Cell-based immunotherapy of glioblastoma multiforme (Review)

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Abstract. Glioblastoma multiforme (GBM) is the most aggressive and lethal primary glial brain tumor. It has an unfavorable prognosis and relatively ineffective treatment protocols, with the median survival of patients being ~15 months. Tumor resistance to treatment is associated with its cancer stem cells (CSCs). At present, there is no medication or technologies that have the ability to completely eradicate CSCs, and immunotherapy (IT) is only able to prolong the patient's life. The present review aimed to investigate systemic solutions for issues associated with immunosuppression, such as ineffective IT and the creation of optimal conditions for CSCs to fulfill their lethal potential. The present review also investigated the main methods involved in local immunosuppression treatment, and highlighted the associated disadvantages. In addition, novel treatment options and targets for the elimination and regulation of CSCs with adaptive and active IT are discussed. Antagonists of TGF-β inhibitors, immune checkpoints and other targeted medication are also summarized. The role of normal hematopoietic stem cells (HSCs) in the mechanisms underlying systemic immune suppression development in cases of GBM is analyzed, and the potential reprogramming of HSCs during their interaction with cancer cells is discussed. Moreover, the present review emphasizes the importance of the aforementioned interactions in the development of immune tolerance and the inactivation of the immune system in neoplastic processes. The possibility of solving the problem of systemic immunosuppression during transplantation of donor HSCs is discussed.

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1. Introduction

Glioblastoma multiforme (GBM) is the most aggressive and lethal primary brain tumor (1). Globally, it constitutes >50% of all diagnosed glioma cases and 20% of all central nervous system (CNS) tumors. In Europe and North America, the frequency of GBM is 3-5 cases per 100,000 individuals, mostly affecting non-Hispanic males (2). Notably, the average age of affected patients is 62 years. The tumor is characterized by invasive growth, intensive angiogenesis and an unfavorable prognosis. When following a modern protocol of complex treatment, the median survival of patients is ~15 months (1,3). GBM radiation resistance is attributed to plasticity and marked proliferation abilities of cancer stem cells (CSCs), which are the main catalyst of neoplastic processes. Irradiation and chemotherapy are unable to eliminate CSCs effectively (4); thus, development of fundamentally novel approaches to GBM treatment are required, and immunotherapy (IT) has great potential.

IT has demonstrated a high level of efficiency in treating hemoblastoses, melanomas, lung, prostate and bladder cancer (5). IT effectively damages CSCs, disrupts their interaction with local cellular microsurroundings and the extracellular matrix (ECM), and controls further actions of CSCs, such as inhibiting the interaction between non-tumor cells and CSCs, thereby suppressing tumor progression and prolonging the patients' life. However, unlike other cancer types, GBM is protected from direct interaction with the immune system by the blood-brain barrier (5,6). GBM cells do not express numerous unique antigens, create immunosuppressive microsurroundings, and express anti-inflammatory cytokines and inhibitors of the immune checkpoint (6), thereby affecting the immune response. The ability of these cells to suppress the immune system locally classifies GBM as a 'cold' tumor that is almost completely resistant to IT.

On the other hand, high radiation doses and chemotherapy inevitably cause myelosuppression (7), followed by severe

systemic immunodeficiency (8) that is intensified by glucocorticosteroids (9), which are used for preventing cerebral edema during all stages of GBM treatment. A combination of local and systemic immunosuppression limits the antitumor potential of IT, and interferes with its ability to control CSCs (7-9). Thus, a novel systemic approach to GBM treatment is required, that combines classic methods of antitumor therapy, IT and innovative biomedical technologies that would be aimed at overcoming local and systemic immunosuppression.

The present study aimed to review systemic solutions for the issues associated with immunosuppression that interfere with the antitumor potential of cell-based IT used in patients with GBM.

2. Summary on GBM

The majority of patients with GBM require surgery to achieve brain decompression, minimize hydrocephalus and lower the risk of fatal complications (10). Using microsurgical equipment, modern neuro-navigation and intra-operative neuro-monitoring increases the chances of effective tumor removal, and minimizes the risk of damaging key functioning areas of the brain (11). However, GBM eradication via surgery is not possible due to brain tissue infiltration with cancer cells; therefore, the main treatment focus is chemoradiotherapy (12).

Traditionally, γ-radiation of 60 Gy is used, including 2 Gy daily at 30 fractions for 6 weeks (13), together with chemotherapy, where temozolomide (TMZ) is considered the gold standard for the treatment of GBM. Treatment options may be extended by combining γ-radiation with proton (14) or boron neutron capture therapy (15), the use of hyperbaric oxygen therapy, consuming a ketogenic diet or pharmacological support with Olaparib (16), kynurenine pathway metabolites (17) or other inducers of genomic instability (18). Tumor-treating fields increase the treatment effect (11,19) and improve the patient's condition.

Current treatment options for GBM are relatively ineffective. In the majority of cases, despite the efforts of medical experts, tumor relapse occurs in 4-6 months following removal (20). In the case of recurrent GBM, patients with ~70 on the Karnofsky Performance Scale and 0-1 on the Eastern Cooperative Oncology Group scale may be reoperated (21). Further rounds of irradiation are rarely used; however, brachytherapy and radiosurgery are considered promising options (22). Chemotherapy may help extend a patient's life, and TMZ or lomustine are often used with bevacizumab (23). Treatment with procarbazine, lomustine, vincristine and platinum-based medications (24) remain limited. Best supportive care is recommended for all patients with recurrent GBM, as the associated prognosis is unfavorable, and the median survival is ~15 months (25) due to the coordinated combination of local factors and systemic mechanisms.

3. Treatment resistance factors

At the genomic level (26), treatment resistance is caused by a unique range of genetic mutations, including the loss of heterozygosity on human chromosome 10q, the homozygous deletion of p16INK4a and mutations in Rb and TP53 genes, cyclin-dependent kinases and the tyrosine kinase signaling pathway. Based on the aforementioned genetic mutations shown in The Cancer Genome Atlas (27), four subtypes of GBM have been defined: Proneural, neural, classical and mesenchymal. The first subtype is characterized by the hyperexpression of transcription factor SOX2, oligodendrocyte transcription factor 2 and platelet-derived growth factor genes. The second subtype is characterized by the expression of glial cell-derived neurotrophic factor, brain-derived neurotrophic factor, insulin-like growth factor 1 and other neuron-related genes. The third subtype is characterized by mutations in the EGFR gene, and mutations in the neurofibromin gene are found in the fourth subtype. For the last 10 years, this classification system has expanded based on the data from genome-wide association studies (28,29), and significant differences in the molecular landscapes of cancer cells in primary and recurrent tumors (30) have been described, indicating a high plasticity of GBM cells.

