

## MINI-SYMPOSIUM: Immunotherapy of Gliomas

**Dendritic Cell Therapy of High-Grade Gliomas**Stefaan Van Gool<sup>1</sup>; Wim Maes<sup>2</sup>; Hilko Ardon<sup>3</sup>; Tina Verschuere<sup>2</sup>; Sofie Van Cauter<sup>4</sup>; Steven De Vleeschouwer<sup>3</sup><sup>1</sup> Laboratory of Experimental Immunology, and Department of Child & Woman, Catholic University of Leuven, Leuven, Belgium.<sup>2</sup> Laboratory of Experimental Immunology, Catholic University of Leuven, Leuven, Belgium.<sup>3</sup> Laboratory of Experimental Immunology, and Department of Neurosciences, Catholic University of Leuven, Leuven, Belgium.<sup>4</sup> Department of Radiology, Catholic University of Leuven, Leuven, Belgium.**Keywords**

clinical trials, dendritic cells, glioblastoma, high-grade glioma, immunotherapy, translational medicine.

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**Abstract**

The prognosis of patients with malignant glioma is poor in spite of multimodal treatment approaches consisting of neurosurgery, radiochemotherapy and maintenance chemotherapy. Among innovative treatment strategies like targeted therapy, antiangiogenesis and gene therapy approaches, immunotherapy emerges as a meaningful and feasible treatment approach for inducing long-term survival in at least a subpopulation of these patients. Setting up immunotherapy for an inherent immunosuppressive tumor located in an immune-privileged environment requires integration of a lot of scientific input and knowledge of both tumor immunology and neuro-oncology. The field of immunotherapy is moving into the direction of active specific immunotherapy using autologous dendritic cells (DCs) as vehicle for immunization. In the translational research program of the authors, the whole cascade from bench to bed to bench of active specific immunotherapy for malignant glioma is covered, including proof of principle experiments to demonstrate immunogenicity of patient-derived mature DCs loaded with autologous tumor lysate, preclinical *in vivo* experiments in a murine orthotopic glioma model, early phase I/II clinical trials for relapsing patients, a phase II trial for patients with newly diagnosed glioblastoma (GBM) for whom immunotherapy is integrated in the current multimodal treatment, and laboratory analyses of patient samples. The strategies and results of this program are discussed in the light of the internationally available scientific literature in this fast-moving field of basic science and translational clinical research.

**INTRODUCTION**

High-grade gliomas (HGGs) consist of World Health Organization (WHO) grade III and WHO grade IV neoplasms: anaplastic/malignant gliomas respectively glioblastomas (GBMs). While GBMs are the most frequent and most malignant of these tumors, they still belong to the category of orphan diseases, with a yearly incidence of about three to four per 100 000 adults (42) and two per million children (120). The treatment for these patients consists primarily of surgery in order to debulk the tumoral mass for symptomatic relief and to obtain tissue for histological diagnosis, as well as radiochemotherapy to induce optimal local tumor control. In spite of improved surgical and radiotherapeutic techniques, and the addition of temozolomide to the multimodal treatment strategy, the

prognosis of patients with GBM remains poor: the median overall survival (OS) is about 15 months, with 88% of patients dead within 3 years (117, 118). Indeed, even after maximal treatment with surgery, radiotherapy and chemotherapy, relapse is universal and is believed to be due to the extensive spread of tumor cells into surrounding regions of the brain (68, 21). At the time of relapse, the prognosis is particularly poor, with reports of 100% mortality within 1.5 years (13). As such, there is obviously a need for more effective new therapies. One of the particular challenges with classical chemotherapeutic strategies is overcoming the blood-brain barrier. Therefore, much of today's preclinical research is focused on alternate approaches, such as more selective therapies, which specifically target intracellular signaling pathways or surface molecules (44, 84), antiangiogenesis strategies (46) and especially

immunotherapy. Acknowledging that different aspects of immunotherapy for malignant gliomas have been extensively reviewed in the last couple of years (149, 97, 66, 144, 94, 96, 28, 41, 27, 116, 2, 3, 133, 102, 17, 140, 38, 79, 141), the aim of the present paper is to present our own translational research program efforts as a disease-specific paradigm for active specific immunotherapy, rather than contributing one more encyclopedic review of dendritic cell (DC) therapy overall.

## PRINCIPLES OF DC THERAPY IN GLIOMA

### Active specific immunotherapy

Immunotherapy of HGGs covers a broad field comprising several approaches. These include passive immunotherapy with monoclonal antibodies (mAb) (132), passive immune stimulation with cytokines (restorative) (86, 11, 148, 145, 39), treatment with stimulated T cells (adoptive) (113, 106, 100) and active specific immunotherapy. In the latter approach, DCs are most commonly used to stimulate the immune system, given their role as the most powerful antigen-presenting cells (APCs) known to date. DCs are a subset of white blood cells, critical to most aspects of adaptive immunity due to their central role as specialized APCs in the initiation phase of T cell responses (83, 54). Typically, DCs reside as immature cells in almost every organ and tissue at the interface of potential pathogen entry sites. In this state, they continuously sample antigens. This sampling, however, results in effective antigen presentation only when DCs are also triggered by danger signals derived from pathogens, tissue damage or signs of inflammation. As danger-triggered DCs start to mature and get activated, they up-regulate chemokine receptors, which guide them to draining lymph nodes. There, the mature DCs are capable of inducing primary T cell responses due to their high levels of major histocompatibility complex (MHC), adhesion and costimulatory molecule expression. Only mature DCs show the typical dendritic morphology with multiple cytoplasmic extensions (7). Unlike other APCs like macrophages, monocytes and B cells, DCs are able to present and cross-present the antigenic peptides in the context of both MHC Class II and Class I molecules, respectively (110, 109). In this way, they can prime not only CD4+ T helper cells, but also CD8+ cytotoxic T cells (23). Both effector cell types are believed to be necessary to induce an effective cell-mediated immune response (34, 72, 121). Direct recognition of tumor cells by unprimed T cells, in contrast, induces T cell anergy rather than immunity as the costimulatory molecules are missing on the tumor cells (127, 126).

DCs are not only sentinels in the adaptive immune response, but have also been shown to be strong activators of natural killer (NK) cells and NK T cells (31), thus linking the innate and adaptive immune responses. In this way, both tumor cells with and without expression of MHC Class I molecules can theoretically be killed (8). All these particular characteristics make DCs the perfect adjuvants in active specific immunotherapeutic strategies, in which one aims to induce a specific immune response *in vivo* (6, 136, 119, 45, 59). Gliomas have been shown to express several tumor-associated antigens (TAAs), including epidermal growth factor receptor isoform III (85), glycoprotein 240 (69), tenascin (128), survivin (139), squamous cell carcinoma-associated reactive antigen for cytotoxic T cells 1 (57), the alpha-2 chain of interleukin 13 receptor

(IL-13R $\alpha$ 2 chain) (93), melanoma-associated antigens like tyrosinase, tyrosinase-related protein 1 and 2, gp100, melanoma antigen (MAGE)-1 and MAGE-3 (18, 115, 75, 76) and Immunoselected Melanoma-2 (AIM-2) (77), all extensively reviewed by Dunn *et al* (35). Moreover, GBM patients exhibit circulating tumor-specific CD8+ T cells (122). Until now, however, a universally expressed glioma TAA with a critical downstream cell survival-related function has not been identified. Therefore, just targeting the known TAAs using individual peptides would inherently lead to immune escape because of the positive clonal selection of antigen-loss variants (48). In other words, those tumor cell clones that do not express the particular targeted TAA (anymore) will escape immune rejection and thus have an important proliferation advantage as compared with the cell clones that still express the targeted TAA. That heterogeneity in TAA expression in gliomas represents the main reason why most investigators favor the use of whole-tumor cell antigens as a source of TAAs to load the DCs. For this, acid-eluted membrane peptides (73, 147, 74), apoptotic bodies after gamma-irradiation (130), tumor lysates (142, 143, 146, 135, 134, 25, 111, 30) and fusion of DC with tumor cells (64, 65) have all been used to load DCs. Of note, Khan *et al* (63) isolated proteins out of paraffin-embedded tissue to load the DCs. To our knowledge, no tumoral RNA has been used to load DCs for administration to patients thus far. In a comparative analysis, the loading with apoptotic bodies or total tumoral RNA seemed to be superior to loading with lysate or fusion of HGG cells with DCs (95). However, loading with apoptotic bodies of HGGs has also been shown to increase the risk of inducing tolerogenic DCs via the cyclooxygenase-2 (COX2) pathway (4). Loading DCs with specific antigens that play key roles in tumor biology has also been attempted (138, 19, 20). Because glioma cancer stem cells might represent the therapy-resistant fraction of HGGs, cancer stem cell-redirected immunotherapy has been explored (99, 98). In our translational research program, we developed patient-derived autologous mature DCs loaded with lysate of the autologous HGG (DCm-HGG-L) for *in vitro* investigation and for clinical applications. In our murine model, we used DCm loaded with lysate of the GL261 murine glioma tumor cells or with total RNA of GL261 cells (DCm-GL261-RNA). The GL261 glioma cell line is derived from Methylcholantrene-induced glioma tumor cells in C57BL/6J mice.

