Glioblastoma-Derived Mechanisms of Systemic Immunosuppression

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Nearly 40 years have passed since initial studies documented abnormalities of the cellular immune response in patients with malignant brain tumors.[1,2] Early observations regarding defects in circulating T-cell populations, blunted responses to mitogenic stimulation in vitro, and aberrant delayed type hypersensitivity reactions were confirmed by numerous subsequent experiments.[3] However, despite this long history of study, there is poor understanding of the underlying factors responsible for suppressing effective antitumor immunity in affected individuals.

Throughout the 1970s and 1980s, dedicated efforts were made to outline characteristic changes of the circulating T-cell compartment in patients with malignant glioma and to define the generators of the observed immunosuppressive effect. This initial experimentation was limited by a primitive understanding of the various components and functional characteristics of the cellular immune response. Associated with this lack of basic knowledge was a paucity of experimental tools for detailed study of cells involved with cellular immunity. Thus, further detailed experimentation into the source of suppression of cellular immunity associated with glioblastoma (GBM) was restricted. During the 1990s, the literature documented a shifting of research interests towards tumor-specific vaccines and other immunologically based therapies, driven by continued failures of conventional adjuvant therapies. As with many immunotherapeutic strategies, these research efforts have been largely unsubstantiated in the clinical realm. Although recent work with dendritic cell-based vaccines has shown some potentially promising results,[4] there is no effective option for a vaccine-based therapeutic approach in patients with GBM. A full discussion of these strategies is outside the scope of this article; however, the consideration of prior immunotherapeutic failures is relevant to a discussion of glioma-derived immunosuppression. More specifically, tumor-associated factors involved with suppression of cellular immune responses in patients with GBM are likely to similarly affect any antitumor immunity generated via immunotherapeutic strategies. Therefore, it is important to renew efforts towards elucidating the biologic basis of the observed suppressive effect.

During the late 1980s, the growing acquired immune deficiency syndrome (AIDS) epidemic resulted in a significant dedication of research money and effort to increasing understanding of cellular immunity, most particularly in T-cell biology. The usefulness of specific T-cell markers for phenotypic and functional characterization, and the development of powerful experimental techniques such as flow cytometry and enzyme-linked immunosorbent assay (ELISA)-based cytokine detection, allowed for more detailed study of newly described populations of immunologically relevant cells with widely divergent functional activities. More recently, increasing understanding of the complex interactions involved with regulation of the immune response, including the influence of various cell-surface proteins and

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secreted factors, has provided new opportunities to revisit concepts proposed by pioneering researchers in brain-tumor immunology from prior decades.

This article summarizes critical data that contribute to current theories of the immunosuppressive effects of malignant glioma, focusing on experimentally relevant conditions that either support or preclude the likelihood that an individual factor may play in glioma-associated immunosuppression. Most early research in the field focused on proteins proposed to be expressed on the cell surface or secreted by glioma cells; this article focuses on several key proteins in each group that have been most closely studied. More recently, work has outlined abnormalities inherent to the various monocytic populations in patients with GBM, and the presence and potential functional relevance of regulatory lymphocytes in these patients. In most cases, these factors have been investigated as potential suppressors of activated T cells, putatively responsible for the functionally relevant component of antitumor immunity. This article provides a systems-based framework for understanding potential sources of the glioma-specific immunosuppressive phenomenon, based on a review of prior experimental results and viewed in the light of current immunologic knowledge of the endogenous regulators of cellular immunity.

CAVEATS IMPORTANT TO CONSIDER WHEN EVALUATING PRIOR WORK

Several caveats should be kept in mind when reviewing the results of prior experimentation in glioma immunology. Recent insight into the endogenous suppressors of cellular immunity, increasing awareness of the complexity of regulatory circuits in immunologic systems, and more common acceptance of the limitations of glioma cell lines and animal models require more critical retrospection of prior experimentation implicating a wide spectrum of glioma-associated factors in the suppression of antitumor immunity.

Cellular heterogeneity is a sine qua non of GBM, with histologic analysis demonstrating an amalgam of presumed tumor cells, reactive astrocytes, tumor-associated microglia, trapped neurons, and various cells infiltrating from the peripheral circulation (including lymphocytes and macrophages).[5] Study of bulk (ie, ''unsorted'') tumor specimens does not take into consideration potentially significant differences in expression patterns amongst these varied cell types, rendering RNA and protein analysis of limited usefulness. These differences are of critical importance when attempting to categorize phenotypic and functional characteristics of cells involved with the cellular immune response. Although immunohistochemistry (IHC) can provide some specificity of cellular expression and subtumoral anatomic localization, this technique is not particularly conducive to the concurrent detection and quantitation of multiple markers necessary for identification and subclassification of relevant regulatory cells.

