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Immune therapeutic targeting of glioma cancer stem cells

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Abstract

Glioblastoma multiforme (GBM) is a lethal cancer that responds poorly to radiotherapy and chemotherapy. Glioma cancer stem cells (gCSCs) have been shown to recapitulate the characteristic features of GBM and to mediate chemotherapy and radiation resistance. Immunotherapeutic targeting of this cell population holds therapeutic promise but must be considered in the context of the immunosuppressive properties mediated by the gCSC. Recent findings have indicated that this goal will be challenging because the gCSC can suppress both the innate and adaptive immune systems by a variety of gCSC-secreted products and cell-membrane interactions. In this review article, we will attempt to reconcile the disparate research findings regarding the potential of immune targeting of the gCSC and propose several novel solutions.

Keywords

Glioma cancer stem cells; STAT3; glioblastoma multiforme; immunotherapy

Introduction

Brain tumor immunotherapy holds unrealized therapeutic promise that must overcome the challenge of tumor-mediated immunosuppression, in which the cancer stem cell (CSC) may play a key role. The CSCs are a heterogeneous group of undifferentiated tumor cells defined by their capacity for self-renewal, multipotency, high tumorigenicity at low cell numbers, and capacity to recapitulate the phenotypic and histological characteristics of the original tumor [1]. CSCs have been identified in a wide variety of hematological and solid malignancies, including lung, breast, prostate, colon, and brain cancers [2-8], and are believed to play important roles in tumor initiation, proliferation, progression, invasion, metastasis, recurrence, and resistance to therapy. The identifying features of the CSCs isolated from human gliomas (gCSCs) [9, 10] include the formation of nonadherent neurospheres *in vitro*, the capacity for multilineage differentiation along astrocytic, oligodendroglial, and neuronal lines [11], and the ability to induce tumors *in vivo* that recapitulate the features of human malignant gliomas. The expression of stem cell markers

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such as CD133 [12] and nestin [13], are also commonly observed, but are not definitive determinants of “stem cells”.

The current standard of care for glioblastoma multiforme (GBM) consists of surgical resection, if feasible, temozolomide administration, and irradiation [14]—modalities that are effective against proliferating and differentiated tumor cells. The gCSCs, however, perhaps as a result of their undifferentiated and quiescent state, slow cell cycling, enhanced DNA repair capability, and expression of antiapoptosis genes [15], are highly resistant to these treatment modalities [16, 17]. This failure of current treatments to eliminate the gCSC population may explain the grim near-inevitability of recurrence and progression in patients with GBM, as the surviving gCSCs are capable of reconstituting the original tumor. Thus, it would seem that successful long-term treatment of GBM will require the elimination or suppression of gCSCs and that a strategy of specifically targeting gCSCs may hold promise as a paradigm for the development of future novel therapies for GBM.

Immunological approaches against GBM have been attempted in the past, but to date, none have been curative [18-30]. Although GBMs express tumor-associated and tumor-specific antigens [31] that are recognized by the host's immune system [32, 33], they are capable of mediating a profound degree of immunosuppression, both systemically and within the tumor microenvironment [34-36], allowing tumor cells to escape host immune surveillance, and thwarting current attempts to use immunotherapies against GBM. Thus, to successfully employ immune therapy against gCSCs, two conditions must be met. First, the host immune system must be able to recognize and distinguish gCSCs in order to mount an effector response against them. Second, the immunosuppression mediated by the GBM and/or gCSCs must be circumvented. It is therefore necessary to define the antigens preferentially expressed within gCSCs relative to normal cells [37] and to determine the immunologic properties of gCSCs. This article will describe recent data concerning the immunological properties of gCSCs and explore potential strategies for immunotherapeutic approaches targeting gCSCs and reversing GBM-mediated immunosuppression.

