The efficacy of dendritic cell based immunotherapy in addition to conventional treatment for N-GBM and recommendations for the ideal protocol

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1. Abstract

Gliablastoma multiforme is one of the most common CNS tumors and its conventional treatment in comparison with other tumors is associated with an poor prognosis. One of the latest developments in search of a better treatment for this tumor, arose in the form of active immunotherapy based on dendritic cells. This therapy treats patients with these antigen presenting cells from the patient, after they have been primed with antigens derived from the autologous tumor to induce a glioblastoma-specific immune response. One of the objectives of this review was to find out if this new therapy improves the clinical outcomes of newly diagnosed glioblastoma patients in addition to standard treatment compared to the conventional therapy alone. The second goal of this study was to control if the variance in clinical outcomes between studies that investigated the effect of additional DC therapy can be explained by variances in various steps of the protocol of these studies. After selecting 12 studies that matched specific selection criteria, a meta-analysis was performed to address both questions. It demonstrated that DC therapy in addition to standard treatment appears to be beneficial, however the comparison between both types of treatment turned out to be uninterpretable, as result of selection bias. In addition, only the step of administration time after surgical resection in the protocol was able to explain the differences between studies with high and low median OS scores. The administration interval appeared to be positively associated with median OS. This review concludes that future DC therapy studies should standardize selection criteria and subject their control groups to similar selection criteria. Finally, it recommends that these studies should implement an administration time of the vaccine in their protocol that is outside of the time frame directly after SR, or within the six weeks after radiotherapy.

2. Introduction

The primary malignant brain tumor grade IV astrocytoma or glioblastoma multiforme (GBM) is with its yearly incidence of 3.5 adult patients per 100000 inhabitants not only the most common CNS tumor (15%), but also the most prevalent primary malignant brain tumor (55%)[1,2]. In addition, GBM is the most lethal type of brain tumor with a median overall survival (OS) of three months without standard treatment[3]. The conventional treatment for GBM is maximal surgical resection of brain tumour, followed by radiation with 60 Gy of fractionated radiation therapy (RT) and chemotherapy with temozolomide (TMZ) during and after RT (the Stupp regimen)[3,4]. The survival of the patient is correlated with the extent of the surgical resection[5]. More extensive resection while retaining quality of live has been made possible through the development of intraoperative MRI[5]. The remaining tumor cells are targeted by radiation and chemotherapy. External beam radiation therapy uses a two- to three-cm margin, since most recurrences are within this area of the tumor[5].The primary chemotherapy for GBM is TMZ, which is an alkylating agent that transfers alkyl groups to guanine bases inducing DNA damage and cellular death[6]. This standard GBM therapy increases the median OS to 14.6 months, with a 2 year survival rate of 27.2% and a 3-year survival of 10%, which remains to be a dismal prognosis[3,5,7]. Most of these patients die as result of recurrence of the tumour, which is also known as recurrent GBM (R-GBM)[1].

Despite the advancements in surgical techniques and increased efficacy in treating non-CNS tumors, there is hardly any profound improvement in the conventional treatment of GBM[8]. This lack of progress can be explained by the characteristics of GBM such as, its invasiveness, location, resistance to radiation as well as chemotherapy[7]. The tumor

infiltrates the brain in a diffuse way, which prevents a complete surgical resection of the tumor[7]. Furthermore, the brain is protected by blood brain barrier (BBB), which complicates GBM targeting of chemoreactive agents. In addition, the unresected parts of the tumor contain specific cell populations, including cancer stem cells (CSCs), that are resistant against radio- and chemotherapy[7]. CSCs are characterized by self-renewal, low proliferation, multipotency and tumor growth under critical conditions[6]. These cells are able to regenerate the tumor population and develop essential supportive structures[7]. Therefore, it is suggested that the incapacity of eliminating all CSCs by standard GBM treatment is partly responsible for the recurrence of GBM. This is supported by findings that demonstrated that patients with increased numbers of proliferating CSCs have a lowered OS[6].

These substantial shortcomings of conventional GBM treatment made way for novel GBM therapies to enhance the efficacy of GBM treatment. Ideally, this novel treatment reaches the entire volume of the CNS due to the diffuse character of GBM. Second, this treatment has a minimal toxicity to the healthy tissue and prevents treatment resistance. Lastly, it retains its capacity to destroy tumors to counteract recurrent tumor growth[8]. Immunotherapy is an emerging GBM treatment, which may meet all these criteria, since it has the potential to target tumors with cellular-level accuracy, generate a long-term immune surveillance against cancer cells and has minimal risk or side effects[3,5,7]. In result GBM immunotherapy received increased amounts of interest, which led to the development of numerous different types of GBM immunotherapy and multiple clinical trials to test their efficacy as additional treatment next to conventional treatment. Due to the favorable results in other types of cancer, active immunotherapy based on dendritic cells (DCs) has become one of the most investigated GBM immunotherapies[8]. To understand the mechanism and potency of DC-immunotherapy for GBM, background information concerning the immune system, including dendritic cells, GBM and its immunosuppressive actions and immunotherapy for GBM will be given after this introduction.

Next to the great potential of DC immunotherapy for malignant gliomas, it has also been heavily criticized due to its low therapeutic efficacy, 15.6 %, in terms of generating a complete or partial response, which is defined by WHO criteria, or Response Evaluation Criteria In Solid Tumors (RECIST)[9]. Next to therapeutic efficacy, the effect of treatments can also be measured based on other clinical outcomes, such as median OS as well as progression free survival (PFS).

Therefore, the first aim of this review is to further complete our understanding concerning the efficacy of DC based immune therapy in addition to standard treatment for N-GBM patients by comparing the median OS and PFS between both treatments. There are multiple clinical studies that analysed the efficacy of DC therapy and their results demonstrate large differences in median OS. The second research question will answer which studies have the lowest and highest clinical outcomes, as the differences between these studies may help to improve the efficacy of DC therapy in the future. A possible explanation for these differences could be the limited amount of patients due to the rarity of the disease, and/or the heterogeneity of GBM resulting in interindividual differences. On the other hand, there has been no consensus about the perfect protocol for DC immunotherapy up to this point, which is reflected by the finding that none of the clinical trials used similar protocols. Thus, I hypothesized that this discrepancy in clinical outcomes at least partially depends on different protocols of these studies. Based on this hypothesis, this review investigated the effect of the variants for four constituents of the protocol, DC maturity, maturation cocktails, antigen type and administration time, on the clinical outcomes. These steps in protocol of DC therapy will be explained in the part 3.4 of the background information. If this analysis confirms my hypothesis, it will also help to elucidate which

variant of each component in the protocol is most beneficial for the clinical outcome. Based on these results, this paper aims to identify the ideal protocol for GBM DC-immunotherapy.

Remarkably, there has been no study that investigated the differences in the protocols and their relationship with clinical outcomes of these different studies. Therefore, I performed a meta-analysis for all published studies that report the efficacy of dendritic cell therapy on N-GBM in humans to create a systematic overview of the differences between these clinical trials.

3. Background information

3.1. The immune system

The main function of the immune system is to distinguish between the self and non-self, so foreign intruders can be recognized and destroyed[10]. The immune system can be separated into two types: the innate and the adaptive immune system. The innate immune system, which consists of macrophages, monocytes, neutrophils, natural killer (NK) cells, basophils, eosinophils and complement, is able to recognize pathogen-associated molecular patterns (PAMPs) by using pattern recognition receptors (PRRs), such as Toll-like receptors (TLRs)[10]. The adaptive immune system becomes activated, whenever T and/or B lymphocytes interact with an antigen presented by antigen presenting cells (APCs)[10]. The most active APCs are dendritic cells (DCs), which are a subpopulation of leukocytes that are derived from $CD34^+$ bone marrow progenitor cells and play a key role in the immune surveillance and antigen presentation of the adaptive immune system[7]. Immature DCs circulate throughout the body to scan the local environment with its receptors for inflammatory mediators, damaged tissue or microbial pathogens. Whenever a DC encounters one of them, it matures and becomes activated[5,8]. In result the DC endocytoses the foreign protein, maximally upregulates MHCs, T-cell costimulatory molecules, cytokines and chemokines that allows migration to T-cell rich areas of lymphnodes to present the tumor antigen via MHC I, or MHC II molecules to naïve T cells[5,8]. This will then induce an antigen specific $CD4^+$ T helper cell or $CD8^+$ T cells immune response, respectively. Activated $CD8^+$ cytotoxic T lymphocytes (CTLs) are able to recognize peptide complexes presented in the context of human leukocyte antigen (HLA) major histocompatibility complex (MHC) class I molecules on tumor cells via T cell receptors (TCRs)[10,11]. Together with a costimulatory signal mediated by CD28 binding to its ligand B7, CTLs induces a cytotoxic mediated cell death[10]. Activated $CD4^+$ T helper cells recognize antigens presented on HLA MHC II class molecules, whose interaction results in cytokine release and recruitment of other immune cells[10]. After activation $CD4^+$ T helper cells can differentiate into multiple effector subtypes, such as Th1 and Th2, where each type has a different function. $CD4^+$ Th1 cells secrete pro-inflammatory cytokines such as interferon-γ, interleukin-2 (IL-2), IL-12, IL-15, lymphotoxin and tumor necrosis factor alpha (TNF-α). These cytokines have been demonstrated to induce a potent anti-tumor effect[12]. $CD4^+$ Th2 cells produce cytokine profile that includes IL-4, IL-10 and IL-13 that are associated with stimulating the humoral immune response. The cytokines of these two $CD4^+$ subtypes work in an antagonistic manner and an imbalance between the responses of both populations towards the Th2 response is associated with pathological states, such as GBM[12]. Healthy controls on the other hand have been demonstrated to have a more Th1 polarised immune response[13]. To prevent autoimmunity, as result of binding foreign antigens that resemble antigens from the body itself,

the immune system has a few safety mechanisms[10]. They are represented by the upregulation of membrane negative costimulatory molecules cytotoxic T-lymphocyte associated antigen 4 (CTLA4) and program cell death 1 (PD-1) proteins, whenever T-cells are activated[10]. CTLA4 and CD28 compete for B7 binding and PD-1 binds to its ligand PD-L1, binding of either one of them will induce a signal cascade that inhibits continued T-cell activation[10]. An additional mechanism based on the activation of regulatory T cells (Tregs) is able to supress the immune system by inhibiting the activity of $CD4^+$ T cells, $CD8^+$ T cells, DCs and NKS cells[10]. These Tregs express CD4, CD25, CTLA4, glucocorticoid-induced tumor necrosis factor receptor (GITR) and are regulated by transcription factor forkhead box protein 3 (FOXP3)[10]. Together with other immune regulatory pathways, such as immune suppressive cytokines and myeloid-derived suppressor cells, Tregs have been associated with generating and maintaining tumor resistance[10]. These immunosuppressive actions of cancer provide a framework for the potency of immunotherapy as cancer treatment.