The use of glioma cell lines has been a mainstay of research evaluating expression patterns of immunologically relevant markers from malignant brain tumors. However, recent comparative analyses have suggested that there are in fact dissimilarities between commonly used glioma cell lines and direct ex vivo tumor specimens. A seminal series of experiments, recently reported by Howard Fine's group,[6] used comparative chromosomal analysis and microarray expression profiling between a large number of primary GBM specimens and a range of commonly used glioma cell lines (including U87, U251, and T98G). They found that the overall spectrum of genomic alterations differed substantially between primary tumors and the tested cell lines. Their analysis demonstrated that observed expression patterns and genomic abnormalities within tested glioma cell lines were more congruent to abnormalities observed in other nonglioma cell lines, suggesting that selection pressure of in vitro culture resulted in activation or alteration of common ''transformation'' pathways. Similar analysis of immunologically relevant markers in short passage cultures of fresh human GBM specimens has demonstrated a rapid shift in the expression of immunologically relevant markers, including downregulation of major histocompatibility complex (MHC)-I and increased expression of TGF-β.[7] Recent data supporting the role of ''cancer stem cells'' found at high frequencies in human GBM and proven to harbor a tumorigenic phenotypic in xenograft models mandate that future in vitro study includes these cell populations.[8,9] Cells with stemlike characteristics are notoriously sensitive to culture conditions for maintenance of a limited-differentiation phenotype, a consideration which sets in vitro experimentation with these populations apart from standardized culture cocktails used in most prior in vitro glioma experimentation. Although such observations do not obviate the results of prior studies investigating the expression of immunologically relevant factors through in vitro culture, these studies mandate that the results of in vitro testing are further confirmed in more biologically relevant systems.

Investigators have used many animal models for comparative analysis in the study of immune responses to malignant brain tumors. These models have been primarily developed in rodents, using stereotactic injection of glioma cell lines into the brains of test animals, allowing for consistent and predictable tumor growth. However, these models often bear little pathologic and immunologic similarity to human GBM. Many of the commonly used rat glioma lines grow as ''pushing masses'' rather than infiltrating neoplasms, and formed tumors often do not demonstrate the pathologic hallmarks of human malignant glioma.[10] From an immunologic standpoint, these models are often more representative of a transplant system, in some cases even demonstrating genetic mismatch between transferred cell lines and the host animal (rendering the immunologic relevance of these models limited at best).[11] In addition, it is possible that the procedure involved with stereotactic injection of tumor cells may alter the immunologic microenvironment of the brain, providing an increased ''danger'' signal and promotion of a proinflammatory environment. More recently, xenograft systems using the injection of dissociated human GBM specimens or human glioma cell lines into immunoincompetent rodents has become a commonly accepted method for studying glioma biology.[12] Deficiencies in immunity that allow for engrafting of tumor in these models concurrently prevent any meaningful study into tumor-specific immune responses. In addition, the evaluation of expression patterns of tumors initially derived from human glioma cell lines subsequently xenografted into immunodeficient animals confirms a closer correlation of derived tumors with other long-term cultured cell lines, rather than ex vivo human glioma specimens.[6] More recently, tumor models driven by retroviral targeting of white-matter progenitor cells in rodents have been developed that closely recapitulate the pathologic hallmarks of human GBM (including pseudopallisading necrosis, microvascular proliferation, and infiltrative growth).[13,14] Preliminary studies have suggested that the frequency and phenotype of tumor-infiltrating lymphocytes (TIL) within these lesions more closely resemble profiles of TIL within human GBM than do other rodent models[15–17] (B. Killory and D. Fusco, unpublished data, 2009). Although preliminary, such models may be instrumental in the further study of relevant immune responses to malignant brain tumors.

Although in vitro and animal data are important components of ongoing immunologic research in glioma, increased emphasis must be placed on the further development of more immunologically relevant models and renewed study of fresh human tissues. This article focuses on immunoregulatory factors explored or confirmed to be present within human tissue.