The glioma cancer stem cell hypothesis

When first formulated, the glioma cancer stem cell hypothesis proposed a model in which the gCSC population within the GBM was responsible for tumor perpetuation and identifiable by the expression of stem cell markers such as CD133 [8, 38]. However, further investigation demonstrated that CD133 negative GBM cells can also give rise to tumors and can possess the hallmark features of gCSCs [39]. Instead, gCSCs are defined empirically by their capacity for self-renewal, pluripotency, tumorigenicity at low cell numbers, and recapitulation of the phenotypic features of primary GBM [40]. This clarifying distinction can serve to resolve disparate research findings between groups such as ours that have characterized these cells based on this more stringent criterion and found them to be immune suppressive [41, 42] and others that have relied only on the property of *in vitro* neurosphere formation and demonstrated immune eradication of the gCSC. At our present state of knowledge, neither neurosphere formation [43] nor CD133 expression, nor any other *in vitro* property, alone or in combination, should be viewed as necessary or sufficient for classification as a gCSC.

The stability of the gCSC phenotype also remains to date undetermined. The original gCSC hypothesis viewed the gCSC as a fixed phenotype of a specific population of cells whose eradication would entail the eventual destruction of the entire tumor. More recent evidence suggests that factors such as hypoxia can induce glioma cells to de-differentiate and acquire stem-like properties [44]. This raises the possibility that “stemness” is a dynamic property that many glioma cells may potentially adopt, depending on circumstance. If this is true, targeting gCSCs in isolation should not be considered a panacea for GBM, since even after successful eradication of gCSCs, other glioma cells may acquire gCSC properties and reconstitute a population of gCSCs.

The argument that gCSCs are resistant to immunological targeting

GBM immunosuppression and gCSCs

Immunosuppression in GBM is mediated by multiple redundant and overlapping mechanisms. Immunosuppressive cytokines are secreted into the tumor microenvironment and released into the systemic circulation. Immune cells are recruited into the tumor microenvironment and induced to assume immunosuppressive phenotypes. Immune effector cells such as primed CD8⁺ cytotoxic T cells and activated macrophages that enter the tumor microenvironment have their effector functions inhibited or rendered anergic, or they are induced to undergo apoptosis or to assume immunosuppressive phenotypes, resulting in a failure to eradicate the tumor, as has been extensively reviewed in [45].

The gCSCs inhibit adaptive immunity

The gCSC can inhibit adaptive immunity through both direct cell contact and by the secretion of inhibitory cytokines. We recently found that gCSCs expressed major histocompatibility complex (MHC) I and the inhibitory costimulatory molecule B7-H1 but showed minimal to no expression of MHC II, CD40, CD80, or CD86 [42]. The lack of activating costimulatory molecules, such as CD40, CD80, and CD86, and other molecules involved in antigen presentation, prevents the activation of T cells capable of targeting the gCSCs. Additionally, the gCSC surface expression of the co-stimulatory inhibitory molecule B7-H1, previously shown to be expressed in gliomas [46], mediates cell-to-cell contact-dependent immunosuppression of autologous T cells, which reduces glioma cell immunogenicity by suppressing T-cell effector cytokine production and activation [42]. The gCSCs have also been shown to express only low levels of the molecules required for effective antigen presentation, including β -molecule-required proteasome subunits, immunoproteasomes, and transporter molecules [47]. These findings are not entirely surprising since glioma-elaborated transforming growth factor- β (TGF- β) and prostaglandin E₂ (PGE₂) have previously been shown to down regulate MHC II expression, and antigen-processing [48].

In addition to cell-contact mediated immune suppressive mechanisms, we have shown that the secretion of galectin-3 by gCSCs induces apoptosis in both naïve and activated T cells [42]. Galectin-3 has previously been shown to function as an immune suppressor by inhibiting T cells and promoting tumor growth [49]. Moreover, the *galectin-3* gene is one member of the 9-gene profile that predicts survival outcome of GBM patients [50]. Both

Galectin-3 and the signal transducer and activator of transcription 3 (STAT3) have previously been shown to be expressed in GBMs [51-54] and this may be secondary to the presence of gCSCs. Or alternatively, tumor-mediated immune suppression is not likely exclusive to only the gCSC population and multiple redundant immune suppressive mechanisms are likely to be shared between CSC, astrocytes, and neural progenitor cells [55]. However, some may be unique to the gCSC population such as the recruitment of immune suppressive macrophages (M2) into the tumor microenvironment which appears to be specific to the CSC and not the bulk tumor or CD133 tumor depleted bulk GBM [30].

