT	11.0 (1.7-37.7)	76	19.4 (3.9-42.2)	30 / 41	25.7 (10.5-42.2)	27 / 18
PP	20.4 (2.3-37.7)	100	nyr (10.26-39.67)	25 / 14	26.8 (10.5-37.7)	15 / 7

Table 5.8 Clinical results

Median values and ranges are given for PFS, OS and FU.

AT, as-treated; FU, follow-up; ITT, intent-to-treat; MGMT, O^6-methylguanine-DNA methyltransferase; mo, months; nyr, not yet reached; OS, overall survival; PFS, progression-free survival; PP, per protocol; 6mo-PFS, PFS at 6 months

Rescue therapy	No. of patients
Surgery	11
TMZ	8
PCV	5
HGG-IMMUNO-2003 trial	3
Gleevec/Hydrea	2
Cediranib (Regal trial)	2
Radiosurgery	2
Carboplatin-Etoposide	1

Table 5.9 Rescue therapy at time of progression
No., number; PCV, procarbazine-lomustine-vincristine; TMZ, temozolomide

Thirteen patients (17%) were assigned to RPA class III, 50 patients (65%) to class IV and 14 patients (18%) to class V. Eleven patients (85%) in RPA class III were treated per protocol, 25 patients (50%) in class IV and 3 patients (21%) patients in class V.

A total resection of the tumor was performed in 51 of the 77 patients (66%). According to RPA classification, total resection was performed in 54%, 66% and 79% of patients for classes III, IV and V respectively. Of the 51 patients with a total resection, 7 patients (13%) belonged to RPA class III, 33 patients (65%) to class IV and 11 patients (22%) to class V. Of the 26 patients with a subtotal resection, 6 patients (23%) belonged to RPA class III, 17 patients (65%) to class IV and 3 patients (12%) to class V.

Grade of resection was taken into account as prognostic variable, and further analyses based on the EORTC RPA classes were done as well. In **Fig. 5.4**, Kaplan Meier curves are shown for PFS and OS based on ITT analysis. **Fig. 5.5** and **Fig. 5.6** show Kaplan Meier curves for PFS and OS, based on RPA classification and grade of resection respectively (ITT analysis).

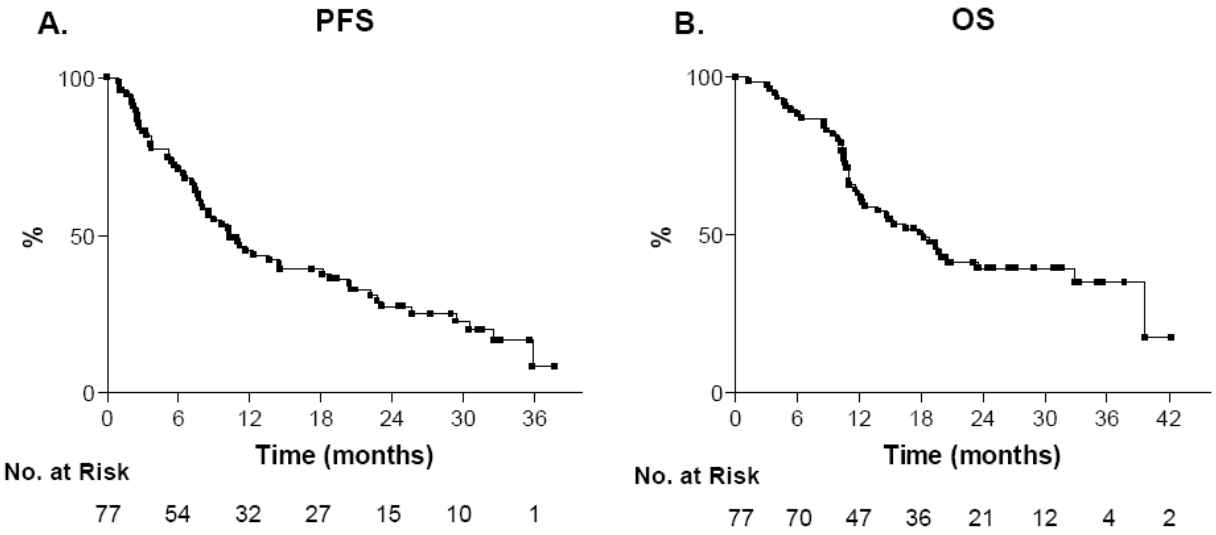


Fig. 5.4 Progression-free survival (PFS) and overall survival (OS) based on intent-to-treat (ITT) analysis
Median PFS (A) and median OS (B) were 10.4 and 18.3 months respectively.

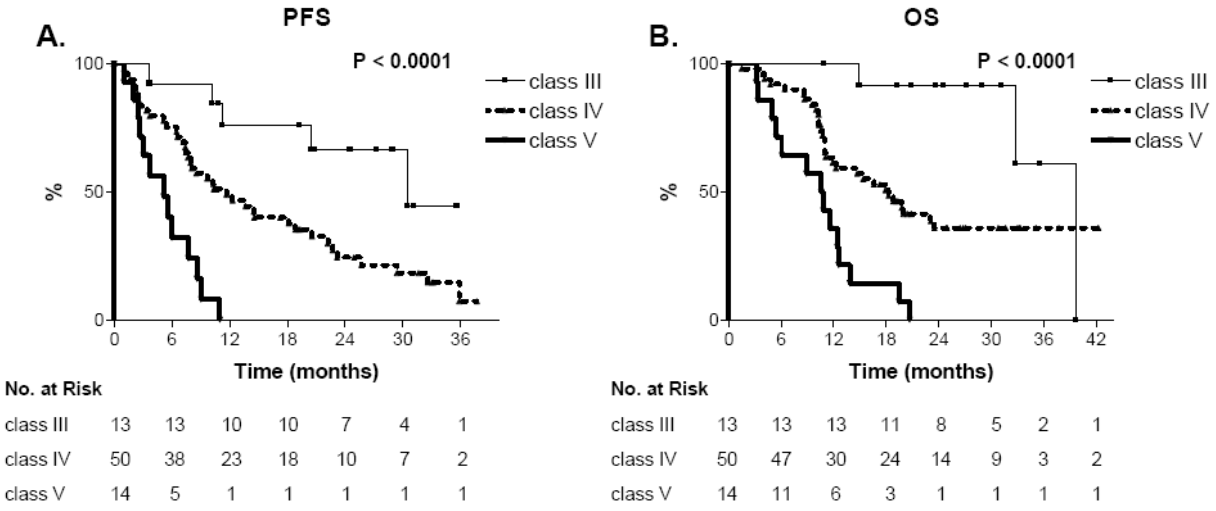


Fig. 5.5 Progression-free survival (PFS) and overall survival (OS) based on RPA classification

Data depicted are based on ITT analysis. Median PFS (A) based on RPA classification was 30.5, 11.7 and 5.2 months for classes III, IV and V respectively. 6mo-PFS was 92%, 72% and 43% for the same classes respectively. Median OS (B) was 39.7, 18.3 and 10.7 months for RPA classes III, IV and V respectively. (ITT, intent-to-treat; RPA, recursive partitioning analysis; 6mo-PFS, PFS at 6 months).

MGMT-status was determined in 48 of the 77 patients (62%). Unmethylated MGMT-status was found in 19 patients, and 29 patients had a methylated MGMT promoter (**Table 5.8**). Of the 29 patients with a methylated MGMT-status, 6 patients had a weak methylation of the MGMT promoter. This can be interpreted in a dual way: 1. only part of the tumor cells have methylation of the MGMT promoter; 2. all tumor cells have a methylated promotor of MGMT and the results reflect merely the high content of non-tumoral cells in the sample. The second option seems to be more probable and therefore we interpreted 'weak methylation' as 'methylated MGMT-status' for analysis. **Fig. 5.7** shows Kaplan Meier curves for PFS and OS, based on MGMT-status.

In 32 of the 51 patients with a total resection (63%), and in 16 of the 26 patients with a subtotal resection (62%), MGMT-status could be determined. 17 (53%) and 12 (75%) patients had a methylated promoter of MGMT, for the subgroups of total and subtotal resection respectively. Unmethylated MGMT-status was found in 15 (47%) and 4 (25%) patients, for both subgroups respectively.

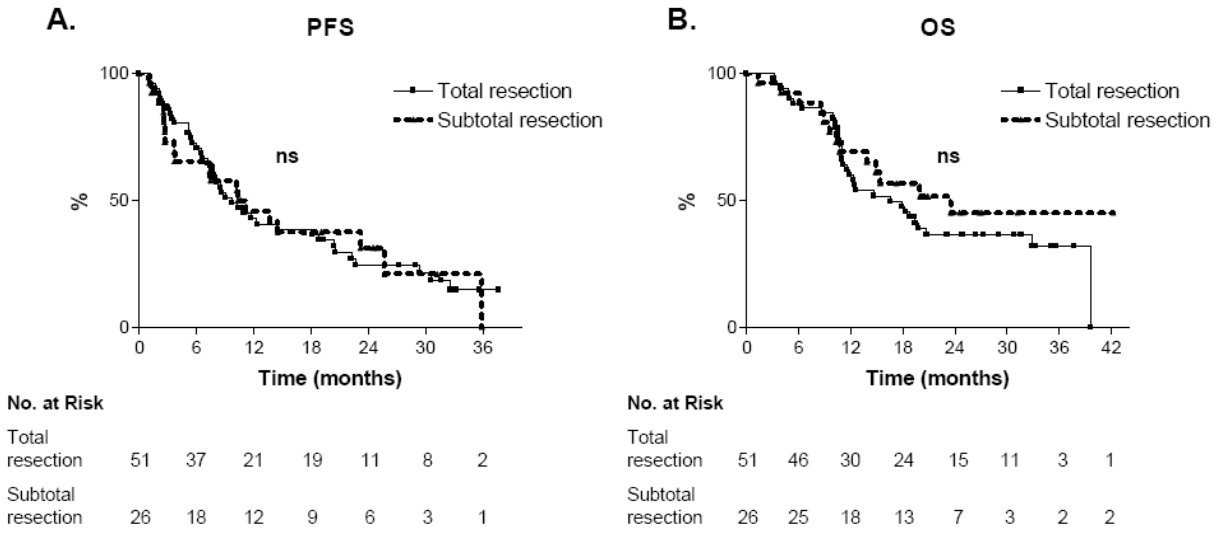


Fig. 5.6 Progression-free survival (PFS) and overall survival (OS) based on grade of resection

Data depicted are based on ITT analysis. Median PFS (A) was 9.7 and 10.8 months for total and subtotal resection respectively. Median OS (B) was 16.6 and 23.5 respectively (ITT, intent-to-treat; ns, not significant; RPA, recursive partitioning analysis; 6mo-PFS, PFS at 6 months).

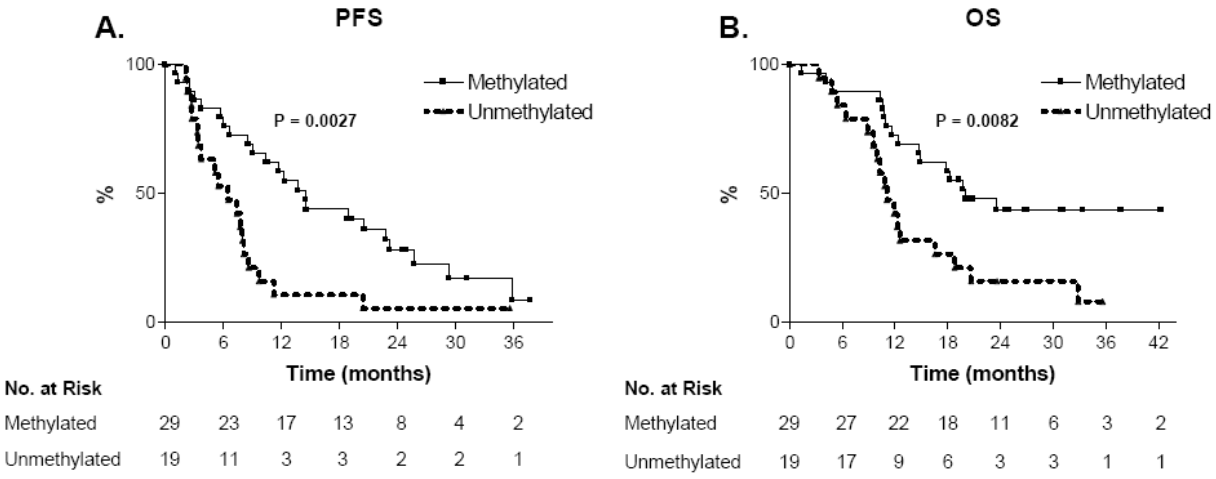


Fig. 5.7 Progression-free survival (PFS) and overall survival (OS) based on MGMT-status

Data depicted are based on ITT analysis. Median PFS (A) was 14.5 and 6.5 months for methylated and unmethylated MGMT-status respectively. Median OS (B) was 20.0 and 11.1 months for methylated and unmethylated MGMT-status respectively (ITT, intent-to-treat; MGMT, O^6-methylguanine-DNA methyltransferase).