### Experimental rodent models for malignant glioma

#### Hurdles associated with current animal models

Over the last 4 decades, hundreds of reports of heterotopic and orthotopic rat and mouse GBM models have been published. It is extremely important to be aware of some fundamental differences between those models, for example, between models exploiting spontaneous tumor formation in genetically engineered mice *versus* engrafted tumor models that are established by implantation of primary tumor cells or tumor cell lines. Although spontaneous tumor models are mimicking the clinical situation of gliomagenesis much better than the engrafted models, the main drawbacks are the poor reproducibility, low tumor penetrance, prolonged latency for tumor formation and the need for advanced *in vivo* imaging techniques. On the other hand, because engrafted models lack the

stepwise genetic changes occurring during tumor progression, many of them are well circumscribed, 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 immunotherapy, as long as the models are studied in immunocompetent animals (16, 43, 22).

### Data from the literature

Below, we briefly summarize the most relevant data on the murine GL261 glioma model, as this model is also used in our translational research program. Ni *et al* underscored the immunogenicity of the GL261 tumor cells by treating mice with intracranial glioma with tumor extract-pulsed cloned DCs. Cured animals showed increased delayed-type hypersensitivity (DTH) responses to GL261 cells, with long-term tumor protection (89). Aoki *et al* showed that pulsing DCs with a complex of tumor extract with cationic liposomes induces an antitumor immune response against intracerebral glioma in which CD8+ T cells are involved (5). Protective immunity against intracranial glioma growth obtained through immunization with either lysate- or RNA-loaded DCs was reported by Insug *et al*, while adding recombinant IL-12 to the vaccine regimen further improved its efficacy (58). A survival benefit of combining vaccination with lysate-pulsed DCs and interferon (IFN)- $\beta$  gene therapy was demonstrated by Saito *et al* (112). Similarly, the group of Okada *et al* revealed that the sequential intratumoral delivery of an IFN- $\alpha$  encoding adenoviral vector and DCs induced long-term survival and specific cytotoxic T lymphocyte (CTL) activity (123). Furthermore, the same group showed that intratumoral administration of DCs, genetically engineered to secrete IFN- $\alpha$ , enhances the efficacy of peripheral vaccines with cytokine gene-transduced tumor cells (70). Kjaergaard *et al* observed complete tumor regression of established intracranial tumors with infiltration of both CD4+ and CD8+ T cells using vaccines created through electrofusion of DCs and irradiated tumor cells (67). Survivin, a member of the apoptosis inhibition family of proteins, has been the GL261 TAA of choice for Ciesielski *et al*. In particular, the authors exploited the xenogeneic differences between human and murine survivin sequences to develop a more immunogenic tumor vaccine (19, 20). The efficacy of systemic immunotherapy with DCs loaded with GL261 antigens was confirmed by Pellegatta *et al*. 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 (99, 98). Recently, Grauer *et al* illustrated the pronounced impact of FoxP3+ regulatory T cells (Tregs) in this model and suggested that Treg elimination is a prerequisite for successful eradication of established glioma using tumor lysate-pulsed DCs (50, 51).

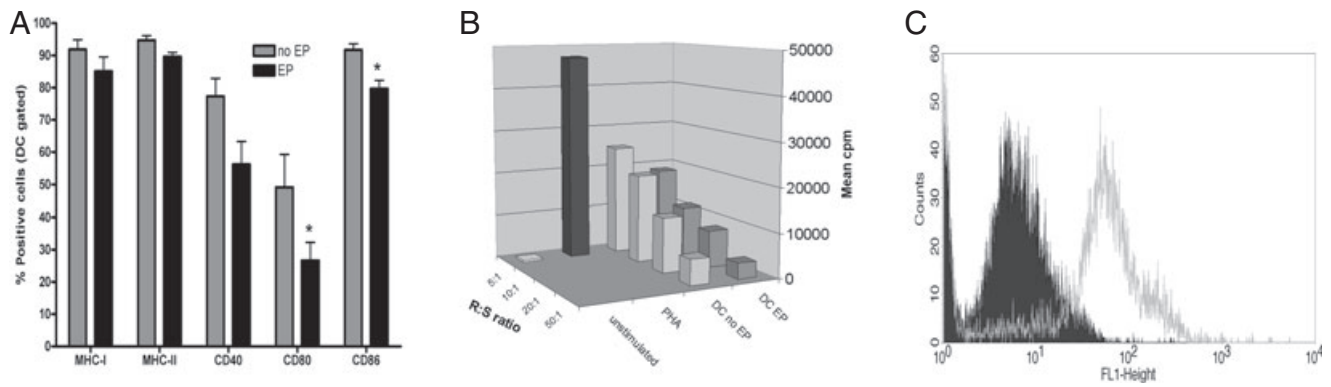
### The concept of DC treatment in the orthotopic GL261 mouse model

We implemented the concept of active glioma immunotherapy with RNA-loaded DCs in the GL261 murine model step-by-step. The rationale for loading DCs with total RNA from tumoral origin is to be able to provide DC immunotherapy for those glioma patients where insufficient tumor tissue is available to produce an optimal

amount of DCm-HGG-L, for example, in patients with a small tumor mass of a relapsed tumor located in a previously irradiated area. Moreover, RNA molecules are translated into proteins by the DCs themselves and those proteins are subsequently processed in the endogenous pathway of antigen processing, resulting in optimal priming of CD8+ T cells. Researchers have dedicated a lot of effort to the improvement of RNA transfection methods. Three techniques have been investigated: passive transfection, lipid-mediated transfection and electroporation (47, 53). Using the latter technique, immature DCs are transfected with 15  $\mu$ g of total tumoral RNA per million DCs through exponential decay electroporation (300 V and 150  $\mu$ F) with a GenePulser electroporator (Bio-Rad, Nazareth, Belgium) (82, 81). By now, electroporation has proven to be the method of choice for the introduction of exogenous RNA molecules into DCs. It does not require additional reagents and is compatible with clinical use (37). With this technology, improving the intrinsic characteristics of DCs such as cytokine, chemokine and costimulator expression through transfection with additional mRNA species becomes possible and seems very promising (1, 12).

Initially, we focused on testing of our cellular product (murine DCs electroporated with total RNA from GL261 tumor cells, DCm-GL261-RNA) in an experimental *in vitro* design. The procedure of electroporation caused only minor changes in the DC phenotype (Figure 1a), and did not alter the stimulatory capacity of DCs in a mixed lymphocyte reaction (Figure 1b), while the expression of enhanced green fluorescent protein at the protein level could be measured (Figure 1c). *In vitro*, DCm-GL261-RNA were able to stimulate T cells to become specific effector T cells with cytotoxic activity against the GL261 target cells (Figure 2). We noticed a nonspecific T cell cytotoxic activity when stimulated with mock-electroporated DCm or DCm electroporated with total RNA from Lewis lung cancer (DCm-LCC-RNA). Nevertheless, specificity of the induced immune response could be demonstrated *in vivo* (81). The data obtained from these *in vitro* stimulation experiments confirmed that DCs loaded with tumor antigens in the form of RNA molecules are capable of inducing a T cell-mediated antitumor immune response. These results are consistent with published preclinical data from our group (82, 81) and others (10).

Since *in vitro* systems merely partially mimic the complex tumor microenvironment and cell interactions *in vivo*, we proceeded to validate our findings in the *in vivo* experimental mouse glioma model (82, 81). In this model, we hope to unravel new mechanisms governing glioma immunology that can eventually be exploited in translational oncology practice (Figure 3a). We opted for prophylactic immunization because others reported the GL261 model to be highly aggressive, necessitating additional intervention for curative settings (40, 78). Moreover, this model reflects the clinical therapeutic setting, at least locally in the brain, in which DC vaccination is given at a stage of minimal residual disease after (sub)total resection and not at the time of bulky disease. We adapted the model in order to monitor tumor growth consecutively with bioluminescence imaging (BLI) (82). We have shown that there is a strong correlation of the BLI signal with *ex vivo* measured tumor volumes. Moreover, implementing optical imaging in our experimental glioma model resulted in a more detailed characterization of GL261 glioma behavior *in vivo*, demonstrating midline crossing of tumor cells, along with the observation of an initial tumor cell adaptation phase before consistent tumor progression.

