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FOCUSSED RESEARCH REVIEW

Immunotherapy with dendritic cells loaded with glioblastoma stem cells: from preclinical to clinical studies

Gaetano Finocchiaro[1] · **Serena Pellegatta**[1]

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Abstract Different approaches have been explored to raise effective antitumor responses against glioblastoma (GBM), the deadliest of primary brain tumors. In many clinical studies, cancer vaccines have been based on dendritic cells (DCs) loaded with peptides, representing one or more specific tumor antigens or whole lysates as a source of multiple antigens. Randomized clinical trials using DCs are ongoing, and results of efficacy are not yet available. Such strategies are feasible and safe; however, immune-suppressive microenvironment, absence of appropriate specific epitopes to target, and cancer immunoediting can limit their efficacy. The aim of this review is to describe how the definition of novel and more specific targets may increase considerably the possibility of successful DC immunotherapy. By proposing to target glioblastoma stem-like cells (GSCs), the immune response will be pointed to eradicating factors and pathways highly relevant to GBM biology. Preclinical observations on efficacy, and preliminary results of immunotherapy trials, encourage exploring the clinical efficacy of DC immunotherapy in GBM patients using high-purity, GSC-loaded DC vaccines.

Keywords Dendritic cells · Immunotherapy · Glioblastoma · Cancer stem-like cells · NIBIT 2014

Abbreviations

b-FGF	Basic-fibroblast growth factor
CSCs	Cancer stem cells
CUSA	Cavitation ultrasonics surgical aspirator
DCs	Dendritic cells
EGF	Epidermal growth factor
GBM	Glioblastoma
GMP	Good manufacturing practice
GSCs	Glioblastoma stem-like cells
HIF-1α	Hypoxia-inducible factor-1α
IFN-γ	Interferon-γ
NK	Natural killer
NS	Neurospheres
OS	Overall survival
PBMCs	Peripheral blood mononuclear cells
PFS	Progression-free survival
TAA	Tumor-associated antigen
TGF-β	Transforming growth factor
TMZ	Temozolomide
TNF-α	Tumor necrosis factor-alpha
VEGF	Vascular endothelial growth factor

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✉ Gaetano Finocchiaro
gaetano.finocchiaro@istituto-besta.it

✉ Serena Pellegatta
serenapellegatta@gmail.com

[1] Unit of Molecular Neuro-Oncology, Fondazione I.R.C.C.S. Istituto Neurologico C. Besta, Via Celoria 11, 20133 Milan, Italy

Dendritic cell immunotherapy for glioblastoma

Glioblastomas (GBMs) are the most frequent of primary malignant brain tumors. In about 90 % of cases, GBM

appears de novo; in other cases, GBM derives from lower-grade gliomas.

During the last 10 years, there has been a growing appreciation of the role that immune escape plays during tumor development [1]. Preclinical data support the evidence that cancer cells can be eliminated by the immune system; afterward cancer creates a situation of equilibrium with immune system by multiple strategies that eventually lead to escape [2]. Attempts to reverse this situation, reeducating the immune system to destroy cancer, met limited success [3].

Different approaches have been explored to raise effective antitumor responses against GBM; in particular, immunotherapy strategies have been based on autologous dendritic cells (DCs) loaded with glioma-associated peptides or whole tumor lysate.

Pioneering preclinical studies showed the efficacy of DCs in prolonging survival of established rat gliomas when pulsed with tumor cell mutants [4], in inducing a specific antitumor immune response and enhancing CD4+ and CD8+ T lymphocyte infiltration when pulsed with acid-eluted tumor antigens from 9L rat glioma cells [5]. Other preclinical investigations for the development of therapeutic vaccines against malignant gliomas, based on the use of DCs, have been carried out in rats [6] as well in mice [7–14].