Cancer cell plasticity is mostly due to epigenetic damage. Notably, in 2016 the World Health Organization (25) selected the isocitrate dehydrogenase (IDH)1\2 mutations as the main determinant of prognosis for patients with GBM, as these mutations result in an excess of 2-hydroxyglutarate in cancer cells that causes hypermethylation of the genome (31). This includes hypermethylation of the promoter regions of O6-methylguanine-DNA methyltransferase, which is responsible for the repair of damaged DNA and the subsequent adaptation to radiation and chemotherapy. The IDH-mutant GBM often affects patients of a young age, developing from a diffuse or anaplastic astrocytoma and becoming localized in the frontal cortex (32). The IDH-wild-type GBM constitutes up to 90% of cases, develops *de novo*, and is localized in parietal, occipital or temporal areas of the brain. The median survival of patients with IDH-mutant GBM is 23 months, and for those with IDH-wild-type GBM is 13 months.

At the cellular level, treatment resistance is associated with CSCs (33) that have a large combination of genetic and epigenetic alterations. These cells were initially discovered in 1997, when Bonnet and Dick (34) described the cell hierarchy of acute myeloid leukemia based on a primitive CSC. These cells have since been described for lung (35), breast (36), ovarian (37) and colon (38) cancer, GBM and other malignant tumors. CSCs are characterized by plasticity (39), are capable of infinite self-renewal and have the highest proliferation rate among all GBM cells. Only ~100 cells are required for GBM development in mammals (40), highlighting their oncogenic potential. The ability to repair DNA in a fast and effective manner renders CSCs resistant to treatment (41) and chemotherapy (42).

CSCs acquire a number of new oncogenic properties by interacting with neural stem and progenitor cells residing in the subventricular zone (43-45) and other germinal centers of the human brain. Activation of primitive self-organization mechanisms of CSCs (46,47) allows them to actively interact with other stem and differentiated cells (48), enabling the exchange of genes, chromosomes and whole nuclei (49), thereby increasing the viability of the tumor and its resistance to treatment.

The main requirement (50) for CSCs to activate their interaction with the ECM is the increased production of key cytokines for the remodeling phase of inflammation, including TGF-β. Under normal conditions, TGF-β synthesis begins at the periphery of the inflammatory area, and its source is

M2-activated macrophages and other immunocytes. This cytokine inhibits proliferation and induces apoptosis in pathologically altered cells, thereby triggering remodeling (51). Mutations in either SMAD or death domain-associated protein 6 signaling pathways allows GBM cells to escape apoptosis (52). Moreover, GBM cells self-activate TGF-β and increase the levels of its synthesis based on autocrine induction.

TGF-β inhibits inflammation, triggers ECM repair, increases the interaction between cancer cells and the ECM (53), creates a niche for cancer cells (54), hinders T cell proliferation, suppresses antigen presentation by macrophages, inhibits the expression of major histocompatibility complex (MHC) class II antigens by dendritic cells (DCs), increases the synthesis rate of atypical human leukocyte antigen molecules and activates M2 microglia (55) (Fig. 1). Subsequently, as a result of inhibiting T cells via the production of programmed cell death protein (PD)-1 and cytotoxic T lymphocyte-associated protein 4 by the tumor, the exhausted phenotype of T lymphocytes is created to interact with regulatory T cells, M2-macrophages and myeloid-derived suppressor cells (56). Thus, CSCs are isolated from the immune system, creating an optimum living environment and increasing the production of kynurenine pathway metabolites in the tumor (57,58); these metabolites act as negative controls of local inflammation. However, the ability of GBM to resist all existing types of treatment is not only due to local factors, but also immunosuppressive mechanisms.

Key factors of systemic immunosuppression in GBM are radiation and chemotherapy (59). Following chemoradiation treatment *in vitro* in GBM cells, these cells produce increased levels of IL-10, IL-6, prostaglandin E2 and other immunosuppressive factors (60). Moreover, the supernatant obtained during cell culturing directly suppressed the proliferation of $CD4^+$ and $CD8^+$ T lymphocytes (61). After receiving 30 fractions of radiation, lymphocytes circulating in the blood flow accumulated an average radiation dose of 2.3 Gy, while the average amount of $CD4^+$ cells in the patients' body decreased by two-fold following chemoradiation, and remained low for a year (62). As patients are recommended radiation treatment (75 mg/m^2/day) along with six subsequent cycles of TMZ treatment (150-200 mg/m^2/day), subsequent adjuvant therapy with chemotherapeutic drugs leads to the inhibition of immunopoiesis in the red bone marrow (63-65). The use of corticosteroids (9,66,67) that have an adverse effect on the survival of patients with GBM is another factor of systemic immunosuppression.

Therefore, local immunosuppression and systemic immunodeficiency create optimal conditions for CSCs to fulfill their lethal potential, and a combination of these factors decreases the survival rate of patients.

4. Immune privilege of the CNS

Despite notable advances in the IT of malignant neoplasms, the application of IT in brain tumors has only been an option in recent decades (5). For >50 years, the brain was considered an immune-privileged organ (68), due to its lack of a lymphatic system and isolation from other tissues by the blood-brain barrier. This notion was based on the research by Medawar (69), who demonstrated the potential allogeneic graft acceptance in a rodent brain, while other grafts were rejected by the immune system. Furthermore, immunocyte functions in the brain have previously been associated with cells of resident microglia that originate from the yolk sac, which maintain their population through proliferation without any interaction with the immune system cells (70).

It is considered that microglia serve a key role (71) in antigen presentation to immunocytes in numerous diseases of the CNS. Tumor growth in the brain is accompanied by recruitment of microglial cells into the neoplastic tissue (72), where they interact with T lymphocytes and other immune cells that can freely penetrate the damaged blood-brain barrier, or penetrate the tumor via the cerebrospinal and interstitial fluid. The potential of antigen-presenting cells to use the lymphatic vessels to enter the deep lymph nodes of the neck has previously been demonstrated, where they interact with T and B cells contributing to the immune response (73,74). Therefore, the brain is an organ with both strong immune supervision and immune response. Preliminary vaccination with graft antigens (75) inhibits the potential of graft acceptance by the brain, which is the dominant principle of IT for the treatment of CNS tumors.