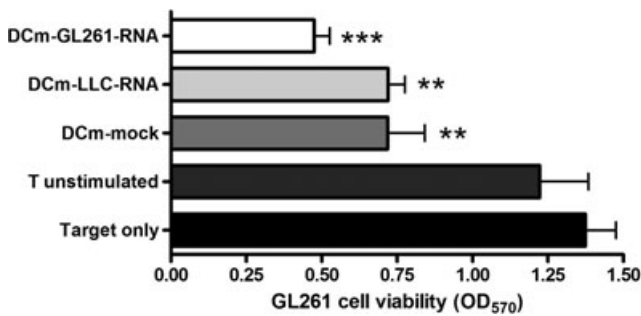


**Figure 1.** Electroporation of DCs with RNA-molecules is feasible without loss of cell function *in vitro*. **A.** Immature DCs were left untouched (no EP) or electroporated (EP) and immediately afterwards matured with 1 µg/mL (*Escherichia coli*) lipopolysaccharide for 24 hours. Mature DCs were analyzed by flow cytometry for surface marker expression. EP significantly lowered the expression of CD80 and CD86 on DCs (overall analysis of variance  $P < 0.001$ ,  $*P < 0.05$ ). **B.** The capacity to stimulate allogeneic cells was assessed in a mixed lymphocyte reaction in 96 well format with total  $2 \times 10^5$  splenocytes from naive BALB/C mice as responder (R) cells and DCs from C57BL/6 mice as

stimulator (S) cells. Unstimulated cells and phytohemagglutinin (PHA) were used as negative and positive controls, respectively. Responder to stimulator ratios (R : S) ranged from 5:1 to 50:1. After 4 days of culture, thymidine incorporation was measured by pulsing cultures for 18 hours with 1 µCi [<sup>3</sup>H] thymidine per well. Mean counts per minute (cpm) are depicted. **C.** One million DCs were electroporated with 1 µg mRNA encoding enhanced green fluorescent protein (eGFP) and eGFP expression (grey curve in FL1-Height histogram) was assessed by flow cytometry 24 hours after electroporation. The filled curve represents nonelectroporated DCs.

Vaccination with DCm-GL261-RNA prolonged survival and could cure nearly half of the treated animals subjected to subsequent tumor challenge (81). As shown in Figure 3b, we could not detect a difference in outcome in this model when the mice were treated with DCm-GL261-RNA vs. DCm-GL261-L (median sur-

vival of 36 and 34 days, respectively, compared to 21 days for untreated mice). Mapping of weight loss and tumor-induced neurologic deficit clearly paralleled the survival data. Finally, histological analysis revealed that DCm-GL261-RNA vaccination resulted in infiltration of lymphocytes and nonlymphoid cells, such as macrophages, particularly at the interface of the tumor mass with the normal brain parenchyma (81). These findings are in agreement with the data previously reported by Insug *et al* (58).

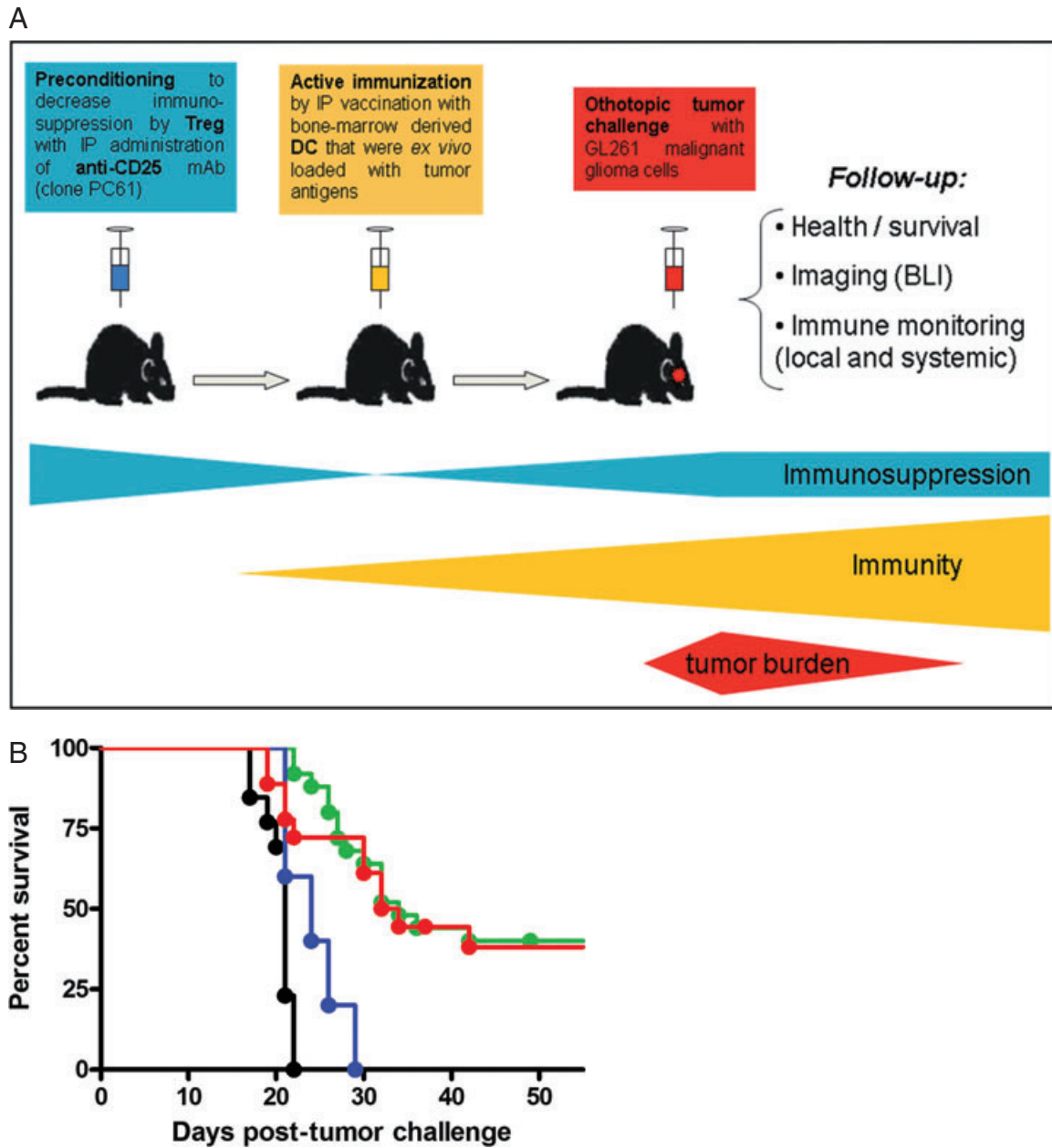


**Figure 2.** RNA-loaded DCs induce a T cell-mediated antitumor immune response *in vitro*. T cells pooled from spleen and lymph nodes of a naive C57BL/6 mouse were stimulated *ex vivo* (two stimulation rounds with DC : T cell ratio of 1:10 with addition of 20 U/mL of IL-2) with either mock-loaded DCm (DCm-mock), DCm electroporated with total RNA extracted from Lewis lung carcinoma cells (DCm-LLC-RNA) or DCm electroporated with total RNA extracted from GL261 glioma cells (DCm-GL261-RNA). Unstimulated T cells were used as the control. Stimulated T cells were then used as effector cells and coincubated with GL261 tumor cells in an effector to target ratio of 10:1. Tumor cell viability was assessed by measuring the metabolization of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to formazan (measured at 570 nm) by living cells after 2 days of co-culture. Pooled results (n = 3) are depicted as optical density values at 570 nm (OD<sub>570</sub>) and compared to GL261 target cells without effector cells. Overall analysis of variance  $P < 0.001$ ;  $**P < 0.01$ ,  $***P < 0.001$ .

**Which cells are players in anti-GL261 mouse glioma immunotherapy?**

Why vaccination only results in tumor protection for half of the treated mice is still not completely understood. Longitudinal imaging of tumor-bearing mice with BLI revealed that within the animals that eventually succumbed, distinct patterns of nonresponding and partially responding mice can be discriminated (82). We hypothesize that there is a very delicate balance between antitumor immune responses on the one hand (either endogenous or induced/boosted by the DC vaccine) and natural and tumor-induced immune suppression on the other. In responding mice, it is apparent that treatment with DCs shifts this balance away from tolerance and toward immunity, resulting in protection against tumor growth. In contrast, in mice refractory to DC vaccination, DCs might actually induce or expand immune-suppressive Tregs and/or immune-suppressive and tumor-promoting myeloid-derived suppressor cells (MDSCs) (103).