OBSERVATIONS OF CELLULAR IMMUNITY IN PATIENTS WITH MALIGNANT GLIOMA

What is known about cellular immunity and the antitumor immune response in patients with malignant glioma? T cells can be found within human GBM specimens, although these cells are present at low frequencies and are primarily restricted to the perivascular spaces.[15,18] These intratumoral populations also demonstrate a skew from the expected CD8+ (''effector'') phenotype towards a more dominant CD4+ representation.[15,19] Studies have also demonstrated the presence of a limited repertoire of T-cell receptor expression on TIL within GBM specimens, and the presence of a predominantly CD45RA-negative (or ''memory'') phenotype, suggesting that there may be an antigen-specific expansion of T-cell subsets within these lesions.[15,20,21] In addition, tumor antigen-specific T cells can be generated from bulk peripheral blood mononuclear cell (PBMC) samples from affected patients, providing evidence for potential tumor-specific cellular immunity.[18] However, these potentially tumor-specific T cells are ultimately ineffective at providing any benefit in regards to tumor control. Certain T-cell abnormalities can be temporarily reversed following tumor resection, and subsequently return with tumor recurrence, potentially confirming an association between tumor and the suppressive phenotype rather than a global defect in cellular immunity.[22]

Circulating T cells in patients with glioma-associated immunosuppression seem to be globally abnormal through in vitro analysis. Although not systemically immunocompromised, these patients generally exhibit a loss of CD4+ cells, resulting in a CD4/CD8 ratio closer to 1 (rather than the usual 2:1) and a total mild lymphopenia. CD4+ and CD8+ T cells are handicapped in their response to in vitro mitogenic stimulation, a phenomenon that has been partially attributed to decreased production of and hyporesponsiveness to interleukin 2 (IL-2).[3] Aside from widely reported anomalies of T-cell number and function in patients with GBM, further detailed analyses of various signaling pathways involved with T-cell activation have demonstrated significant abnormalities in expression levels and poststimulation phosphorylation patterns of proteins downstream from the T-cell receptor, including PLCγ1, pp100 and p56lck.[23]

The aforementioned findings offer critical insights into the nature of GBM-associated immunosuppression. First, T cells can enter the tumor microenvironment and potentially respond specifically to antigen, but these cells are somehow blocked in their ability to expand into effector populations and provide tumor clearance. Second, considering that most peripheral/circulating T cells are never physically exposed to tumor cells, and assuming that affected patients are not globally immunocompromised, the presumed tumor-specific immunosuppressive factor must extend far beyond the tumor microenvironment. Third, considering that relative immunosuppression can be transiently reversed following tumor extirpation, and returns with tumor recurrence, the tumor itself provides a driving force behind the generation of the immunosuppressive effect.

SUPPRESSIVE SURFACE MARKERS EXPRESSED ON GLIOMA CELLS

Several studies have explored the expression of surface markers on GBM cells with putative suppressive effects on cellular immunity. The most widely explored of these factors is likely Fas ligand (FasL or CD95L). Fas (CD95) is constitutively expressed on activated T cells in CD4 and CD8 compartments, and the Fas/FasL system is known to be an important regulator of activated T-cell populations. Binding of Fas and FasL is believed to be critical in mediating activation-induced cell death (AICD). AICD is essential for downregulating activated T-cell populations at the termination of immune responses, for maintaining T-cell homeostasis, and in the prevention of autoimmunity.[24] Although FasL is primarily expressed on the surface of activated T cells, several groups have documented the expression of FasL on the surface of glioma cells.[25–28] The resulting hypothesis focuses on the potential killing of activated tumor-specific T cells through cell-cell contact with FasL-expressing glioma cells, thereby resulting in effective escape from the cellular immune response. To provide potential functional relevance for this hypothesis, Ichinose and colleagues[26] correlated the expression of FasL in 9 of 14 GBM specimens with decreased numbers of TILs in relevant samples. Yu and colleagues[19] performed double-staining IHC for FasL and endothelial cell markers, finding that approximately 30% of tested GBM samples demonstrated positive FasL staining on tumor vasculature. In contrast to the findings of Ichinose and colleagues,[26] these investigators did not find any correlation between FasL expression and extent of lymphocytic infiltration, although they did identify a shift in CD4/CD8 ratios in positive samples. Frankel and colleagues[29] identified increased levels of soluble FasL in fluid aspirated from cystic malignant gliomas. Surface-bound FasL can be cleaved by metalloproteinases, resulting in a soluble yet functional form of the protein.[24] Soluble FasL within cystic aspirates from astrocytoma was effective at inducing cell death in cultured Jurkat cells.[29] These investigators suggested that elevated FasL in cyst fluid is representative of concentrations in the tumor extracellular microenvironment and therefore potentially effective at killing infiltrating tumor-specific T cells.