5. IT and CSC control

The presence of specific antigens in cancer cells is a key element of successful IT (76). Since 2009, the National Cancer Institute in the USA has regularly updated this list of antigens (5,6), which include IL-13 receptor α2 (IL13Rα2) and HER2. IL13Rα2 is expressed by 60-80% of GBM cells, is absent in healthy CNS cells, yet is often found in kidney cells. HER2 is expressed by >80% of GBM cells, but is also present in healthy tissues (77). The first phase of a clinical trial that aimed to target these antigens (78) demonstrated that the size of the tumor lesion and the associated risk level may be reduced; however, there was no significant increase in the survival rates of patients. In this case, IT may not be successful, as it is not specifically targeting CSCs, which have almost no specific antigens. Therefore, further investigations into the molecular targets are required to eliminate CSCs.

In order to specifically target CSCs, a number of proteins have been targeted, including heat shock proteins (79-81), telomerase reverse transcriptase (82), Wilms' tumor protein (83,84), glycoprotein 100 (85), tyrosinase-related protein 2 (86), ephrin type-A receptor 2 (87,88), A2B5 protein (89,90), SOX family of transcription factors (91) and cytomegalovirus phosphoprotein 65 (92), among others. Alternative methods include the delivery of specific antigens to CSCs, using adenoviruses, lentiviruses, parvovirus and recombinant polioviruses DNX-2401 and PVS-RIPO (93-95). However, the relative treatment success associated with these methods remains low. Selective elimination of CSCs in GBM is currently not an option, and therefore requires the development of novel and more systemic approaches.

CSCs are characterized by the heterogeneous nature of molecular genetic landscapes (96) not only in cell clones, but also in singular cells (97), and these have previously been extracted from tumor biopsy samples. Numerous immunocytochemical markers of CSCs (98-100) and metabolic pathway maps of cancer cells have been described, and notable differences have been demonstrated between patients with primary

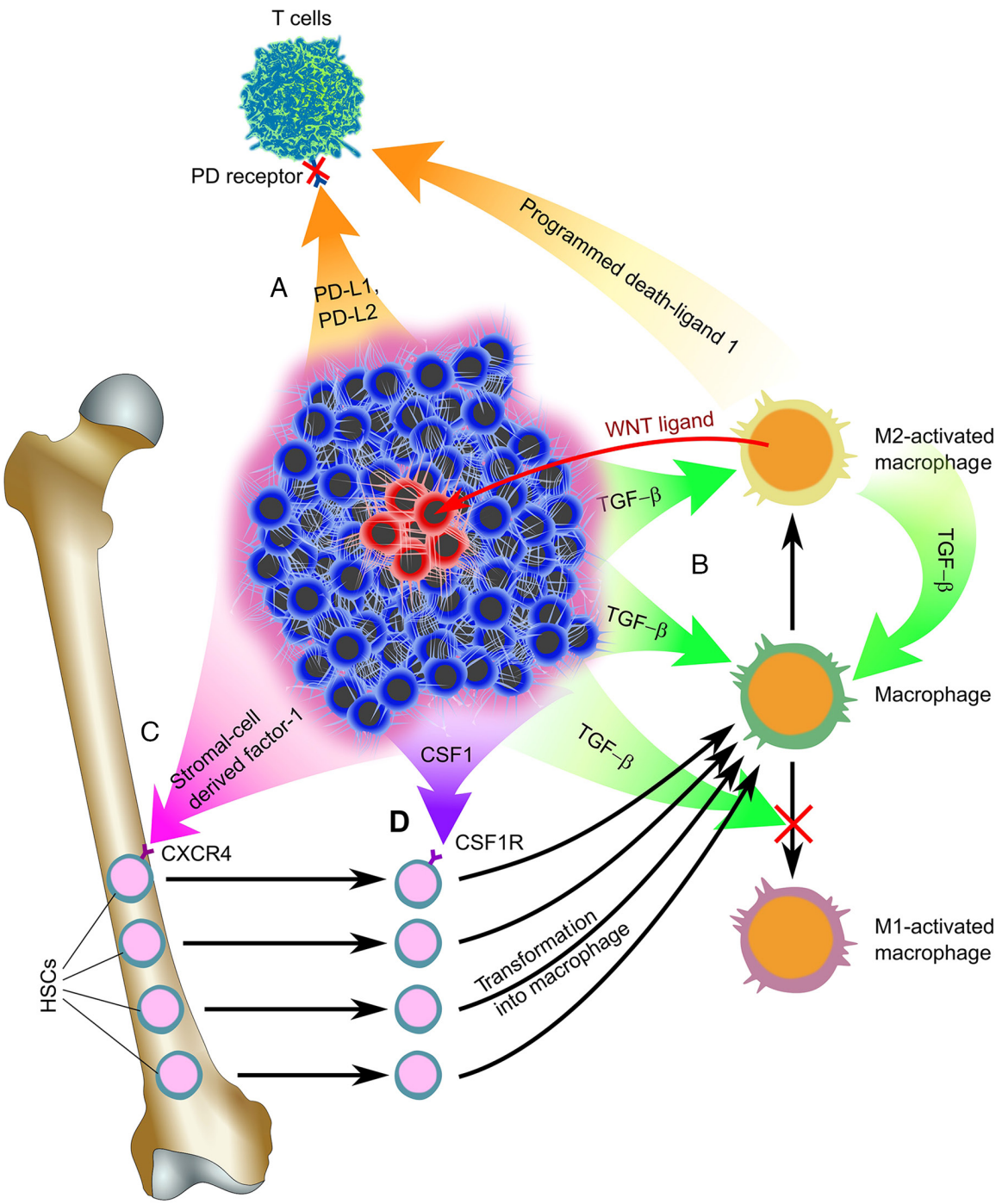

Figure 1. Mechanisms of interaction between glioblastoma multiforme cells and the immune system. (A) Tumor cells produce PD-Ls (PD-L1 and PD-L2) that bind to the PD-1 receptor on the surface of T cells, which causes T cell anergy and blocks adaptive immunity. (B) The tumor produces a large amount of TGF-β, which activates macrophages along the alternative (M2) pathway; in response, macrophages intensively produce TGF-β, which prevents their activation along the classical pathway and turns off active immunity. (C) Tumor cells and damaged tissues produce a large amount of stromal cell-derived factor-1 (also known as C-X-C motif chemokine ligand 12), which is a chemokine of the C-X-C subfamily that interacts with the CXCR4 receptor on the membrane of HSCs and monocytes attracting them into the tumor, thereby enhancing immunosuppression and suppressing inflammation. (D) The production of CSF1 by tumor cells activates CSF1R on the membrane of normal stem cells and monocytes, which transforms them into macrophages activated by TGF-β through the alternative pathway. PD, programmed cell death; PD-L, PD ligand; HSCs, hematopoietic stem cells; CSF1, colony stimulating factor 1; CSF1R, CSF1 receptor; CXCR4, C-X-C chemokine receptor type 4.