Our data provided a rationale to investigate in-depth the mode of action of DC treatment and to implement strategies that are aimed at lowering the detrimental immune suppression within the tumor microenvironment. In order to study the individual role of separate cell populations, we used depleting mAb to selectively remove the targeted cell population (Figure 3a). When CD8+ T



**Figure 3.** Immunotherapy in the GL261 experimental orthotopic glioma mouse model. **A.** Overview of the prophylactic DC vaccination model. Adult C57BL/6 mice are orthotopically challenged with  $5 \times 10^5$  syngeneic GL261 malignant glioma cells injected 1.5 mm posterior and 1 mm lateral from the bregma at a depth of 3 mm under stereotactic guidance. Read-out consists of monitoring general health parameters (weight and neurological symptoms), overall survival analysis, *in vivo* bioluminescence (BLI) requiring GL261 cells expressing luciferase in white mutant C57BL/6.Cg.Tyrc-2J/J mice, and detailed immune monitoring both systemically (in blood, spleen and tumor-draining cervical lymph nodes) and locally within the brain. Immunotherapy in our preventive treatment setting consists of either active immunization, decreasing regulatory T cell (Treg)-mediated immune suppression, or combined treatment. Active immunization is performed through intraperitoneal (IP) vaccina-

tion on day 14 and day 7 before tumor challenge with one million mature bone-marrow derived CD11c+ DCs that were loaded *ex vivo* with tumor antigens (in the form of total RNA). In some experiments, animals were preconditioned with a single intraperitoneal injection (250  $\mu$ g) of the depleting anti-CD25 monoclonal antibody (clone PC61). This *in vivo* depletion of CD25-expressing cells was performed 1 week before the first vaccination with DC (or 3 weeks before tumor challenge if no DC vaccination was administered). **B.** Pooled survival data represented as Kaplan-Meier graph. Overall log rank  $P < 0.001$ . Survival curves of animals vaccinated with DCm-GL261-RNA (green curve,  $n = 27$ ), DCm-GL261-L (DC loaded with GL261 lysate) (red curve,  $n = 19$ ), DCm-splenocyte-RNA (blue curve,  $n = 5$ ) and untreated animals (black curve,  $n = 13$ ) are depicted.

cells were removed, tumor-challenged mice had worse outcomes as compared with the control-challenged mice, suggesting that the injection of GL261 cells as such induces a minor endogenous CD8<sup>+</sup> T cell response in the immunocompetent hosts. When CD8-depleted mice were subsequently treated with DCm-GL261-RNA, the survival was still prolonged as compared with CD8<sup>+</sup>-depleted or control-untreated animals, but none of the mice ultimately survived. These data suggest that besides a major role of CD8<sup>+</sup> T cells in inducing the antitumoral immune effect leading to long-term survival, other immune cells stimulated via DC vaccination play a role as well. When Tregs were depleted via anti-CD25 mAb, all mice survived the subsequent tumor challenge, without or with DC treatment. Although the injection of anti-CD25 mAb only transiently depleted circulating CD4<sup>+</sup>CD25<sup>+</sup>FoxP3<sup>+</sup> Tregs, we found an increased amount and specific antitumoral activity of brain-infiltrating lymphocytes in these Treg-depleted animals, even stronger than in the animals that were treated with DCm-GL261-RNA. Whereas an infiltration of MDSCs was found in the control tumor-challenged mice, similar to published data from others (49, 125), we found an increased infiltration of macrophages with M1 phenotype in the Treg-depleted animals, releasing or unmasking an endogenous tumor rejecting immunosurveillance. Finally, upon rechallenge of long-term surviving mice after Treg depletion and/or DC vaccination, only those mice that were treated with DC vaccination depicted a prolonged antitumor protective immune response. Upon rechallenge, the CD25-depleted surviving animals that were not vaccinated showed mortality rates similar to untreated mice. All together, our data suggest that there is a complex interaction between different cell populations: tumor cells, effector T cells, memory T cells, Tregs and macrophages with different functional phenotypes. Most likely all these cells interact with each other, and the net result of complex interactions determines the outcome of the mice upon vaccination.

### Human DCm-HGG-L

In preparation for potential clinical use, we studied the loading of human DCs with tumor lysates *in vitro*, as well as the potency of DCm-HGG-L (26, 29). DCs were differentiated out of monocytes, obtained after two adherence cycles of peripheral blood mononuclear cells. Differentiation was induced during 6 days with medium change and addition of IL-4 and Granulocyte/Macrophage Colony-Stimulating Factor (GM-CSF) at day 1, with 30% medium and 100% cytokine refreshments at days 3 and 5. Loading with lysate was performed at day 6, after which, maturation stimuli were added to the cultures for one day: IL-1 $\beta$ , tumor necrosis factor (TNF)- $\alpha$  and prostaglandin E2 (PGE2). The phenotypic and functional characteristics of these so-called early mature DCs have been described (26, 29). After two cycles of stimulation, the first stimulation in the presence of IL-6 and IL-12 and the second in the presence of IL-4 and IL-7, DCm-HGG-L were able to induce T cell proliferation. Upon stimulation, a shift towards Th1 cytokine production was observed. Finally and most importantly, DCm-HGG-L induced tumoricidal and tumor-suppressive activity by the T cells, which was antigen specific, MHC specific and MHC restricted, pointing to CD8<sup>+</sup> cytotoxic T cell activity. These cells were prepared as cellular product to be injected intradermally in the shoulder region of patients.

It is important to note here that multiple steps in the production process, as well as the administration of the DC vaccine, are amenable to significant improvement. In fact, no comparative clinical data are currently available pertaining to the optimal cellular source of DCs, cytokine cocktail to induce DC differentiation, source of tumor material, maturation cocktail, amount of cells to be injected, volume in which the cells should be suspended, route of injection, site of injection, schedule for vaccination, method for boosting the immune response, and combinations with other antitumoral and immunomodulatory treatment strategies. Still, progress has been made over the last few years, and currently, most groups use DCs that were matured *ex vivo*, injecting these DC vaccines intradermally or directly into lymph nodes.

## DESIGN OF THERAPEUTIC STUDIES

### Selection of patients

Upon literature search, three case reports and thirteen phase I, phase I/II or phase II trials have been published on DC vaccination of glioma patients since 2000 (Table 1). 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 derived regarding the optimal immunotherapeutic strategy, nor would it make sense to perform a meta-analysis on these data. Already in our first reports (25, 111), we stressed the importance of a minimal residual disease setting for adjuvant postoperative DC vaccination in HGG patients. In the largest series published to date (30), we confirmed the importance of a gross total resection prior to DC vaccination for GBM patients 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 directed to 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 (142, 146, 143), which one would expect to have a negative effect on the generation of an effective immune response.

In order to avoid this confounding variable, our trial has been restricted to patients with relapsed HGG, who could obtain a total or near-total resection and be rapidly weaned from corticosteroids within 1 to 2 weeks after surgery. There are multiple arguments supporting the need for these restrictions. First, because there are major immune-suppressive mechanisms at play within the tumor microenvironment (33) and even systemically (36), only a gross-total resection results in a clinically effective recovery of normal immune system function (107). Second, we have not been able to produce good-quality DCs out of monocytes isolated at the time of corticosteroid treatment. Third, we have previously faced an overwhelming peritumoral inflammatory reaction in one patient with bulky residual tumor (111). In this patient, the vaccine-induced inflammatory immune reaction occurred with a progressively increasing delay after the vaccine injection. This is compatible with the hypothesis that the growing tumor and, as such, the increasingly induced immune suppression counteracts the vaccine-induced

**Table 1.** Overview of reported clinical trials. Abbreviations: CTL = cytotoxic T lymphocyte; DC = dendritic cell; DTH = delayed-type hypersensitivity; GBM = glioblastoma multiforme; IFN $\gamma$  = interferon- $\gamma$ ; IL-4 = interleukin-4; JAM = just another method; MHC = major histocompatibility complex; mo = month; NK = natural killer; no. = number; OS = overall survival; pat = patient; PBMC = peripheral blood mononuclear cell; PFS = progression-free survival; qPCR = quantitative polymerase chain reaction; rHL-12 = recombinant human interleukin-12; RTE = naive recent thymic emigrant T cell; TTP = time to progression; TTS = time to survival; w = week.

Author	Year	No. of patients (type of trial)	Antigen source	Administration	Treatment schedule	Immune response	Clinical response
Liau <i>et al</i> (73)	2000	1 (case report)	Allogeneic MHC class I-matched GBM peptides	Intradermal	3 vaccinations	T-cell proliferation	None
Yu <i>et al</i> (147)	2001	9 (phase I)	Tumor-specific MHC-I associated peptides	Subcutaneous	3 vaccinations at 2-week intervals	Systemic CTL cytotoxicity against tumor (n = 4) (JAM assay)	Prolonged survival compared to control group
Kikuchi <i>et al</i> (64)	2001	8 (phase I)	DC fusion with autologous glioma cells	Intradermal	3–7 vaccinations at 3-week intervals	Increase in NK cells in PBMCs (n = 4); increased IFN $\gamma$ in supernatant (n = 6)	Mixed response (n = 1); steroids during vaccination (n = 5); 2 minor responses
Yamanaka <i>et al</i> (142)	2003	10 (phase I-II)	Autologous tumor lysate	Intradermal and/or intratumor (Ommaya)	1–10 vaccinations at 3-week intervals	Increase in NK cells in PBMCs (n = 5); positive DTH reaction to tumor lysate (n = 3); increased T-cell mediated antitumor activity (n = 2); Elispot IFN $\gamma$	Minor response (n = 2)
Wheeler <i>et al</i> (135)	2003	17 (phase I) 17 (phase II)	Autologous tumor lysate	Not reported	3 vaccinations at 2-week intervals 3 vaccinations at 2-week intervals; fourth vaccination 6 weeks after third (n = 10)	Not reported (study on CD8+ RTE)	Not reported
Yu <i>et al</i> (146)	2004	14 (phase I-II)	Autologous tumor lysate	Subcutaneous	3 vaccinations at 2-week intervals	IFN $\gamma$ release in PBMCs (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 <i>et al</i> (25)	2004	1 (case report)	Autologous tumor lysate	Intradermal	6 vaccinations; w1, w3 and further every 4 weeks	Positive DTH reaction to tumor lysate	Long-lasting tumor-free survival (>7 years after vaccination)
Rutkowski <i>et al</i> (111)	2004	12 (phase I)	Autologous tumor lysate	Intradermal	2–7 vaccinations; w1, w3 and further every 4 weeks	Positive DTH reaction to tumor lysate (n = 6)	Partial response (n = 1); tumor-free survival (n = 2; 5 years after vaccination)