After exposure to gCSC-conditioned medium *in vitro*, T cells obtained from peripheral blood of healthy donors exhibit comprehensive depression in cellular immune function, including minimal proliferation in response to mitogen or polyclonal stimulation and substantial reduction of IL-2 and IFN- γ production [42]. This is identical to the response of PBMCs obtained from GBM patients [56]. Furthermore, incubation of healthy donor PBMCs with gCSC-conditioned supernatant medium markedly expanded the CD4+FoxP3+ Treg population [42], and this was in part mediated by gCSC-elaborated TGF- β 1. This expansion of the Treg population in GBM patients has also been well documented previously in both the peripheral blood [57] and with respect to infiltrating the tumor [58, 59]. Cumulatively, these data indicate that the gCSC employs a variety of cell surface and secreted products to markedly inhibit adaptive immune responses.

The gCSCs inhibit innate immunity

Microglia are CNS-resident macrophages and constitute the dominant infiltrating immune cell population in GBM. Although activated microglia are effective mediators of innate immunity, capable of phagocytosis, cytotoxicity, antigen presentation, and promotion of inflammation [60, 61], this function is impaired within the context of malignant gliomas. For example, glioma-infiltrating microglia do not express the costimulatory molecules necessary for effective antigen presentation [58] and are unable to secrete proinflammatory cytokines [62]. Instead, they can actually support GBMs by mediating angiogenesis, proliferation, and tissue invasion [63] and become similar to other tumor-associated macrophages found in many other malignancies [64]. The factors that induce this polarization in the GBM microenvironment to an immunosuppressive and tumor-supportive phenotype (M2) have not been clearly defined.

We have recently demonstrated that gCSCs play a role in this subversion of microglial activity. In addition to chemokine C-C motif ligand 2 (CCL2), the gCSCs also produce soluble colony stimulating factor 1 (sCSF-1), TGF- β 1, and macrophage inhibitory cytokine 1 (MIC-1). The gCSC-elaborated CCL2 and sCSF-1 recruit circulating monocytes into the GBM. Then the sCSF-1 and TGF- β 1 polarize the monocytes towards the immunosuppressive M2 macrophage phenotype with up regulation of STAT3 and down modulation of the proinflammatory STAT1 [30, 65, 66]. The gCSC-elaborated MIC-1 also markedly inhibits phagocytosis [30] and the macrophage proinflammatory cytokine, tumor necrosis factor- α [67]. The gCSC-exposed macrophages also showed increased secretion of the immune suppressive cytokines IL-10, TGF- β 1, and IL-23, and increased capacity for inhibiting T-cell proliferation [30]. Thus, the gCSCs play an important role in recruitment of

monocytes to the GBM, inhibition of effector function, and polarization towards immunosuppression of innate immunity.

The gCSC tumorigenicity associates with immunosuppression but not CD133 expression

In order to ascertain whether gCSC-mediated immunosuppression associated with tumorigenic potential and CD133 surface expression, we studied three different gCSCs that had either minimal (4%; gCSC 1), moderate (14%; gCSC 2) or high expression (79%; gCSC 3) of CD133. CD133 expression levels did not correlate with the degree of gCSC-mediated T-cell immunosuppression. For example, gCSC 2, with moderate levels of CD133 expression, exerted the strongest inhibition of T-cell proliferation (Fig. 1A), induced the highest level of Foxp3⁺ Tregs (Fig. 1B), and demonstrated the greatest percentage of increased T-cell apoptosis (Fig. 1C), suggesting that CD133 is not a marker for the immunosuppressive properties of gCSCs. Interestingly, we did observe that there was an association of CD133 level with gCSC-produced galectin-3 (Fig. 1D). However, we have found that the degree of immunosuppression, as reflected by the degree of inhibition of T-cell proliferation (Fig. 1E), induced Tregs (Fig. 1F), or enhanced T-cell apoptosis associated directly with gCSC tumorigenicity as measured by animal survival, whereas the expression of CD133 did not correlate with tumorigenicity (Fig. 1H). These findings are consistent with those of other recent reports [39, 68].