3.2. Glioblastoma multiforme (GBM)

Gliomas are classified by the world health organisation (WHO) into 4 histological grades based on their increasing degrees of undifferentiation, anaplasia and aggressiveness[14]. High-grade gliomas or malignant gliomas encompass both grade III and grade IV gliomas. This review focuses on the glioblastoma, also known as GBM (grade IV), whose histology is characterized by pleomorphic cells, mitotic activity, intravascular microthrombi, necrosis with, or without cellular pseudopalisading and/or microvascular proliferation (MVP)[6]. Although all gliomas are known for their tumor infiltrating capacity, glioblastomas have a distinctively fast infiltrative growth[6]. The infiltration of glioblastomas into the surrounding brain parenchyma is restricted by the CNS and therefore they do not metastasize[14].
Glioblastomas show high heterogeneity on a molecular level. Genome-wide expression studies categorised glioblastoma into 4 transcriptional subclasses: classical, mesenchymal, proneural and neural[14]. The classical subclass distinguishes itself by chromosome 7 amplifications, chromosome 10 deletions, *EGFR* amplification, *EGFR* mutations, *Ink4a/ARF* locus deletion[14]. The mesenchymal glioblastoma shows a high frequency of *NF1* mutation/deletion and high expression of *CHI3L1*, *MET* and genes involved in the tumor necrosis factor and nuclear factor-κB pathways[14]. The proneural subclass displays alterations of *PDGFR* and mutation in *IDH1* and *TP53*, which is similar to the gene expression of lower-grade gliomas and secondary glioblastomas, which are lower grade gliomas that recur as glioblastomas[14]. Neural glioblastomas express neuronal markers, whereas the other subclasses do not. In contrast, there are many mutations and alterations that overlap between the subclasses like *PTEN* loss, but there are also numerous very rare mutations that differ within the subclasses themselves[14]. These mutations are part of underlying mechanism of immunotherapy for GBM that relies on targeting characteristics of cancer that distinguishes tumor cells from healthy cells. However, not every characteristic is accompanied with a specific antigen expression by the tumor cell, which is required for the recognition by immune cells. In result multiple studies identified glioblastoma associated antigens (GAAs) that can be targeted by immune system: ER-2, TRP-2, gp100, MAGE-1, IL-13Rα2 and AIM-2, EGFRvIII[7]. How these GAAs are used in immunotherapy to treat GBM will be discussed later.
The most important characteristic of GBM and other cancers, which supports immunotherapy as treatment, is its suppression of the immune system. Patients with GBM exhibit alterations in systemic immune response, such as decreased T-cell responsiveness, increased circulating regulatory T cells, defective monocytes and DC functions[7].

Several immunosuppressive cytokines, including transforming growth factor beta (TGF-β), prostaglandin E2 (PGE2) and interleukin-10 (IL-10), interleukin-6 (IL-6) and VEGF, are highly expressed in GBM patients, whose effects may contribute to these defects (figure 1)[15]. In addition, glioma cells change the local immune response to prevent immune cell effectors at the tumor site, which involves CD70, FasL, gangliosides, HLA-G, PDL-1, IDO and TGFβ 1-3. An additional way of glioma cells to further supress the immune response is to produce a chemokine profile, which recruits immunosuppressive cells, such as Tregs cells and myeloid-derived suppressor cells (MSDC)[4]. Furthermore, cancer stem cells (CSCs) have been demonstrated to act as immunosupressors in GBM. Next to CSCs there are also glioma-infiltrating microglia (GIM) that represent a third of the cells of the tumour mass and support tumor invasion and proliferation[4]. Most of these immunosuppressive actions in GBM have also been demonstrated in other types of cancer. In addition, observations showed that a stronger immune response is beneficial for the survival of GBM patients. For example, the survival of patients with primary GBM has been found to be positively correlated with tumor infiltration of cytotoxic and helper T cells[10]. In line with this observation, another study observed a negative correlation between the degree of immunosuppression and survival in glioblastomas[10]. This raises the question why there has not been similar success in the development of immunologic therapies for GBM as for other types of cancer.

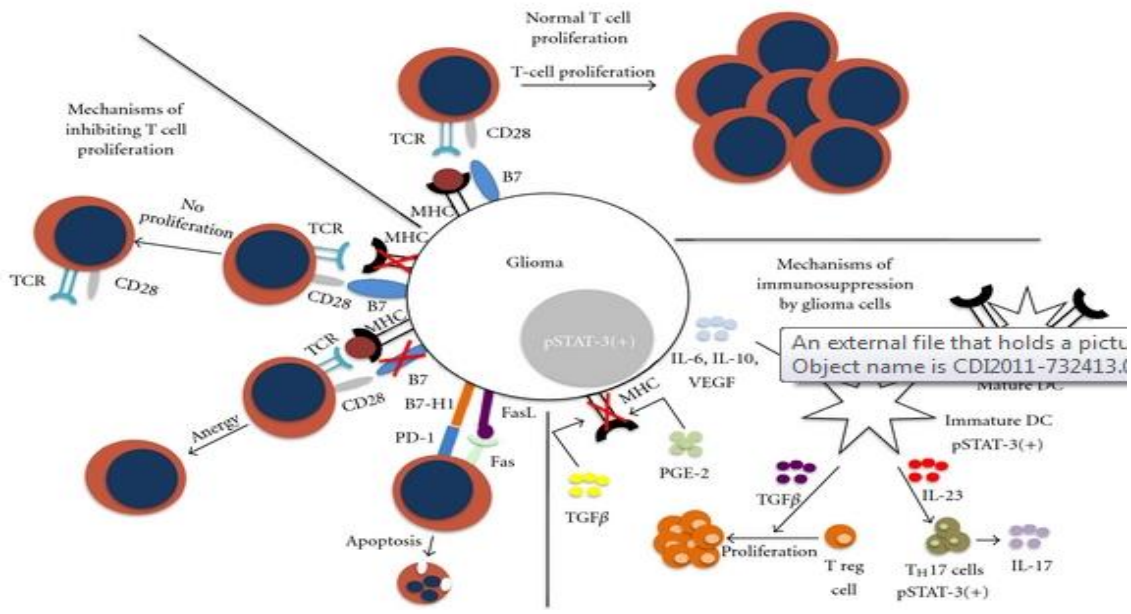

Fig.1. This picture presented in the study of Jackson et al. (2011) represents the mechanisms of normal T cell proliferation, mechanisms of GBM to inhibiting T cell proliferation and induce immunosuppression. Normal proliferation of T cells requires costimulatory activation of the T cell receptor as well as B7-CD28 interaction. The decreased T-cell responsiveness may be explained by the downregulation of MHC and costimulatory B7 molecules by gliomas as well as the upregulation of B7-H1 and FasL that activate apoptotic pathways after interaction with T-cells, reducing the amount T-cell in the environment of gliomas. A multitude of different cytokines produced by glioma cells contribute to its immunosuppressive activities. PGE2 and TGFβ downregulate the expression of MHC molecules, which results in lowered antigen presentation and subsequently decreased T cell proliferation. IL-6, IL-10, VEGF are capable of activating STAT-3, which leads to the proliferation of specific immature DCs that are unable to function as APCs. The secretion of TGFβ and IL-23 of these immature DC contribute to the proliferation of Treg cells and activation of Th17 cells, whose inflammatory activity has been demonstrated to be tumorigenic in other types of cancers[15].

3.3. Immunotherapy for GBM

A possible contributor to the current absence of an effective FDA approved immunotherapy for GBM, might have been the lack of interest as result of the long standing assumption that immunotherapy was not effective in treating brain cancers, due to an immune-privileged brain. This immune privilege was based on the blood brain barrier (BBB) and the brain specific characteristics, such as the absence of lymphatic drainage system, low levels of APCs, less HLA presentation compared to other tissues and low amounts of circulating T-cells[3,4,10,15]. The BBB is created by endothelial cells that form the walls of capillaries in the brain and spinal cord. The BBB is responsible for a stable fluid microenvironment to optimize neural functioning and protect the CNS from damaging agents from the peripheral circulation[16]. Together with those other specific brain features, the brain appeared to be protected against peripheral immune reactions and immunotherapy[3]. However recent studies have led to major revisions concerning the immune system of the brain. Microglia, macrophages and dendritic cells have been found to function as powerful APCs in the CNS[15]. CNS-associated antigens flow in cerebrospinal fluid through Virchow-Robin perivascular spaces to nasal and cervical lymphnodes[15]. Certain activated T-cells in these nodes express integrins, like α4β7, that induce CNS tropism, allowing T-cells to cross the BBB[15]. Antibodies have also been found in the brain, although their concentrations were lower than in the plasma[15]. Finally, the BBB of GBM patients appears to be disorganized with a asymmetric structure of brain capillaries into the tumor, a dysfunction of tight junction between the endothelial cells and a decrease in BBB-associated pericytes, which demonstrates with all the other findings that the brain of GMB is not completely immunological privileged[4]. Consequently, immunotherapy emerged as highly potent treatment option for GBM, since immune cells can be activated by peripheral antigen exposure expressed by APCs from the vaccine, subsequently migrate through the bloodstream to cross the BBB and induce an immune reaction against tumor cells[3].
Based on these findings, numerous different types of immunotherapy for GBM have been developed and tested in preclinical as well as in clinical trials, such as immunotherapeutic agents (ipilimumab, steroids, lymphodepleting cytotoxic agents), peptide vaccines (that target ER-2, TRP-2, gp100, MAGE-1, IL-13Rα2 and AIM-2, EGFRvIII), dendritic cell vaccines, heat shock protein vaccines, autologous tumor cells vaccines, gene transfer mediated in situ vaccines and adoptive immunotherapy[5,7]. This paper will however focus on dendritic cell immunotherapy for primary GBM, since it is the most frequently used vaccination protocol to treat GBM patients.

3.4. Immunotherapy using dendritic cells (DCs)

DC-immunotherapy relies on the priming dendritic cells to induce an anti-tumoral immune response[3]. These DCs are generated ex vivo, which requires collecting a large number of peripheral blood mononuclear cells (PBMCs) from the patient by apheresis. Then monocytes are isolated by elutriation, CD14 antibody selection, or selection of adherent cells after overnight culture on plates[17]. Subsequently these cells are cultured with GM-CSF and IL-4 to promote differentiation into immature DCs. Then DCs are primed with glioblastoma associated antigens (GAAs),. Examples of GAAs are defined or synthetic tumor peptides, whole autologous tumor cell (ATC) lysates, nucleic acids that encoding tumor antigens, apoptotic or necrotic tumor cells, DNA or mRNA derived from ATCs, tumor antigens via genetically modified viral systems or alternatively fusing DCs with tumor cells[5,11]. Whenever one requires mature sensitised DCs for their vaccine, immature DCs are matured in the presence of different cocktails, which can include

IL1β, IL-6, TNFα, PGE2, LPS, polyriboinosinic polyribocytidylic acid (poly I:C), IFNα and/or IFNγ[4,8]. The maturation of DC can be analysed based on their surface marker expression, cytokine profiles, migratory capacity, allogeneic and autologous T cell stimulatory capacity and their specific cytotoxicity against tumor antigens[18]. Mature DCs are then administered to patients in a weekly or biweekly fashion via intradermal, subcutaneous, intranodal, intracranial via an ommaya reservoir, intravenous or intratumoral routes, with a dose range between 10^6 and 10^9 DCs per dose[3,11, 19]. The total number of doses varies between clinical trials. In response patients generate a T-cell population that recognizes the tumors that express these antigens[3]. A simplified version of DC-immunotherapy is illustrated in figure 2, which is a modified figure presented in the study of Xu et al. (2011)[2]. This treatment does not only affect the initial tumor, but may also induce a memory immune response that protects the body from recurring tumors in the future[3].

The first clinical trials that tested DC immunotherapy in lymphoma and melanoma showed promising effects, such as development of antitumor immune responses in all patients without an autoimmune response and some patients showed partial and even complete tumor regressions[20,21]. This evidence provided the backbone for the increase in multiple clinical trials to test DC vaccines for other types of cancer[8]. Up to this point the only DC vaccine approved by the FDA is Provenge, which uses an autologous dendritic cell vaccine to treat hormone-resistant metastatic prostate cancer[5]. However, there are more than 200 trials treating more than 3000 cancer patients with DC-immunotherapy[4]. Only the treatments with DC vaccines for malignant melanoma, prostate cancer, renal cell cancer and malignant glioma with DC vaccines reached or finished phase 3 clinical trials[9].