5.2.3 Discussion

Based on the results in the 8 pilot patients (**Chapter 5.1**),[57] we fully integrated autologous DC-based tumor vaccination in the multimodal standard primary treatment, consisting of maximal safe neurosurgical resection, external beam radiotherapy and concomitant TMZ chemotherapy, and TMZm chemotherapy, in 77 patients with newly diagnosed GBM.[3]

Median OS based on ITT analysis was 18.3 months from leukapheresis, which compares favorably to the survival data reported by Stupp *et al.* with a median OS of 14.6 months.[3] More recent studies, with other new treatment strategies for GBM (e.g. Talampanel, poly-ICLC, or cilengitide treatment with standard radiochemotherapy, or 5-aminolevulinic acid guided glioma resection),[135-137] point to a median OS in the range of 18-20 months, in line with our findings. However, as final step in the clinical development of DC-based tumor vaccination, a prospective randomized clinical trial, which has recently started (HGG-2010 trial), is needed to confirm the possible beneficial activity of immunotherapy integrated in the primary multimodal treatment for patients with GBM.

Based on ITT analysis, 6mo-PFS was 70.1%: as such, this result coincided with the assumption in our power analysis (**Chapter 5.2.1.1**). When we take RPA classification into account, 65% of the patients belonged to RPA class IV, 18% to class V and 17% to class III. As one could expect, outcome improved significantly with lower RPA classification. Median OS was 39.7, 18.3 and 10.7 months for classes III, IV and V respectively. These data compare favorably to the RPA class-related survival estimates and RPA class-adjusted outcome in the EORTC 26981/22981-NCIC CE3 Trial,[97] in which median OS in the radiotherapy / TMZ arm of the study was 21.4, 16.3 and 10.3 months for the respective classes. In our study, 6mo-PFS was 92%, 72% and 43% for RPA classes III, IV and V respectively. This shows on the one hand that the prognostic factors used for the EORTC RPA classification also hold true for this new treatment regimen including tumor vaccination. On the other hand, tumor vaccination integrated into standard radiochemotherapy seems to improve survival, especially in the patients belonging to RPA class III.

Patients that were treated per protocol had a better clinical outcome than as-treated patients and even more so than ITT patients. This is only logical, reflecting a positive selection of patients receiving the full treatment regimen. In the same line of thought, more patients in RPA class III were treated per protocol as compared to classes IV and V (85%, 50% and 21% respectively).

Surprisingly, no clear benefit of total resection over subtotal resection was seen in this group of patients, although grade of resection was a strong, independent predictor of the outcome in the recurrent HGG patients that were vaccinated.[59] This might be explained by the fact that in the subgroup of patients with subtotal resections, other prognostic factors were more favorable; of the patients for whom MGMT-status could be determined, 75% had a methylated MGMT-promoter in the subgroup of subtotal resection, as compared to only 53% in the subgroup of total resection. Also, for the patients with subtotal resections, more patients belonged to lower RPA classes as compared to patients with total resections. One could, on the other hand, hypothesize that the impact of grade of resection in recurrent HGG patients treated with immunotherapy is greater due to the fact that the residual tumor cells in these patients have proven themselves to be resistant to chemo- and radiotherapy. Thus, these residual recurrent tumor cells might be more aggressive than the 'original' tumor cells. A subtotal resection in recurrent GBM would therefore result in a more aggressive residual tumor as compared to a subtotal resection in newly diagnosed GBM.

Based on MGMT-status one can divide the patient population into two subgroups; patients with a methylated MGMT-status had a significantly better PFS and OS as compared to patients with an unmethylated status. These results confirm that methylation of the MGMT promoter is a favorable prognostic factor in GBM.[9,132-134] Of the 48 patients in whom MGMT-status was determined, 29 patients (60%) had a methylated MGMT promoter. This percentage is higher than in the EORTC 26981/22981-NCIC CE3 Trial.[132] In that trial, the MGMT promoter was methylated in 45% of the patients treated with radio- and chemotherapy, and in whom MGMT promoter methylation status could be determined. However, according to the literature, MGMT methylation frequency in newly diagnosed GBM patients, varies from 19% tot 68% as determined by Methylation-Specific Polymerase Chain Reaction.[138]

Thirty-eight serious adverse events (NCI CTC grade III, IV and V) were reported in 30 patients (39%), including 19 hematological serious adverse events (hematotoxicity) in 18 patients (23%). Hematological adverse events were most likely the result of concomitant and maintenance TMZ therapy. However, Stupp *et al.* reported grade III or IV hematological toxic effects in only 16% of patients.[3] One patient died (NCI CTC grade V) due to an overwhelming infection in the postoperative period, even before radiochemotherapy or vaccination was started. This death was therefore not related to the treatment regimen.

5.3 GENERAL DISCUSSION

The integration of immunotherapy within the standard postoperative therapy for patients with a newly diagnosed GBM is based on the presumed mutually beneficial effect of the conventional treatment strategies and immunotherapy. Each aspect of the presented concept is believed to play a major role in the global results of this approach. First of all, maximal safe surgery is performed to induce a state of minimal residual disease as a starting point for the following therapies. Not only has it been shown that the extent of resection has a major impact on the benefit of postoperative radiochemotherapy in GBM,[5] but extent of resection is a strong, independent predictor of the outcome for patients with relapsed malignant glioma, treated with postoperative, adjuvant DC vaccination.[59] However, this was not the case for newly diagnosed GBM patients treated with immunotherapy.

Many antitumoral strategies, such as radiotherapy, kill tumor cells by apoptosis and the resulting apoptotic bodies form a good source of cross-presented antigens, which might further lead to cross-priming of T cells in an appropriate pro-inflammatory environment. In 1984, North *et al.*[127] already showed an elimination of local regulatory T cells (*avant la lettre*) in irradiated brain tumor areas. Recently, Kjaergaard *et al.*[80] showed that long-term survivors in a murine brain tumor model only occurred in the group of animals that received both radiotherapy and DC-based vaccines. The data showed an irradiation-induced upregulation of MHC molecules on tumor cells, thereby making them better targets for CTL.

TMZ chemotherapy during radiotherapy might be of benefit for the subsequent immunotherapy. First, chemotherapy affects the size of the lymphocyte pool slightly, thereby allowing thymic-independent antigen-driven T cell regeneration within the context of T cell homeostasis.[126,129] The concept of tumor-specific immunization at the time of immune reconstitution after chemotherapy has been demonstrated in several animal models. [126,129] Specifically of importance to a regimen with 6 weeks TMZ, it has been shown that TMZ affects the attraction of tumor-specific Treg cells into the tumor cavity by blocking CCL2 production by the tumor cells.[139] Although a relative enrichment of Treg cells in peripheral blood has been described at the end of radiochemotherapy, whilst the total CD4+ T cell population is diminished,[43] one might assume that the pro-inflammatory condition, induced by radiotherapy,[140] counteracts Treg cell functionality in the periphery.[141,142]

Immunotherapy itself can increase the sensitivity of GBM tumor cells to chemotherapeutics like TMZ. Wheeler *et al.*[119] already suggested an increased susceptibility of GBM to

chemotherapy in patients pre-exposed to DC based vaccination. Their group even published on a presumed molecular mechanism of this synergy based on preferential targeting of tyrosin related protein 2, a chemoresistance mechanism, by cytotoxic T cells as an explanation.[120] The idea of combining these treatment modalities is not restricted to neuro-oncology.[122,123] Recently, Masucci *et al.*[121] showed an advantage of combining TMZ with restorative immunotherapy using IL-2 in melanoma patients.

Overall, it is hypothesized that the combination of radiotherapy, chemotherapy and immunotherapy could potentiate the cumulative antitumoral activity, when applied in a well designed strategy. In this chapter, we advocate such a strategy of autologous DC-based immunotherapy as add-on therapy, but fully integrated in the multimodality treatment of surgery, radiotherapy and chemotherapy in patients with newly diagnosed GBM. For this radio-chemo-immunotherapy, we provide feasibility data in terms of clinical responses, as well as an acceptable QOL.

The use of boost lysates after the first 4 induction vaccines with loaded DC, is based on the data from Jouanneau *et al.*.[143] They showed that multiple vaccinations with DC were efficient to induce an immune response in an orthotopic mouse model, but did not elicit optimal long-term survival. In contrast, injection of DC for priming, followed by boosts with tumor cell lysate alone, generated the most effective antitumoral effects (CTL and humoral responses). Also, we have shown in relapsed GBM patients that vaccination with boost lysates can result in substantial numbers of long-term survivors after relapse.[59]

CHAPTER 6. IMMUNE MONITORING

6.1 CD127dim EXPRESSION AS Treg CELL MONITORING TOOL IN VACCINATED GLIOMA PATIENTS [a]

Breaking immunological tolerance to cancer, in this case HGG, is a difficult process that is counteracted by many immunosuppressive mechanisms.[144] It has been clearly demonstrated, both in human and rodent studies, that Treg cells play an important role in tumor immunology, more specifically in tumor immune escape mechanisms. They are very potent suppressors of endogenous and induced antitumoral immune responses and can hence be detrimental to immunotherapy.[12,43,83,84,145] Up till now, the best marker for Treg cells is a unique transcription factor, termed Foxp3 (forkhead box p3).[146] Importantly though, as Foxp3 is an intracellular protein, it cannot be used to separate human Treg cells for functional studies or *in vivo* expansion for cellular therapy, thereby limiting its use in the human setting. However, recently Liu *et al.*[147] showed that IL-7 receptor alpha subunit (CD127) expression inversely correlates with Foxp3 expression and suppressive function of human CD4+ Treg cells in patients with longstanding type 1 diabetes mellitus.
Our aim was to validate CD127 as a reliable flow cytometric marker for Treg cells in HGG patients treated with DC vaccination in an immune monitoring assay.

6.1.1 Material and Methods

6.1.1.1 Subjects

Blood samples were obtained from 4 healthy subjects and 11 HGG patients who were treated with immunotherapy.[59] In the patients, blood samples were taken at different points during their vaccination schedule. Approval by the local ethics committee was obtained, as well as patients' written informed consent before the start of immunotherapy.

[a] Ardon H, Verbinnen B, Maes W, Beez T, Van Gool S, De Vleeschouwer S. Technical advancement in regulatory T cell isolation and characterization using CD127 expression in patients with malignant glioma treated with autologous dendritic cell vaccination. J Immunol Methods 2010;352:169-173.

6.1.1.2 Antibody staining and FACS analysis

PBMC were isolated from blood samples by Lymphoprep (Lucron Bioproducts BVBA, Sint Martens-Latem, Belgium) density gradient centrifugation and stored in liquid nitrogen in 20% HSA containing 10% dimethylsulfoxide. For each experiment part of the PBMC was thawed, washed and resolved in RPMI 1640 medium containing 10% human AB serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Lonza, Verviers, Belgium). The following mAb were used for staining and sorting: PE-conjugated anti-CD127 (hIL-7R-M21), allophycocyanine (APC)-conjugated anti-CD25 (M-A251), peridinin chlorophyll protein (PerCP)-conjugated anti-CD4 (SK3). Mouse IgG1 was used as isotype control staining. All mAb were purchased from Becton Dickinson (BD Biosciences, Erembodegem, Belgium). Alexa Fluor® 488-conjugated anti-Foxp3 (PCH101) was purchased from eBioscience (eBioscience Inc., San Diego, CA, USA). Staining was performed according to the manufacturer's instructions and modified as follows: 1-2 x10^5 PBMC per sample were suspended in 100 µL staining buffer. Non-specific staining was blocked with human AB serum and cells were subsequently surface-stained with 5 µL fluorescence-conjugated specific mAb. Cells were gently mixed and incubated for 30 min at 4°C. After washing of the cells with PBS containing 0.5% bovine serum albumin, cells were fixated for 60 min using 1 mL Fix/Perm buffer. Afterwards, cells were permeabilized in Perm buffer in two washing steps. Non-specific intracellular staining was blocked with human AB serum and cells were subsequently stained with Alexa Fluor® 488-conjugated anti-Foxp3 (eBioscience). After 30 min incubation at 4°C, cells were washed twice with Perm buffer and then resuspended in 200 µL fixation buffer. Acquisition was performed on a FacsCanto flow cytometer (BD Biosciences) with Cellquest analysis software.