Kikuchi <i>et al</i> (65)	2004	15 (phase I-II)	DC fusion with autologous glioma cells	Intradermal	Vaccination + rhIL-12	Positive DTH reaction to tumor lysate (n = 15); increased cytotoxic activity (n = 2); increased intracellular IFN $\gamma$ in CD8+ T cells (n = 1)	Partial response (n = 4); mixed response (n = 1)
Liau <i>et al</i> (74)	2005	12 (multicohort dose-escalation study)	Acid-eluted tumor associated peptides (autologous)	Intradermal	$\leq 3$ vaccinations	CTL response (n = 6)	Median TTP 19.9 mos—OS 18 to >58 mos—median OS 35.8 mos
Yamanaka <i>et al</i> (143)	2005	24 (phase I-II)	Autologous tumor lysate	Intradermal or intratumor (Ommaya)	1–10 vaccinations at 3-week intervals	Positive DTH reaction to tumor lysate (n = 8); positive IFN $\gamma$ Elispot (n = 7)	Partial response (n = 1); minor response (n = 3); significantly increased median survival
Okada <i>et al</i> (92)	2007	2 (A) / 5 (B)	IL-4 transfected fibroblasts + apoptotic glioma cells (A) / autologous tumor lysate (B)	Intradermal	2 vaccinations with 2-week interval	A: increased T cell reactivity; B: no response	A: clinical and radiological response in both patients; B: no response
Wheeler <i>et al</i> (134)	2008	34 (phase II)	Autologous tumor lysate	Subcutaneous	3 vaccinations at 2-week intervals; fourth vaccination 6 weeks after third	Post-vaccine antigen-directed IFN $\gamma$ response in PBMCs (qPCR-based assay) (n = 17); DTH-test resulted in cutaneous GBM in 1 pat (DTH was subsequently discontinued)	Significant positive correlation between post-vaccine response magnitude on one hand and TTS and TTP spanning chemotherapy on the other hand
Walker <i>et al</i> (130)	2008	13 (phase I)	Single-cell suspension of autologous tumor cells	Intradermal	Priming phase consisting of 6 vaccinations at 2-week intervals; further vaccinations at 6-week intervals	Increased T cell infiltration in post-vaccination tumor specimens compared to pre-vaccination specimens (n = 3)	9-month survival (9/13); 12-month survival (6/13); 18-month or longer survival (3/13)
De Vleeschouwer <i>et al</i> (30)	2008	56 (phase I-II)	Autologous tumor lysate	Intradermal	Cohort comparison	Positive DTH reaction to tumor lysate (9/21 at time of diagnosis and 9/17 after 2 vaccinations)	PFS 3 mos; OS 9.6 mos; 2-year OS 14.8%; total resection is a predictor for better PFS; younger age and total resection are predictors for better OS in univariable analysis; tendency towards improved PFS when faster DC vaccination schedule with tumor lysate boosting was applied



**Table 2.** Vaccination schedule in the different cohorts of the cohort comparison trial HGG-IMMUNO-2003 for patients with relapsed HGG. Abbreviations: W = Week; DC = injections with DCm-HGG-L; L = injections with HGG-L without DC; Dark gray area = the time schedule of the induction vaccinations; Gray area = the start of the maintenance boost vaccinations; . . . = Boost vaccines were given each 4 weeks for cohort A and B. After three 4-weekly lysate injections, further boosts were given each 12 weeks for cohort C and D.

W	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	. . .
A	DC		DC				DC				DC				DC			4
B	DC		DC		DC		DC		DC				DC				DC	4
C	DC	DC	DC	DC				L				L				L		12
D	DC	DC	DC	DC				L				L				L		12

immune reaction. This observation pointed to a tumor-specific reaction induced by the vaccine, but maybe also to the gradually shifting balance in favor of tumor-induced immune suppression.

In our initial experiments, we detected an effective antitumoral reaction only 4 months after the start of vaccination (25). This observation and the finding of an increasing tumor-mediated immune suppression (111) brought us to design an accelerated and intensified schedule of induction vaccinations to establish immune control before tumor regrowth occurs and induces tumor-associated immune suppression (Table 2). Two DCm-HGG-L vaccines were administered within 3 weeks in cohort A; five DCm-HGG-L vaccines were administered within 9 weeks in cohort B, whereas four DCm-HGG-L vaccines were administered within 4 weeks in the cohorts C and D.

### Selection of outcome measures

Already before the consensus on study design and clinical end points in DC vaccination was published by the Cancer Vaccine Clinical Trial Working group (56), we designed a cohort comparison trial in which small cohorts of at least 20 patients at a time were treated, with small modifications in DC-based vaccination strategies implemented in a stepwise fashion from one cohort to the next. As such, each cohort served as a control for the next cohort, enabling the investigators to detect or sometimes validate stepwise improvements in the vaccine. The available reports in the literature on DC-based vaccination in patients with malignant glioma result in an overall clinical response rate of 11.4% based on the Macdonald criteria (80), ranging from 0% to 33%, taking into account that in 21% of all reported patients, clinical response was not within the scope of the study (28). Overall, this is still in accordance with the 5%–10% reported clinical responses upon DC-based vaccination in other malignancies, such as melanoma (71). Immunological responses, assessed by a wide variety of immunomonitoring tools, have been demonstrated in 50% of reported cases, ranging from 20% to 100%. Only recently, Wheeler *et al* (134) found stronger evidence between immunological responses and survival in vaccinated glioma patients. 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 pretreatment prognostic variables might have more impact on outcome than any (new) potentially active therapy or treatment strategy. To that end, recursive partitioning analysis (RPA) as originally described and validated for newly diagnosed malignant gliomas by the Radiation

Therapy Oncology Group (RTOG) in 1993, provides us with an excellent model of prognostic classes of pretreatment and treatment-related patient’s variables (24). Using this RPA classification, 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, 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 response with increased OS is at least incomplete. Even immunomonitoring tools directed toward the affected organ, that is, the brain, will have to take into account that the presence of tumor-infiltrating lymphocytes and other immune effectors do not unequivocally correlate with a good or a bad outcome (35). Proof of efficacy of immunotherapy asks for impact on OS in patients with HGG, of whom the different prognostic risk factors are clearly categorized.

### Nature of DCs

All clinical reports have used monocyte-derived DCs for their trials. The methodology for DC preparation is now fairly well established, and gives a sufficient yield of DCm for injections into patients (124). Most of the older trials have used immature DCs (73, 147, 142, 135, 146, 74, 134), while others have used maturation stimuli like TNF- $\alpha$  (64, 65), penicillin-killed *Streptococcus pyogenes* (OK-432) (143), monocyte-conditioned medium (130), IFN- $\gamma$  and TNF- $\alpha$  in combination with IL-4-secreting fibroblasts (92). Only one phase I study focused on the dose of DCs and could not find any dose-limiting toxicity (74).

In order to guide the use of DCm-HGG-L stepwise into clinical practice with the goal of improving clinical outcome, we set up a cohort comparison trial (HGG-IMMUNO-2003) for end-stage patients with relapsed HGG, in which each cohort is designed based on recent knowledge and experience and aimed to provide results that can be compared with earlier cohorts of patients. A cohort comparison approach has been published with the aim to define improved chemotherapeutic control of HGG in childhood (137). Within our approach, we varied some parameters, including not only the vaccination schedule but also the maturation stimuli, while other principle parameters were preserved. All patients received freshly cultured monocyte-derived DCs loaded with autologous HGG lysate (DCm-HGG-L). The monocytes were obtained after plastic adherence and were differentiated with IL-4 and GM-CSF. In cohorts A, B and C, maturation during 24 hours was induced with TNF- $\alpha$ , IL-1 $\beta$  and PGE2, a cocktail, which is

essentially based on the so-called Jonuleit cocktail (60). However, we opted to omit IL-6 from the cocktail, as it is currently known to play a major role in the induction of a Th17 phenotype T cell response (131). As a next step, PGE2 was omitted in cohort D based on recent discussions on the activity of PGE2 at the time of maturation. For instance, PGE2 had already been linked to the induction of a DC2 type years ago (62). Because of its importance mainly for the induction of the mobility of DCs (114), it was kept in the classical maturation cocktail. In more recent studies, however, PGE2 has been shown to induce indoleamine 2,3-dioxygenase (IDO) activity in human DCs, thereby creating a tolerating DC phenotype (9). Moreover, PGE2 up-regulates CD25 on DCs, the latter believed to be a marker of strong DC maturation, while on the other hand representing a marker that is shed and thereby consumes the IL-2 needed for autocrine T cell activation. CD25 expression was analyzed on 226 samples of mature DCs prepared in our hands, and the median CD25 expression was 15% (range: 0% to 76%). Because incompletely matured DCs themselves play a role in tolerance induction (87), we wanted to apply a recently described method to induce *in vivo* DC maturation after preparation of the injection site with imiquimod (88, 108). Imiquimod binds to Toll-like receptor 7 and induces strong DC maturation and activation. Its role in the generation of immune responses in a preclinical *in vivo* model of HGG has been described (104). In cohort D, we combined *ex vivo* maturation with TNF- $\alpha$  and IL-1 $\beta$  with *in vivo* maturation with imiquimod.