Based on preclinical results, several international research groups have worked on the development of therapeutic vaccines based on the use of DCs loaded with glioma-derived tumor antigens, also providing data on safety and efficacy in clinical trials using autologous tumor lysate-loaded DCs [15–20]. Yu et al. [20] showed that the ex vivo differentiation of DCs and exposure to tumor lysate antigens could induce an immune response after vaccination of malignant glioma patients. Yamanaka et al. [21] have demonstrated that DC vaccination elicits systemic cytotoxicity detected by IFN-γ expression in response to tumor lysate. Furthermore, intratumoral cytotoxic T cell infiltration was detected in several recurrent GBM patients. This study also showed an increase in tumor lysate-reactive CD8+ T cells after vaccination, due to DC influence on patient immune system. Liau et al. [22] used acid-eluted GBM peptide-pulsed DCs, instead of tumor lysate-loaded DCs, and obtained similar promising results. Their study showed both a low toxicity profile at all dose levels tested and a measurable peripheral antitumor T cell response in half of the newly diagnosed or recurrent GBM patients, but not correlated with clinical outcome. Most of these studies demonstrated the ability of an active immunotherapy strategy to generate antigen-specific cytotoxicity in brain tumor patients and indicate the possible therapeutic relevance of DC therapy for glioma.

More recently a phase III clinical study called DCVax-L aimed to evaluate immunotherapy efficacy in GBM. Results on the efficacy will be available in the near future [23].

Two clinical studies, DENDR1 and DENDR2 (DENDR1—EUDRACT No 2008-005035-15; DENDR2—EUDRACT No 2008-005038-62) including, respectively, the treatment of first diagnosis and recurrent GBM patients with DCs loaded with autologous tumor lysate are currently active in our institution. Results of many studies [24], including ours, provided evidence for feasibility and safety of DC-based GBM immunotherapy, however failed to provide convincing evidence of efficacy, raising a number of clinical and biological issues to be addressed in order to increase the potential of these strategies [25].

Our first results obtained on a group of recurrent GBM patients demonstrated that the response of NK cells correlates with significantly prolonged survival. Increased frequency and activation of NK cells correlated with increased progression-free survival (PFS) and overall survival (OS) of patients. Two important factors could affect the efficacy of DC immunotherapy: the tumor volume at the time of vaccine, as observed by us and others [26, 27], and the immunosuppressive environment generated by the tumor, as indirectly evaluated on peripheral blood. In our patients, we investigated serum levels of TGF-β, VEGF, and IL-12 and we found an inverse correlation of patient survival with these immune-suppressive factors and a positive correlation between increased PFS and IL-12 levels, a cytokine involved in IFN-γ production by NK cells.

Some evidence suggests that immunotherapy can achieve a better success when used in combination with standard radio-chemotherapy [28, 29]. In particular, during chemotherapy-induced lymphopenia, vaccines could activate specifically the immune system by expanding a small number of tumor-specific lymphocytes [30]. In addition, radio-chemotherapy can enhance immunogenicity of dying tumor cells [28]. We have results of preliminary analysis on 22 patients enrolled in the DENDR1 study, in which patients affected by primary GBM after radiotherapy and chemotherapy with temozolomide (TMZ) received three intradermal injections of mature DC before adjuvant chemotherapy. The subsequent four injections were performed 17 ± 3 days after each cycle of adjuvant TMZ. Our initial data indicate that clinical benefit is associated with an increased activation of peripheral NK and NKT cells rather than CD8+ T cells [31, 32]. Additional data showed that differential sensitivity of NK and CD8+ T cells to TMZ administration may partly explain these results (paper in preparation).

Glioblastoma stem-like cells

An initial concept of cancer stem-like cells (CSCs) implied a hierarchical model of cancer in which only a minority of cells identified by specific markers (CD133 being the most studied) was considered responsible for tumor development [33]. Subsequently, this CSC model was challenged by observations that more permissive animal models allowed a much larger fraction of tumor cells to be tumorigenic [34]. Also, the reliability of CD133 and other CSC markers was questioned by experiments showing that also CD133-negative cells can be tumorigenic [35]. Remarkably, the switch to stem cell programs may rely on epigenetic rather than genetic changes, allowing cells to adapt faster to environmental challenges, without the need of numerous cell generations required for advantageous mutations to prevail [36]. Thus, hierarchical model of CSCs initially proposed for GBM and other cancers should probably be substituted by a more flexible concept of cancer stem-like cells, a subpopulation of cells fitter than others for tumor adaptation to environmental (and possibly therapeutical) challenges thanks to the exploitation of stem cell programs. Notably, Chen et al. [37] observed that "cancer cells are not all alike within the tumor mass." Self-renewing cells with different features and capabilities exist within an individual lesion and may represent a reservoir of CSCs. GSCs in particular have been reported to share biological features with neural stem cells (NSC), like capacity for self-renewal and differentiation accompanied by the expression of marker proteins (e.g., nestin, CD133, SOX2, SSEA-1).