6.1.1.3 Isolation of CD4+CD127hi and CD4+CD127dim cells

Human CD4+ T cells were isolated from PBMC by negative selection (indirect magnetic labeling) using the MACS® CD4+ T cell isolation kit II from Miltenyi Biotec (Miltenyi Biotec B.V., Utrecht, The Netherlands), according to the manufacturer's instructions and modified as follows: total PBMC were counted, washed and resuspended in 40 µL buffer per 10^7 cells. 10 µL biotin-antibody cocktail was added per 10^7 cells. Cells were mixed and incubated for 10 min at 4°C. Then 30 µL buffer and 20 µL anti-biotin microbeads per 10^7 cells were added. Cells were mixed again and incubated for an additional 15 min at 4°C.

Next, cells were washed once and resuspended in 500 µL buffer per 10^8 labeled cells. Magnetic separation was done using a MACS® Separator and LD Column (Miltenyi Biotec). The effluent was collected, representing the enriched CD4+ T cells (94±1.4% purity by FACS).

Next, CD4+ cells were counted, washed and resuspended in 100 µL buffer per 10^7 cells. 5 µL anti-CD127 PE (BD Biosciences) was added, cells were mixed and incubated for 30 min at 4°C. After washing once, cells were resuspended in 80 µL buffer per 10^7 cells and 20 µL anti-PE microbeads (Miltenyi Biotec) was added. Cells were mixed again and incubated for an additional 15 min at 4°C. After incubation, cells were washed once and resuspended in 500 µL buffer per 10^8 labeled cells. Magnetic separation was done using a MACS® Separator and LD Column (Miltenyi Biotec). The effluent was collected, representing the enriched CD4+CD127dim T cells (77±3.2% purity by FACS). Retained cells were eluted outside the magnetic field, representing magnetically labeled CD4+CD127hi cells (94±1.7% purity by FACS). All steps were performed on ice and cold buffer was used in every step.

6.1.1.4 Suppression assays

Suppression assays were performed as mixed lymphocyte reactions (MLR) in flat-bottom 96-well culture plates, using PBMC from both a healthy subject and 3 HGG patients. 2 x10^5 responder PBMC were stimulated with 2 x10^5 allogeneic mitomycin-treated PBMC. MACS-sorted CD4+CD127hi or CD4+CD127dim cells from the same source as responder PBMC were added at different ratios: 6 x10^4 (1:1), 3 x10^4 (1/2:1) or 1.5 x10^4 (1/4:1) cells. Cells were cultured in a final volume of 200 µL medium per well. Culture medium was RPMI 1640 supplemented with 10% human AB serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Lonza). T cells were incubated for 7 days at 37°C in 5% CO_2. Cultures were pulsed with 1 µCi [^{3}H]thymidine/well and harvested 16 h later. Triplicate cultures were set up for every condition tested. [^{3}H]thymidine incorporation was measured with a Tri-Carb® 2100TR Liquid Scintillation Counter (PerkinElmer Life Sciences, Inc., Zaventem, Belgium) and results are expressed as counts per minute.

6.1.1.5 Statistical analysis

Data are represented as mean ± standard error of the mean. The Pearson correlation coefficient (r) was obtained through linear regression analysis. Statistics were calculated with Prism software 4.0a (GraphPad Software Inc., San Diego, CA, USA).

Considering CD127dim status of the cell as a positive test result and Foxp3+ status as the golden standard for the Treg status of that cell, sensitivity, specificity, type I and II errors, accuracy, and positive and negative predictive values were calculated: CD127dimFoxp3+, true positive; CD127dimFoxp3-, false positive; CD127hiFoxp3+, false negative; CD127hiFoxp3-, true negative. 95% confidence intervals (95% c.i.) of all correlation indices are provided.

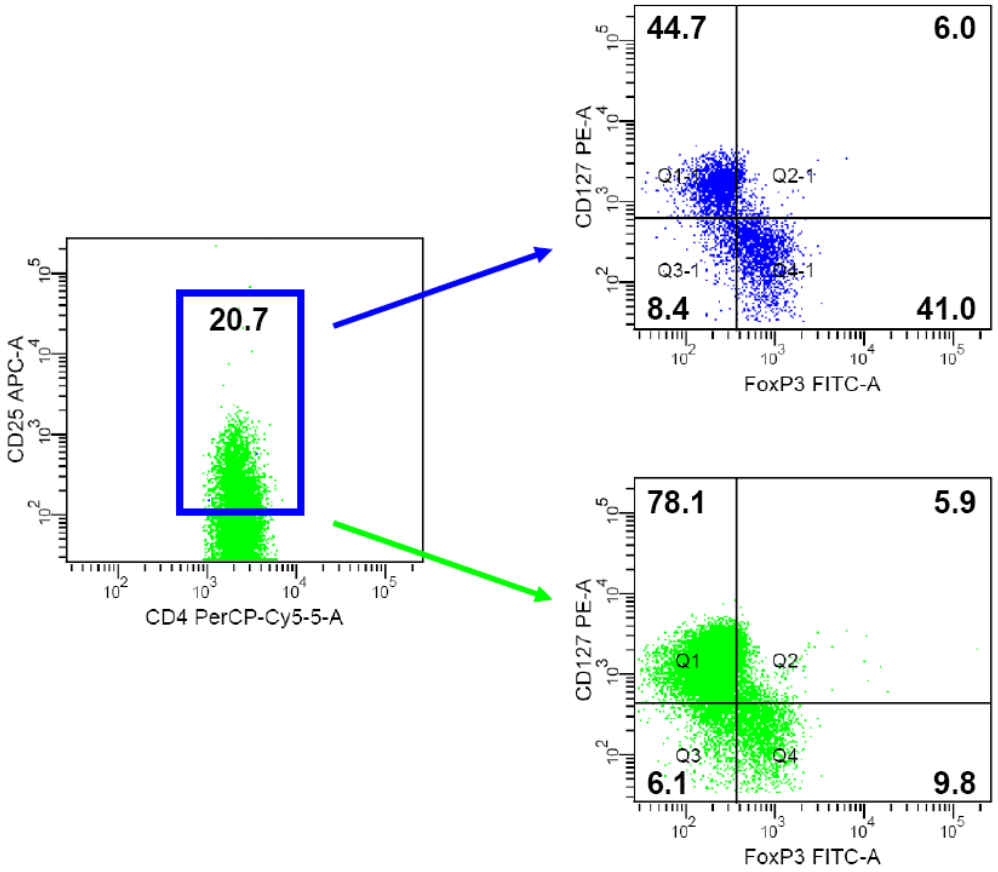


Fig. 6.1 Flow cytometric analysis of CD4+ and CD4+CD25+ cells

Two distinct populations are evident in both CD4+ (lower right scatter plot) and CD4+CD25+ cells (upper right scatter plot): CD127dimFoxp3+ versus CD127+Foxp3- cells. Representative scatter plots are shown (n = 15). Expression of CD4 and CD25 was assessed on total peripheral blood mononuclear cells (a lymphocyte gate was used). Expression of Foxp3 and CD127 was assessed in CD4+ and CD4+CD25+ gated cells.

6.1.2 Results

6.1.2.1 CD127 expression inversely correlates with Foxp3 expression in CD4+ and CD4+CD25+ cells

FACS analyses on PBMC from the healthy subjects (n = 4) and HGG patients treated with immunotherapy (n = 11) were conducted to determine the relationship between CD4, CD127, Foxp3 and CD25, using multi-parameter flow cytometry (**Fig. 6.1**). Within the CD4+ population, and even more clearly within the CD4+CD25+ cell population, two distinct subpopulations could be distinguished based on CD127 and Foxp3 expression: Foxp3+ cells displaying CD127dim expression (35.3±2.6%) and Foxp3- cells displaying CD127hi expression (43.7±2.3%). The sensitivity and specificity of CD127dim expression for Treg cells within the CD4+CD25+ population were 74.3% (95% c.i. 68.5-80.0) and 79.2% (95% c.i. 75.4-83.0) respectively. Type I error (false positive) and type II error (false negative) were 11.5% (95% c.i. 9.1-13.9) and 10.9% (95% c.i. 8.7-13.1) respectively. Accuracy of the CD127dim test was 77.6%, positive predictive value 74.6% and negative predictive value 80.1%. We found a significant positive correlation between Foxp3 expression and CD127dim expression both in the CD4+ and CD4+CD25+ population (**Fig. 6.2**).

6.1.2.2 CD4+CD127dim cells are potent suppressors of allogeneic MLR

Next, we wanted to check the suppressive activity of the CD4+CD127dim cells to confirm their Treg cell status. Therefore, we examined the ability of CD4+CD127dim T cells versus CD4+CD127hi T cells to suppress an allogeneic MLR. The results showed that the CD4+CD127dim T cell subset suppressed the MLR in a dose-dependent manner, in contrast to CD4+CD127hi T cells (**Fig. 6.3**).

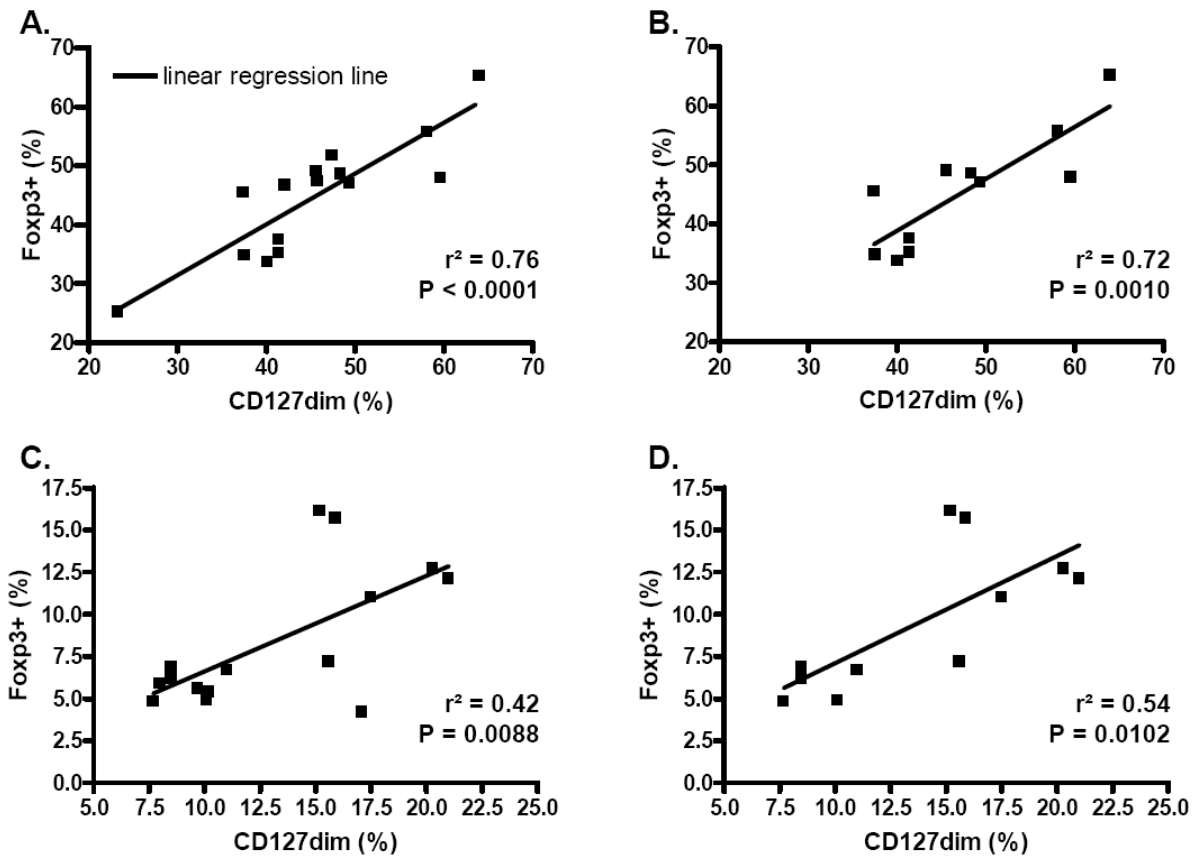


Fig. 6.2 Correlation between CD127dim and Foxp3 expression

Graphs showing a positive correlation between CD127dim expression and Foxp3 expression in different cell populations: A. CD4+CD25+ cells (all samples; *n* = 15); B. CD4+CD25+ cells (patients' samples; *n* = 11); C. CD4+ cells (all samples; *n* = 15); D. CD4+ cells (patients' samples; *n* = 11). The Pearson correlation coefficient (r) was obtained through linear regression analysis. The 95% confidence intervals are 0.64-0.96, 0.50-0.96, 0.21-0.87 and 0.24-0.93 for figures A, B, C and D respectively.