and recurrent GBM. In addition, cancer cells that are immunopositive for the main CSC marker, CD133 antigen (101), do not always demonstrate properties of CSCs, while CD133$^-$ cancer cells may demonstrate these (102). However, the development of a systemic approach to CSCs should focus on the role of these cells in the pathogenesis of GBM.

Proliferation is a key property of CSCs, and intensive proliferation is accompanied by a notable increase in cancer cell number, leading to an increased oxygen consumption and hypoxia development (103). Long-term hypoxia is detrimental for all cancer cells, including CSCs (33,40). These cells can survive by triggering angiogenesis and providing

Table I. Completed clinical trials of CAR-T cell therapy in patients with GBM.

ClinicalTrials.gov identifier	Phase	Antigen	Type of GBM	CSC-targeted therapy
NCT02664363	I	EGFRvIII-CAR-T cells	Newly diagnosed WHO Grade IV malignant glioma	No
NCT03283631	I	EGFRvIII CAR-T cells	Recurrent GBM	No
NCT02209376	I	Autologous CAR-T cells redirected to EGFRvIII-receptor	EGFRvIII + GBM	No
NCT01109095	I	CMV-specific cytotoxic T lymphocytes expressing CAR targeting HER2	Newly diagnosed and recurrent GBM	No
NCT03726515	I	EGFRvIII CAR-T cells + pembrolizumab	Newly diagnosed, MGMT-unmethylated GBM	No
NCT01454596	I-II	EGFRvIII CAR-T Cells	EGFRvIII + GBM	No

GBM, glioblastoma multiforme; CSC, cancer stem cell; CAR, chimeric antigen receptor, WHO, World Health Organization; v, variant; CMV, cytomegalovirus; MGMT, O^6-methylguanine-DNA methyl-transferase.

the corresponding tumor with a blood supply, and this is only possible through interaction with the ECM (48). Previous cell proteome studies have demonstrated that increased synthesis of proteins associated with the ECM-receptor interaction signaling pathway is a key difference between CSCs and differentiated GBM cells (53). Proteome profiles of CSCs and normal stem cells are considered similar (104).

Thus, the optimum targets for managing CSCs may include cell-surface receptors, such as integrin V, integrin β3 and integrin β1, as well as other components of the ECM-receptor interaction signaling pathway. Optimum targets may also include ECM components secreted by CSCs, such as collagen type VI α1, laminin β1, fibronectin 1 and tenascin. Targeting these elements may inhibit the development of the CSC niche, and disrupt the corresponding intercellular interactions mediated by these cells (105). This method may be possible using the technologies of adaptive and active IT.

6. Adaptive IT

Adaptive IT involves the extraction of the patient's immune cells in order to activate them to tumor antigens, and subsequently return them to the patient's body. An example of this process involved the vaccination of the patient with the antigens of destroyed cancer cells, along with Bacillus Calmette-Guérin antigens (106). Furthermore, subsequent extraction of T cells was carried out, and these were additionally stimulated with IL-2 *ex vivo,* and injected into the patient. In a previous study, vaccination with cancer cell antigens was accompanied by injection of granulocyte-macrophage colony-stimulating factor (GM-CSF), and T cells were subsequently obtained from the blood, activated *ex vivo* with *Staphylococcus aureus* antigens and returned to the patient (107). The use of immunocytes from patients with GBM may not be successful, as these immunocytes have been exhausted by anti-inflammatory cytokines, inhibitors of immune checkpoints and other reprogramming factors produced by GBM cells, as well as by the systemic immune suppressing effect of antitumor treatment. Thus, their cytotoxic effect is relatively low (108).

A further example of adaptive IT involves intravenous or intracranial injection of genetically modified T cells equipped with chimeric antigen receptors (CAR), where the areas of antigen-recognition domains, consisting of monoclonal antibodies, are connected with the areas of intercellular signaling domains of T lymphocytes (109). A previous study using rat models and monovalent CAR-T cells targeting IL-13Rα2, IDH1-R132H, HER2 or EGFR variant (v) III demonstrated tumor regression, but the clinical application of CAR-T cells (110) is yet to be fully established.

Previous data highlighting 6 clinical trials using CAR-T cells in patients with GBM that have reached phase I are displayed in Table I. The aforementioned trials are limited by the small number of participants, the involvement of patients with recurrent and refractory GBM who have received multiple chemotherapy cycles, limited observation periods and treatment that does not target CSCs (111). Additionally, the combination of CAR-T cell therapy and immune checkpoint inhibitors have been used in patients with first-time diagnoses and recurrent GBM (112). Thus, this method has no clear potential. Moreover, despite a wide range of side effects, the life expectancy of patients with GBM who take part in clinical trials exceeds the life expectancy of those who are treated according to the standard protocol (113). The heterogeneous nature of GBM cells predetermines the need for creating multivalent CAR-T cells that are capable of eliminating cancer cells that express HER2, ephrin type-A receptor 2, IL13Rα2 and other antigens (114). Multivalent CAR-T cells that have the ability to inhibit key components of the ECM-receptor interaction signaling pathway in CSCs may act as a potential treatment option for managing CSCs.

7. Active IT

Active IT stimulates the development of antitumor immunity following injection of peptide vaccines, including tumor cell vaccines (TCVs) and DC vaccines (DCV). An example of a

peptide vaccine is rindopepimut, that targets cancer cells expressing a mutant peptide of EGFRvIII (115-117). In March 2016, the phase III ACT IV trial was terminated as the overall survival rate of patients was not increased (118). This may be associated with a lower antigen load due to previous surgery, influence of PD-ligand 1 (PD-L1) or the immunosuppressive effect of radiation and chemotherapy. This peptide vaccine may still be used in complex personalized treatment of patients with GBM cells that express the EGFRvIII antigen.