Finally, our observation that gCSCs possess immunosuppressive properties brings into question what the appropriate murine models ought to be for studying human malignancies. Previous studies by Quintana [69] demonstrated an increasing frequency of tumorigenic/stem cells after their transplantation into highly-immunocompromised mice, which increased the investigators' ability to detect tumorigenic melanoma cells by several orders of magnitude. Use of this type of a model system would negate or minimize a potentially defining characteristic of these cells, i.e., immunosuppression. We found that the degree of immunosuppression exerted by the gCSCs directly associated with their tumorigenicity and lethality in murine model systems, suggesting that immune suppression should be considered a characteristic of gCSCs.

The gCSC immunosuppression is mediated by STAT3

STAT3 is activated by phosphorylation on the tyrosine residue in the transactivation domain in response to signaling via Janus kinase 2, which is activated by a wide variety of cytokines and growth hormones, including IL-6 and epidermal growth factor (EGF). Phosphorylated STAT3 (p-STAT3) translocates to the nucleus and regulates transcription of immunosuppressive factors such as IL-10 [70, 71] (which suppresses Th1-mediated cytotoxic immune responses and is essential for Treg function [72]), vascular endothelial growth factor (which inhibits dendritic cell maturation and activation by inhibiting costimulatory molecule expression [73]), PGE₂ (which induces the immunosuppressive Th17 cells [74, 75]), and TGF- β (which induces Tregs, inhibits T-cell proliferation, and down modulates the IL-2 receptor [34]). The STAT3 pathway has been shown to be a key molecular hub in tumor-mediated immunosuppression by inhibiting macrophage activation [76, 77] and polarization to the effector M1 phenotype, while promoting polarization to the immunosuppressive M2 phenotype [78]; moreover, it can reduce the cellular cytotoxicity of

natural killer cells and neutrophils, as well as the expression of MHC II, CD80, CD86, and IL-12 in dendritic cells, rendering them unable to activate T cells and to generate antitumor immunity [79].

STAT3 is activated in many cancers, including GBM [80, 81], inducing both tumorigenesis [82] and immunosuppression [83]. Recently, Sherry et al. [84] demonstrated that STAT3 is up regulated in gCSCs. The growth and self-renewal of the gCSC was dependent on this pathway. In addition, we found that gCSCs secrete a variety of products that induce p-STAT3 expression in immune cells. The gCSC-mediated inhibition of T-cell proliferation and activation, T-cell apoptosis, Treg induction, inhibition of macrophage phagocytosis, and induction of macrophage IL-10 secretion, can all be blocked by the inhibitors of p-STAT3 [30, 41]. Thus, these findings indicate that the STAT3 signaling pathway is a central regulatory hub, mediating multiple mechanisms of immunosuppression, and that gCSCs promote immunosuppression by activating pSTAT-3 (Fig. 2).

The argument that gCSCs can be targeted with immunotherapy

Vaccination strategies against gCSCs

As potentially critical mediators of tumor initiation, progression, and recurrence, as well as mediators of resistance to chemotherapy, radiation therapy, and host immune responses, gCSCs are an important therapeutic target. The existence of immunosuppressive properties in gCSCs [41, 42] raises the issue of whether these cells can be targeted successfully by immunotherapy strategies such as vaccination. Several recent studies have suggested that vaccination may, indeed, be a feasible approach [85-87]. Specifically, Pellegatta et al. [86] have demonstrated that dendritic cell immunotherapy could be used to target murine GL261 glioma cells. This highly perpetuated cell line was characterized as “stem-like” based on the ability of the cells to form neurospheres and to express nestin, but the defining characteristics of what constitutes a cancer stem cell is currently being debated. The ubiquitous characterization of many of the human glioma cell lines as “cancer stem cells” needs to be regarded cautiously and with reservation because *in vitro* perpetuated cell lines can be highly divergent from the *in vivo* parental cells in both phenotype and function.