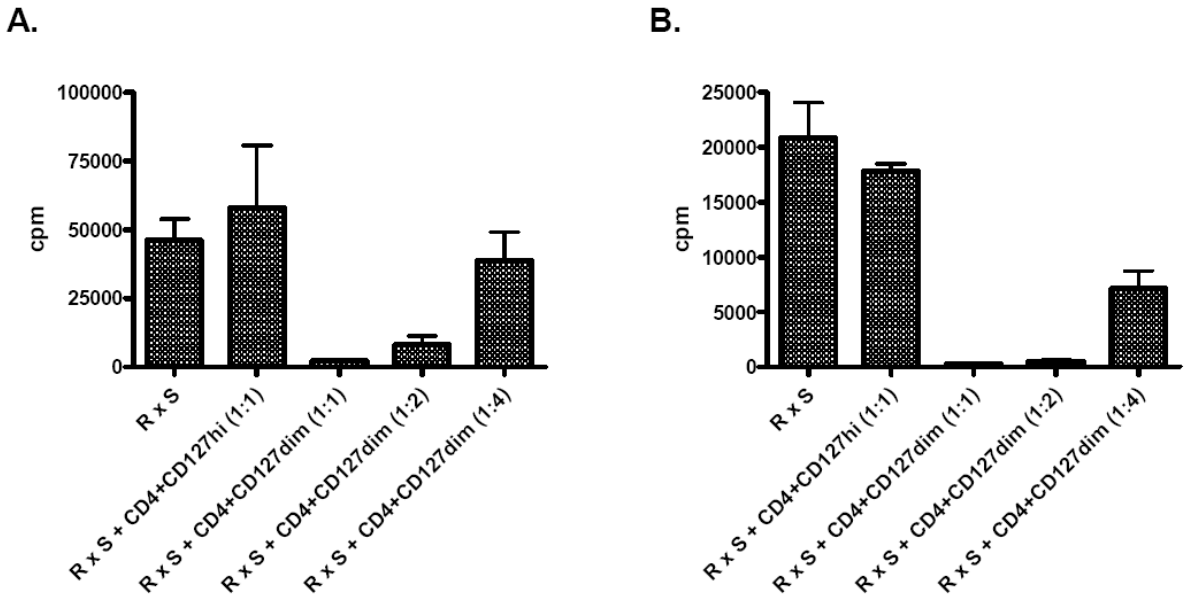


Fig. 6.3 Suppressive function of CD4+CD127dim cells in allogeneic MLR

Graphs showing dose-dependent suppressive function of CD4+CD127dim cells: A. graph of one experiment in a healthy subject; B. graph representative of 3 experiments in HGG patients. 2 $x10^5$ responder peripheral blood mononuclear cells (PBMC) (R) were stimulated with 2 $x10^5$ allogeneic mitomycin-treated PBMC (S). CD4+CD127hi or CD4+CD127dim cells were added at different ratios: 6 $x10^4$ (1:1), 3 $x10^4$ (1:2) or 1.5 $x10^4$ (1:4) cells (cpm, counts per minute; MLR, mixed lymphocyte reaction; R, responder; S, stimulator).

6.1.3 Discussion

For effective antitumoral immunity in HGG patients, it is important to have an immunological environment in which tumor tolerance can be overcome. Malignant gliomas attempt to counteract this immune response by a wide variety of tolerance-inducing cells and immunosuppressive mechanisms.[144] In this perspective, Treg cells have been recognized as key players that are capable of suppressing T cell-mediated antitumoral immunity.[12,43,83,84,145] Fecci *et al.*[43] found an increased Treg cell fraction amidst a diminished CD4+ compartment in HGG patients. The same study showed that a depletion of Treg cells *in vivo* led to glioma rejection in a mouse model. This was also shown by Grauer *et al.*[83] in a murine glioma model. Jacobs *et al.*[145] did not find an increase in systemic Treg cells in HGG patients, but they showed that intratumoral Treg cells are strong suppressors of effector T cells. However, Heimberger *et al.*[28] did not find a correlation between intratumoral Treg cells and survival in HGG patients. Also, Dunn *et al.*[12] stated that there is no support in the literature for an unequivocal correlation, either positive or negative, between the presence of TIL and survival.

On the other hand, Treg cells might play a more decisive role in patients actively treated with immunotherapy and it would be interesting to correlate Treg cells and survival in HGG patients treated with DC vaccination. Grauer *et al.*[84] showed that elimination of Treg cells was essential for an effective vaccination with tumor lysate-pulsed DC in a murine glioma model. Maes *et al.*[85] demonstrated in the same model that Treg cell depletion was superior to DC vaccination when survival was considered, but that only DC vaccination could induce a memory response resulting in a long-term survival upon rechallenge of the mice with orthotopic glioma tumor cells.

To optimize the immune monitoring in HGG patients treated with immunotherapy, we investigated whether CD127 expression is an appropriate and easily assessable marker for Treg cells. Within DC vaccinated HGG patients, we could show a significant positive correlation between Foxp3 expression and CD127dim expression both in the CD4+CD25+ and entire CD4+ population, as previously shown by Liu *et al.*[147] in type 1 diabetes mellitus patients. The sensitivity of CD127dim expression as marker for Foxp3+ Treg cells within the CD4+CD25+ population was 74.3%, with a specificity of 79.2%. We also confirmed the suppressive capacity of CD4+CD127dim cells in a functional assay; CD4+CD127dim cells suppress an allogeneic MLR in a dose-dependent manner, in contrast to CD4+CD127hi cells. We did not include CD25hi expression as isolation marker for Treg cells, since Liu *et al.*[147] showed that suppressive activity was independent of CD25, as both the CD4+CD127dimCD25+ and CD4+CD127dimCD25− T cell subsets suppressed a MLR. Moreover, we showed a positive correlation between Foxp3 expression and CD127dim expression in the entire CD4+ population, independent of CD25 expression, although less pronounced than in CD4+CD25+ cells.

Based on these results, we conclude that CD127dim expression can be used as an easily detectable marker for functional Treg cells in HGG patients treated with immunotherapy. Compared to intracellular Foxp3 staining, CD127 surface staining is an easy way to monitor Treg cells and this offers an important practical advantage over Foxp3 staining.

6.2 IMMUNE MONITORING IN THE PERIPHERAL BLOOD

Flow cytometry is the most routinely used tool in the monitoring of cellular immune responses and was also applied by us to monitor immune responses in vaccinated GBM patients in the pilot and HGG-2006 trial (**Chapter 5**). Monitoring results were compared with clinical data.

6.2.1 Material and Methods

6.2.1.1 Pilot trial: Elispot, flow cytometry and delayed type hypersensitivity reaction [a]

In the pilot trial, immune monitoring was performed at 3 different levels. First, DTH reaction was tested at the first and the fourth vaccination. For this, 100 µL crude tumor cell lysate and 100 µL control PBS/HSA were injected intradermally. After 48 and 72 hours redness and induration were assessed. DTH reactions were judged as positive if the average perpendicular measurement of the induration exceeded 5 mm.

Second, blood samples were obtained at times of leukapheresis, vaccine 1, vaccine 4 and vaccine 7. In each whole blood sample, the phenotype of circulating T cell populations was determined by FACS: total CD3+ population, and the CD4+ and CD8+ subpopulations, as well as the activation markers HLA-DR on CD3+ cells and CD25 on both subpopulations. For this, FITC- and PE-labeled mAb were purchased from BD Biosciences Pharmingen (San Jose, USA).

Finally, PBMC from each blood sample were cryopreserved and thawed together at the end of the immunotherapy for use in an Elispot assay. A positive response was defined as an at least twofold increase in number of antigen-specific spots after the fourth and seventh vaccination, as described by Banchereau *et al.*.[148] The protocol was adapted, based on the manufacturer's instructions (Mabtech, Nacka Strand, Sweden). In brief, 96-well polyvinylidene difluoride membrane plates (MAIPSWU10; Millipore, Bedford, MA, U.S.A.) were treated with 70% ethanol (50 µL per well) for 1 min and washed with PBS prior to coating. Next, plates were coated overnight (4°C) with coating antibody (1-D1K, 15 µg/ mL, Mabtech). After blocking, 2 x10^5 viable PBMC per well were seeded in the presence of phytohemagglutinin (1 µg/mL),

[a] Ardon H, Van Gool S, Lopes IS, Maes W, Sciot R, Wilms G, et al. Integration of autologous dendritic cell-based immunotherapy in the primary treatment for patients with newly diagnosed glioblastoma multiforme: a pilot study. J Neurooncol 2010;99:261-272.

serum-free medium (CTL-test^{TM}, Cellular Technology Ltd., Aalen, Germany), autologous tumor cell lysate or protein extracted by ethanol precipitation, and incubated for 24 h (37°C, 5% CO_2) in a final volume of 100 µL. Cells were washed away and detection antibody (7-B6-1-biotin, 1 µg/mL, Mabtech) was added (overnight, 4°C). Streptavidine-ALP (Mabtech) was added for 1 h after which substrate solution was added (AP conjugate substrate kit, Bio-Rad, Nazareth Eke, Belgium). Spots were counted with Immunoscan and Immunospot software. Antigen-specific spots were calculated after subtracting the background spots of unstimulated PBMC. All conditions were done in triplicates.

6.2.1.2 HGG-2006 trial: Flow cytometry

In the HGG-2006 trial, immune monitoring was performed by flow cytometry. Blood samples were obtained at the same points in time as in the pilot trial (**Chapter 6.2.1.1**). PBMC from each blood sample were cryopreserved and thawed together at the end of the immunotherapy for flow cytometric analyses. For each blood sample, the phenotype of circulating cell populations was determined by FACS: CD4+ and CD8+ subpopulations, as well as Treg cells based on CD4+CD127dim expression, and NK cells based on CD3-CD56+ expression. CD127dim expression was used in the monitoring of Treg cells based on our experimental results (**Chapter 6.1**).[149] Also, the activation marker CD25 was determined on the CD8+ and Treg cell subpopulations. For this, FITC-, PE- and PerCP-labeled mAb were purchased from BD Biosciences Pharmingen (San Jose, USA).

Based on the FACS data, relative counts (ratios) of the different cell populations could be determined; CD4+ and CD8+ T cell ratios were expressed on the total lymphocyte gate, as were NK cell ratios. Treg cell ratios were expressed on total CD4+ cells. Also, absolute cell counts of the different cell populations could be calculated. Ratios and absolute cell counts, at the different points in time during treatment, were then compared for each of the cell subpopulations, and changes were stated as increase or decrease (immunological profiles). Data at the time of leukapheresis (LF) were compared with data at the time of the first vaccine (V1), to evaluate the effect of radiochemotherapy, and with data at the time of the seventh vaccine (V7). Next, data at the time of V1 were compared with data at the time of the fourth vaccine (V4) to evaluate the induction phase of the vaccination. Furthermore, data at the time of V4 were compared with data at the time of V7 to evaluate the maintenance phase of the vaccination.

6.2.1.3 HGG-2006 trial: Statistical analysis

Two approaches were used to verify if there was any information in the immunological profiles to predict PFS and OS. In a first approach, Cox regression models[a] were used to verify if the immunological profiles contained any information for the prediction of PFS and OS. Considered predictors were the change in cell counts/proportions at time of LF and at time of V1, and the change in cell counts/proportions at time of V1 and at time of V4. To express this change (i.e. increase or decrease), ratios were used (LF/V1 and V1/V4) as well as binary variables indicating if there was change or not. Ratios were available for 4 different cell types: CD4+ cells, CD8+ cells, NK cells and Treg cells. Univariable, as well as various multivariable models were fitted. Due to the high correlation between CD4+ and CD8+ subpopulations, information from both counts were never combined in the same model. Likelihood-ratio tests were used in each model, to verify if there was any information in the considered predictor(s).