We subsequently moved to a third major change in our strategy, besides changing the vaccination schedule and maturation cocktail, by introducing boost vaccines with lysate only, starting with cohort C. The rationale for this was a publication from Jouanneau *et al* (61), clearly demonstrating in the murine GL261 model that optimal survival was reached when priming of the immune system was performed with DCm loaded with tumor antigens, whereas for boosting purposes, only lysate was used. Although not elaborated extensively, one might hypothesize based on these results and our own results in the murine model (81) that profound and/or protracted DC vaccination might result in a higher proportion of Tregs and MDSCs that ultimately down-regulate the global immune reactivity, while HGG-L injection more indirectly maintains the ongoing immune response and the induction of memory T cell responses. Hence, from cohort C on, boost vaccines were given using crude tumor lysates. In cohort D, the lysates were injected intradermally at sites pretreated with imiquimod cream.

### Injection site

At the time of the design of most clinical trials so far, no knowledge was available if and which lymph nodes would be the optimal destination of injected DCs. Later on, however, data became available that priming of T cells by DCs within the cervical lymph nodes induced an integrin homing pattern toward intracerebral locations (15). Other preclinical *in vivo* brain tumor models clearly showed an enrichment of tumor-specific Tregs in the blood and cervical lymph nodes (40). Although intradermal injections in the cervical lymph node region are feasible, we have thus far opted to inject the DCm-HGG-L and HGG-L in the ventral region of shoulder and upper arm, with the idea of targeting the axillary lymph nodes for T cell stimulation.

As pointed out by Tuyaerts *et al* (124), the question of whether DCm-HGG-L 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 DCs ultimately reaches the T cell area of lymph nodes (129). However, taking into account the documented failure rate of even experienced radiologists injecting DCs under ultrasound guidance, and the desire to spread DC vaccination technology to more centers in order to perform large-scale clinical trials, we pragmatically favored the intradermal route for DC injection, similar to most researchers in the field. One particular issue that should become of major interest is the local injection of DCm-HGG-L directly into the tumor region. In their study, Yamanaka *et al* (143) showed some benefit from intratumoral plus intradermal injections of DCs as compared with only intradermal injections, although one should note that this interesting observation is 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 DCs.

### 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.

We have also identified a potential role of imaging to monitor immune reactions in the tissue surrounding the resection cavity or residual tumor lesion (25). 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 effects of immunotherapy of HGG.

## RESULTS

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, immunologically or oncologically (Table 1). In all trials, it seemed to be feasible to administer DC-based vaccines. Moreover, all reports conclude that the toxicity is minimal except for the case reported by our group where we observed an overwhelming inflammatory reaction around a residual tumor (111). Of major importance, none of the reports describe autoimmune phenomena induced by DC vaccination.

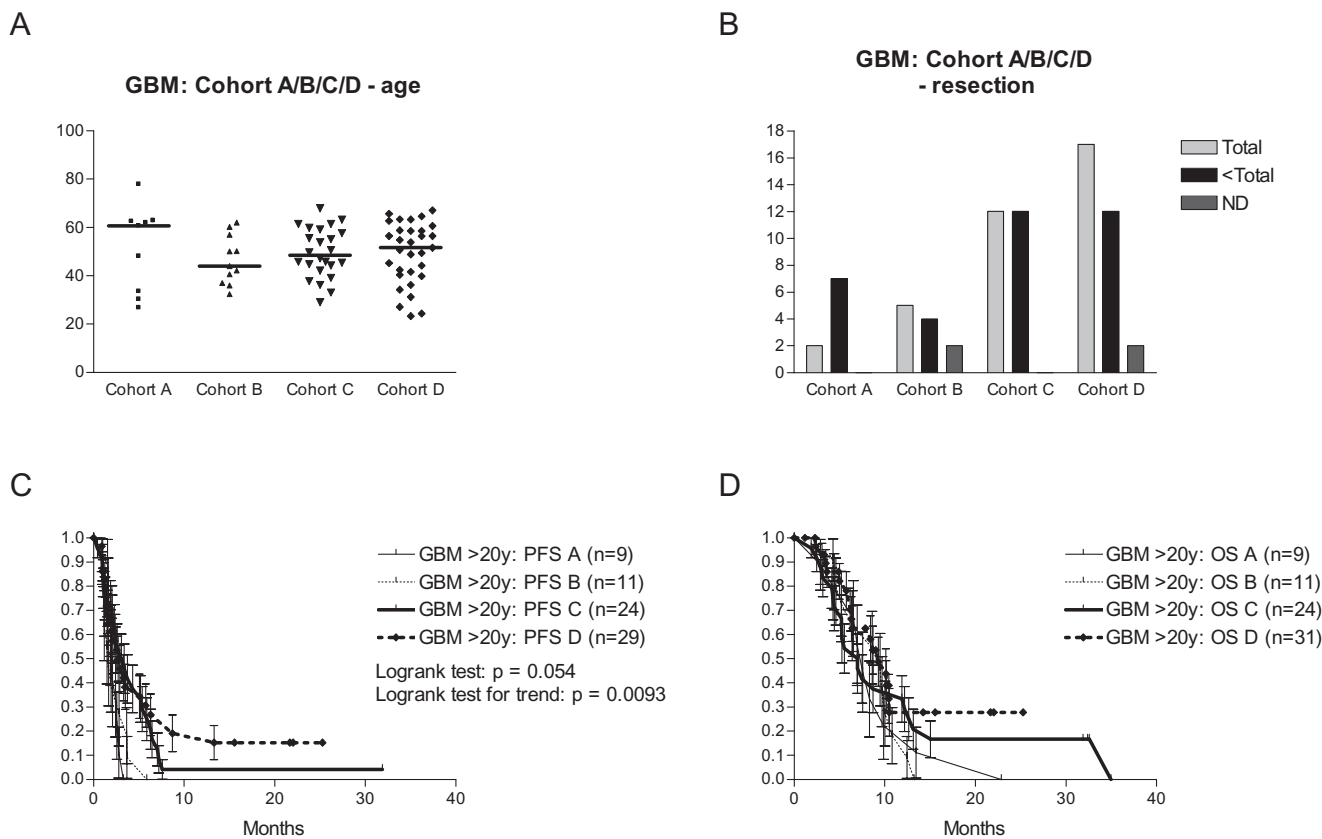
### Data from the literature

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. Moreover, immunological responses are not detected in all patients. Seven studies (142, 65, 25, 111, 143, 134, 30) report positive DTH reactions to tumor lysate in only a subset of treated patients. Liau *et al* (73) described a patient, in which a strong T cell proliferative

response occurred against the allogeneic tumor peptide preparation that was used to pulse the DCs for vaccination. Similarly, an increase in systemic CTL activity was seen in a subset of treated patients in five studies (147, 142, 146, 65, 74). Other immune monitoring assays that have been used are expression of CD8+ antigen-specific T cell clones (146), increase of intracellular IFN- $\gamma$  in CD8+ T cells (65) and an increase in NK cells (64, 142). Also IFN- $\gamma$  release/response by peripheral blood mononuclear cells (PBMCs) has been assessed in five studies. Kikuchi *et al* (64) measured IFN- $\gamma$  in supernatans of a culture of PBMCs with autologous glioma Enzyme-Linked Immuno Sorbent Assay. IFN- $\gamma$ -Elispot assays were used by Yamanaka *et al* (142, 143), while Wheeler *et al* (134) and Yu *et al* (146) used quantitative polymerase chain reaction assessment of IFN- $\gamma$  responsiveness. T cell infiltrates in tumor tissue taken after vaccination was documented by several groups (73, 147, 142, 146, 74, 130). A correlation between immunologically measurable effects and clinical outcome was reported in several papers (74, 135, 134). In the study by Wheeler *et al* (134), a significant progressive (logarithmic) association was found between time to survival after inclusion and postvaccine IFN- $\gamma$  enhancement. This allowed them to propose that single-vaccine response metrics can quantitatively reflect inhibition of tumor progression by T cells in GBM patients.