The safety of DC-based immunotherapy has been investigated thoroughly in many phase I clinical trials[9]. Although local reactions at the administration site, such as pain, rash and itch, were common, they were mild and self-limiting[9]. Systemic side-effects (pyrexia, malaise, and other influenza-like symptoms) can occur, but more important is that systemic grade 3-4 toxicity (US National Cancer Institute-Common Terminology Criteria) was rarely found, when DC vaccines were administered as monotherapy[9]. Another concern about DC vaccines is the possibility of autoimmunity, although such reaction has not been documented so far. This threat is based on exposing dendritic cells to specific antigen sources that also contain peptides from non-tumoral glial cells and therefore could theoretically induce autoimmune encephalomyelitis[8]. Nonetheless, the risk of inducing autoimmunity which can take up to many years to develop is subordinate to the immediate threat posed by GBM malignancy[8].

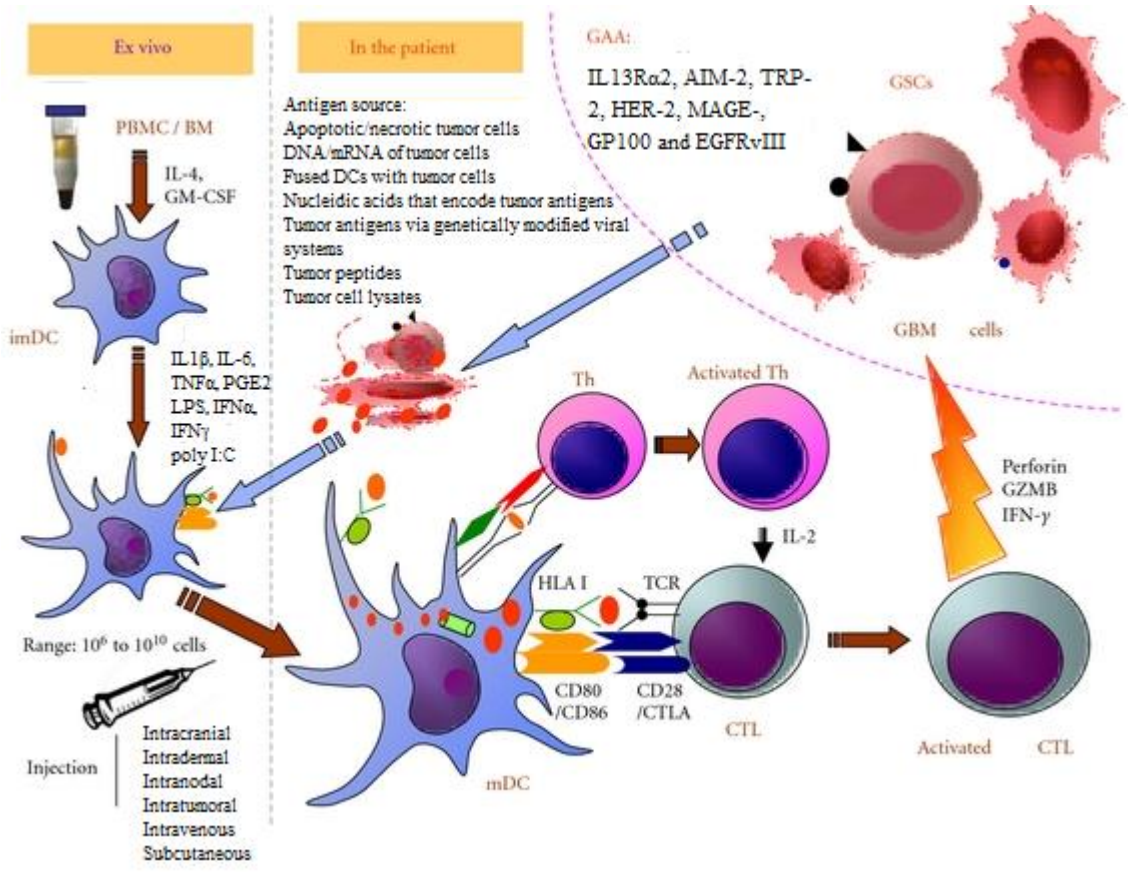

Fig.2. Immunotherapy based on DCs for GBM. After isolating PBMCs from the patient, these cells are exposed to IL-4 and GM-CSF *ex vivo*, which induces differentiation into immature DCs. Subsequently these immature DCs are cultured with maturation cocktails, which contain cytokines, such as IL1β, IL-6, TNFα, PGE2, LPS, IFNα, IFNγ and poly I:C. To induce a patient's specific anti-GBM immune response, DCs require priming of glioblastoma associated antigens (GAAs) derived from (processed) autologous tumor cells, including apoptotic tumor cells, DNA/mRNA of the tumor cells, fused DCs with tumor cells, nucleic acids that encode tumor antigens, tumor antigens via genetically modified viral systems, tumor lysates and tumor peptides. After cultivating, between 10^6 up to 10^{10} DCs are used as vaccine and are injected via an intracranial, intradermal, intranodal, intratumoral, intravenous, or subcutaneous way. These DCs induce activation and proliferation of $CD8^+$ CTLs and $CD4^+$ Th cells. Activated $CD8^+$ CTLs recognize and eradicate tumor cells, whereas activated $CD4^+$ Th cells increase the ability of DCs to activate $CD8^+$ CTLs through the interaction between CD40 on DCs and CD40 ligand on activated $CD4^+$ Th cells. In addition, $CD4^+$ Th cells are involved in maintaining and proliferating CTLs by secreting IL-2[2].

The efficacy of DC vaccines can be assessed on by their immunological response, overall objective response rate, progression free survival and overall survival. However, since the early 1980's the FDA determined that the criteria for approval of cancer drugs should rely on more direct evidence of clinical benefit such as PFS and OS. Aguille et al. (2014) reviewed all published clinical trials that documented OS of patients with melanoma, advanced renal cell carcinoma (RCC), prostate cancer and malignant glioma that received DC therapy[9]. Apart from the negative effects on OS (-8.6 months and -2.3 months) of two DC therapies for melanoma, the other 36 clinical trials showed an increase in the median OS of at least 2 months and maximally 25.8 months, compared to the median OS of patients treated with the standard therapy. The median OS specifically for malignant glioblastoma patients ranged from 9.6 months to 38 months, where only one of 15 studies score below the median OS of 14.6 months of N-GBM patients that received golden standard treatment known as the Stupp regimen[3]. However, these results did not consider the differences

between grade III and grade IV astrocytomas, or the difference between N-GBM and R-GBM patients. This is an additional reason, why I performed a systematic review of all published articles that report the efficacy of dendritic cell therapy on N-GBM in humans.

3. Meta-analysis

4.1. Selection of studies

The references were selected based on the search methods of Anguille et al. (2014) and Bregy et al. (2013),while limiting the search to the years between 1993 and 2014. After removing duplicates, the records of these articles were screened on active DC immunotherapy that included a separate median OS for N-GBM patients, which resulted in 12 clinical trials with 121 N-GBM patients. Then, I analysed the characteristics of the patient- and control population, treatment, diagnosis, protocol of the DC therapy and clinical outcomes of the selected studies. The specifics of the patients and treatment as well as clinical outcomes per study are outlined in table 1. The characteristics of the DC treatment protocol for each study, including time of administration, APC type, volume, maturation cocktail, antigen, dosage and site of administration, have been summarized in table 2.

4.2. The effect of additional DC immunotherapy on the clinical outcomes of N-GBM patients

The effect of DC therapy was examined by comparing the median OS, or PFS of N-GBM patients that received DC therapy in addition to conventional treatment with N-GBM control patients that only received conventional treatment. The overall survival in these 12 studies are defined as the length of time from either the date of diagnosis, or from the time of follow up after vaccination to the time of death, or end of the study. Table 2 shows that additional DC therapy of all studies with exception of the study performed by Chang et al. (2011) increased the median OS in comparison with the median OS of the conventional treatment of 14,6 months reviewed by Stupp et al. (2005)[34]. The studies performed by Cho et al. (2012), Jie et al. (2012), and Vik-Mo et al. (2013) included their own control group and all three studies demonstrated an increase in median OS compared to their control groups months[29,30,33]. This first impression of the effect of DC therapy on the median survival of patients compared to conventional treatment based on the information provided by these studies appears to be quite promising; however there are some matters that need to be considered. First of all, the comparison between medians only gives a simple impression, but it is not a statistical proved method for comparisons. Second, all 12 studies selected their patients for DC therapy on elaborate selection criteria of which the majority has not been included in the selection criteria of the control studies of conventional treatment studies, such as a greater score than 70 on the Karnofsky performance status (KPS), which is an established good prognostic factor for GBM[34,35]. These different selection criteria of all 12 studies compared to the study of Stupp et al. (2005), summarized in supplementary table 1, lead to a selection bias between these two types of studies[34]. Therefore, it would be more appropriate to compare the boxplots of the DC therapy patients of all 12 studies with control patients that were included in a three of these studies, which were selected on identical criteria as their DC group. However the studies of Sampson et al. (2009) and Jie et al. (2012) did not publish the individual results of their patients[24,30]. After examining the study of Cho et al. (2012), four of 18 patients and six patients out of 16 of the DC

group and control group respectively were included in the study, although they did not met their selection criteria as they did not complete the conventional treatment[29]. After removing these patients from our dataset, I adapted the number of patients, the median PFS and OS scores for this study in table1. In result a group of 91 patients that received additional DC therapy and 20 patients that received conventional treatment remained. An overview of the difference between additional DC therapy and conventional treatment in OS of both groups is reflected by boxplots depicted in figure 3. This figure demonstrates that the effect of additional DC treatment on survived time is as expected not as great as demonstrated before. Nonetheless, this difference in survived time between the combined group DC treated patients of 10 clinical N-GBM studies and the combined patients of the control groups of the clinical N-GBM studies of Cho et al. (2012) and Vik-Mo et al. (2013) is significant ($p=0.004$)[22-24,26-29,31-33].

Table 1

Clinical outcomes for N-GBM patients and control group measured by Med. PFS and med. OS and

Author and year	Type of trial	Nr. of patients	Avg age + range (yrs.)	Diagnosis	Treatment history	Med. PFS for N-GBM	Med. OS N-GBM patients	Med. OS control group	Increase med. OS
Yu et al. (2001)[22]	CT	9	49 (28-77)	N-GBM (7) N-AA (2)	SRT + EBRT	7.7 mo. (4/7 patients)	16.3 mo.		
Yu et al. (2004)[23]	CT	14	44.7 (28-61)	R-GBM (9) R-AA (3) N-GBM (1) N-AA(1)	SR+SRS (2), chemo		33.3 mo.		
Sampson et al. (2009)[24]	CT	12	43.8 (34-58)	N-GBM	TR + RT		22.8 mo.		
Ardon et al. (2010)[25]	CT	8	50.4 (31-62)	N-GBM	STR(6)/TR(2)	18 mo.	24 mo.		
Chang et al. (2011)[26]	CT	17	44.7 (18-69)	R-GBM (6) R-AA (1) R-MO (1) N-GBM (8) N-MO (1)	SR +RT		13.6 mo.		
Fadul et al. (2011)[27]	CT	10	60 (48-78)	N-GBM	STR(7)/PR(2)/TR(1) +EBRT + chemo	9.5 mo.	28 mo.		
Prins et al. (2011)[28]	CT	23	51 (26-74)	R-GBM (8) N-GBM (15)	SR+EBRT +chemo		35.9 mo.		
Cho et al. (2012)[29]	CT	18	52.1 (14-70)	N-GBM	TR(14)/STR(4) +RT (15)+ GKRS (11) + chemo (16)	9.5 mo. (control 8 mo.)	24.5 mo.	14 mo.	10.5 mo.
Jie et al. (2012)[30]	CT	13	40.2 (29-51.4)	N-GBM	TR(10)/STR (3) + RT + chemo	11.9 mo. (control 7.8 mo.)	17 mo.	10.5 mo.	6.5 mo.
Valle et al. (2012)[31]	CT	5	66 (50-73)	N-GBM	Fluorescence guided surgery + steroid discontinuation + RT + chemo	16.1 mo.	27 mo.		
Phuphanich et al. (2013)[32]	CT	21	52 (26-79)	R-GBM (3) N-GBM (17) N-BsG (1)	SR+RT+chemo	16.9 mo.	57.6 mo.		
Vik-Mo et al. (2013)[33]	CT	7	56.2	N-GBM	SR + RT + chemo	24.8 mo. (control 8.4 mo.)	27.1 mo.	20.9 mo.	6.2 mo.