Murine and rat models of malignant gliomas and human GBMs contain a fraction of cells with stem-like features (GSCs) that may be responsible for glioma recurrence [38–40]. It was found by several groups, including ours, that GBM populations enriched with GSCs can give rise to gliomas resembling closely the original tumor and rather different from the experimental gliomas generated by brain injection of established cell lines [33, 40]. These GSCs have been found to preserve genetic alterations of their originating tumor and are tumorigenic in nude mice. Genetic analysis performed on GSCs growing in the presence of mitogenic factors compared with adherent cells cultured in the presence of serum and with xenograft gliomas showed that alterations (e.g., loss of heterozygosity, LOH of 10q and 9p) present in the original specimens were maintained in GSCs and xenograft gliomas but not in adherent cells, which underwent significant changes during passages [41, 42].

Growing progress has been made in unraveling the molecular heterogeneity of GBM, pointing to three subtypes characterized by different molecular alterations: proneural, proliferative, and mesenchymal [43]. Recent data have shown that tumor microenvironment can favor the amplification of cancer cells exploiting stem cell programs for survival: Hypoxia appeared one of the major drivers in these processes [44]. Cells in the tumor microenvironment, such as macrophages/microglia, may play a crucial role in this process through TNF-α and NF-kB by inducing plasticity between the proneural and mesenchymal subtypes observed in GBM [45].

During the last years, the TCGA project has helped to dissect the molecular complexity of GBM addressing issues of genetic heterogeneity both among different patients and within the same patient specimen [46]. Heterogeneity indicates that several different genetic clones coexist within the tumor and represent one of the factors underlying limitations in GBM treatment and poor prognosis [47, 48]. The concept of GSCs has been reshaped based on these observations: It has been reported that GSCs are genetically heterogeneous and cannot be identified as a clonal entity [49].

We believe that if cancer stemness is a temporary, inducible condition, in the frame of tumor plasticity, it is indeed possible that CSC is replenished after immune attack. This may imply that CSC targeting may slow down but not eliminate tumor growth. To some degree, immune memory may circumvent this, but we think it should be acknowledged that CSC immunotherapy may not be a magic bullet.

Immunological characterization of glioblastoma stem-like cells

The identification of GBM subpopulations expressing stem cell programs provided the background for immunological studies aimed at their targeting.

Di Tomaso et al. [50] found that GSCs, but not their paired serum-grown tumor lines, inhibited T cell proliferation of healthy donors. Wei et al. [51] also found that the cancer-initiating cells inhibited T cell proliferation and activation, induced regulatory T cells, and triggered T cell apoptosis. These immunosuppressive properties were markedly diminished when the STAT3 pathway was blocked in the cancer-initiating cells. The same authors reported that inhibition of T cell proliferation and activation, induction of regulatory T cells, and T cell apoptosis were mediated by B7-H1 and soluble galectin-3. These immunosuppressive properties were diminished by inducing differentiation of the cancer-initiating cells [51]. A further, intriguing, set of observations was reported by the same group. They found that hypoxia potentiated GSC-mediated inhibition of T cell proliferation and activation, induced FoxP3+ T cells, and inhibited macrophage phagocytosis. These immunosuppressive effects were mediated by STAT3 and its transcriptionally regulated products HIF-1α and vascular endothelial growth factor (VEGF). Inhibitors of STAT3 and HIF-1α