The development of TCVs involves recruiting immune cells into the blood stream and presenting them with specific antigens, followed by stimulation with pro-inflammatory cytokines that determine the main type of intercellular interactions in the neoplastic lesion. Immunization is performed simultaneously with injecting GM-CSF, and immunocytes are subsequently extracted from the patient's body, activated with *Staphylococcus* enterotoxin A antigens, multiplied in the culture medium with IL-2 and returned to the patient. Autologous or gene-modified allogeneic cancer cells that are either treated with ultrasound or deactivated with radiation, are used as antigens for TCV creation (6). One TCV has been developed with genetically modified cancer cells producing GM-CSF (119). In addition, a vaccine from radiation-treated autologous cancer cells with inactivated allogeneic cancer cells producing GM-CSF has also been described (55).

Stimulation of GM-CSF (106) is one of the crucial conditions of the effective application of TCVs. In a recent study (120), a GM-CSF injection in animals with implanted glial brain tumor was accompanied by the recruitment of mononuclear $CD45^+$ cells into the blood flow. The subsequent injection of bacterial lipopolysaccharide and IFN-γ enriched the tumor tissue with markers of M1-activated microglial cells, increased the number of antigen-presenting $CD86^+$ cells, and decreased the amount of TGF-β and IL-10 in the tumor, thus destabilizing one of the main mechanisms underlying local resistance to treatment. Notably, such stimulation increased the life expectancy of the experimental animals. Furthermore, stimulation of healthy animals along with the subcutaneous injection of dead cancer cells eliminated the chance of graft acceptance, or notably increased the period of tumor development in rats following the intracranial injection of cancer cells (55,121).

Previous data detailing 78 clinical trials for TCV suggested that only 30 biomedical products passed phase I. These studies were characterized by the small number of patients, lack of a unified approach to selecting patients and complete disregard for local immunosuppression and systemic immunodeficiency. Moreover, only two studies targeted CSCs (NCT00846456 and NCT01171469). Notably, the results of these clinical trials demonstrated that the average life expectancy of patients that received TCV was significantly increased, compared with the control group. However, no biomedical product that is currently on trial can be categorically classified as TCV, as a part of the immunocytes returned to the patient following stimulation with cancer cell antigens includes DCs, which present the antigens with MHC class I and II molecules to T cells. This induces immune aggression in the tumor tissue (122).

DCV is one of the most important elements of adaptive tumor IT. These vaccines are created in a traditional manner, for example, cancer cells are lysed, and incubated with mononuclear $CD45^+$ cells of the red bone marrow and the corresponding cytokine mixture that contains IL1 or IL2, GM-CSF and IFNγ (123). DCs attached to the surface of the plate are washed, stimulated with pro-inflammatory cytokines and returned to patients with GBM. DCs migrate from the patient's blood flow into the lymphatic system, and subsequently move into the regional lymph nodes, where they stimulate the proliferation and differentiation of T lymphocytes, enabling an antitumor immune response (124).

DCV-based IT is one of the most promising methods of GBM treatment (125), and in certain cases the median survival of patients is 525 days, compared with a median survival of 380 days for patients who have been treated according to the standard protocol (126). Notably, 52 clinical trials of DCV are currently being conducted, and only 14 have been completed (Table II). Myeloid, lymphoid and plasmacytoid DCs are used to generate DCVs (68). Myeloid DCs are of bone marrow origin from a common hematopoietic CD34(+) stem cell. More mature progenitors of such cells, circulating in the blood, have the morphology of monocytes, but the CD34 antigen is absent on their membrane, while markers of myeloid differentiation CD11c, CD13, CD14 and CD33 are present. Plasmacytoid DCs are of lymphoid origin and are characterized by the absence of myeloid markers; however, they express CD4, CD45RA, BDCA2/CD303, BDCA4/CD304, MHC II antigens and co-stimulatory molecules CD80, CD86 and CD40, and contain a large number of IL3 receptors (70,75). Creation of a DCV (99) is possible using such potential antigens as autologous live cells of GBM, allogeneic cancer cells, lysates of cancer stem CD133(+) and differentiated CD133(-) cells of GBM, as well as a combination of autologous cancer cells, which were previously killed with irradiation, with lysates of allogeneic cancer stem CD133(+) cells. However, a brief analysis of the corresponding results indicated clear differences in the number and composition of the used immunocytes, different vaccination methods, a lack of uniform criteria for evaluating the effectiveness of the vaccine and disregard for issues associated with local immunosuppression and systemic immunodeficiency.

8. IT and molecular-based therapy

Solving issues associated with local immunosuppression and systemic immunodeficiency is complex. The increased synthesis of TGF-β by GBM cells is a key element of local immunosuppression. Previous studies have focused on suppressing TGF-β synthesis with pharmacological agents, such as trabedersen (127) and galunisertib (128), but the use of TGF-β inhibitors alone (129) for the management of local immunosuppression was insufficient. Blockade of the signaling axis colony stimulating factor 1 (CSF1)\CSF1 receptor (CSF1R; also known as macrophage colony-stimulating factor receptor) is one of the most important ways of regulating local immunosuppression. CSF1, through CSF1R, induces the differentiation of hematopoietic stem cells (HSCs) and monocytes into tumor-associated macrophages, which under the influence of TGF-β are activated along the alternative M2 pathway and markedly increase the synthesis of this cytokine (130). In this regard, certain prospects may be associated with the combination of TGF-β inhibitors with CSF1R antagonists (131) such as pexidartinib, emactuzumab and cabiralizumab.

Table II. Completed clinical trials of dendritic cell vaccines for the treatment of GBM.