Acronyms: AA = anaplastic astrocytoma, BSG = brain stem glioma, chemo=chemotherapy, GKRS =gamma knife radiotherapy, Med.= median, MO = malignant oligodendroglioma, mo. =months, N- =newly diagnosed, OS= overall survival, PFS= progression free survival, R- = recurrent, RT = radiotherapy, SEBRT = external beam radiotherapy, SR = surgical resection, SRS = stereotactic radiosurgery, SRT = stereotactic radiosurgery, STR =sub-total resection, TR =total resection.

Table 2
Details of patient, treatment and protocol characteristic of selected articles to review the effect of DC therapy on N-GBM

Author and year	No. of pts.	Diagnosis	Time of admin	APC type	Volume	DC maturation cocktail	Antigen	Dosage	Site of admin
Yu et al. (2001)[23]	9	N-GBM (7) N-AA (2)	After RT	Auto DC	10^6	-	ATCP (MHC I peptides)	1/2 wks up to 3 vacc.	s.c in deltoid organ
Yu et al. (2004)[24]	14	R-GBM (9) R-AA (3) N-GBM (1) N-AA(1)	Immediatly after SR	Auto DC	10^7-10^8	-	ATH	2/wks for 3 wks	i.d.
Sampson et al. (2009)[24]	12	N-GBM	12-16.8 wks after histological diagnosis	AMDC	28-86 x 10^6	TNF-α, IL-1β and IL-6	EGFRvIII-specific peptide conjugated to keyhole limpet hemocyanin	1/2 wks (3x)	i.d. in upper thigh, 10 cm below the inguinal ligaments
Ardon et al. (2010)[26]	8	N-GBM	9 wks post SR	AMDC	1-12x10^6 med. 4.1x10^6	TNF-α, IL-1β and PGE2	ATL	EBRT+ chemo (TMZ) (6 mo.) fb. DC loaded ATCP 1/wks for 4 wks fb. Chemo (TMZ) + booster ATCP	i.d. in upper arms (lymph node region)
Chang et al. (2011)[26]	17	R-GBM (6) R-AA (1) R-MO (1) N-GBM (8) N-MO (1)	8 wks post SR	AMDC	1-6x10^7	Maturation medium	ATC	1/wk (x4) + 1/2 wks (2x) + 1 month (4x)	s.c. in either axilia
Fadul et al. (2011)[27]	10	N-GBM	6-7 wks post-RT	AMDC	1 x 10^7	TNF-α and PGE2	ATL	1/2 wks (3 vaccins)	i.d. in bilateral cervical lymph nodes.
Prins et al. (2011)[28]	23	R-GBM (8) N-GBM (15)	7-30 wks post SR	Auto DC	-	-	ATL	1/2 wks 3 vaccins) + booster vaccine administered if pts had no toxic effects to the first 3 DC vaccines	I.d. below axilia

Table 2 (continued)

Author and year	No. of pts.	Diagnosis	Time of admin	APC type	Volume	DC maturation cocktail	Antigen	Dosage	Site of admin
Cho et al. (2012)[29]	14	N-GBM	4-8 wks post SR	AMDC	2-5 x 10^6	-	ATC	1/wk (4x) +1/2 wks (2x) + 1/month (4x)	s.c. bilaterally in subaxillary region
Jie et al. (2012)[30]	13	N-GBM	3 wks post SR	AMDC	6 x 10^6	IL-1β, TNF-α and PGE2	Heat-shocked ATC	1/wk (2x) + 1/2 wks (2x)	s.c. in groinal lymph nodes
Valle et al. (2012)[31]	5	N-GBM	Before RT (2/5) and 3 wks after RT (3/5)	AMDC	3.4-10 x 10^6	TNF-α, IFN-α and Poly I:C	ATL	1/month (2x) + 1/ 2 mo. (4x) + 1/3 mo. (until the end of available doses)	-
Phuphanich et al. (2013)[32]	21	R-GBM (3) N-GBM (17) N-BsG (1)	11.8-49.6 wks	Auto DC	-	TNF-α	ATCP: MAGE1, AIM-2, TRP-2, gp-100, HER-2, IL23Rα2	1/2 wks (3 vaccines)	i.d. in axilia
Vik-Mo et al. (2013)[33]	7	N-GBM	First wk after completion RT/chemo	AMDC	10^7	-	Autologous GSC-mRNA	2/wk + 1/wk (3x) + TMZ or vaccine every other wk	i.d.

Acronyms: AA = anaplastic astrocytoma, AMDC= autologous mature dendritic cell, ATH= autologous tumor homogenate, ATC = autologous tumor cell, ATCP = autologous tumor cell peptide, ATL= autologous tumor lysate, BSG = brain stem glioma, CT = clinical trial, GKRS =gamma knife radiotherapy, GSC = glioblastoma stem cell, i.d.= intradermal, MO = malignant oligodendroglioma, mo. = months, PR = partial resection, pts = patients, R- = recurrent, RT=radiotherapy, s.c.= subcutaneous, SR=surgical resection.

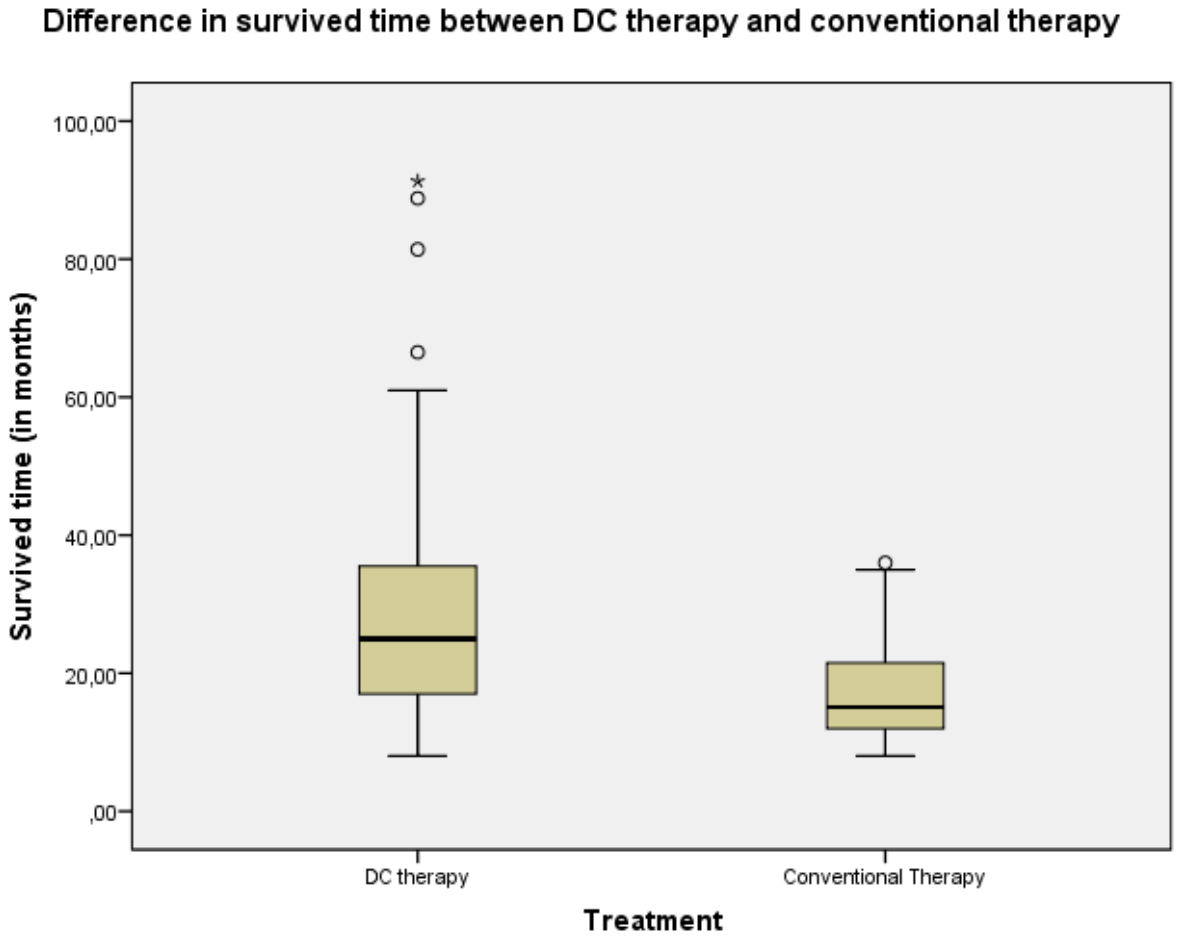

Fig. 3. These boxplots illustrate the difference in survived time between patients that received additional DC therapy and patients that only received conventional treatment. The DC therapy boxplot represent the survived time of the combined group DC treated patients of 10 clinical N-GBM studies[22-24,26-29,31-33]. The conventional therapy boxplot reflects the survived time of the combined group of patients that received conventional treatment from the control groups of Cho et al. (2012) and Vik-Mo et al. (2013) studies[29,33].

The comparisons in survived time the PFS of all DC therapy studies were analysed in a similar way. Eight out of 12 studies reported the PFS of N-GBM patients, which describes the time period between the surgical resection and appearance of the first symptoms of tumor recurrence or disease[22,26,27,29-33]. The PFS of all patients that received additional Dc therapy in these eight studies (n=65) were compared with combined group of patients treated with conventional therapy alone that were included in the study of Cho et al. (2012) and the study of Vik-Mo (2013) (n=20)[22,26,27,29-33]. The results of these comparisons are demonstrated in the form of boxplots and they show that there is a differences in PFS between additional DC therapy and conventional therapy alone (figure 4). The difference in PFS between the combined group of DC treated patients of eight studies and combined patients of the control groups is significant (p=0.019).