Consistent with the finding of Pellegatta, Xu et al. [87], using the 9L cell line, demonstrated that antigen-specific T-cell responses could be generated from dendritic cell vaccinations using “cancer stem cell”-associated antigens. They found that there was an increase in tumor-associated antigens expressed on the “cancer stem-like cells” and that these antigens could be used to trigger effector responses from CD8+ T cells, based on IFN- γ production, but they did not present cytotoxicity functional data. Of note, these investigators used irradiated cell lysates in the dendritic cell vaccination, which is likely to alter the immunological properties. In a very recent study by Brown et al., antigen-specific CD8 T cells were shown to be able to directly lyse “cancer stem-cell” targets [85]. This contrasts with our finding that gCSCs are capable of inducing T-cell apoptosis [42]. It is possible that the clonotypic tumor-specific T cells used by Brown et al. may be more resistant to gCSC-mediated apoptosis than the naïve T cells we examined. Alternatively, cytolytic T cells may still render lysis of the target but then rapidly undergo their own demise, similar to a bee delivering its fatal sting. Interestingly, Brown et al. found many of their “cancer stem cells”

capable of expressing MHC II, whereas we found MHC II levels to be low [42], which may also account for the discrepancy. Di Tomaso et al. [47] also found that autologous cytotoxic T-cell responses against gCSCs could be generated *in vitro* in at least some GBM patients, but the efficiency of these responses was low and correlated with MHC II expression on the gCSCs.

Reconciliation of disparate research findings

We have observed that passage number markedly affects the immunosuppressive properties of perpetuated gCSCs. Specifically, the secretion of immunosuppressive cytokines by gCSCs and the ability of gCSC-conditioned medium to induce M2 macrophages diminish markedly over time with multiple passages, suggesting a progressive loss of immunosuppressive capability as gCSCs are maintained in tissue-culture conditions (Fig. 3). Indeed, Pellegata et al. [86] reported that neurospheres derived from the GL261 cell line had increased expression of immune stimulatory cell surface markers such as CD80 and CD86, whereas we have found the expression of these two markers in gCSCs recently isolated from human tumor specimens to be low [42]. Because gCSCs are a heterogeneous cell population, and immunosuppressive properties provide no survival benefit in a tissue-culture environment, selective pressures may favor the loss of immunosuppressive phenotypes over time. An alternate hypothesis would be that factors specific to the *in vivo* environment of gCSCs that are not present *in vitro* are necessary for maintaining immunosuppressive phenotypes over the long term. In either case, the instability of the immunosuppressive phenotype in gCSCs may mean that animal model systems using established glioma cell lines do not accurately replicate the immune interactions pertinent to tumors arising *de novo* and that a vaccination strategy effective against the former may not be so with the latter. Furthermore, some of these vaccination strategies have been conducted in immunocompromised murine model systems, and this would artificially influence the complex interaction between the adaptive and innate immune system and potentially confound the data.

As recurrence and persistence are hallmark features of gliomas, it is clear that the intrinsic immune system of the patient, unaided, is usually unable to eradicate the gCSCs that are the progenitors giving rise to recurrence and progression. The gCSCs may be more than capable of immunosuppression sufficient to allow escape from the host's natural immune surveillance, but this does not necessarily mean that an immune response properly primed by an external therapeutic manipulation will not be able to overcome this immunosuppression and still successfully target and kill the gCSCs. Several clinical trials of a peptide vaccine targeting the EGF receptor variant III (EGFRvIII) have suggested therapeutic efficacy in patients with malignant glioma [21, 24]. Because EGFRvIII expression may be enriched in CD133+ gCSCs [88], EGFRvIII-targeted therapies may help glioma patients to mount a more specific immune response against gCSCs.

Mechanisms for inhibiting the immunosuppressive properties of gCSCs

Ultimately, within malignancies there exists a balance between immune stimulation by tumor antigens and immune suppression mediated by tumor cells that promotes escape from

immune surveillance. Tumorigenesis and recurrence occur in a setting in which the latter overrides the former. If tumor-mediated immunosuppression can be successfully inhibited, then the balance may tipped back in favor of immune stimulation, allowing the host's immune system to more easily eradicate the tumor, either on its own or in conjunction with immunostimulatory therapies.