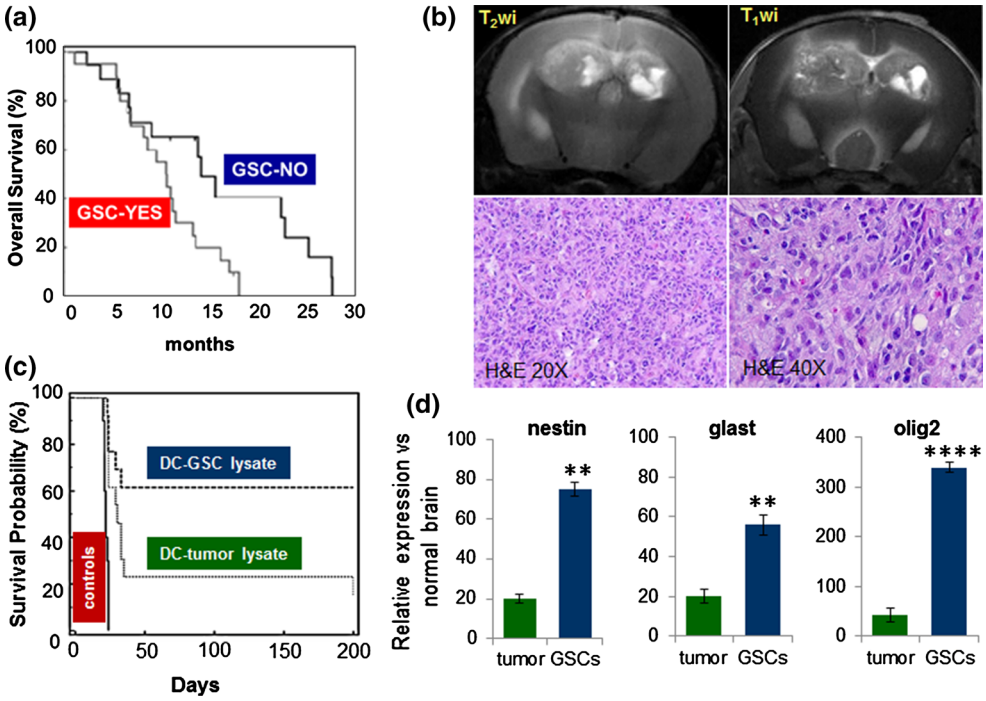

Fig. 1 **a** Kaplan–Meier analysis showing that in vitro NS formation is associated with decreased survival in patients ($p = 0.018$). **b** Magnetic resonance imaging performed on nude mice 55 days after tumor implantation (*upper panel*) and histological analysis performed on xenograft gliomas (*lower panel*). **c** Kaplan–Meier analysis curves showing that DCs loaded with GSC lysate protect from GL261-GSC glioma a larger fraction of mice compared with DCs loaded with whole tumor lysates. **d** Real-time PCR performed on GSCs reveals that expression of nestin, GLAST, and OLIG2 is 8.1, 2.6, and 3.7 higher, respectively, compared to tumor (**$p < 0.001$, ****$p < 0.0001$)

down-modulated the hypoxia-induced immunosuppressive effects of GSCs [52]. GSCs were also found to modulate innate immunity in glioblastoma by inducing immunosuppressive macrophages/microglia, and this capacity could again be reversed by inhibiting phosphorylated STAT3 [53]. Interestingly, NK and CD8+ T cells were also described as being able to recognize and kill GSCs [54, 55]. In particular, GSCs were also described as competent in processing and presenting specific antigens. Consequently they could be recognized and killed by CD8+ cytolytic T cells [56].

All these findings support the relevance of GSCs as targets for immunotherapy and suggest that given their potential for immune suppression, conditions for GSC antigen presentation to DCs ex vivo should be carefully evaluated.

Neurospheres as in vitro GBM model

GSCs can be also obtained by established cultures from murine gliomas, like GL261. GL261-GSCs have similar features to human GSCs [38].

The generation of spheres growing in the absence of serum and in the presence of growth factors (mostly EGF and b-FGF) has been considered as the appropriate way to grow CSCs in vitro. Thus, in case of GBM, the term neurospheres (NS) has been considered as synonymous of CSC. NS may mirror much more closely than previous, serum-based glioma cell lines, the actual biology of GBM [42]: They are always tumorigenic in immune-deficient hosts, and the tumors they form in these hosts are much more representative of human GBM [41]. More important, the potential for GBM to form NS is associated with increased aggressiveness and decreased survival in patients, as shown by Pallini et al. [57] and confirmed by our own data (Fig. 1a). We confirmed the in vivo tumorigenicity (Fig. 1b) of our NS and their ability to maintain in vitro most genetic alterations of the original tumor and many features of their original subclassification [43, 58].

GSCs as a more specific target for DC immunotherapy

There are four important issues to consider in designing effective cancer vaccine: identify potent tumor rejection antigens; stimulate an effective antitumor immune response; avoid autoimmune pathology; and prevent immune evasion. The identification of a specific marker

could be mandatory; however, this urgency conflicts with the evidence that strategies directed against one antigen only may have limited efficacy. A vaccine targeting epidermal growth factor receptor variant III (EGFRvIII, a constitutively activated and immunogenic mutation present in GBM) showed a promising gain in survival, but also evidence of immune escape, as patients lost EGFRvIII expression at recurrence [59]. Strategies aimed at minimizing immune escape should include the immune targeting of pathways essential for tumor perpetuation. Based on present knowledge, GSCs may represent a reproducible source of potential antigens at the core of tumor capacity for perpetuation that are otherwise "diluted" in the tumor lysate used in current immunotherapy protocols for DC loading.