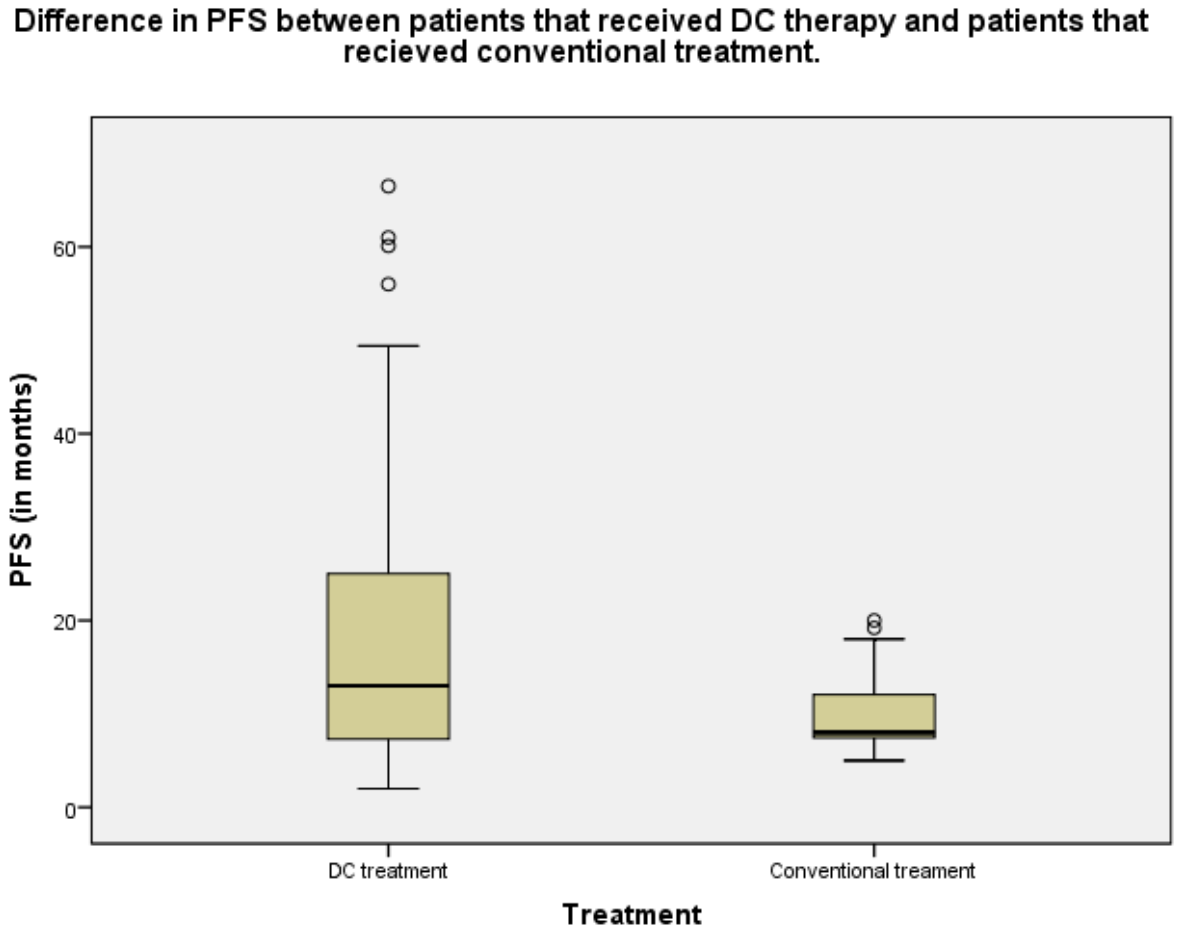

Fig. 4. These boxplots illustrate the difference in PFS between patients that received additional DC therapy and patients that only received conventional treatment. The boxplot of DC treatment represent the PFS of a combined group of patients of 9 clinical N-GBM studies[22,26,27,29-33]. The boxplots of conventional treatment reflects the PFS of a combined group patients from the control groups of the Cho et al. (2012) and Vik-Mo et al. (2013) studies[29-33].

4.3. The difference in clinical outcomes between all studies

Figures 3 and 4 have demonstrated that DC therapy, in addition to conventional treatment, appears to be beneficial for the OS and PFS of N-GBM patients. When analysing the increase in median PFS and OS between different studies in table 2, the fluctuation in these clinical outcomes between different studies stands out. To find out which studies demonstrated the strongest effect of DC therapy a scatterplot was created for the average PFS and OS of the DC group and if present control group with their confidence interval of 95% for each study (figures 5 and 6). The study of Jie et al. (2012) did not include the OS of individual patients, nor did it report the confidence interval of the average OS, which explain the absence of its confidence interval in figure 5[30].The study of Yu et al. 2004 only included one N-GBM patient, which is the reason for their omission in figure 5[23]. The most important

finding of figure 5 is that the average OS of control groups of Cho et al. (2012) and Vik-Mo et al. (2013) do not differ from their DC groups, since their confidence interval overlaps with more than 50 percent[29,33]. Furthermore, the mean OS demonstrated in the studies of Prins et al. (2012) and Phuphanch et al. (2013) are greater than the found means in the studies of Vik-Mo et al. (2013), Fadul et al. (2011), Sampson et al. (2009) Chang et al. (2011), Yu et al. (2001) and Cho et al. (2012)[22,24,26-29]. In addition, the mean OS of shown in the studies of Ardon et al. (2010) and Fadul et al. (2011) are higher than the ones of the studies of Yu et al. (2001) and Cho et al. (2012)[22,25,27,29].

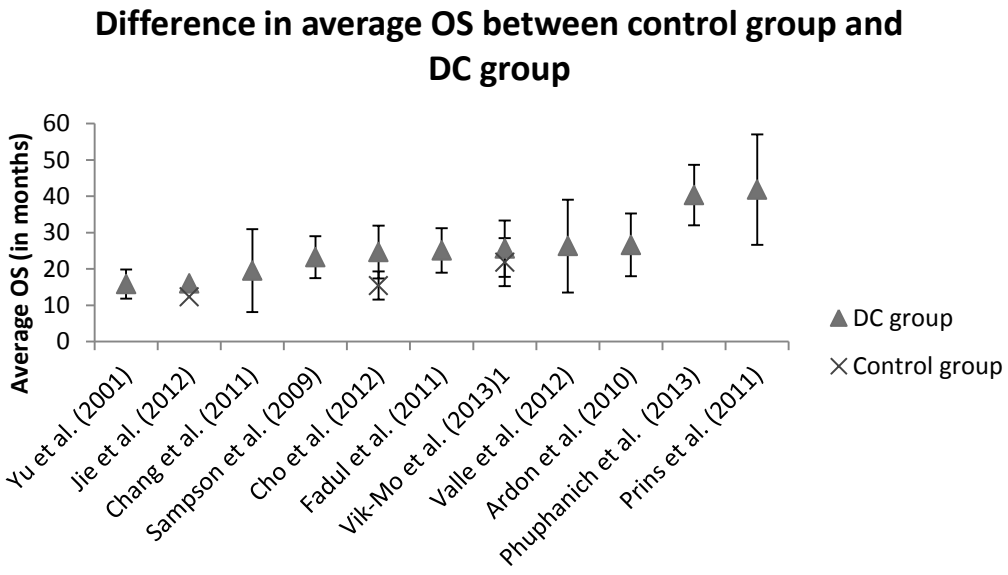

Fig. 5. This scatterplot shows the differences in average OS between 11 clinical studies that tested the effect of DC with error bars that represent the 95% confidence interval[22,24-33]. This scatterplot also includes the average OS and its 95% confidence interval of the control groups that were included in the studies of Cho et al. (2012) and Vik-Mo et al. (2013)[29-33].

An overview of the average PFS from patients treated with additional DC therapy as well as the average PFS from patients treated with conventional treatment alone are depicted in figure 6. In contrast to the average OS, the differences in PFS are smaller between studies, since the study of Phuphanich et al. (2013) is the only one that shows an higher average PFS than the studies of Yu et al. (2001) and Jie et al. (2012)[22,30,32]. Additionally, none of DC therapies scored higher on average PFS compared to the control group that were included in three studies. The fact that the lower limit of the study of Valle et al. (2012) stretches below zero months can be explained by the low amount of patients (n=3)[31].

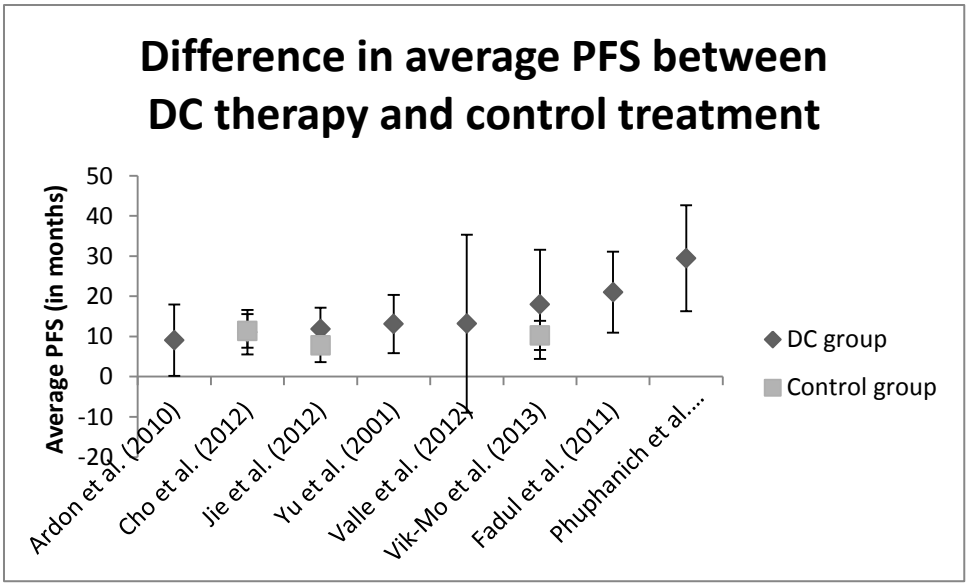

Fig.6. This scatterplot shows the differences in average PFS between eight clinical studies that tested the effect of DC, using error bars that represent the 95% confidence interval[22,26,27,29-33]. This scatterplot also includes the average PFS and its 95% confidence interval of the control groups that were included in the studies of Jie et al. (2012), Cho et al. (2012) and Vik-Mo et al. (2013)[29-33].

Based on the results of figures 5 and 6 together, the 12 studies can be split up into three groups: low, mid and high. The low group, scores clearly on lower on average PFS and survived time, compared to the scores of the high group, whereas the scores of the mid group are equal to one or both other groups. The low group is represented by the studies of Yu et al. (2001) and Jie et al. (2012) and the high group consists out of the studies of Prins et al. (2011) and Phuphanich et al. (2013)[22,28,30,32].

4.4. The difference in DC therapy protocols

In the upcoming part of this review, the effect of differences within four components of the DC-immunotherapy protocol on clinical outcomes will be analysed and discussed. I have chosen to compare the protocols based on the median OS, since median OS has been reported in all 12 studies, whereas median PFS has only been reported in eight studies. On top of that, there are more studies discussing DC protocols based on their effect on OS than PFS. Therefore I additionally investigated if there might be certain aspects in the protocol of these studies that differentiates the low group from the high group. In this investigation the results of the study of Yu et al. (2004) have been excluded, since comparisons in clinical outcomes based on only patient are not representative[23].

4.4.1. The effect of DC maturity on the median OS of N-GBM patients

One of the first crossroads in DC therapy protocols is the question whether to use immature DCs, or

mature DCs. Table 1 shows that eight trials specifically mentioned the usage of autologous mature dendritic cells for their treatment, whereas the four other trials did not specify the type of autologous DCs. Three out of these four studies did not include a maturation step in their protocol, only the study of Phuphanich et al. (2013) added a cytokine TNF-α, which is associated with the maturation of immature DCs[32]. Although the maturation of DCs with TNF-α is suboptimal compared to other maturation cocktails, the DCs in this study are at least partially matured and is therefore placed with the other studies that used matured DCs[17,18]. On the other hand, the study of Cho et al. (2012) and Vik-Mo et al. (2013) used matured DCs without including a maturation step in their protocol, meaning that there were no cytokines added to immature DCs[29,33]. Both studies analysed the DC and lymphocyte cell markers, which is normally performed to examine the maturation, but only the study of Vik-Mo et al. (2013) show the results of their analysis[29,33]. The study of Cho et al. (2012) has been marked in figure 7, due to the uncertainty concerning the maturation state of their DCs[29]. After creating a median line based on the median OS of the 11 trials, the differences between the studies that used autologous mature DCs (AMDCs) and unspecified autologous DCs were investigated (figure 7).