A simple way to reduce tumor-mediated immunosuppression is by a gross-total resection of tumor, which eliminates the bulk of the immunosuppressive tumor microenvironment in which immunosuppressive cytokines are most concentrated, as well as most of the immunosuppressive tumor-infiltrating cells, such as Tregs, and M2-polarized macrophages/microglia. Elimination of the tumor mass effect also allows for withdrawal of immunosuppressive adjuvant treatments such as steroids. An example of the benefit of surgery in the context of immunotherapy has been shown in GBM patients without a bulky and actively progressing disease who responded better to dendritic cell based vaccines [22]. It is for this reason that many recent clinical trials of immunotherapies in GBM patients are conducted on patients who first receive a complete surgical resection of their tumor.

Clearly, therapies that target and/or reverse gCSC immunosuppression are needed. The existence of multiple overlapping and redundant mechanisms of immunosuppression suggests that focusing on any single mode of immunosuppression alone may not be effective and probably accounts for many failures of targeted immunotherapy strategies in the past. More likely, an effective strategy will need to be able to reverse multiple modes of immunosuppression simultaneously. For this reason, the STAT3 pathway garners interest. As an important regulatory hub underlying a wide range of immunosuppressive pathways in both the adaptive and innate immune systems, inhibition of p-STAT3 holds the promise of being able to simultaneously target multiple modes of immunosuppression. Furthermore, because pSTAT-3 is also directly involved in tumorigenesis and proliferation, STAT3 inhibition can also target tumor growth directly. Inhibition of STAT3 has been shown to reverse glioma-associated immunosuppression and to inhibit tumor growth [89]. Specifically, WP1066, a small molecular inhibitor of STAT3, can be administered orally, has excellent central nervous system penetration, and can activate effector T cells, induce T-cell stimulating cytokines [83], and inhibit Tregs [90, 91]. Recently, we showed that STAT3 inhibition by WP1066 and siRNA in gCSCs can diminish gCSC induction of p-STAT3 in immune cells, reduce Treg induction, restore T-cell function, and induce T-cell proliferation [41]. The pSTAT3 blockade in gCSCs also reverses gCSC-mediated inhibition of macrophage phagocytosis, gCSC-mediated induction of IL-10 secretion in macrophages, and it diminishes the degree of gCSC-mediated M2 polarization by increasing the activity of the M1 phenotype regulator, STAT1 [30].

Furthermore, STAT3 inhibitors may exert direct anti-gCSC activity. Sherry et al. [84] showed that blocking STAT3 in gCSCs leads to inhibition of growth and neurosphere formation, but did not result in apoptosis, probably because gCSCs are relatively resistant to apoptosis. In addition, loss of expression of the CSC markers, nestin and olig2, was observed upon inhibition of STAT3. These investigators suggested that STAT3 inhibition suppresses the self-renewal properties of gCSCs in culture but does not fully execute the

differentiation of these cells. This would indicate that STAT3 blockade also has the potential to reverse resistance to chemotherapy and radiation therapy mediated by the gCSC.

Future directions

Studies revealing the immune properties of gCSCs are limited. A greater understanding of the immune biology of gCSCs and their interactions with the host immune system will lead to the elucidation of novel targets and new treatment regimens to enhance the efficacy of the current immunotherapeutic approaches. Detailed studies are warranted to identify new tumor-associated antigens expressed by gCSCs to be targeted for immunological approaches as well as to clarify the dependence of gCSCs on specific signaling pathways in contrast to other stem cell populations. Vaccination strategy with dendritic cells loaded with gCSCs has been shown to be effective in animal tumor model systems with different cell lines, but it will require more investigations to better understand the mechanism of effect before being translated to human studies. Therapeutic approaches targeting the key factors allowing the tumor to evade the immune system have been studied in an attempt to reverse the immunosuppression and treat the glioma. Although the STAT3 inhibitors WP1066 [41], STA-21, and S31-201 have been shown to be effective in reversing immunosuppression mediated by gCSCs, other inhibitor molecules such as JSI-124 [92], NSC 74859 [93], and AZD1480 [94] can be tested for efficacy against gCSCs. In addition to the STAT3 pathway, other signaling pathways, including Wnt, hedgehog, Notch, the HOX family, Bmi-1, PTEN, telomerase, and efflux transporters, are involved in self-renewal and differentiation of gCSCs [95-97]; thus, these molecules may also be favorable targets for suppression of gCSCs by glioma immunotherapy. In addition, treatment strategies inducing the differentiation of CSCs can make them more vulnerable to these therapies [98]. Also, recent studies have demonstrated an association between human cytomegalovirus (HCMV) infection and malignant glioma [99-101] and have shown that HCMV nucleic acids and proteins expressed in malignant gliomas promote glioma cell invasiveness [102]. The role of HCMV in malignant glioma biology and its possible interaction with gCSCs should be further investigated. Targeting HCMV could be a new treatment strategy to eliminate cancer stem cells in glioma patients. Finally, since our knowledge of the immunologic properties of gCSCs is increasing, novel approaches can continue to be identified and improved when they are administered with other immunotherapeutic modalities such as chemotherapy or radiation therapy.