ClinicalTrials.gov identifier	CSC-targeted IT	Type of GBM	Phase of clinical trial	Median survival time, months
NCT02049489	Yes	Recurrent GBM	I	No data
NCT00323115	No	Newly diagnosed GBM	I	28.0
NCT00576537	No	All types of GBM	II	No data
NCT00846456	Yes	Newly diagnosed and recurrent GBM	I-II	23.0
NCT00576641	No	All gliomas and GBM	I	No data
NCT00626483	No	Newly diagnosed GBM	I	No data
NCT01006044	No	Newly diagnosed GBM	II	23.4
NCT03615404	No	Newly diagnosed and recurrent GBM	I	No data
NCT01213407	No	Newly diagnosed	I	No data
NCT02820584	No	Recurrent GBM	I	No data
NCT00612001	No	All gliomas	I	No data
NCT01280552	No	All type of GBM	II	11.2
NCT00890032	No	Recurrent GBM	I	No data
NCT00068510	No	All type of gliomas	1	No data

GBM, glioblastoma multiforme; CSCs, cancer stem cells; IT, immunotherapy.

Table III. Completed clinical trials of immune checkpoints inhibitors for the treatment of GBM.

ClinicalTrials.gov identifier	Study title	Phase of clinical trial	Median of survival
NCT02798406	Multi-center, open-label study of a conditionally replicative adenovirus (DNX-2401) with pembrolizumab (Keytruda®) for recurrent glioblastoma or liposarcoma	II	No results posted
NCT03493932	Cytokine microdialysis for real-time immune monitoring in glioblastoma patients undergoing checkpoint blockade	I	No results posted
NCT02550249	Neoadjuvant nivolumab in glioblastoma (neo-nivo)	II	No results posted
NCT03636477	A study of Ad-RTS-hIL-12 with veledimex in combination with nivolumab in subjects with glioblastoma; a substudy to ATI001-102	I	No results posted
NCT02327078	A study of the safety, tolerability, and efficacy of epacadostat administered in combination with nivolumab in select advanced cancers (ECHO-204)	I\II	No results posted
NCT02335918	A dose escalation and cohort expansion study of anti-CD27 (varlilumab) and anti-PD-1 (nivolumab) in advanced refractory solid tumors	I\II	No results posted
NCT02529072	Nivolumab with dendritic cells vaccines for recurrent brain tumors (AVERT)	I	~4 years from study initiation

PD-1, programmed cell death protein 1; Ad-RTS-hIL-12, human interleukin-12 vector.

In addition, local immunosuppression is caused by the production of PD-L1 and other molecules from cancer cells (132-134), which inhibit receptor activation on cytotoxic T lymphocytes (135) allowing cancer cells to take control of the immune system. Notably, following PD-L1 stimulation, resident tumor microglia with M2 activation not only increase the levels of these ligands, but also induce monocyte-derived macrophages that inhibit the immune response and promote tumor growth (136). Nivolumab, an inhibitor of PD-1 receptors, is able to direct the tumor microglia to the M1-phenotype and alter the microsurroundings of cancer cells (137). However, the combination of TGF-β antagonists with agents that prevent neutralization of T lymphocytes remains inefficient (138). Only seven clinical trials of immune checkpoint inhibitors in the complex treatment of glioblastoma have been completed worldwide to date (Table III).

Bevacizumab is a monoclonal antibody that targets vascular endothelial growth factor (139-141), and when used

together with TMZ, has been indicated to increase the average life expectancy of patients with GBM from 6.5 to 9.5 months. Nivolumab is more effective than bevacizumab (142), but a combination of these agents allows the treatment of cerebral edema without corticosteroids (6,143), which enhances the potential of IT. However, a significant increase in the life expectancy of patients is yet to be achieved, which highlights issues associated with these treatment methods that are more profound. Nevertheless, despite the small number of completed clinical trials (Table III), immune checkpoint inhibitors are among the most promising drugs for immunochemotherapy of GBM.

9. HSCs and IT

The results of previous studies have demonstrated that patients with GBM with a high level of leukocytes exhibit an improved prognosis following the use of antitumor vaccines (144), inhibitors of immune checkpoint (145-147) and other immunotherapeutic agents. This indicates the impact of systemic immunodeficiency caused by the damage to the bone marrow tissues following radiation and chemotherapy. Systemic solutions involve transplantation of HSCs. In cases of unimpaired hematopoiesis, the proportion of HSCs in the blood of a healthy individual does not exceed 0.01%, and stimulation with GM-CSF increases the number of HSCs in the blood >100-fold, which allows HSC extraction for further use in the reconstruction of the immune system (148). However, this task is difficult to achieve. Following radiation and several courses of chemotherapy, the bone marrow of patients becomes exhausted (132,133), and it is impossible to obtain an adequate quantity of HSCs for transplantation. This issue may be resolved with the transplantation of autologous HSCs obtained from the patient, that have been cryopreserved prior to disease occurrence, but only a number of patients have this option.

Restoring the immune system of a patient using autologous HSCs may not be the only option, as the development of novel antitumor immunity may counter high levels of tumor aggression. In the entire existence of both mammals and humans, infective agents have posed serious threats; however, life expectancies were never long enough for cancer development. Thus, evolution has led to the development of a number of mechanisms for eliminating pathologically altered cells (such as inflammation, apoptosis and autophagy), but only if their number is relatively small and it does not interfere with cell or tissue homeostasis (149).

HSCs are progenitors of the immune system, as well as coordinators and stabilizers of regeneration processes. Proliferation speed and the number of these cells decreases with age, thus reducing the levels of immune protection and increasing the number of pathologically altered cells in the body. Notable increases in the number of cancer cells are observed during the rapid invasive growth of GBM, resulting in the destruction of neurons and glial cells, inflammation, cerebral ischemia and the intensified production of chemo-attractants that stimulate the migration of HSCs into the brain (150).