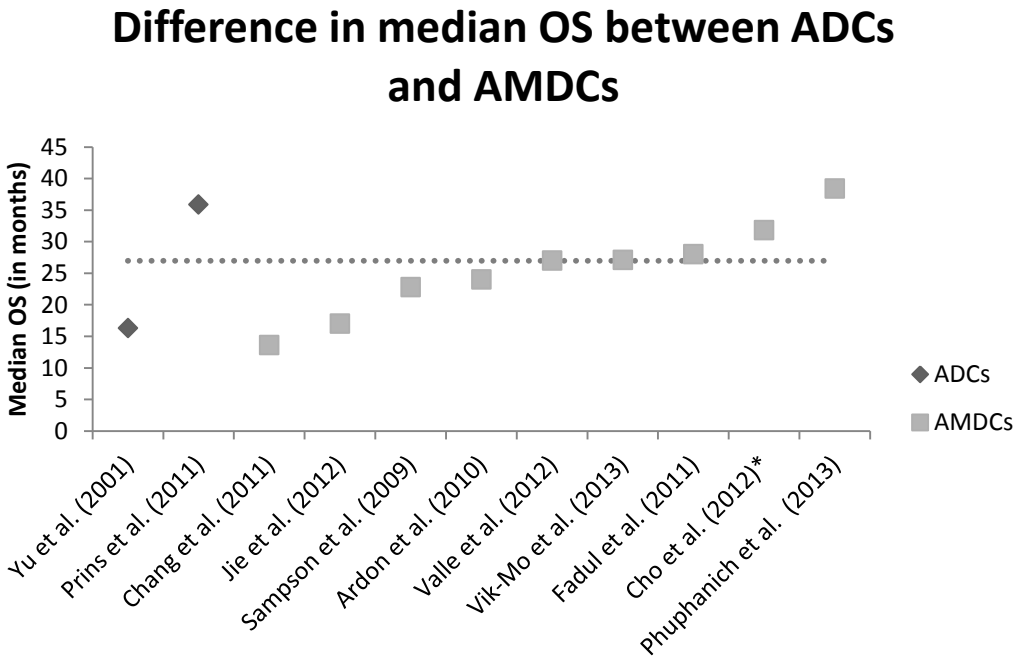

Fig. 7. The figure shows the differences in median OS between two studies that used unspecified autologous dendritic cell (ADC) types and nine studies that used autologous mature dendritic cells (AMDC)[22,24-33]. The dashed line represents the median OS of all 11 studies together. The asterisk (*) is placed by the study of Cho et al. (2012) and Phuphanich et al. (2013), due to the uncertainty of the maturation state of their DCs[29,32].

The median of OS of the trials with unspecified autologous DC is not distinctively different from to the median OS of trials that utilised AMDCs. Five out of nine AMDC studies score equal (1) or below (4) the median OS of all 11 studies[24-26,30-31.] The groups AMDC studies also have two studies that do

not score higher than 1 month above the median OS of all studies together[27,29,33]. The scores of ADCs on median OS are opposite from each other with the study of Yu et al. (2001) scoring below and the study of Prins et al. (2011) scoring above the median OS of all 11 studies[22,28].
These results are in line with to the current absence of clinical evidence that argues for using immature or mature DC in the DC therapy protocol for N-GBM patients. Nonetheless, they were opposite to my expectation based on relevant literature, as I thought that the median OS would be higher when patients are treated with mature DCs compared to the patients treated with immature DCs. For example, there are clinical studies that treated R-GBM and melanoma patients with DC therapies, which concluded that mature DCs showed better survival and are superior in activating immunological responses respectively, than those vaccinated with immature DCs[36,37]. In addition, immature DCs have been associated with a direct role in establishing a tumor resistance[36]. Based on these findings, the median OS of N-GBM patients that received DC therapy do not appear to depend on the state of maturation of DCs.

4.4.2. Effect of maturation protocol on median OS

There are multiple ways to mature DCs. There are factors (TNFα, LPS, CD40L, IFNα and IFNγ*)* that can mature DCs by themselves, as well as factors (PGE2, IL1β, IL-6 and I:C) that are combined in a cocktail with similar maturation effects[17]. Each protocol generates mature DCs with different phenotypes and stimulatory abilities. Table 2 shows that 5 different maturation mixtures were used in six studies of the selection [24,25,27,30-32]. The study of Chang et al. (2011) has been excluded from further analysis, since it does not mention the constituents of its maturation medium[26].
Figure 8 demonstrates the contrast in median OS between different maturation protocols of these six studies and the median OS of all six studies as a reference[24,26,27,30-32]. First of all, it shows that studies with a combination of antigens in the maturation mixture the studies score lower than studies that used one type of cytokine in its maturation protocol. Second, this figure demonstrates that the combination of cytokines of TNF-α, IFN-α and Poly I:C as well as the combination of TNF-α and PGE2 score higher on median OS than both other combinations. Most importantly, the maturation protocol of the high group differentiates itself from the low group by not including IL-1β nor PGE2 next to TNF-α.

The majority of these results are in contrast with my assumptions that are based on relevant literature. The first finding is for example contrary to the genetic profiles that demonstrated that the use of a cocktail of cytokines is the best way to mimic the *in vivo* situation, where multiple danger signals induce the maturation of DCs[17]. According to one of the most cited explanations for limited efficacy of DC therapy, which suggests that the incapability of DC vaccines to induce a Th1-polarised response is the result of insufficient DC maturation, the other results are also counterintuitive[17]. For example the cytokine of maturation protocol with the highest median OS is TNF-α, which is associated with inducing a Th-2 polarised immune response, whereas the cocktail that also included IFN-α and poly

I:C, which are associated with inducing an immune response that is strongly biased towards the Th1 immune response, belonged to a study of the middle group[17,38,39]. The cytokine combination of the maturation protocol belonging to the study with the lowest score is more conform to the previous explanation, since PGE2 has similar effects on $CD4^+$ T cell differentiation as TNF-α, although the maturation cocktails also included IL-1β that has been demonstrated to induce maturation of DCs towards a Th2 as well as Th1 polarised immune response[17]. Supporting the latter finding, IL-1β has been identified as an IL-12 inducing agent in DCs, which suggests that this cytokines would contribute also to a more Th1 biased immune response, when included in a maturation cocktail[40].

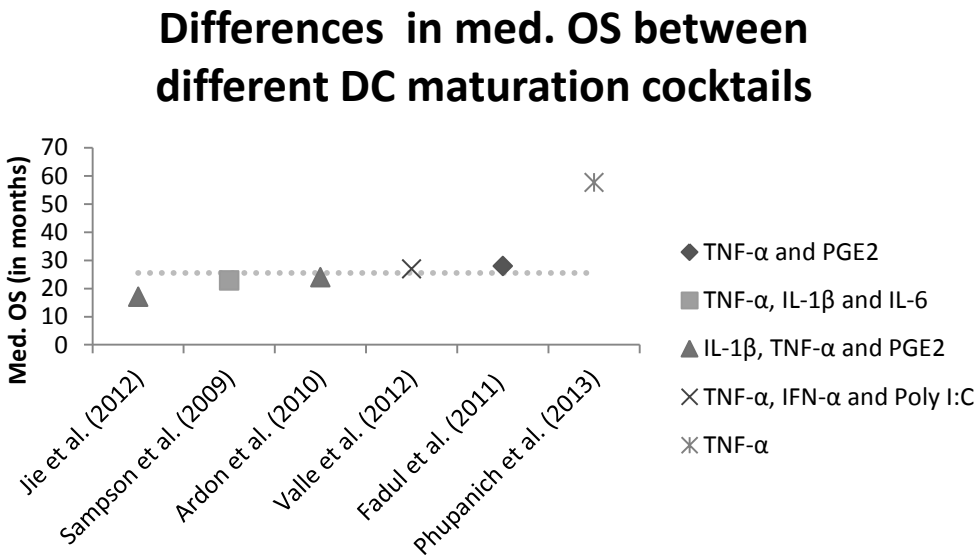

Fig. 8. This scatterplot shows the difference in median OS between different types of DC maturation cocktails of six clinical N-GMB studies[24,26,27,30-32]. The dashed line represents the median OS of all six studies.

4.4.3. Effect of antigen type on median OS

The next step in the protocol of DC immunotherapy is choosing the type of antigenic preparations out of the wide range of different types for priming the immature or mature DCs population to induce a tumor specific immune response. By loading these preparations on DCs, the DCs are primed with antigens. The selected 11 studies used 6 different GAAs: ATLs, ATCs, heat-shocked ATCs, specific glioblastoma stem cell (GSC) antigens, autologous tumor cell peptides (ATCPs) and synthetic peptides[22,24-33]. The majority of these antigen types are based on the removed autologous tumor of the patient. These autologous tumor cells (ATC) can be cultured with the DC directly[28,29], or be first inactivated by heat-shock[30], irradiation[27] or by freeze-thawing cycles[26,28,31] (ATL). The study of Vik-Mo (2013) and Phuphanich et al. (2013) both used their antigens to specifically target the cancer stem cells in gliablastoma[32,33]. The study of Vik-Mo (2013) performed a sphere-forming assay on the ATCs, which allowed isolation and expansion of GSCs and from these cells they isolated and amplified GSC mRNA[33]. The potential of DCs treated with some of these specific GSC mRNA have been

demonstrated *in vitro* as well in a case study[41,42]. Based on the same principle, the study of Phuphanich et al. (2013) tried to target specifically GSCs of N-GBM patients by loading autologous tumor cell peptides (ATCPs), IL13Rα2, AIM-2, TRP-2 and HER-2, which are overexpressed on glioblastoma derived cancer stem cells, on DCs together with glioblastoma associated peptides MAGE-1 and gp100[32,43]. The study of Yu et al. (2001) cultured their DCs with ATCPs that are associated with MHC I[25]. Lastly, the study of Sampson et al. (2009) pulsed DCs with a synthetic EGFRvIII-specific peptide conjugated to keyhole limpet hemocyanin that targets the tumor-specific mutation of EGFR expressed in most GBMs[24].The median OS for each antigen type of all 11 studies and the median OS for all 11 studies together are illustrated in figure 9.

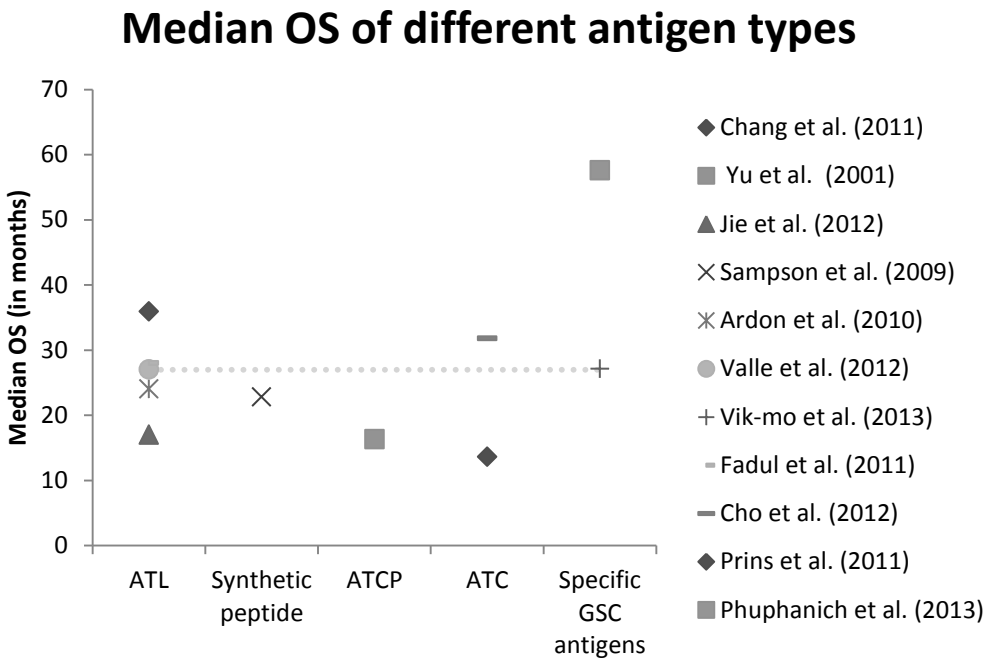

Fig. 9. This scatterplot demonstrates the median OS for each antigen type loaded on the DCs for each of the 11 clinical N-GBM studies[22,24-33]. The dashed line represents the median OS of all 11 studies together.