Based on review of the literature, at least three groups in the United States, one group in Europe, one in Japan, one in Asia and one in Australia are actively performing clinical trials. All these trials are small, and include a heterogeneous group of patients with regard to histology and/or time of vaccination. Some studies included patients with all subtypes of HGGs (64, 147, 142, 146, 130, 92, 134), while others focused solely on patients with GBM (73, 25, 111, 30, 135, 74). Some studied patients with relapsed HGG (73, 64, 142), while others included patients with a first diagnosis of HGG, either exclusively (147) or combined with patients treated at recurrence (135, 146, 74, 130, 92, 134). Only the European group reported on children with relapsed GBM (25, 111, 30). 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* (147) reported that seven patients with newly diagnosed GBM who received three biweekly intradermal vaccinations with peptide-pulsed DCs had median survival times of 455 days as



**Figure 4.** HGG-IMMUNO-2003 protocol: DC vaccination for adults with relapsed GBM. Patients with relapsed GBM were reoperated (removal of as much tumor tissue as feasible) and received subsequent immunotherapy. Patients were divided among four consecutive cohorts. **A.** The age (expressed in years on the y-axis) for the four cohorts is shown, and

median age is indicated. **B.** The number of patients with total resection or less than total resection of relapsed GBM is shown for each cohort (ND = not documented). **C.** Progression-free survival (PFS) for the four cohorts of patients. **D.** Overall survival (OS) for the four cohorts of patients.

compared with 257 for 42 nonvaccinated 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 vaccinations at the time of relapse in eight GBM patients, and made a similar comparison with 26 historical control patients (146). 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 group demonstrated that vaccine responders exhibited more favorable clinical outcomes relative to nonresponders. Moreover, the vaccine-induced responses elicited therapeutic benefits primarily by sensitizing tumors to subsequent chemotherapy (134). In the phase I study reported by Liao *et al* (74), the patients with stable tumors or no residual disease at time of DC vaccination compared favorably even with historical/concurrent data as the best prognostic subgroup of GBM patients treated during the same time period. In contrast, Okada *et al* (92) did not show any benefit in progression-free survival (PFS) in patients treated with DCs loaded with autologous tumor lysate, in combination with IL-4 producing fibroblasts (92). In the study on recurrent HGG patients reported by Yamanaka *et al* (142), radiologic responses were observed in the group of five patients who received intratumoral plus intradermal vaccinations, while no response was detected in the five patients who were only injected intradermally.

### **Clinical experiences on a large group of patients with recurrent GBM**

In our clinical practice, we have implemented immunotherapy with DCm-HGG-L for the treatment of patients with relapsed HGG, as a stepwise process using a cohort-comparison study concept (HGG-IMMUNO-2003). Children above the age of 3 years, adults and elderly patients up to 70 years with relapsed HGG can be included into the trial, if as much as possible and at least 1.5 cm<sup>3</sup> viable tumor material is available in sterile, dry and frozen (−80°C or liquid N<sub>2</sub>) condition, if (sub)total resection is documented after operation, if the patients can be tapered off of corticosteroids within 1 week after operation and if pathology is confirmed. By following this strategy, we have been able to compare each cohort within the trial with the other cohorts. Thus far, two major issues in the complexity of DC immunotherapy have been addressed: the vaccination schedule and the maturation cocktail.

We have analyzed a subgroup of the first 75 adults with relapsed GBM who were treated according to the four cohorts described earlier. As shown in Figure 4, there was no difference in median age or in extent of resection among the patients in the four cohorts. The median PFS and OS times of the total group were 2.6 and 8.62 months, respectively, with a 2-year survival of 13.6% [standard error of the mean (SEM) = 4.6]. Complete resection (n = 39) vs. partial or subtotal resection (n = 36) improved the median PFS from 1.87 to 3.51 months (log rank test:  $P = 0.004$ ), and the OS from 6.97 to 10.08 months (log rank test:  $P = 0.0168$ ). We reported earlier that the extent of resection, but not the Karnofsky index nor

the age, was an independent risk factor in a multivariate analysis (30). Interestingly, we observed a stepwise improvement in the PFS curves from cohort A to cohort C by shortening the interval between the induction DC vaccines, with further improvements noted by changing the maturation cocktail in cohort D (Figure 4). The median PFS for the four cohorts was: 1.94, 1.67, 3.23 and 2.72 months, respectively; the PFS at 2 years was 4% in cohort C and 15.3% in cohort D. Although the median OS did not differ significantly between the four cohorts, we noticed a 2-year survival of 16.7% (SEM = 7.6) in cohort C and of 27.8% (SEM = 9.9) in cohort D.

Considering the whole subgroup of 88 children and adults treated for relapsed GBM with new surgery and immunotherapy, the younger long-term surviving patients published earlier (30) illustrate a level 1c medical evidence of clinical efficacy ([http://www.cebm.net/levels\\_of\\_evidence.asp](http://www.cebm.net/levels_of_evidence.asp)). This high level of evidence points to the change from 100% mortality of relapsed GBM after resection alone (90) toward a situation where at least some patients survive the disease after subsequent immunotherapy (the intervention). The patient published in 2004 (25) is the longest disease-free survivor in a series of 88 patients with relapsed GBM (children and adults), with a follow-up of 86 months. The significant shift in PFS of adults treated with immunotherapy in the consecutive cohorts of patients further illustrate level 2b efficacy. The latter efficacy level points to significance in individual cohort studies without randomization.

Further analysis is currently being performed on a subgroup of the first 117 adult patients with (all types of) relapsed HGG according to allocation in the different RPA classes I to VI as described by the RTOG (manuscript in preparation). The RPA classes reflect the integrated importance of age, pathology, mental status, Karnofsky performance score, extent of resection and intensity of radiation therapy. The latter factor is irrelevant for the patients with relapsed HGG as radiotherapy could not be implemented anymore. With a median follow-up of 20.8 months, 80% of patients were allocated to classes III to V and a highly significant difference in OS was noted according to RPA class. With median survival times for classes I to VI of more than 36 months (to date), 14.2, 14.0, 10.6, 6 and 4 months, respectively, as well as 2-year survival rates (after start of vaccination) of 100%, 30.8%, 25%, 11.5%, 0% and 0% for these classes, these results are comparable with survival data of newly diagnosed HGG patients in the original RTOG database, even though no additional radiotherapy was performed in the relapsed setting. Moreover, we could demonstrate that the use of imiquimod resulted in a significant survival benefit in patients in class III and as such, this might be the first report on the significant and relevant clinical advantage of the use of Toll-like receptors (TLR) agonists in tumor vaccination strategies.

### **MRI of patients who obtained DC vaccination for relapsed GBM**

Radiologic follow-up is routinely performed with serial MRI scans in GBM patients. In some cases, no contrast enhancing mass is seen. However, others display contrast enhancement along with variable perilesional edema and mass effects. The differentiation between a vaccine-induced inflammatory immune reaction and early tumor relapse remains challenging as the radiological characteristics of both entities are similar. As such,

**Figure 5.** MRI findings during and after DC vaccination for relapsed GBM. Transverse T1-weighted MR images after contrast administration, obtained 1 day after debulking surgery for relapsed GBM (early postoperative period), 6 weeks after the first vaccine (6 w after V1), 6 months after the first vaccine (6 m after V1), 9 months after the first vaccine (9 m after V1) and after long-term follow-up (LT follow-up), 3 to 6 years after the first vaccine according to the case (3–6 years after V1). Case 1 is a patient with a resection in the left frontal lobe. MR imaging showed discrete contrast enhancement around the resection cavity 6 weeks after administration of the first vaccine. No contrast enhancement was

documented in follow-up imaging. Case 2 is a patient after surgery in the right frontal lobe. Transient contrast enhancement was seen at 6 and 9 months after the start of the vaccination therapy. Follow-up MR scans showed subsequent resolution of the contrast enhancement. Case 3 shows follow-up MR images in a patient with surgery in the left frontal lobe. Over consecutive time points, the resection cavity collapsed and a comparable area of contrast enhancement has remained stable for 3 years. Case 4 is a patient with a resection in the right frontoparietal region. Subsequent MRI shows an increasing region of contrast enhancement, suggestive of tumor relapse/progression.

only further follow-up with repeat imaging and clinical correlation makes it possible to distinguish these two possibilities. Furthermore, a PET scan with the radiolabeled amino acid methionine may help, as this technique provides metabolic data for the lesion in question (25).

Four different MRI patterns at follow-up are illustrated in Figure 5. All patients presented with relapsed GBM and received vaccination therapy after neurosurgical resection. Case 1 is a 36-year-old man with a recurrent tumor in the left frontal lobe. MRI at 6 weeks after the first vaccine revealed perioperative contrast enhancement, which retrospectively most likely represented surgical changes, given the resolution of the enhancement on consecutive images. The patient still has no further clinical or radiographic evidence of recurrence at 3 years. Case 2 is a 16-year-old woman with no clear changes on MRI until 4 to 6 months after the start of immune therapy, when a large area around the resection cavity revealed contrast enhancement, albeit with no significant mass effect or edema. Subsequent imaging showed resolution of the contrast enhancement with no evidence of recurrence on the last MRI, taken 6 years later. The patient remains clinically stable at 86 months after treatment. Case 3 is a 59-year-old man with stable clinical and radiological disease for over 3 years, although at 6 weeks following administration of the first vaccine, MRI showed contrast enhancement in the area around the resection cavity. Over time, the surgical cavity collapsed, while the contrast enhancement remained relatively unchanged. Case 4 is a 60-year-old man with rapid clinical deterioration 6 to 7 months after the first vaccine. Parallel to the clinical evolution, contrast enhancement was observed in the area around the resection cavity, 6 months after administration of the first vaccine. Consecutive MRI revealed a significant increase in contrast enhancement with progressive midline shift, indicative of mass effect.