The key mechanism underlying the recruitment of HSCs into the tumor lesion is carried out by hypoxia-inducible factors a group of ligands that activate the production of >80 cytokines by damaged tissues. Key cytokines involved

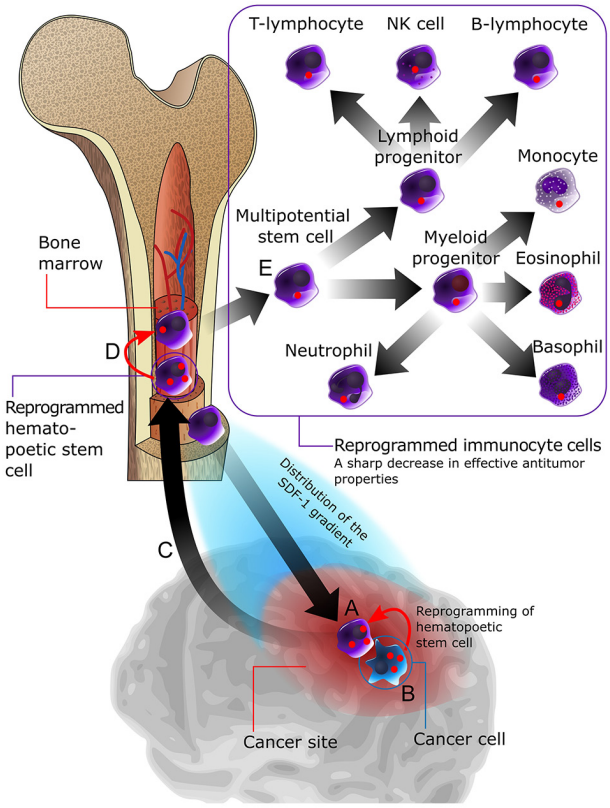

Figure 2. Reprogramming of healthy HSCs when interacting with CSCs of GBM allows the tumor to avoid being targeted by the immune system. (A) HSCs with C-X-C chemokine receptor type 4 on their cell membrane migrate from the bone marrow into the tumor, along with a concentration gradient of SDF-1. (B) Reprogramming of HSCs when interacting with CSCs. (C) Migration of reprogrammed HSCs back to the bone marrow and reprogramming of further HSCs. (D) Appearance of reprogrammed progenitor cells and (E) reprogrammed immunocytes that are non-reactive towards CSCs and cancer cells of GBM. HSCs, hematopoietic stem cells; CSCs, cancer stem cells; GBM, glioblastoma multiforme; SDF-1, stromal cell-derived factor-1.

in this process include monocyte chemoattractant protein-1 and stromal cell-derived factor-1α, which interact with C-C motif chemokine 2 and C-X-C chemokine receptor type 4 receptors on the HSC membrane, and induce their migration to the tumor lesion (151). Results of a previous study demonstrated that following 3 days of HSC injections into rats with implanted C6 gliomas, the transplanted cells migrated into the tumor and were visible in the blood vessels of glioma (152). In addition, following 4 and 5 days of the experiment, these cells penetrated areas of invasive growth and necrotic segments of neoplastic tissue, where they aggregated and interacted with cancer cells.

Briefly, HSCs adhere to cancer cells and interact with them, which has been demonstrated by the aggregation of fluorescent stain in cancer cells that is bound to the HSC cytoplasmic proteins during this interaction (153). Results of a previous *in vitro* study revealed that aggregation of the stain in the cancer cells decreased the adhesion to the culture plate surface and eliminated the interaction with the ECM, due to TGF-β stimulation and reduced proliferation of GBM cells (154). This effect became more pronounced when the cancer cell: HSC cell ratio reached 1:3, indicating the antitumor potential of HSCs. In natural conditions, aggregation of normal stem cells in a damaged