DCs loaded with GL261-GSCs was significantly more effective than DCs loaded with serum-cultured GL261 at inducing immune rejection of highly malignant gliomas that are otherwise lethal in about 1 month. With these experiments, we provided a proof of principle that the use of a cellular population enriched in GSCs for DC loading may increase the efficacy of antiglioma (and possibly antitumor) immunotherapy [38]. Similar results have been obtained in a model of rat GBM [60].

In characterizing expression profiles of GL261-GSCs, we have found that expression of a group of radial glia genes is up-regulated. Four of these genes are also overexpressed by human GBM NS, encoding proteins involved in important biological tumor functions and conferring important characteristics of GSCs.

During these years, we confirmed the relevance of some of these genes related to radial glia and their involvement in GBM biology.

In particular, we found that FABP7 is highly expressed by GBM NS and is involved in proliferation and invasion of GBM cells. FABP7 was also found involved in response to radiotherapy, as this treatment caused increased tumor migration. Treatment with PPAR antagonists affected FABP7 expression and decreased migration ability of NS also after irradiation [61].

We also observed that GLAST, the neural stem cell marker and with relevant action in glutamate trafficking, is highly expressed in the plasma membrane of GSCs. Recently we showed that immunization with GLAST peptides efficiently promotes specific antitumor response in the murine glioma GL261, preventing the tumor progression in 40 % of immunized mice [62]. Remarkably, in preliminary experiments performed on human GSCs, we have seen that GLAST seems to be involved in glutamate release and enrichment or inhibition of its expression impact on aggressiveness in vivo and on invasion in vitro and in vivo.

More recently we observed that SOX2, a transcription factor functionally essential for normal stem cells, can represent a good target for immunotherapy. SOX2 was found crucial for tumor initiation by murine oligodendroglioma, and an immunotherapy strategy with SOX2 peptides, in combination or not with chemotherapy, was able to induce a specific antitumor response and to prolong survival [63].

Interestingly, in another preliminary set of experiments, we treated GL261 glioma-bearing mice with subcutaneous injections of 1 million bone marrow-derived DCs pulsed with 50 micrograms of lysate from gliomas or from GL261-GSCs on days 7, 14, and 21 after tumor implantation. The tumor lysate was obtained by sonication of explanted GL261 gliomas or GL261-GSCs, and murine DCs were prepared as previously described [38]. We have found that mice vaccinated with DCs loaded with GSC lysate survive longer than others vaccinated with tumor lysate (Fig. 1c). Using Real-time PCR, we found that three genes related to neural stem cells: nestin, OLIG2 [38, 64, 65], and GLAST [38, 62], were up-regulated in GSCs compared to whole tumor (Fig. 1d) (unpublished data).

A first evidence of the safety and feasibility of GSC targeting by immunotherapy in terms of progression-free survival (PFS) was reported in a clinical study on GBM patients where DCs were transfected with mRNA derived from GSCs. Median PFS was 694 versus 236 days, $p = 0.0018$ compared to matched controls [66]; however, no significant difference was observed in overall survival between the two groups. Seven patients were treated receiving GSC mRNA-transfected DCs. No adverse autoimmune events were observed, and an immune response activation was induced in all treated patients.

GSCs were obtained after mechanical and enzymatic dissociation of GBM specimens and cultured using the standard medium DMEM/F12 containing mitogenic factors EGF and b-FGF, leukemia inhibitory factor, B27 supplement, heparin, penicillin/streptomycin (pen/strep), and HEPES, as previously described [40, 67].