A second approach consisted of two steps. First, changes in all cell counts[b] were used to cluster[c] the patients[d]. Due to the low number of subjects, the number of clusters was fixed on 3. Secondly, we verified if there was a relation between cluster membership and survival (PFS/OS) using log-rank tests.

All analyses were performed using SAS software, version 9.2 of the SAS System for Windows (Copyright © 2002 SAS Institute Inc.) SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA.

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[a] Assumptions of linearity and proportional hazard were verified. Patients who died without progression were considered as censored in the analysis of OS. Note that the changes are included in the model as baseline covariates, since there was no subject information on the moment of the administration of the vaccines.

[b] Variables were transformed (-1/X) to reduce the effect of outliers.

[c] More specifically, a non-hierarchical disjoint clustering method based on Euclidean distances was used. This kind of clustering method is called a k-means model, since the cluster centers are the means of the observations assigned to each cluster.

[d] To handle the missing data a single imputation was performed. Note that the strategy assumed that the data are multivariate normally distributed and the missing data are missing at random. That is, the probability that an observation is missing can depend on the observed variable values of the individual, but not on the missing variable values of the individual.

6.2.2 Results

6.2.2.1 Pilot trial [a]

An increase in the proportion of CD8+CD25+ T cells was noted during the course of vaccination in 6 out of 7 patients tested (**Table 6.1**). Two of those patients (#3 and #7) showed a positive skin test (DTH), and an increase in IFN-γ-producing tumor antigen-reacting T cells was observed in 5 patients (#1, #3, #5, #6 and #7).

Patient	Skin test	Elispot (V1 compared to V4)	Elispot (V4 compared to V7)	CTL[1] (V1 compared to V4)
1	negative (V1 + V4)	increase in IFN-γ-producing cells	persistent increase	increase in CTL
2	negative (V1)	no increase	no increase	not done
3	positive (V1 + V4)	increase in IFN-γ-producing cells	persistent increase	increase in CTL
4	negative (V1 + V4)	no increase	no increase	increase in CTL
5	not done [2]	increase in IFN-γ-producing cells	persistent increase	increase in CTL
6	negative (V1 + V4)	increase in IFN-γ-producing cells	not done	increase in CTL
7	positive (V1 + V4)	increase in IFN-γ-producing cells	no increase	increase in CTL
8	delayed positive [3]	no increase	no increase	no increase

Table 6.1 Immune response

CTL, cytotoxic T lymphocytes; IFN-γ, interferon-γ; V, vaccine

[1] CTL = CD8+CD25+ cytotoxic T lymphocytes

[2] not enough tumor lysate

[3] positive reaction at injection sites 6 months after V1

In 6 patients, a DTH skin test with autologous tumor homogenate was performed at time of the first and fourth vaccination (**Table 6.1**). In patient #2, the skin test was only performed at the first vaccination and in patient #5 there was not enough tumor material available to do a skin test (all tumor lysate was used for vaccination). Two patients (#3 and #7) had a positive reaction within 72 hours after intradermal injection at the first, as well as the fourth vaccination. In these patients the control intradermal injections with PBS/HSA were negative. Patient #3 was progression free at the end of the FU period. Patient #7 had a recurrence of the

[a] Ardon H, Van Gool S, Lopes IS, Maes W, Sciot R, Wilms G, et al. Integration of autologous dendritic cell-based immunotherapy in the primary treatment for patients with newly diagnosed glioblastoma multiforme: a pilot study. J Neurooncol 2010;99:261-272.

primary tumor at 17 months FU. In patient #8, a delayed positive reaction at multiple injection sites occurred 6 months after the first vaccination. This patient had a recurrence of the primary tumor at 26 months FU.

The results of the Elispot assay (n = 8) showed an increase in IFN-γ-producing tumor antigen-reacting T cells between the first and fourth vaccination in 5 out of the 8 patients (**Table 6.1** – **Fig. 6.4**). In 3 of these 5 patients (#1, #3 and #5), a more than twofold increase in tumor specific IFN-γ production persisted after 3 cycles TMZm (**Table 6.1** – **Fig. 6.4**). Two of the 5 patients with an increase in tumor specific IFN-γ production at the fourth vaccination, had positive skin tests (#3 and #7).

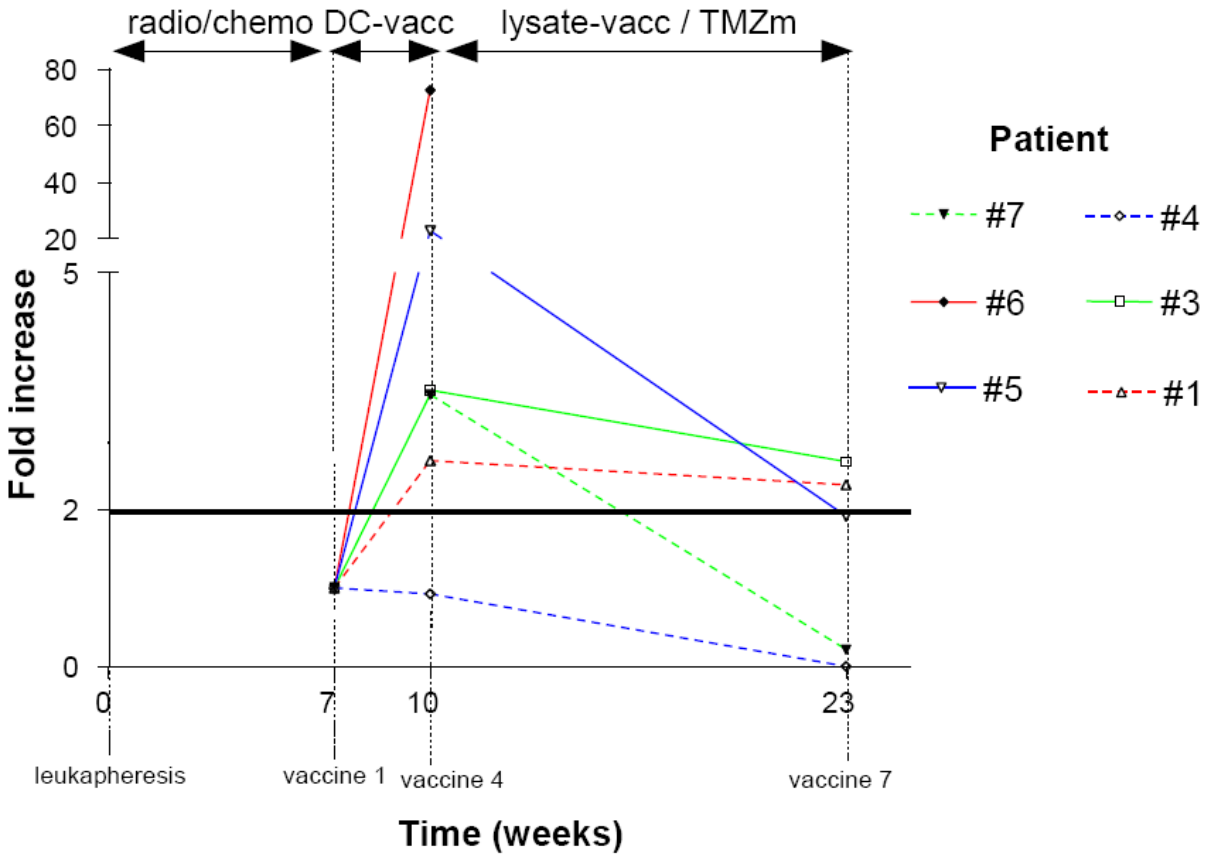

Fig. 6.4 Assessment of the number of specific tumor-antigen reacting T cells using Elispot

The number of interferon (IFN)-γ producing tumor antigen-reacting T cells is depicted during the vaccination schedule in 6 out of 8 patients. Two patients (#2 and #8) had more spots in the control condition than in the experimental condition at all time points (results not shown). In 5 patients, an increase in tumor specific IFN-γ production can be seen between the first and fourth vaccination. In 3 of them, a more than twofold increase in tumor specific IFN-γ production persisted after 3 cycles maintenance temozolomide chemotherapy (TMZm) (vaccine 7). The fold increase was calculated with the following formula: ((SFC at time x) - (SFC in control condition at time x)) / ((SFC before treatment) - (SFC in control condition before treatment)) (DC-vacc, dendritic cell vaccination; lysate-vacc, boost vaccination with tumor cell lysate; radio/chemo, radiochemotherapy; SFC, spot-forming colonies).

6.2.2.2 HGG-2006 trial

Based on the FACS data (**Chapter 6.2.1.2**), patterns of increase and decrease in the different cell ratios and absolute cell counts could be determined, rendering information on immunological responses and profiles. Based upon combination of increase/decrease in different cell ratios and absolute cell counts, a further subdivision was made into subgroups/clusters. For example, one could have an increase in Treg cells and a decrease in NK cells, as compared to an increase in Treg cells and an increase in NK cells. In this way, clusters were made with NK, Treg and CD8 cells. Absolute cell counts are depicted in **Fig. 6.5**.

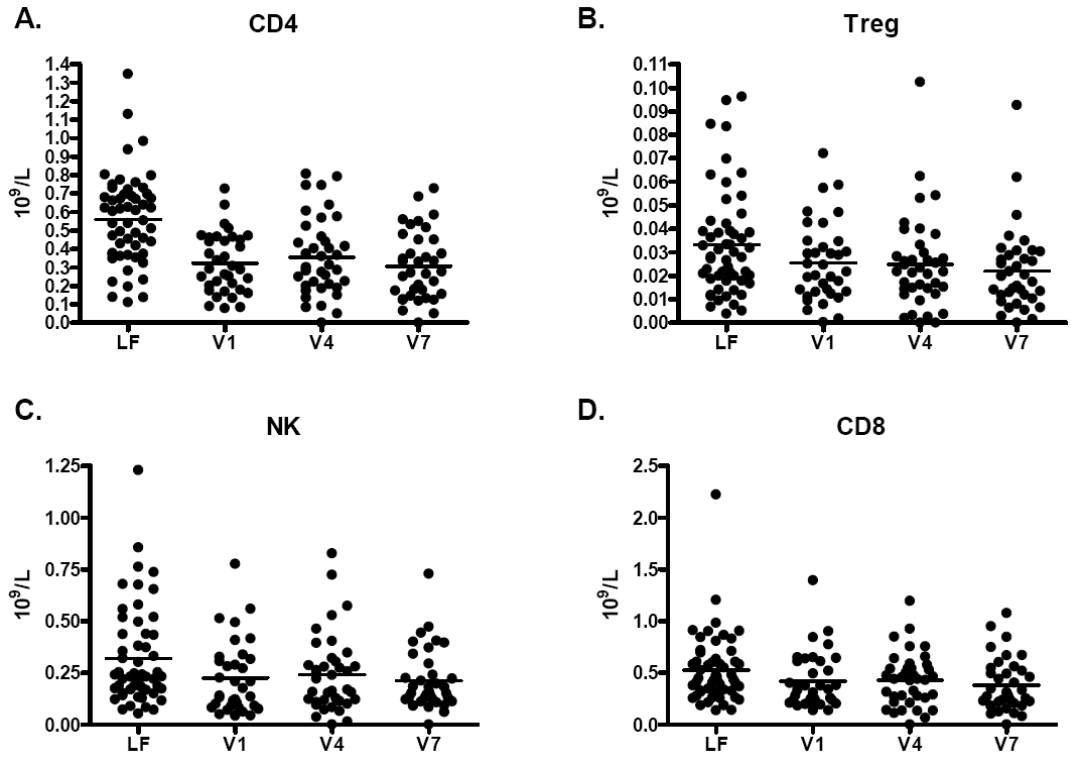

Fig. 6.5 Absolute cell counts of cell populations

At set points in time during vaccination, blood samples were obtained and absolute cell counts were determined (LF, *n* = 54; V1, *n* = 35; V4, *n* = 38; V7, *n* = 36). Median values for absolute cell counts of CD4 cells (A) were 0.5782, 0.2916, 0.3069 and 0.2732 x10^9/L at LF, V1, V4 and V7 respectively. Median values for absolute cell counts of Treg cells (B) were 0.02788, 0.02166, 0.02175 and 0.01858 x10^9/L at LF, V1, V4 and V7 respectively. Median values for absolute cell counts of NK cells (C) were 0.2316, 0.1574, 0.1947 and 0.1534 x10^9/L at LF, V1, V4 and V7 respectively. Median values for absolute cell counts of CD8 cells (D) were 0.4408, 0.3408, 0.4400 and 0.2932 x10^9/L at LF, V1, V4 and V7 respectively (LF, leukapheresis; NK, natural killer cells; Treg, regulatory T cells; V1, vaccine 1; V4, vaccine 4; V7, vaccine 7).