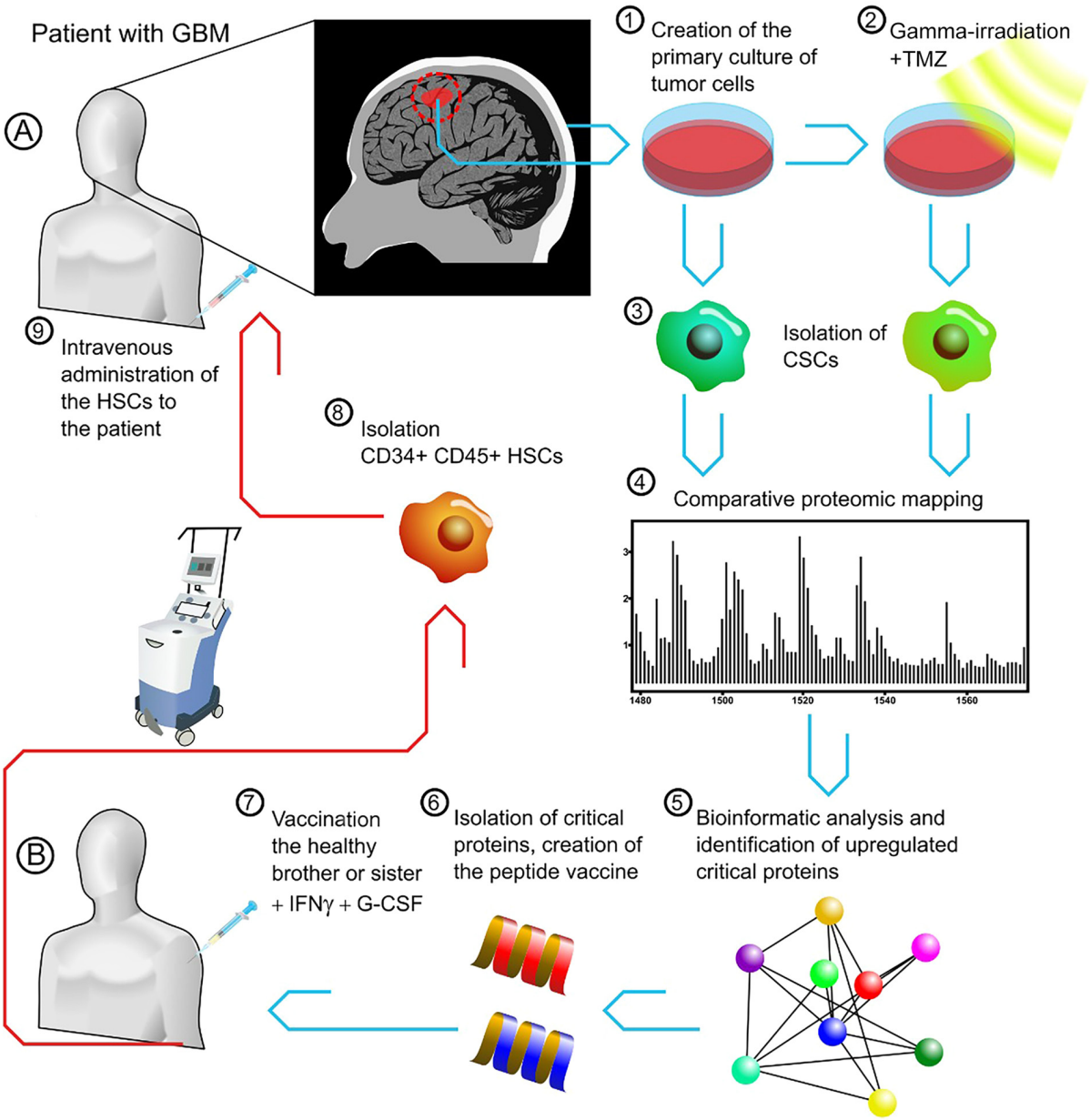

Figure 3. Hypothetical scheme for immunotherapy of GBM using donor HSCs. (A) Patient with newly diagnosed GBM who has been treated according to the standard protocol. (B) Healthy donor is a sibling of a patient with GBM. Step 1: Obtaining a primary culture of GBM cells from cancer tissue which was removed from the patient's brain during surgery. Step 2: γ-irradiation of the culture of primary GBM cells *in vitro* at a dose of 60 Gy, with the addition of TMZ to the medium. Step 3: Cultivation of cells of primary and irradiated GBM on serum-free media with subsequent isolation of CSCs from glioma spheres. Step 4: Comparative proteome mapping of primary and irradiated CSCs. Step 5: Bioinformatic analysis of cellular proteomes with the identification of proteins that are maximally upregulated in irradiated CSCs. Step 6: Isolation of proteins upregulated in irradiated CSCs and creation of a peptide vaccine. Step 7: Vaccination of individual B with a peptide vaccine based on upregulated proteins of irradiated CSCs, in combination with an injection of G-CSF and IFN-γ. Step 8: Isolation of CD34(+) CD45(+) cells from the bloodstream of individual B. Step 9: Subsequent transplantation into the bloodstream of patient A. GBM, glioblastoma multiforme; CSCs, cancer stem cells; G-CSF; granulocyte colony-stimulating factor; HSCs, hematopoietic stem cells; TMZ, temozolomide.

area results in M2-polarization of macrophages, suppression of antigen presentation, increased production of IL-4 and other anti-inflammatory cytokines, accompanied by the apoptosis of pathologically altered cells, clearance of the dead cells and debris of the phlogogenic site, and ECM remodeling (155). Thus, disruption of the interaction between CSCs and the ECM induced by HSCs is a powerful regulatory stimulus.

Furthermore, the exchange of the fluorescent stain is not one-sided, indicating the transfer of information in both directions. When HSCs interact with cancer cells, their cytoplasm also displays fluorescent stain aggregation and the transfer of specific proteins, accompanied by the epigenetic reprogramming of HSCs (154). The ability of these cells to effectively restore hematopoiesis and immunopoiesis requires further investigation; however, a previous clinical trial demonstrated that oncologic and autoimmune diseases cause significant changes in the molecular phenotype of HSCs, impacting treatment outcomes and the prognosis of patients (156).

Thus, whether GBM develops only as a result of pathological transformation of neural stem cells in the human brain, or arises as a result of the interaction of normal neural stem cells with HSCs remain to be fully elucidated. During an *in vitro*

experiment, neural stem cells demonstrated a high mobility to cancer cells of different lines, and this level of migration was markedly increased between neural stem cells and GBM cells, which may be attributed to their origin from the same histogenetic source in the central nervous system Additionally, GBM cells demonstrated a high mobility towards neural stem cells, actively interacting with them and exchanging fluorescent stain. HSCs are stem cells of a different origin, but their mobility towards GBM cells is not as active as that of neural stem cells (152) Notably, unlike stem cells in germinal regions of the brain, HSCs play a key part in the mechanisms underlying the memory retention of antigens, managing the processes of immune tolerance and immunocyte activation (157). Following the aforementioned antitumor vaccination, HSCs may inactivate the immune system, leading to ineffective methods for the treatment of GBM (Fig. 2).

Collectively, the aforementioned research demonstrated that transplantation of autologous HSCs to patients with GBM is not a systemic approach to the issue of immunodeficiency. Based on the hypothesis that epigenetic reprogramming of HSCs occurs during their interaction with cancer cells, IT may only be effective following complete regeneration or replacement of the immune system, using allogeneic HSCs of a healthy donor. The allogeneic transplantation of bone marrow should become the basis for a novel approach to GBM treatment. Both the duration and quality of remission for patients with leucosis (158) and multiple myeloma (159), who have received allotransplantation of bone marrow, are significantly improved. This is directly associated with the initiated graft-versus-tumor effect. This approach requires extensive further investigation; however, future treatment options should involve the use of allogeneic HSCs.

10. Conclusion

Modern oncology exhibits a wide range of antitumor products and methods. However, despite all recent scientific advances, the survival rates of patients with GBM still remains low. This issue requires novel systemic solutions, but novel treatment options for patients with GBM should not be confined to medication, radiation or a combination of both methods. GBM treatment success is dependent on the use of modern surgical, radiation and chemotherapy methods. Cytoreductive, cytotoxic and cytostatic therapies are a first treatment stage that must involve CSC management in order to be successful. At present, the only method of managing CSCs is IT, but fulfilling the cytoregulatory potential of IT is only possible following complete restoration of the immune system functions.

HSC transplantation should be performed directly after chemoradiation treatment. Autologous HSCs obtained from the patient prior to disease development should be the first choice for a graft. Therefore, cryopreservation of HSCs is required, to allow potential cancer treatment before its occurrence. If this option is unavailable, transplantation of allogeneic HSCs from a biologically compatible donor may be considered; however, further experimental studies are required.

Subsequent IT should be based on the use of immunocytes from a healthy donor that are targeted against the key proteins of CSCs, with a main focus on components of the ECM-receptor interaction signaling pathway. Following HSC transplantation and restoration of healthy immunopoiesis, TCVs are required, based on CSC antigens, DCVs and multivalent CAR-T cells targeting key elements of the ECM-receptor interaction signaling pathway. Such IT should be used in combination with inhibitors of the immune checkpoint, antagonists of TGF-β and antiangiogenic agents.

The assumption that HSCs are the main tool of the immune memory requires a healthy donor to receive a vaccine based on key CSC antigens prior to obtaining HSCs. This would allow the production of biomedical HSC-based products for personalized proteome-based GBM therapy (Fig. 3). This method requires further experimental and clinical investigation; however, the current review may provide a theoretical basis for the development of novel GBM treatment options.

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Author's contributions

IB designed and wrote the review, and approved the final version of the manuscript. Data authentication is not applicable.

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Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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