If you take the average of the results in median OS for each antigen type, the studies that targeted GSCs have the highest score on median OS; whereas the study that used ATCPs associated with MHC I score the lowest. The averages of the other antigen types appear to be quite similar. The only study that primed DCs with one peptide scores a little below the median OS of all 11 studies[24]. Key is the fact that this figure demonstrates that the studies of the high group did not use similar antigen types, nor did the studies of the low group.

As mentioned before, there is evidence that implies that the low effectivity of conventional treatments is the result of their incapacity to eradicate CSCs in glioblastoma. This also supported by animal

studies that demonstrated that lysates from CSCs induce a stronger immunologic protection compared to ATLs[43,44]. This suggests that the studies where DCs target GSCs will lead to a better median OS compared to DCs that target glioblastoma cells in general[43,44]Although the studies of the high group did not both specifically target GSCs, the average results of each antigen type are in line with this suggestion. Another assumption concerning the effect of antigen types in DC therapy is that a limited selection of GAAs increases the risk of an immune escape, due to tumor expansions by antigen-loss variants[4]. However, the comparison between antigens between these studies proves it wrong, as the study of Vik-Mo (2013) should have scored higher than the study of Phuphanich et al. (2013), since it used a more diverse antigen cocktail to target GSCs. Furthermore, the study of Yu et al. (2001) with one type of antigen does not score extremely lower than the average of other antigen types that used multiple antigens, in exception of the studies that specifically targeted GSCs.

4.4.4. Effect of administration time on median OS

After pulsing the DCs with antigens, the DC-based vaccine is administered to the patient. The moment of administration of the vaccine differs between the 11 studies ranging from directly to 49.6 weeks after surgical resection, which divides the studies in two groups: the studies that treated the patient before radiation and after radiation. The median OS for all 11 studies of both groups are arranged in figure 10 on the x-axis from left to right, based on their administration time after surgical resection (figure 11). The study of Jie et al. (2011) on the outer left side administered its DC the fastest and the study of Phuphanich et al. (2013) with the longest time interval between standard treatment and DC administration is placed on the outer right[30,32]. The results demonstrate that the median OS of studies, which injected their patients directly after surgical resection or directly after completing RT, is lower than the median OS of studies with a longer administration time. The studies of the low group clearly show earlier time of administration (between 4 and 9 weeks) compared to the high group, whose administration interval varied from 7 to 49.6 weeks.

These results are in contrast to view that proposes that DC vaccines should be administered directly after surgical resection, chemo- and/or radiotherapy, to maximally profit from the activated immunologic response that follows from surgical resection[49]. However, they are in accordance with animal and clinical studies that suggest otherwise, as they demonstrate that early follow-up vaccinations are not essential and may even hamper the immune function, due to their activation-induced death of T cells that have been recently activated[49]. There also might be another explanation for the effectiveness of DC therapies that administer their vaccine not directly after completion of standard treatments. This is based on the Kaplan-Meier estimates of the study of Stupp et al. (2005) (figure 12), which shows that the longer the time between the conventional treatment and DC therapy will be, the greater the chance will be that the selected patients have a higher median OS[34].

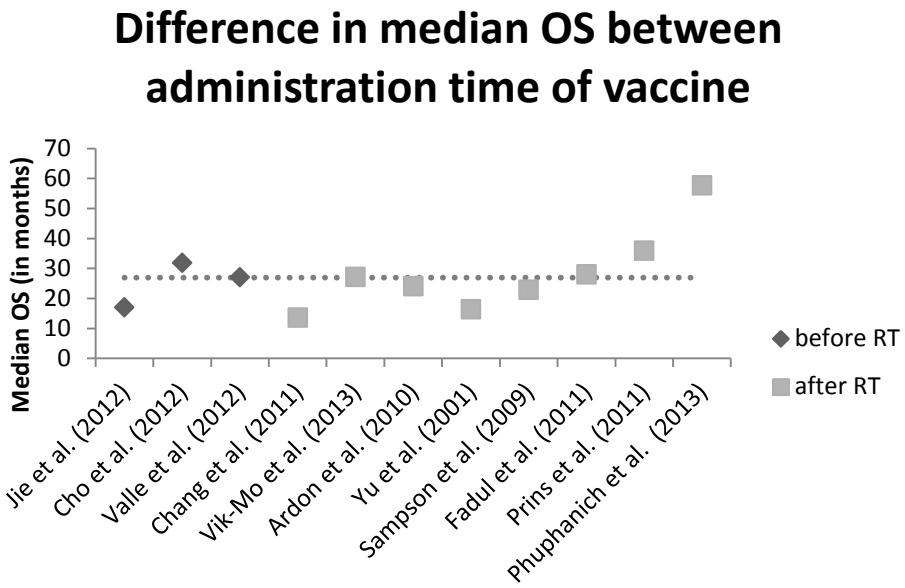

Fig. 10. This scatterplot shows the median OS of 11 selected studies that have been arranged based on their administration time of vaccine after surgical resection. The interval between surgical resection and vaccine administration increases from left to right. Furthermore have the studies been divided based on the administration of the vaccine before or after radiotherapy.

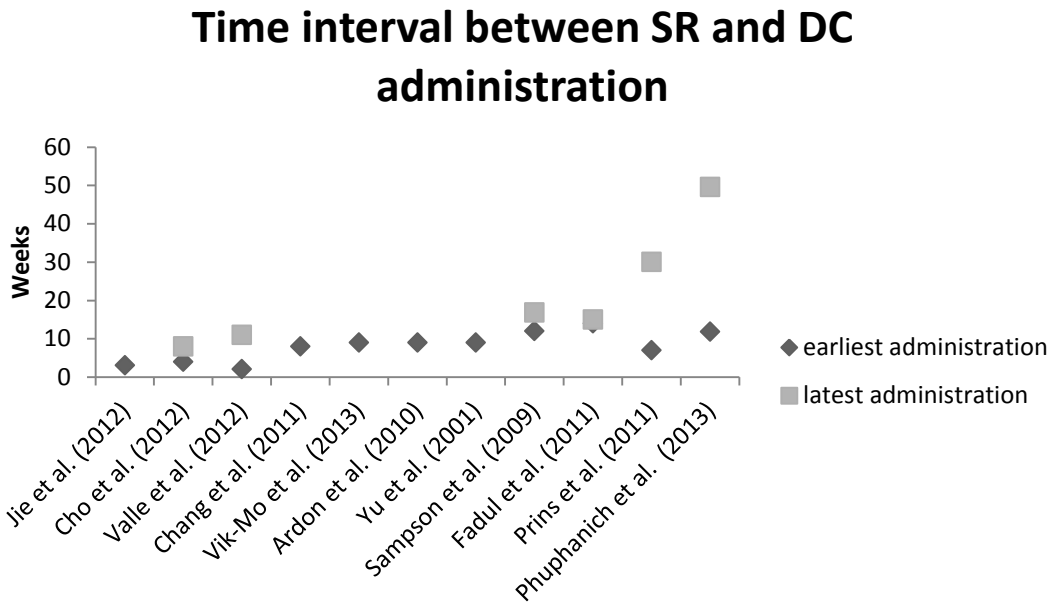

Fig. 11. This scatterplot demonstrated the range in administration time of the DC after surgical resection of the selected 11 studies.

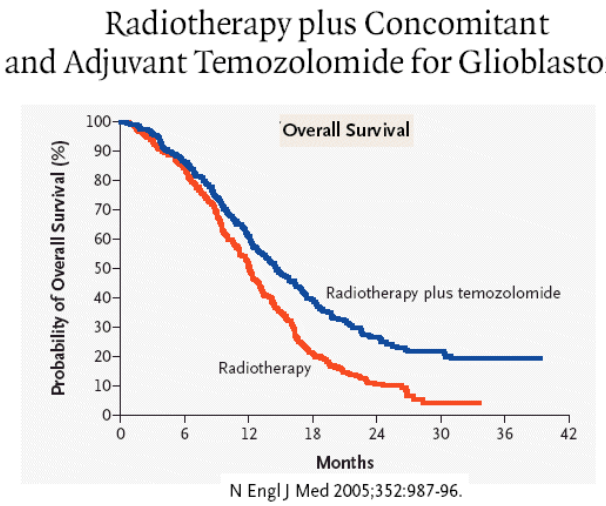

Fig.12. This Kaplan-Meier estimates of the study of Stupp et al. (2005) shows the relation between the chance of survival and the amount of months after receiving radiation therapy alone, or accompanied by TMZ therapy[34].

5. Discussion

This meta-analysis investigated the additional value of DC therapy next to standard treatment for newly diagnosed glioblastoma, by comparing its effect on clinical outcomes in the form of PFS and OS of N-GBM patients with the efficacy of conventional treatment alone. At first glance, the results appear to be according with the high expectations of DC therapy in the field of GBM treatment as all of studies, in exception of one, score above the clinical outcomes of conventional treatments reviewed by Stupp et al. (2005)[34]. However, there are some factors that have to be taken into account when comparing these treatments, as they could play an important role in this apparent increase. One of them is the selection criteria of clinical DC therapy studies. All of the investigated studies in this review selected their patients based on multiple inclusion and exclusion criteria, which have not been included in most of their control studies that investigated the effect of conventional treatment. All these criteria, such as completion of RT/standard treatment, no tumor progression during/after RT or conventional treatment, life expectancy greater than 3/6 months, withdrawal from the study when operated due to tumor recurrence, a greater KPS score than 60, together with the low numbers of patients (5-17) in these studies, will probably have resulted in a selection bias. This bias creates the situation, where the patient population that received additional DC therapy has a better prognosis compared to the selected patients of their conventional treatment control studies. In other words, the positive effects demonstrated in DC therapies compared to the effects of standard treatment based on historical data, might be overrated. Three clinical studies of Cho et al. (2012), Jie et al. (2011) and Vik-Mo et al (2013) also included a control group that received standard treatment that was selected on similar selection criteria as the DC therapy group. These three studies confirmed the presence of

this selection bias, since they did not show a clear difference in PFS and/or OS scores between both treatments[29.30,33]. However, there is a second factor that complicates both comparisons is the limited amount of patients, ranging from five to 17, which prevents profound based on statistical analysis. In a way to overcome this problem I combined all patients that received additional DC therapy or conventional treatment alone of the selected studies. This enabled the best possible way to control if additional DC therapy has any effect based on these data. Thus, I analysed the difference in PFS and OS scores of the patients who received DC therapy of all selected studies together and control patients that were selected on similar criteria as the DC patient group in those studies. The effect of additional DC therapy was smaller than when compared to the median OS/PFS of historical controls, as consequence of the increased mean in clinical outcomes of the control patients of these studies, but it proved to be still significant. The only issue about this result is that even the selection criteria between DC therapy studies differed. Therefore, it remains uncertain if the beneficial effect of DC therapy on clinical outcome also would be demonstrated in a randomized clinical trial with more patients, where patients of both groups are selected on similar criteria.