The expression of nonclassic human leukocyte antigen (HLA) molecules by glioma cells has also been explored as a potential source of immunosuppression. In contrast to classically described MHC-Ia molecules, which are expressed on cells of virtually all tissue types and primarily responsible for peptide presentation to CD8+ T cells, MHC-Ib molecules bind a restricted set of peptides and have been primarily associated with natural killer (NK) cell-mediated immunity through the binding of the CD94/NKG2 class of receptors.[30] The expression of these markers, previously associated with suppression of cellular immunity, has been explored in GBM. The first of these is HLA-G, which has been identified on the surface of several glioma cell lines and a limited subset of fresh glioma samples.[31] A potential functional role for HLA-G in glioma was demonstrated through gene transfer experiments, which suggested that forced expression of HLA-G by U87 glioma cells rendered the transfected cells resistant to killing by alloreactive PBMCs.[31] Additional studies demonstrated that increased levels of soluble HLA-G can be detected in sera from patients with multiple malignancies, including glioma, although a functional role for this finding has not been defined.[32] HLA-E, a second member of the MHC-Ib family, has been similarly associated with glioma. Advanced tumor grade is paralleled by increasing levels of tumor-specific HLA-E expression, although no functional relevance has been provided for this finding.[33] A potentially contradictory association between expression of HLA-E in GBM specimens with increasing numbers of infiltrating CD8+ T cells has been reported,[34] resulting in an unclear impact of these nonclassic HLA proteins on glioma-specific cellular immunity.

More recent data have explored the potential functional relevance of glioma-specific expression of B7-homolog 1 (B7-H1, also known as programmed death ligand 1, or PD-L1). The CD28-B7 family of costimulatory molecules has been

expanded to include several receptor-ligand pairs that have potent regulatory effects on antigen-specific T cells. The classic role for the canonical members of this family, CD28 (expressed on T cells) and B7-1 (expressed on antigen-presenting cells), is to provide necessary costimulation in the process of antigen-specific activation through the T-cell receptor. Newer members of this family, including B7-H1, have been suggested to be critical regulators and suppressors of activated T-cell function.[35] Involvement of B7-H1 in glioma-associated immunosuppression has been hypothesized and explored by several groups. Studies have demonstrated low-level expression of this marker on multiple glioma cell lines.[36,37] In addition, single-color IHC has provided evidence for B7-H1 positivity in the small number of GBM samples tested.[36] Further experimentation demonstrated that coculture of allor-eactive T cells with B7-H1+ cell lines, in the presence of blocking antibodies, can drive increased expression of proinflammatory cytokines and T-cell activation markers. However, no evidence for increased T-cell death was identified in the absence of blocking antibodies, which argues against a baseline functional role in this system.[36] A series of experiments recently reported by Parsa and colleagues[38] identified a link between the commonly identified mutation in phosphatase and tensin homolog (PTEN) and increased expression of B7-H1. Expression levels of B7-H1 were directly correlated with loss of PTEN function, shown to be secondary to increased Akt activity and posttranscriptional upregulation of B7-H1 on the cell surface. PTEN-deleted cells were rendered less susceptible to cytotoxic T lymphocyte-mediated targeting, providing evidence for a potential functional connection between a commonly identified mutation in GBM and the immunosuppressive effect.[38]

Although the expression of immunosuppressive factors on the surface of glioma cells may provide some explanation for local effects on activated T cells, this hypothesis does not adequately define a comprehensive solution to the systemic immunosuppression problem. Again, considering that most circulating T cells do not come into direct contact with tumor cells, additional far-reaching factors are necessary to explain the characteristically global defects in affected individuals.