In our view, targeting gCSCs holds the most promise as one component of a multi-modal treatment strategy. The aim is not simply to just eradicate the gCSC population as if it were some mythic Achilles' heel that would bring down the whole tumor with it, but also to suppress the tumor supportive functions, such as radiation resistance, chemoresistance, and immune suppression, provided by the gCSCs, and if necessary, to prevent or reverse the adoption of gCSC phenotypes by other glioma cells. This in turn would hopefully render the remaining tumor cells more vulnerable to other concurrent treatments, leading ultimately to the successful suppression of the malignancy.

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Fig. 1. The degree of gCSC mediated immune suppression associates with in vivo tumorigenicity but not with CD133 expression

The gCSCs (2×10^6) were cultured in neural stem cell medium for 5 days, and then the supernatant medium was harvested for testing immunosuppressive properties. Additionally, the gCSCs (5×10^4) were intracranially implanted in nude mice (6 per group). **A.** The percentage of inhibited T-cell proliferation does not correlate with gCSC CD133 expression levels. **B.** The number of induced Foxp3+ Tregs does not correlate with the gCSC CD133 expression levels. **C.** Increased T-cell apoptosis does not correlate with gCSC CD133 expression levels. **D.** The amount of gCSC-produced galectin-3 associates with CD133 expression. **E.** The percentage of inhibited T-cell proliferation inversely associates with the mean survival time of gCSC-implanted animals. **F.** The number of induced Foxp3+ Tregs

inversely associates with the mean survival time of gCSC-implanted mice. **G.** Increased T-cell apoptosis inversely associates with the mean survival time of gCSC-implanted animals. **H.** The gCSC CD133 expression levels do not correlate with the mean survival of gCSC-implanted animals.

Fig. 2. Theoretical schema showing relationship between glioma cancer stem cells and the immune system

The gCSCs express p-STAT3, which in addition to inducing gCSC proliferation and self-renewal, is also capable of inducing p-STAT3 expression in a wide variety of immune cell populations via a feed forward mechanism. This up regulation of p-STAT-3 in the immune cells down modulates effector function such as T-cell activation, proliferation, and elaboration of effector cytokine production. The gCSC secretes a variety of factors: macrophage inhibitory cytokine, which inhibits macrophage phagocytosis; transforming growth factor (TGF-β1), which induces Tregs and polarizes the macrophage to an immunosuppressive M2 state; soluble colony stimulating factor (sCSF-1), which recruits circulating monocytes and also mediates differentiation and polarization of the monocyte into M2 tumor-associated macrophages; chemokine (C-C motif) ligand 2 (CCL-2), which enhances Treg tumor infiltration; and galectin-3, which triggers T-cell apoptosis. Additionally, the cell-surface expression of the costimulatory inhibitory molecule B7-H1, which mediates immune suppression by a variety of mechanisms, and the down modulation of the major histocompatibility complex (MHC) further enhance the immunosuppressive properties of the gCSCs.

Fig. 3. Decrease in immunosuppressive properties in gCSCs maintained in vitro over many passages

Comparison of early and late passage gCSCs (n = 4) showing decrease with later passages in (A) the production of TGF-β1 (p = 0.10), and sCSF-1 (p = 0.05), (B) the ability to induce macrophages (MΦ) to secrete the immunosuppressive cytokines IL-10 (p = 0.05) and IL-23 (n = 2, p = 0.05), and (C) the ability to inhibit T-cell proliferation (p = 0.008). Late passages were 15-20 passages after early passages. P-values calculated using student's t-test.