A phase I clinical trial, ICT-107, is ongoing based on a DC vaccine targeting six different GBM-associated antigens, of which four are considered GSC associated, in combination with standard radio-chemotherapy [68]. DCs are pulsed with class I (HLA-A1 and HLA-A2)-restricted peptides from HER2, TRP-2, gp100, MAGE-1, IL13Rα2, and AIM-2. Four of these tumor-associated antigens (HER2, TRP-2, AIM-2, and IL13Rα2) are considered related to GSCs as previously observed in the preclinical rat model 9L [60]. Preliminary observation derived from 21 patients showed that median PFS and OS in newly diagnosed patients were 16.9 and 38.4 months, respectively. In these patients, it was also observed that expression of the four ICT-107 antigens in tumors before vaccination correlated with prolonged PFS and OS. No significant correlation between immune response, evaluated as type I cytokine level evaluation, and survival metrics was revealed in these patients. Nevertheless, results of this phase I study

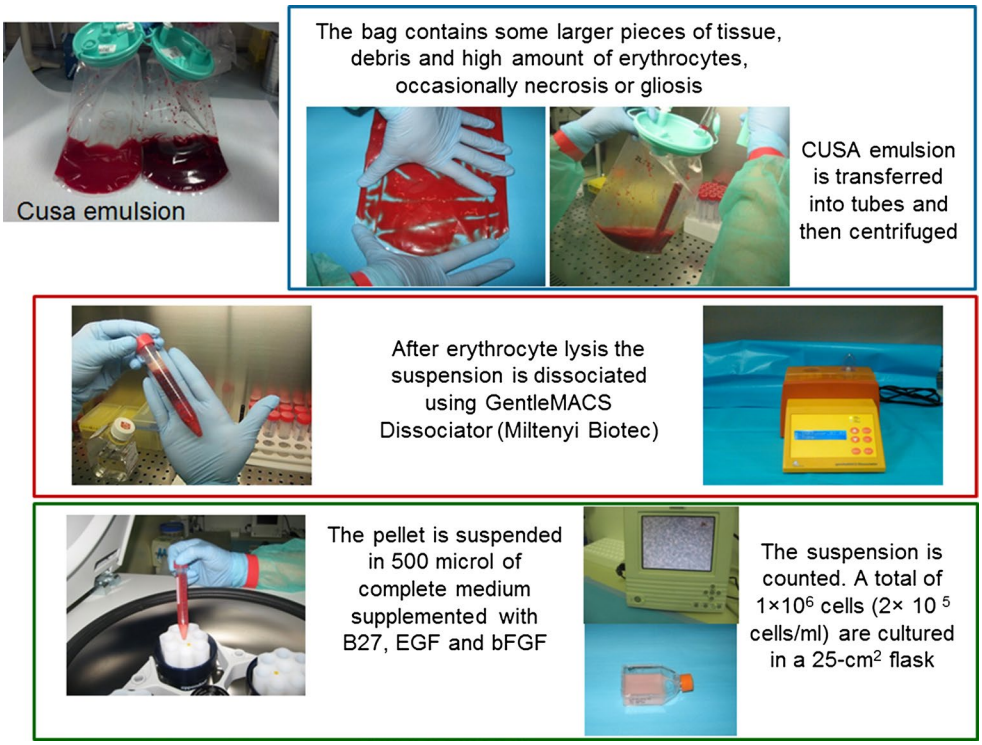

Fig. 2 CUSA processing protocol. Pictures show the key passages performed starting from the CUSA material

of ICT-107 demonstrated the safety and feasibility of TAA-pulsed DCs.

Setup of experimental conditions for expansion of "clinical-grade" neurospheres

Based on our preclinical data and recent clinical evidence, we believe that GSCs may represent a reproducible source of potential antigens.

We have a long-term experience in culturing GSCs from fresh GBM specimens growing in the absence of serum and in the presence of EGF and b-FGF after mechanical and enzymatic dissociation of tumor fragments received immediately after surgery. These GSCs have been used as an in vitro model in many of our published studies [61, 69–72].

In recent years, the cavitation ultrasonics surgical aspirator (CUSA) is preferably used for GBM surgery. CUSA facilitates the removal of large tumors from inside out and delivers an irrigating solution that converts the fragmented tissue into an emulsion and then aspirates the particles directly into a bag. The bag contains tumor fragments, debris and high amounts of erythrocytes, and occasionally necrotic or reactive tissue.