Next, the patterns of increase/decrease were compared to clinical outcome, to see if there was any information in the immunological profiles to predict PFS and OS (**Fig. 6.6**; not all data shown). Univariable, bivariable and multivariable models were tested. Increase (yes/no) was used as predictor, and ratios were used as continuous predictors as well. Using all these different models, we found no evidence that the immunological responses contained any predictive information for either PFS or OS. Also cluster analysis was performed, but none of the performed clustering models resulted in a clustering membership with a statistical significant relation with PFS or OS (data not shown). However, cluster analysis based on the 2 variables with the highest R^2, Treg and NK cell ratios (LF/V1), resulted in the determination of 3 clusters (**Fig. 6.7**). A trend towards a longer PFS was seen from cluster 1 to cluster 3, although this did not reach statistical significance (**Fig. 6.7**).

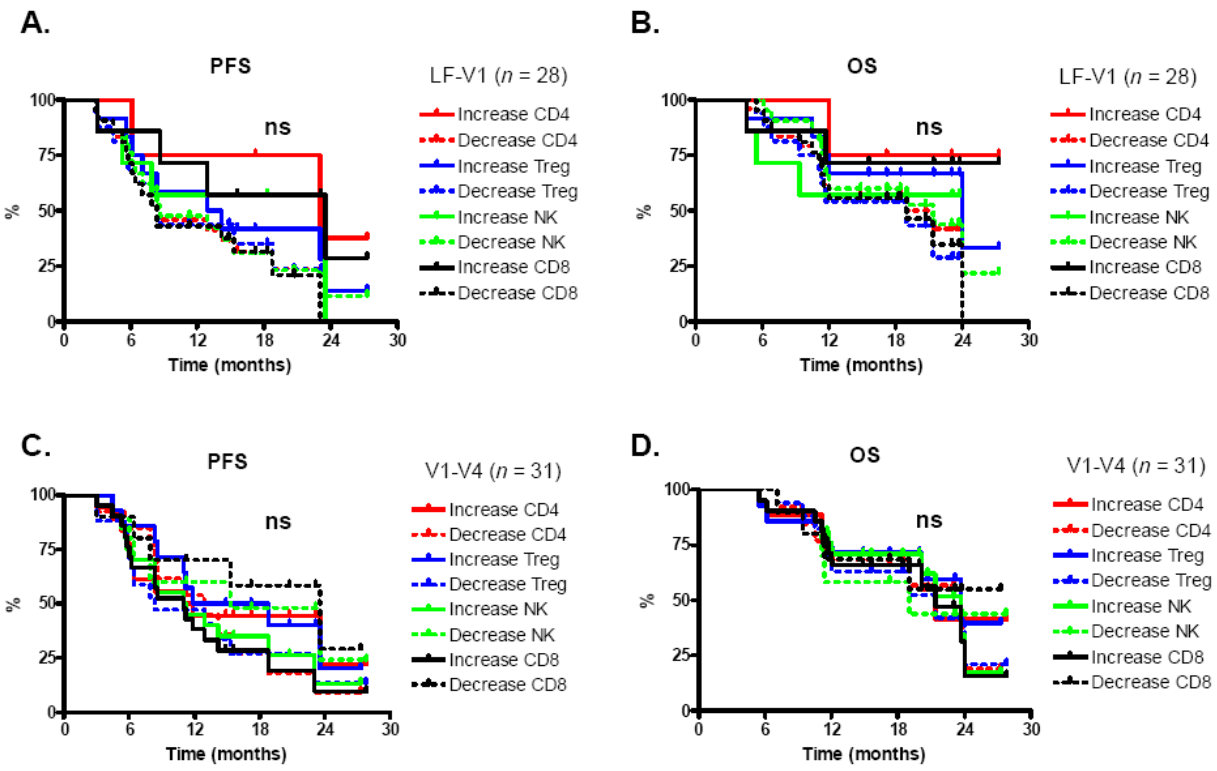

Fig. 6.6 Progression-free survival (PFS) and overall survival (OS) based on patterns of increase/decrease in absolute cell counts of different cell populations

PFS (A) and OS (B) are shown according to changes (increase/decrease) in cell populations (CD4, CD8, natural killer (NK) cells and regulatory T (Treg) cells) between leukapheresis (LF) and first vaccine (V1). PFS (C) and OS (D) are shown according to changes in the different cell populations between first vaccine (V1) and fourth vaccine (V4) (ns, not significant).

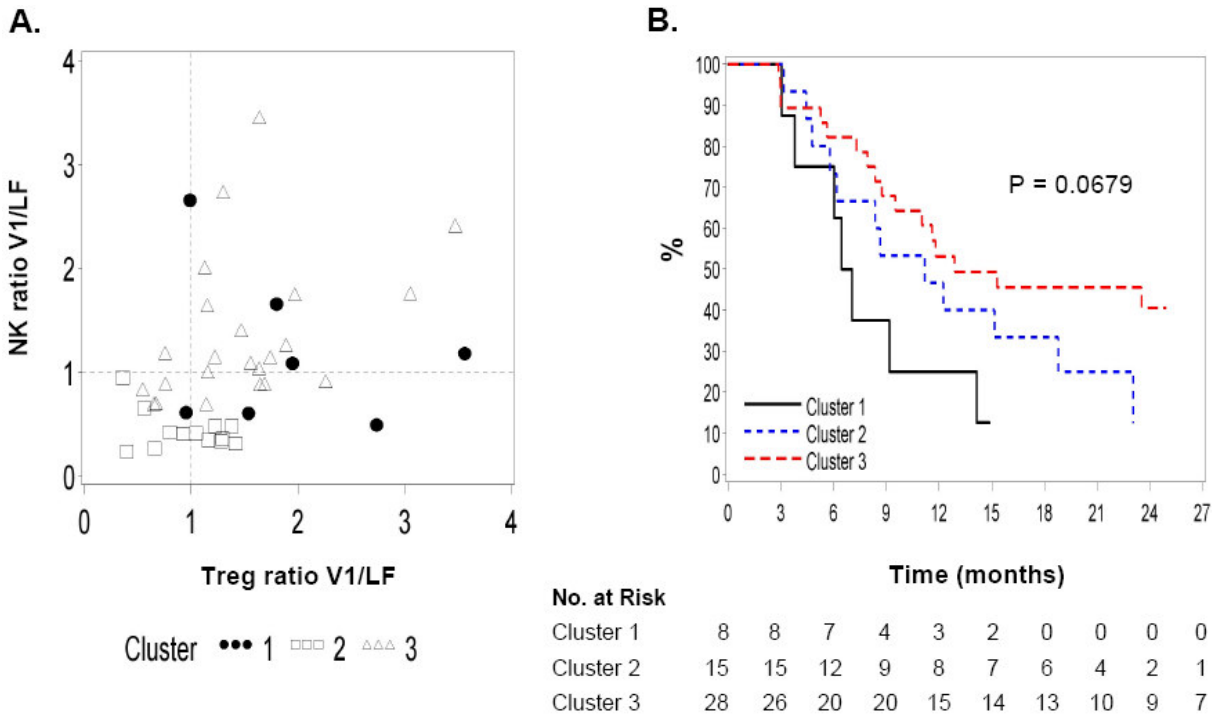

Fig. 6.7 Cluster analysis and impact on progression-free survival (PFS)

A. Cluster analysis based on the 2 variables with the highest R^2, regulatory T (Treg) and natural killer (NK) cell ratios (LF/V1); 3 clusters can be distinguished, depicted by full circles (cluster 1), squares (cluster 2) and triangles (cluster 3). B. PFS for the 3 different clusters, as determined by cluster analysis (LF, leukapheresis; V1, vaccine 1). After visual inspection of the clusters in the 2 provided axes (Treg and NK), they could be termed as immune suppressive (cluster 1), immune neutral (cluster 2) or immune reactive (cluster 3).

6.2.3 Discussion

Both in the pilot and HGG-2006 trial, immune monitoring was used to assess the induction and/or maintenance of vaccine-induced antitumoral immunity in the described close temporal relationship with radiochemotherapy. DTH testing to antigen is one monitoring tool to indicate cellular immunity, although it remains controversial whether or not DTH to autologous tumor can be a reliable correlate of clinical responses. In the pilot trial we could not find any positive correlation between immune reactivity and clinical outcome. In 2 out of 6 patients tested, there was a positive skin test at the first as well as the fourth vaccination, suggesting that radiochemotherapy did not compromise the patients' specific immunity. One patient had a delayed positive reaction at the injection sites, 6 months after the first vaccination.

The results of the Elispot assays in the pilot trial showed a vaccine-induced increase in IFN-γ-producing tumor antigen-reacting T cells in 5 out of 8 patients. This points to the induction of a tumor-antigen-directed immune response. In 3 out of these 5 patients, a more than twofold increase in tumor specific IFN-γ production persisted after 3 cycles TMZm. These data further support the notion that radiochemotherapy does not interfere negatively with immunotherapy and that these treatment modalities can be combined without losing the therapeutic effect of either one. An increase in the proportion of CD8+CD25+ cells within the CD8+ population was noted during the course of vaccination in 6 out of 7 patients. Although very aspecific, this might suggest a re-expansion of activated CD8+ cytotoxic T cells, which is a prerequisite to implement tumor vaccination.

In the HGG-2006 trial, changes in absolute and relative cell counts during vaccination were determined for both effector and suppressor cell populations. These 'immunological profiles' were compared to clinical outcome and we hoped to find predictive immunological response patterns. Unfortunately, using different statistical models, no statistical significant evidence was found that the immunological profiles contained any predictive information for either PFS or OS. However, cluster analysis based on Treg and NK cell ratios (V1/LF) revealed 3 clusters. A trend to increased PFS was seen from cluster 1 to cluster 3, although this did not reach statistical significance. These 3 clusters might depict different response patterns: 1. 'immune suppressive', 2. 'immune neutral' and 3. 'immune reactive' (**Fig. 6.7**). Since these 'response patterns/clusters' are based on changes in cell ratios between leukapheresis and the first vaccine, one could hypothesize on the importance of a differential priming of the immune system by radiochemotherapy, i.e. even before starting the immunotherapy.

A possible explanation for the lack in correlation between immunological and clinical responses – apart from the low number of analyzed data sets – might be that the peripheral immune status does not mirror the immune responses that occur in the tumor itself. Also, using tumor lysate as source of TAA has as disadvantage that there is a lack of specific antigens to be targeted in monitoring assays. This is in contrast to peptide-based vaccines, were a known peptide can be used for monitoring. The discordance between clinical and immunological data is a known problem for this type of treatment, using tumor lysate as source of TAA. This shows the inherent shortcomings of immune monitoring for these types of treatment. Therefore, the use of surrogate immunological endpoints as main parameters to build a treatment strategy upon does not seem to be ideal. The full nature of the estimated beneficial effects of DC vaccination is without any doubt much more complex than any immune monitoring tool at this stage can fully capture.

6.3 IMMUNE MONITORING IN THE TUMOR

We wanted to establish a baseline phenotypical characterization of brain tumor-infiltrating cells (BTIC), which we could then compare to the immune status of the patient as measured in the peripheral blood. As stated above, use of peripheral immune monitoring tools might prove inadequate in case the peripheral immune system does not mirror local events in the brain tumor microenvironment.

6.3.1 Material and Methods

BTIC were isolated out of fresh resection specimens of 10 HGG patients that were not previously treated with immunotherapy. Five of the 10 patients had received corticosteroids in the days preceding the operation because of peritumoral edema with mass effect. In none of the patients did this result in lymphopenia.