In the situation where the effect of DC therapy in addition to conventional treatment would be investigated in a phase 3 randomized clinical trial with abundant patients another problem remains in the form of unstandardized treatment protocols for DC therapy. Thus, the second aim of this review was to find out which treatment protocol should be implemented in this future clinical trial for an optimal clinical outcome. Given that multiple clinical trials that investigated DC therapy for N-GBM showed large differences in their clinical outcomes, I compared the differences in the protocol between trials with the lowest and highest scores on clinical outcomes in search of the ideal protocol of DC-immunotherapy for N-GBM. In result I found two studies that clearly scored higher on median PFS and OS than two other studies, which I defined as the high and low group, respectively. The second step required investigating the effects of variants of various components of the protocol, such as DC maturity, maturation mixtures, antigen type and administration time, in the hope of finding a relation between specifics variants and the low or high group of studies.

In case of DC maturity there is no difference between both groups. Contrary to the literature, the comparison in median OS between ADCs with an unspecified state of maturity and AMDC did not show a benefit for patients treated with AMDCs. A possible explanation might be that DCs without a maturation step do mature at least partially, which was demonstrated in the study of Prins et al. 2012[28]. On the other hand it has also been suggested that not all maturation steps are as efficient in producing fully functional matured DCs[17]. Both explanations suggest that the differences in maturation between ADCs and AMDCs are smaller than expected and gives the impression semi-matured DCs used in are inferior to fully matured DCs. However, the question remains which maturation protocol leads to fully matured DCs and which ones not.

Therefore, the next comparison in median OS was based on different maturation protocols between the studies, since the state of maturation and functionality of DCs has been suggested to rely on

differences in the maturation mixture. The results suggest that TNF-α should be preferred above the combination of TNFα, IL-1β and PGE2 as maturation protocol, whenever one chooses to use matured DCs. The evidence for this statement is not compelling, as it is based on the maturation protocol of one high and one low group study, instead of all four. On top of that, the highest score on median OS belongs to the study that only used TNF-α, a cytokine that has been associated with skewing the Th1/Th2 balance towards to Th2 immune response. This is directly the opposite of what I expected, since I assumed that a maturation cocktail with more cytokines associated with a Th1 than a Th2 immune response would induce a stronger Th1 biased immune response resulting in a higher median OS. In addition, the results are also in contrast with assumption that the use of a maturation cocktail should be preferred over maturation mixture with one type of cytokine, as it mimics the *in vivo* situation better[17]. The comparison between variants of DC as well as maturation protocols shows that the efficacy of DC therapy does not appear to depend on the level of maturation of administered DCs. Next to the incapacity of DCs to induce a Th1 polarised immune response, another explanation for the inability of conventional treatments to treat N-GBM effectively is that standard therapy removes and destroys the tumor in general without specifically targeting and eradicating cancer stem cells, which have been demonstrated to restore the damage of standard treatment. This is only partly confirmed by the results of this meta-analysis, since only one of the two studies of the high group aimed to induce a specific immune response that targets the CSC of glioblastomas (GSC), whereas the antigens used by the low group studies targeted glioblastomas. The composition of the antigen types in the low and high group are in contrast with another assumption, which suggests that DCs should be primed with as many tumor associated antigen to prevent an immune escape as result of heterogeneity of tumors[4]. One of the high as well as the low group used ATLs, which is a more diverse antigen source than ATCPs used in the other studies of both groups. In conclusion it may be that DC therapies that specifically target GSCs are more effective than those that use unspecific glioblastoma antigen sources. In addition, the role of antigen diversity appears to be marginal based on this meta-analysis. Until so far, there is disconcordance between my expectations based on the literature and the findings based on this meta-analysis. A possible explanation may lie in the fact that there are huge discrepancies in the time of administration of DC vaccines between the studies. According to the overview of the effect of administration time on median OS presented in this review, the impression is raised that administration time may fulfil an important role in the survived time of patients. The highest scores in median OS are associated with greatest interval times, whereas the lowest scores are associated with shorter times of administration. This is the only investigated variable of the protocol that clearly separates the low group from the high group. This implies that the time interval between surgical resection or completion of conventional treatment and time of administration is positively related with median OS. However, it has to been taken into consideration that the longer patients had to wait for DC therapy, the greater the probability of overall survival of the selected patients would be, according to the Kaplan-Meier estimate of conventional treatment[34]. This can result in another

selection bias, since studies with a short time interval include patients that would have been excluded in studies with a longer time interval when the patients would have died during this additional time period. However, based on the Kaplan-Meier estimate the group of patients with longest time interval, estimated to be around 3.5 months after RT, would have maximally excluded 5% of the patients that would have been included in a study that would have directly administered the DC vaccine after RT. This percentage would not have resulted in exclusion of even one patient, due to small amount of patients in these studies. Nonetheless it is a factor that has to be taken into account in a future phase 3 clinical trial with more patients. Although these results demonstrate at which time point one should not administer additional DC vaccines, it remains unclear what the best time point is for DC vaccination and whether DC administration is more beneficial before, or after radiotherapy. To answer these questions further investigation would be required.

Taking all results together, I have to conclude that they did not meet my expectations. Although I knew that these studies not fully validated their results, due to small patient numbers or absence of randomization, the promising efficacy of DC therapy in addition of standard therapy is also likely biased as result of selection criteria. It is unclear to what extent this bias affects the apparent potential of additional DC therapy for N-GBM. Therefore, I would recommend future clinical DC therapy studies to standardize the selection criteria and compare their results with the results of conventional treatment that selected their patients on similar criteria to prevent selection bias and get better insight in the efficacy of DC therapy. Another disappointment resulted from the comparisons in protocol between the studies with high and low scores on median OS, as my hypothesis, which suggested that the differences in OS between both groups were the result of different protocols, was only confirmed in one out of four steps in the protocol. So instead of presenting an ideal DC therapy protocol based on 4 different steps, I will solely advise future DC therapy studies to administer their vaccine neither directly after SR, nor within 6 weeks after RT completion to enhance the OS of patients. Although future research is required to further investigate promising variants of the DC therapy protocol, such as maturing DCs with a maturation cocktail that contain multiple cytokines that are associated with inducing a Th1-polarised immune response and the effect of priming DCs in such a way that they induce a GSC specific immune response, to optimise the DC therapy protocol, this review has given a new insight how these experiments should be performed ideally.

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7. Supplementary material

Table 1. Selection criteria of the 12 studies that treated N-GBM patients with DC therapy in addition to conventional therapy compared with selection criteria of conventional alone.

Selection criteria		Authors and year of publication											
		Yu et al. (2001)	Yu et al. (2004)	Sampson et al. (2009)	Ardon et al. (2010)	Chang et al. (2011)*	Fadul et al. (2011)	Prins et al. (2011)	Cho et al. (2012)*	Jie et al. (2012)	Valle et al. (2012)	Phuphanich et al. (2013)	Vik-Mo et al. (2013)
Karnofsky score		≥60	≥60	≥80	≥70	≥70	≥60		≥70	≥60		≥60	
Age			≥18	≥18	>18 and <70	≥17 and ≤70	≥18		≥14 and ≤70	≥20 and ≤60			≥18 and ≤70
ECOG performance status													0-1
Post-operative residual gadolinium contrast enhancing mass size													0-5cm^3
First diagnosis of high-grade glioma, histologically proved					x					x			X
TR or STR of tumor mass, confirmed by assessment of neurosurgeon and postoperative MRI within 72h.					x								

Supplementary table 1 (continued)

Selection criteria	Authors and year of publication											
	Yu et al. (2001)	Yu et al. (2004)	Sampson et al. (2009)	Ardon et al. (2010)	Chang et al. (2011)	Fadul et al. (2011)	Prins et al. (2011)	Cho et al. (2012)	Jie et al. (2012)	Valle et al. (2012)	Phuphanich et al. (2013)	Vik-Mo et al. (2013)
Availability of enough tumor tissue, kept dry in sterile vial at -80C				x								
Life expectancy				>3 mo.					>6 mo.			
Histology confirmed by reference pathology				x								
Written informed consent by patient				x		x	x	x	x		x	
Able to undergo SR for N-GBM confirmed by neuropathology review						x	x					
Have a resected tumor yield $\geq 8 \times 10^7$ tumor cells, after mechanical and enzymatic digestion of tumor tissue						x						
Fit into groups I to IV as defined by Curran's recursive partition analysis			x									
Completed 6 wks of conformal RT with concomitant TMZ						x		x	x		x	
Completed RT before vaccination			x							x		
Maintenance dose of glucocorticoid therapy	Lowest possible	Lowest possible		Perioperative administration tapered within 7d post operatively								
Presence of at least one of the vaccine antigens on the patient's tumor											x	
Withdrawn from study when pt. underwent 2nd surgery due to tumor recurrence										x		

Supplementary table 1 (continued)

Selection criteria		Authors and year of publication											
		Yu et al. (2001)	Yu et al. (2004)	Sampson et al. (2009)	Ardon et al. (2010)	Chang et al. (2011)	Fadul et al. (2011)	Prins et al. (2011)	Cho et al. (2012)	Jie et al. (2012)	Valle et al. (2012)	Phuphanich et al. (2013)	Vik-Mo et al. (2013)
Normal base line haematological parameters	hemoglobin	>9.9 g/dl	>9.9 g/dl		>9 g/dl	x	x	>9 g/dl				x	
	Leukocyte count				>3000/µl								
	Lymphocyte count				>500/µl								
	Granulocyte/neutrophil count	>1000/µl	>1000/µl	>1000/ µl	>1000/µl			>1500/µl					
	Platelet count	>60000/µl	>60000/µl	>50000/µl	>100000/µl			>100000/µl					
	Alanine aminotransferase	<2x the upper limit of normal	<2x the upper limit of normal										
	Aspartate aminotransferase	<2x the upper limit of normal	<2x the upper limit of normal										
	Prothrombin time & Activated thrombo plastin time	No greater than 1.4x normal control, unless therapeutically warranted	No greater than 1.4x normal control, unless therapeutically warranted	No greater than 1.5x normal control,									
Adequate hepatic function	SGPT					x	x	<2.5x the upper limit of norma					
	SGOT												
	Alkaline phosphatase	<2x the upper limit of normal	<2x the upper limit of normal										
	bilurubin							<1.5mg/dL					
Adequate renal function	BUN	<30 mg/dl	<30 mg/dl			x	x	<1.5x institutional normal					
	creatinine	<2 mg/dl	<2 mg/dl										
No allergy to components of DC		x						x		x			
No allergy to gentamicin												x	

Supplementary table 1 (continued)

Selection criteria	Authors and year of publication											
	Yu et al. (2001)	Yu et al. (2004)	Sampson et al. (2009)	Ardon et al. (2010)	Chang et al. (2011)	Fadul et al. (2011)	Prins et al. (2011)	Cho et al. (2012)	Jie et al. (2012)	Valle et al. (2012)	Phuphanich et al. (2013)	Vik-Mo et al. (2013)
No tumor progression during RT			x									
No tumor progression after standard treatment of RT with concurrent TMZ treatment											x	
No pregnancy	x	x	x		x			x	x		x	
No simultaneous treatment according to other clinical trials				x								
No systemic disease associated with an unacceptable anesthetic or operative risk	x	x	x			x	x	x	x		x	
No acute infection requiring active treatment	x	x	x	x	x		x		x		x	
No immunosuppressive disease or infection			x	x	x		x	x				x
No systemic corticosteroid use						within 2 wks before leukapherasis						during the course of vaccination
No concurrent or prior corticosteroid use							within in 10 days of initial vaccination					
No corticosteroid administration >			2mg/d dexamethasone		20mg/d dexamethasone						4mg/d dexamethasone	
No history of an autoimmune disorder	x	x		x	x	x	x		x		x	x
No prior history of other malignancies	excluding basal cell carcinoma and benign tumors	excluding basal cell carcinoma and benign tumors		x	x		x				excluding basal cell carcinoma and benign tumors	
Required use a medically accepted form of birth control	x	x										

All selection criteria, which have been marked in green, are also included in the study of Stupp et al. in 2005 that examined the efficacy of conventional treatment on OS in N-GBM patients[34].