As current “routine” MRI does not enable adequate documentation of the time course and nature of the vaccine-induced inflammatory immune responses, further research is being performed to characterize these responses in both patients and animal models, using advanced magnetic resonance (MR) techniques like MR spectroscopy (32, 52, 91), MR diffusion-weighted (91, 105), perfusion-weighted (91, 105, 55) and diffusion tensor imaging (101). Hopefully, such research will provide clues for early prediction of treatment response and thereby allow for earlier changes in treatment approach as necessary.

### The integration of immunotherapy in the primary multimodal treatment strategy

Based on previous experience, a “cohort C” approach has been integrated into the primary treatment regimen for patients with

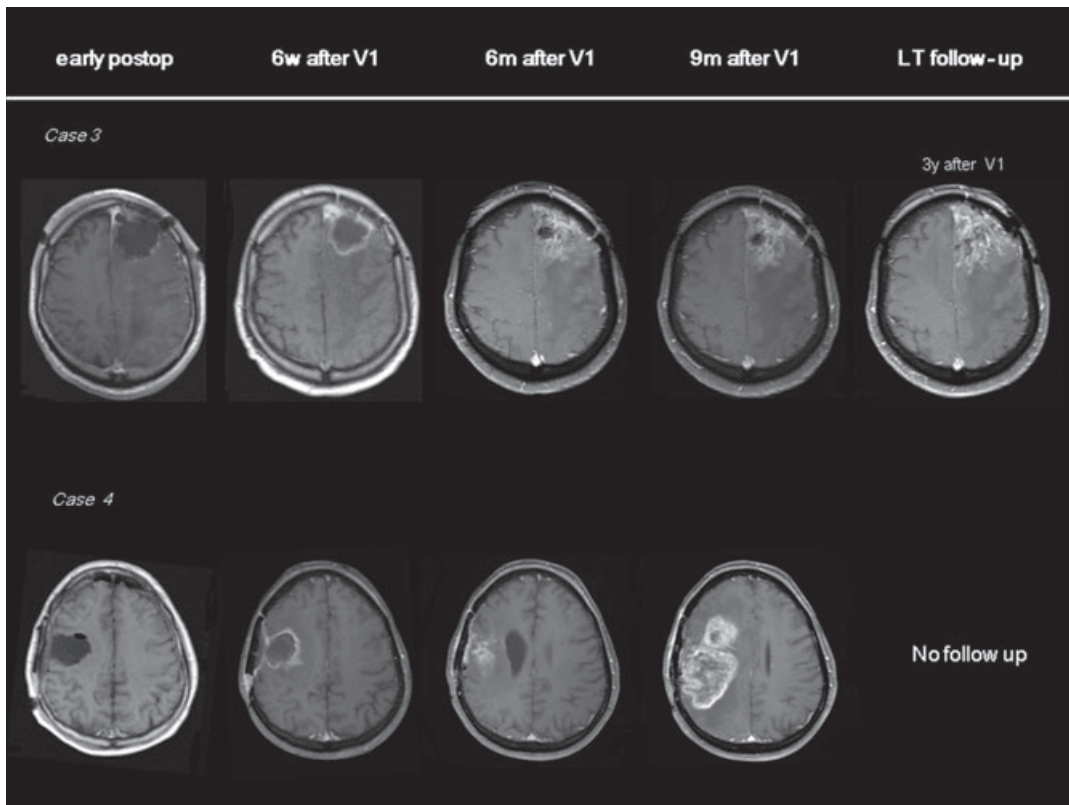
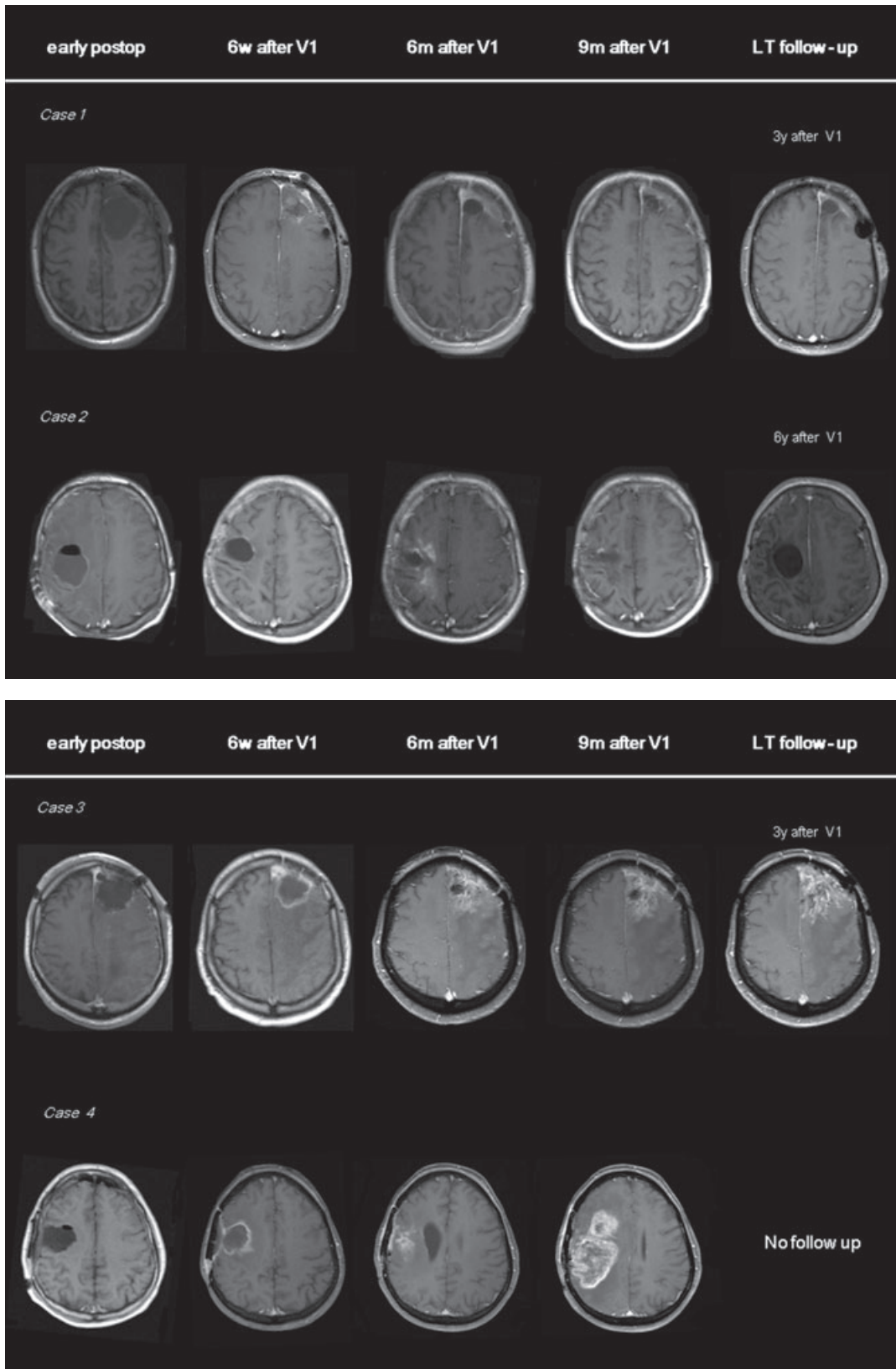
newly diagnosed GBM at our medical center. After radiochemotherapy, four weekly DCm-HGG-L are injected, and lysate boosts are administered at day 8 of the 28 day cycles 1, 2, 3 and 6 of temozolomide maintenance therapy. In total, 66 adults with a primary diagnosis of GBM are available for further analysis, with a median age of 55 years. The data are still preliminary due to short follow-up of recently included patients. Nonetheless, the median PFS and OS times are currently 10.3 and 17.9 months, respectively, the 6-month PFS is 75%, and the 2-year OS is 31.6%. The incidence of side effects has been minimal, in that the registered quality of life has not been affected by therapy and most patients have been treated in an ambulatory setting.

## CONCLUSIONS

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 HGGs cause relatively high community burdens, not only with many years of life lost due to cancer (14), but also because of the major 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. However, timely translation of new concepts into clinical practice should be the primary objective.

With several groups entering into clinical practice in this field, new data are now being generated. However, it is of paramount importance not to discredit this promising approach based on data from poorly designed trials with inappropriate end points. Thus far, the observations with regard to both immunological responses and clinical responses are promising and beneficial effects are reproducible. 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 restricted 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 molecular tumor signature, should be implemented in the near future. For this, collaborative efforts between well-organized and experienced vaccination centers should be established.



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