For the isolation of the BTIC, freshly resected tumor specimens were cut into small pieces in a Petri-dish in 5 mL digestion medium and then incubated for 30 min at 37°C. Digestion medium consisted of RPMI 1640 supplemented with 10% fetal calf serum (Hyclone, Logan, UT, USA), nonessential amino acids (diluted 1:100) (Cambrex), 2 mmol/L L-glutamine, 100 U/mL penicillin, and 200 µg/mL streptomycin (all from Lonza, Verviers, Belgium), β-mercaptoethanol (7 $x10^{-4}$%), 1.25 µg/mL amphotericin B (Sigma-Aldrich, St Louis, MO, USA), 2.5 mg/mL collagenase D and 5 U/mL DNase. After incubation, the cell suspension was passed over a cell strainer (BD) and thoroughly washed. Next, the suspension was centrifuged (400 g, 5 min) and the cell pellet was resuspended in 10 mL percoll 40% (Sigma) at 37°C. This suspension was carefully put on top of 4 mL percoll 70% at 37°C, and next centrifuged for 25 min at 800 g. After gradient centrifugation, the myelin and debris layers were removed and the mononuclear cell interphase was recovered. Cells were washed twice and the isolated BTIC were used for phenotypical characterization by flow cytometric analysis. Comparison was made with fresh peripheral blood samples, which were obtained at time of surgery as well. Flow cytometric analysis was done for the following cell populations, relevant to the effector and suppressor arm of the immune system: CD4+ T cells; CD8+ cytotoxic T cells; CD4+CD127dim(CD25+) Treg cells; CD3-CD56+ NK cells; CD3+CD56+ NKT cells; CD33+CD11b+CD45+CD15-HLA-DR+ MDSC.

6.3.2 Results

Phenotypical characterization of the BTIC using FACS analysis showed that all cell types for which analysis was done, could be found to infiltrate gliomas: CD4+ T cells, CD8+ CTL, NK cells, NKT cells, Treg cells and MDSC. Comparison to the same cell types in peripheral blood samples showed a tendency of suppressor cells (MDSC and Treg cells) to accumulate in the tumor (**Fig. 6.8**). Since numbers are only small, further research has to be done to elucidate these preliminary findings.

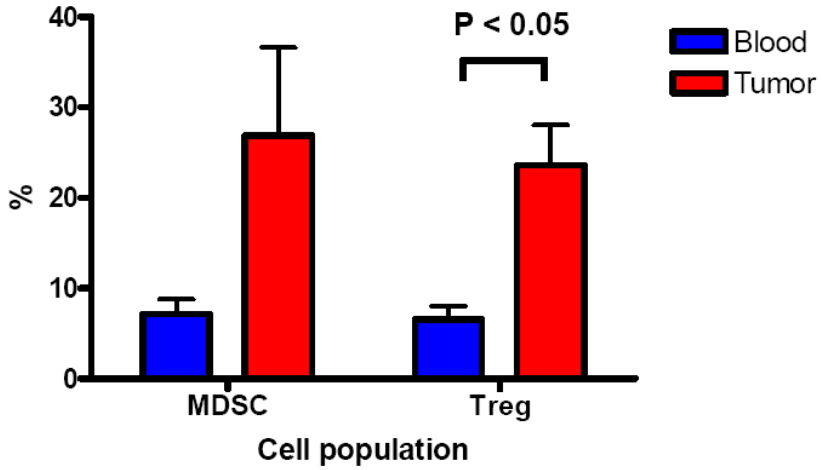

Fig. 6.8 Comparison between brain tumor-infiltrating cells (BTIC) and peripheral blood cells
Results of flow cytometric analyses on patients' samples (n = 10) are shown (MDSC, myeloid-derived suppressor cells (percentage on C45+ cells); Treg, regulatory T cells (percentage on CD4+ T cells)).

6.3.3 Discussion

Although the data on BTIC are preliminary and numbers are only small, the results seem to indicate that the peripheral immune status does not mimic the intratumoral immune reaction. Treg cells and MDSC seem to be attracted to the tumor, probably leading to suppression of the effector arm. This is in accordance with the model of 'cancer immunoediting', in which the tumor escapes the immune system in the final phase. MDSC and Treg cells could enact an immune suppressive function, which would allow the tumor to evade the immune system. Moreover, it is known that HGG can actively suppress the immune system through different immune escape mechanisms, including induction of Treg cells (**Chapter 1.5**).[39,40] Jacobs *et al.*[145] showed that intratumoral Treg cells are strong suppressors of effector T cells. However,

no correlation between intratumoral Treg cells and survival in HGG patients has been found.[12,28]

How these findings relate to immune monitoring in vaccinated patients has to be further elucidated, but one might argue that peripheral immune monitoring tools are not well suited for monitoring the immune response against these tumors. This was illustrated by the lack of correlation between immune monitoring results and clinical outcome in both the pilot and HGG-2006 trials.

Before any definite conclusions can be drawn, further research into BTIC is clearly needed. On the one hand more data have to be obtained on the differences between intratumoral and peripheral immune responses. On the other hand functional assays have to be set up with BTIC to evaluate the possible role of effector and suppressor cells in brain tumor immunological processes.

CHAPTER 7. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

7.1 IMMUNOTHERAPY FOR HGG

In recent years, insights into tumor immunology have been refined and it is now clear that both the adaptive and innate immune system play an important role. Moreover, it has been shown that immunological processes take place within the CNS and the brain should be looked upon as an immune-distinct environment. Many glioma-specific antigens have been described and interactions between gliomas and the immune system are being elucidated: gliomas will grow and evade the antitumoral immune responses if the balance between tumor cell proliferation and elimination tips in favor of immune-resistant cells ('immune escape').

Immunotherapy for patients with HGG is a novel therapeutic approach that opens new opportunities for enhanced survival without major toxicity. This is of particular importance, taking into account that HGG cause a relatively high community burden, not only with many years of life lost due to cancer, but also because of the morbidity from the tumor and subsequent treatments. In the assessment of immunotherapeutic results for patients with HGG, the improvement of PFS and, particularly, the significant increase of OS with satisfactory quality of life are, of course, by far the most important variables, much more than immunologic surrogate markers for response. The studies in preclinical animal models for human gliomas, especially the murine models, are interesting in order to obtain more insight into the basic biology underlying disease evolution and immunotherapeutic mechanisms. Still, timely translation of new concepts into clinical practice should be the primary objective.

To compare survival data between studies within these heterogeneous patient populations, it is essential to obtain simple, homogenous subsets of patients with comparable outcome. To this end, we slightly modified the original RTOG RPA classification and made it applicable to patients with relapsed HGG, treated with DC-based immunotherapy. We could define simple, clinically relevant, prognostic classes and we found long-term survivors after relapse in classes III and IV.

With several groups entering into clinical practice in this field, new data are now being generated. However, it is of paramount importance to study this promising approach based on data from well designed trials with appropriate end points. Thus far, the observations with regard to both immunological responses and clinical responses are promising and beneficial

effects are reproducible. We could demonstrate the clinical feasibility of postoperative DC-based immunotherapy in children with relapsed malignant brain tumors, without major adverse events. Furthermore, we could prove that the integration of DC-based immunotherapy in the standard primary treatment of GBM, consisting of maximal safe surgical resection, radiotherapy and TMZ chemotherapy, is feasible and well tolerated. In addition, survival data compare favorably to results obtained with current state-of-the-art therapy.

Of particular interest is the fact that no induction of autoimmunity or other major toxicities have been observed to date. However, larger and more homogeneous patient groups with more refined inclusion criteria should be studied for more rapid progress in this field. Moreover, confirmatory studies with an appropriate randomization *versus* a control patient group, preferably even stratified for prognostic markers, including molecular tumor signature, should be implemented in the near future. For this, collaborative efforts between well-organized and experienced vaccination centers should be established.

7.2 OPTIMIZATION OF IMMUNOTHERAPY

Although several studies have reported promising results, and activation of the immune system against gliomas seems possible, immunotherapeutic approaches are counteracted by rapid tumor progression and glioma-induced immune suppression. There is a clear need for improvement of the immunotherapeutic strategies and several options exist to enhance the immune response. It has been shown in murine models that co-stimulation of T cells can be enhanced by using agonistic antibodies to co-stimulatory molecules, resulting in a better activation of T cells. The use of antagonistic antibodies to co-inhibitory molecules (such as 'cytotoxic T lymphocyte-associated antigen-4' (CTLA-4) and 'programmed-death-1 receptor') can also lead to a stronger T cell activation.[39,45]

It is also important to target Treg cells that suppress the antitumoral immune response. It has been shown in mouse models that elimination of Treg cells, either in combination with anti-CTLA-4 antibodies or not, results in increased immunity against glioma and a stronger response to DC-based immunotherapy.[84,85] In humans, studies have been carried out using daclizumab (anti-CD25 antibody), ONTAK ('denileukin diftitox') and CD25-specific immunotoxins (LMB-2).[39,150] Clinical responses, however, have been limited. Low-dose metronomic TMZ diminished Treg cells in a rodent glioma model and in melanoma patients, and therefore seems a promising tool to use in glioma patients. Unfortunately, this tool cannot

be combined with DC-based immunotherapy, since metronomic TMZ administration would counteract the induction of an adequate immune response.[151,152] On the other hand, low-dose metronomic oral cyclophosphamide has been propagated as an effective Treg cell-depleting regimen that does not counteract immunotherapy.[153,154]

The local immune suppression by the glioma itself can be targeted via suppressive cytokines and enzymes, such as TGF-β2, that are produced by the tumor. TGF-β is capable of inhibiting T cell and B cell activation and proliferation, it suppresses the activity of NK cells, reduces the production of cytokines like IL-2, IL-6, IL-10, IFN-γ, and suppresses the expression of MHC-II molecules on glioma cells. In preclinical studies, it has been shown that the expression of TGF-β2 by GBM cells can be diminished by exposing them to antisense oligonucleotides against mRNA of TGF-β2. The most advanced antisense oligonucleotide for the therapy of HGG is phosphorothioate-modified AP 12009 (trabedersen), which targets mRNA encoding TGF-β2. AP 12009 is administered intratumorally using convection-enhanced delivery. A series of phase I and II clinical trials has evaluated the toxicity profile and optimal dose of the substance, and a randomized, controlled phase III study is ongoing in patients with recurrent or refractory anaplastic astrocytoma after standard radio- and chemotherapy.[155-157]

Finally, production of DC can be optimized; for example, maturation of DC can be improved by using 'Toll-like receptor' agonists.[39,59,60]

7.3 OPTIMIZATION OF IMMUNE MONITORING

Treg cells can be detrimental to immunotherapy and hence monitoring of these cells is of critical importance in patients treated with DC-based tumor vaccination. To facilitate Treg cell monitoring in HGG patients treated with immunotherapy, we showed that CD127dim expression can be used as an easily detectable marker for functional Treg cells.

Furthermore, the discordance between clinical and immunological data is a known problem for DC-based immunotherapy using tumor cell lysate as source of TAA, and was also evident in our study in newly diagnosed GBM patients. On the one hand, using tumor cell lysate has as disadvantage that there is a lack of specific antigens to be targeted in immune monitoring assays. On the other hand, the lack in correlation between immunological and clinical responses might also be explained by the fact that the peripheral immune status does not mirror the immune responses that occur in the tumor itself, as was indicated by our

preliminary results on BTIC. Immune monitoring assays done on peripheral blood would thus be inadequate and would not render any useful information.

Further research on BTIC might lead to a better understanding of the differences between the immune cells infiltrating the tumor and the circulating cells in the peripheral blood. This might lead the way to new strategies for immune monitoring, as well as an adaptation of immunotherapeutic approaches. Ways to monitor immunological processes in the brain and tumor would be of great value to immunotherapeutic approaches in glioma. Although data are still preliminary, advanced MRI, eventually combined with PET, may soon provide better tools to monitor the effects of immunotherapy for HGG.

Up till now, the use of surrogate immunological endpoints as main parameters to build a treatment strategy upon does not seem to be ideal. The full nature of the estimated beneficial effects of DC vaccination is without any doubt much more complex than any immune monitoring tool at this stage can fully capture.

SUMMARY

In spite of full, state-of-the-art oncological therapy, including maximal safe surgical resection, external beam radiotherapy and chemotherapy, the prognosis of glioblastoma multiforme (GBM) remains poor with a median survival of 14.6 months. Notwithstanding this maximal treatment, relapse is universal. At time of recurrence, prognosis is even worse and virtually all patients die within 18 months after relapse. There is a clear need for well-tolerated long-term treatments that are tumor-specific and able to kill all (residual) tumor cells that infiltrate in the adjacent areas of the brain. The immune system provides us with some promising tools in that regard, and therefore we have investigated the integration of dendritic cell (DC)-based immunotherapy in the current therapeutic concepts, both in newly diagnosed and relapsed GBM patients.