Tissue fragments in CUSA bags after several rounds of spinning ad washing are dissociated using the gentleMACS Dissociator (Miltenyi Biotec) that provides a closed system and reproducible results. We have optimized a gentle and effective protocol starting from appropriate gentleMACS programs, which allow to obtain a high yield of viable tumor cells (Fig. 2). After processing, cell suspensions are cultured as neurospheres in DMEM/F12, B27 supplement, human recombinant b-FGF, and EGF. Using tumor fragments obtained from surgery and combining mechanical dissociation with enzymatic disaggregation on a series of primary GBM, we previously obtained GSCs in 52 % of the cases. This percentage is now increased to 72 % with the use of surgical material from CUSA and the optimized protocol. Proliferation kinetics was studied by plating three primary cell lines obtained in parallel from GBM specimen and from CUSA material at density of 15,000–30,000 cells/cm^2. Cultures were collected every 5 days and the total number of viable cells assessed at each passage by trypan blue exclusion. A long-term proliferation of cell lines at low subculturing stages (3–10 passages) showed that proliferation increased exponentially, but the proliferation index of NS from CUSA materials was higher than from tumor fragments (1.32 vs 1.18, respectively, $p = 0.03$).

This improvement and the use of a closed system providing a wider margin of safety encouraged us to incorporate this process in a clinical trial protocol, DENDR-STEM, a pilot study in patients with recurrent GBM. We have developed a method to generate GSCs from CUSA bags under GMP guidelines using clinical-grade reagents which preserve the exponential growth ability of GSCs. GMP-grade DCs will be derived from PBMCs as previously described [73] and will be loaded with GSC lysate (Fig. 3).

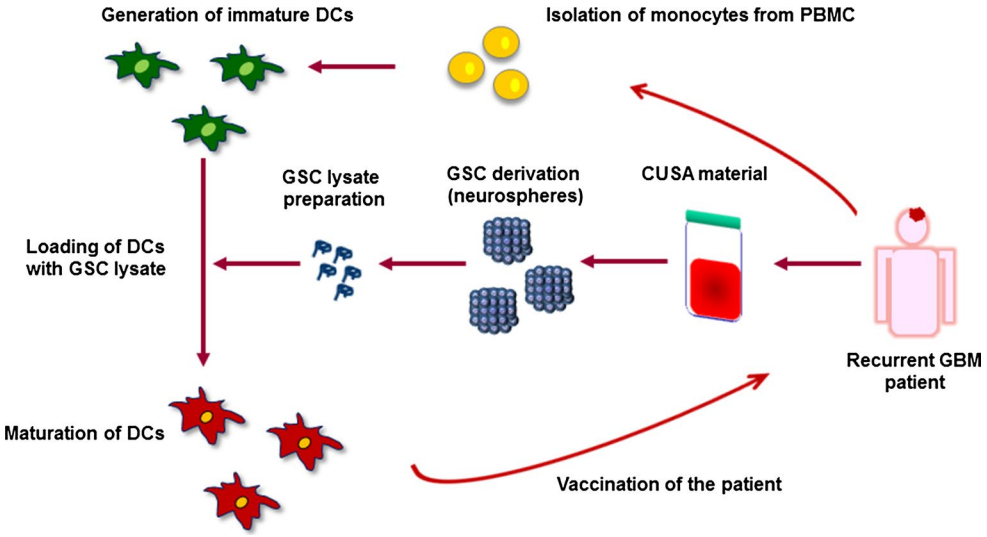

Fig. 3 DENDR-STEM clinical study is based on GSCs as target for DC immunotherapy. The protocol for GSC derivation from CUSA material was revised according to GMP rules

Perspectives

With DENDR-STEM, we propose to test in patients with recurrent GBM the safety and feasibility of GSCs as more specific targets of DC immunotherapy.

However, radiotherapy and chemotherapy can induce mutations in GBM [74]. Consequently tumor at recurrence may have different genetic alterations compared to tumor from which GSC is derived [75]. Frozen specimens of the patients that will undergo the second surgery and also GSCs from such specimens could be amenable in the future to next-generation studies (exome and RNA sequencing). Notably, one patient that we treated at recurrence on a compassionate basis using DCs loaded with the lysate from the primary tumor showed clinical benefit and survived 22 months after vaccination [27]. Data on GBM evolution at recurrence are piling up [75] and will help the design of future clinical trials. Finally, in preclinical studies, we have tested the efficacy of peptide-based immunotherapy targeting GSC-associated antigens [62, 63]. Clinical studies like DENDR-STEM could help the identification of a set of antigens that could provide an immunotherapeutic strategy less labor intensive and expensive than that based on GSC use.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interests.

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