First, we have implemented immunotherapy for the treatment of patients with relapsed high-grade glioma (HGG), as a stepwise process using a cohort-comparison study concept (HGG-IMMUNO-2003). We slightly modified the original RTOG RPA classification, a known model to define prognostic classes based on treatment and pre-treatment prognostic variables, and made it applicable to patients treated in this study. In this heterogeneous group of patients, we could define simple, clinically relevant, prognostic classes using this modified RTOG RPA model. Also, we found long-term survivors after relapse in classes III and IV, which is a remarkable clinical finding, comparing favorably to almost all other trials in (multi-)relapsed patients with HGG.

Based on the already published importance of age in DC vaccination in the patients with relapsed HGG, we analyzed the results in 45 children (18 years and younger) with a recurrent malignant brain tumor, who were treated with autologous DC-based immunotherapy (HGG-IMMUNO-2003). We could demonstrate the clinical feasibility of this approach without major adverse events, even in young children. HGG seem to respond more favorably to vaccination than ependymoma and medulloblastoma/primitive neuro-ectodermal tumor, and – for atypical teratoid-rhabdoid tumor – the addition of immunotherapy to radio- and chemotherapy might be beneficial. Although preliminary, and derived from a heterogeneous patient group, our results on DC-based vaccination support testing of the integration of this innovative immunotherapy approach in new treatment protocols for HGG and atypical teratoid-rhabdoid tumor.

Next, we integrated DC-based immunotherapy in the standard primary treatment of GBM, consisting of maximal safe surgical resection, radiotherapy and temozolomide chemotherapy. In 8 pilot, adult newly-diagnosed GBM patients, tumor vaccination proved to be feasible and well tolerated. The survival data were used to power the phase I/II HGG-2006 trial for patients with newly-diagnosed GBM, in which 77 patients were included. Median overall survival (OS) based on intent-to-treat analysis was 18.3 months, which compares favorably to the survival data reported by Stupp *et al.* with a median OS of 14.6 months. Survival seems to be improved especially in the patients belonging to EORTC RPA class III. The integration of immunotherapy within the standard postoperative therapy for patients with a newly diagnosed GBM is based on the presumed mutually beneficial effect of the conventional treatment strategies and immunotherapy. It is hypothesized that the combination of radiotherapy, chemotherapy and immunotherapy could potentiate the cumulative antitumoral activity, when applied in a well designed strategy. For this radio-chemo-immunotherapy, we provide feasibility data in terms of clinical responses, as well as an acceptable QOL.

Finally, we addressed the issue of immune monitoring in vaccinated, newly-diagnosed GBM patients. Both in the pilot and HGG-2006 trial, we applied flow cytometry on peripheral blood as immune monitoring tool to assess the induction and/or maintenance of vaccine-induced antitumoral immunity in the close temporal relationship with radiochemotherapy. For this, we first validated IL-7 receptor alpha subunit (CD127) dim expression as a marker for human regulatory T (Treg) cells, since these cells have claimed a very prominent role in tumor immunology as potential suppressors of immune responses. Thus, monitoring of Treg cells is essential in patients treated with immunotherapy. 'Immunological profiles' based on flow cytometry were compared to clinical outcome, but no clear correlation between immunological and clinical responses was found. A possible explanation for this lack in correlation might be that the peripheral immune status does not mirror the immune responses that occur in the tumor itself. Therefore, we established a baseline phenotypical characterization of brain tumor-infiltrating cells, which we compared to the immune status of the patient as measured in the peripheral blood. Preliminary results seem to indicate in fact, that the peripheral immune status does not mimic the intratumoral immune reaction. How these findings relate to immune monitoring in vaccinated patients has to be further elucidated, but one might argue that peripheral immune monitoring tools are not well suited for the full monitoring of the immune response against these tumors.

SAMENVATTING

Ondanks een conventionele oncologische behandeling, bestaande uit een maximaal veilige chirurgische resectie, bestraling en chemotherapie, blijft de prognose van glioblastoma multiforme (GBM) slecht met een mediane overleving van 14.6 maanden. Zelfs na deze maximale behandeling treedt in het algemeen een recidief op. De prognose is op dat moment nog slechter en zo goed als alle patiënten overlijden binnen de 18 maanden na recidief. Er is dan ook duidelijk nood aan behandelingen op lange termijn, die goed verdragen worden, tumorspecifiek zijn en in staat zijn om alle (overgebleven) tumorcellen, die het omgevende hersenweefsel binnendringen, te doden. Het afweersysteem biedt ons mogelijkheden die veelbelovend lijken en we hebben de inbouw van op dendritische cellen (DC) gebaseerde immunotherapie in de huidige behandelingsmodaliteiten dan ook onderzocht, zowel in patiënten met een nieuw gediagnosticeerd als recidief GBM.

In de eerste plaats hebben we immunotherapie doorgevoerd als behandeling van patiënten met een recidief hooggradig glioom (HGG) in een studie waarbij verschillende cohorten werden ingesloten met stapsgewijze aanpassingen in de behandeling (HGG-IMMUNO-2003). De originele RTOG RPA classificatie, een gekend model om prognostische klassen te definiëren op basis van prognostische variabelen die afhankelijk zijn van patiëntgebonden en behandelingsfactoren, werd beperkt aangepast zodat deze toegepast kon worden op de patiënten in de studie. Zo konden we in deze heterogene groep van patiënten eenvoudige en klinisch relevante prognostische klassen definiëren gebruik makende van de door ons aangepaste RTOG RPA-classificatie. Daarnaast vonden we in de prognostische klassen III en IV patiënten die langdurig overleefden na het recidief, hetgeen een opmerkelijke bevinding is in patiënten met een (meervoudig) recidief HGG en een resultaat dat beter is dan in bijna alle andere studies bij gelijkaardige patiënten.

Vertrekkende van het al gepubliceerde gegeven dat leeftijd een belangrijke prognostische factor is voor op DC gebaseerde vaccinatie bij patiënten met een recidief HGG, hebben we de resultaten bekeken bij 45 kinderen (jonger dan 18 jaar) met een recidief kwaadaardige hersentumor die behandeld werden met autologe DC-vaccinatie (HGG-IMMUNO-2003). We konden de klinische haalbaarheid van deze aanpak aantonen zonder belangrijke nevenwerkingen, zelfs bij jonge kinderen. Patiënten met een HGG lijken meer voordeel te halen uit deze behandeling dan patiënten met een ependymoma of medulloblastoma/primitieve neuro-ectodermale tumor. Daarnaast lijkt het toevoegen van

immunotherapie aan radio- en chemotherapie voor patiënten met een atypische teratoide-rhabdoide tumor (ATRT) een gunstig effect te hebben. Hoewel onze resultaten preliminair zijn en voortkomen uit een heterogene groep van patiënten, ondersteunen ze verder onderzoek naar de inpassing van deze vernieuwende immunotherapeutische aanpak in nieuwe behandelingsprotocollen voor HGG en ATRT.

In een volgende stap integreerden we op DC gebaseerde immunotherapie in de conventionele primaire behandeling van GBM, bestaande uit maximaal veilige resectie, bestraling en temozolomide chemotherapie. Tumorvaccinatie bleek haalbaar te zijn in een pilootstudie met 8 volwassen patiënten met een nieuw gediagnosticeerd GBM, en werd daarnaast goed verdragen. De overlevingsgegevens van deze studie werden gebruikt om de fase I/II HGG-2006 studie voor patiënten met een nieuw gediagnosticeerd GBM te ontwerpen. In de HGG-2006 studie werden 77 patiënten ingesloten en de mediane overleving was 18.3 maanden (analyse van de volledige groep patiënten). Dit verhoudt zich gunstig tot de mediane overleving van 14.6 maanden die werd gerapporteerd in de studie van Stupp *et al.*, al is deze historische vergelijking als dusdanig moeilijk. Daarom werd ook hier een analyse volgens een gevalideerd prognostisch model uitgevoerd en vooral de patiënten in EORTC RPA-klassen III en IV lijken langer te overleven dan met de conventionele aanpak. Het inpassen van immunotherapie in de gangbare postoperatieve behandeling van patiënten met een nieuw gediagnosticeerd GBM is gebaseerd op de veronderstelde synergistische werking van conventionele therapieën en immunotherapie. Men gaat ervan uit dat de combinatie van bestraling, chemo- en immunotherapie de antitumorale werking van elk kan versterken indien de verschillende therapieën goed op elkaar zijn afgestemd. Van deze radio-chemo-immunotherapie hebben we de haalbaarheid kunnen aantonen met klinische weerslag en aanvaardbare levenskwaliteit.

Ten slotte hebben we het volgen van een immunologisch antwoord in gevaccineerde patiënten met een nieuw gediagnosticeerd GBM geoptimaliseerd. Zowel in de piloot- als in de HGG-2006 studie hebben we flowcytometrie aangewend als middel om het uitlokken en/of onderhouden van een door de vaccinatie geïnduceerde antitumorale afweer te kunnen volgen in bloedstalen van patiënten. Hiervoor hebben we eerst het gebruik gevalideerd van de afwezige of minimale expressie van de interleukine-7 alfa receptor subeenheid (CD127) als kenteken van regulatoire T-cellen bij patiënten met een GBM, aangezien deze cellen een belangrijke rol kunnen spelen in het onderdrukken van immunologische afweerreacties. Het volgen van regulatoire T-cellen bij gevaccineerde patiënten is dan ook essentieel. Op basis van de resultaten van de flowcytometrie konden ‘immunologische profielen’ worden

opgesteld die werden vergeleken met de klinische resultaten. We konden echter (nog) geen duidelijke correlatie vinden tussen de immunologische en klinische gegevens. Een mogelijke verklaring hiervoor zou kunnen zijn dat de immunologische status zoals gemeten in het bloed niet overeenstemt met de immunologische afweerreacties die plaatsvinden in de tumor zelf. In een volgende stap hebben we dan ook een fenotypische beschrijving uitgevoerd van afweercellen die de hersentumor binnendringen, en deze dan vergeleken met de immunologische status in het bloed. Inderdaad lijken de vroegtijdige resultaten erop te wijzen dat de immunologische afweerreacties in de tumor niet overeenstemmen met deze in het bloed. Wat dit betekent voor het volgen van een immunologisch antwoord in gevaccineerde patiënten moet nog verder worden onderzocht, maar het volgen van mogelijke afweerreacties in het bloed is hiervoor waarschijnlijk niet de beste methode.

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CURRICULUM VITAE

Hilko Ardon was born in Lekkerkerk, the Netherlands, on August 22nd, 1978. He received his secondary education at the Stedelijk Gymnasium Breda, the Netherlands, from which he graduated in 1996. In the same year, he started his medical studies at the Katholieke Universiteit Leuven. During his studies, he was a student-researcher at the Center for Surgical Technologies (department of Experimental Gynecology) in 1999 and 2000, supervised by prof. dr. J. Deprest and prof. dr. R. Devlieger. In 2000-2001, he was vice-president of "Medica", the student organization of the faculty of Medicine. One year later, he attended clinical attachments in Pediatrics and Obstetrics & Gynecology at the Queen's University of Belfast, UK. He obtained his medical degree in 2003, magna cum laude, and started a further training in neurosurgery at the University Hospitals Gasthuisberg, Leuven, in the same year, under supervision of prof. dr. C. Plets and prof. dr. J. Goffin. In 2006, he started his PhD project, described in this thesis, at the K.U. Leuven, with prof. dr. S. De Vleeschouwer and prof. dr. S. Van Gool as promoter and co-promoter respectively. In August 2010, he obtained his board certification as a neurosurgeon. Hilko Ardon married Marie Boonen in 2005 and they are the parents of Emma, Finn and Thomas.

LIST OF PUBLICATIONS

- S. De Vleeschouwer, **H. Ardon**, F. Van Calenbergh, R. Sciot, G. Wilms, J. van Loon, J. Goffin and S. Van Gool: Validation of a modified RTOG Recursive Partitioning Analysis model in a large group of patients with relapsed malignant glioma treated by re-operation and adjuvant postoperative Dendritic Cell vaccination (submitted)
- **Hilko Ardon**, Steven De Vleeschouwer, Frank Van Calenbergh and Stefaan Van Gool: High-Grade Gliomas: Dendritic Cell Therapy, in: Hayat, M.A. (Ed.), Tumors of the Central Nervous System, Volume 2. Springer Company (in press)
- **Hilko Ardon**, Stefaan Van Gool, Frank Van Calenbergh en Steven De Vleeschouwer: Immunotherapie bij gliomen: een overzicht van werkingsmechanismen en een stand van zaken van de klinische studies. Tijdschrift voor Neurologie en Neurochirurgie (in press)
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