GLIOMA-SECRETED FACTORS WITH POTENTIAL IMMUNOSUPPRESSIVE EFFECTS

In 1984, Fontana and colleagues[39] presented the first report providing evidence for a factor secreted from cultured glioma cells that inhibited IL-2-induced T-cell proliferation and blocked allogeneic T-cell responses in mixed lymphoid reactions. Over the next several years, continued study from this group, and others, confirmed the immunosuppressive effects of this factor.[40–42] Purification and sequencing of the responsible protein, initially named GBM-derived T-cell suppressor factor (G-TsF), demonstrated significant homology with human transforming growth factor beta (TGF-β). Subsequent study found G-TsF to be identical to TGF-β2.[43,44] Expression of all 3 isoforms of TGF-β by GBM was ultimately documented.[45,46] This family of proteins has been shown to significantly impair activation of T cells and monocytes, and more recently TGF-β has been associated with the generation of CD4+ T regulatory cells (Treg, discussed later). Although most data have focused on the expression of TGF-β by glioma cells in culture, potential in vivo relevance has been provided through IHC analysis of GBM specimens, indicating that areas of high extracellular TGF-β concentration within tumor samples have fewer numbers of infiltrating lymphocytes than do regions with lower levels of TGF-β.[47] Although the secretion of TGF-β provides a compelling explanation for glioma-associated immunosuppression, the potential for systemic effects is unclear. Several studies using comparative analysis of TGF-β levels in serum from GBM patients and controls identified no difference between the 2 groups, suggesting that systemic levels were not significantly altered by the presence of tumor.[48,49]

Tumor-specific production of immunosuppressive prostanoids, most notably prostaglandin E2 (PGE-2), has also been associated with suppression of antitumor immunity. Studies have demonstrated high levels of expression of cyclooxygenase type 2 (COX-2) and arachadonic acid (the substrate for prostanoid production) in malignant glioma. Elevated intratumoral levels of these factors have been correlated with increasing pathologic grade and proliferative behavior. These findings have resulted in some interest in the use of selective COX-2 inhibitors for treatment of GBM.[50] Production of PGE-2 has also been suggested to play a role in GBM-associated immunosuppression, although attempts to demonstrate PGE-2-specific immunosuppressive effects, using coculture experiments with mitogen-driven proliferation of PBMC in the presence of glioma supernatant, have provided mixed results.[51,52] A more recent study suggested that COX-2 expression by GBM, with secondary induction of IL-10 production from intermediary dendritic cells, resulted in the generation of CD4+ Treg cells.[53] This effect could be blocked by selective COX-2

inhibitors and subsequently rescued via addition of exogenous PGE-2, although in vivo correlates of these findings have yet to be provided.

Multiple studies have attempted to link expression of the immunosuppressive cytokine IL-10 with glioma-associated immune inhibition. Early reports used real-time polymerase chain reaction to demonstrate increased levels of IL-10 mRNA within bulk GBM specimens.[54,55] However, subsequent studies using in-situ hybridization confirmed that IL-10 was not expressed by putative glioma cells. Rather, IL-10 expression could be specifically localized to circulating monocytes and tumor-associated microglia/infiltrating macrophages within these tumors.[56–58] The role of immunosuppressive mononuclear cells is discussed in more detail later.

It remains difficult to conceptualize a mechanism by which factors secreted locally by glioma cells could reach serum concentrations sufficient to repress T-cell function globally. In addition, it seems unlikely that such a factor could be subsequently transferred into in vitro, tumor-free studies at a sufficient concentration to exert continued suppressive effects in experimental systems. However, GBM-secreted factors may play an important role in recruiting or driving the development of a secondary immunosuppressive agent.

ABNORMALITIES OF MONOCYTIC POPULATIONS IN PATIENTS WITH GBM

Several studies in the early 1980s suggested that glass-adherent cell populations (notably monocytes) were involved with suppression of T-cell proliferation in response to mitogenic stimulation, secondary to observations that exclusion of these cells from PBMC cultures partially restored proliferative defects.[59,60] Since that time, few studies have focused on the potential immunosuppressive effect of this population, despite the central role that monocytes and their progeny, including the potent antigen-presenting dendritic cells, play in shaping cellular immune responses in various systems. It has been shown that a soluble factor (or factors) secreted by GBM cells in vitro can induce functionally relevant changes in monocyte cultures, including downregulation of IL-12, MHC-II, and the costimulatory molecules CD80 and CD86.[61] These changes are associated with a concomitant increase in IL-10 production,[61] consistent with prior observations identifying monocytic cells as the predominant source of IL-10 within GBM specimens. A recent study by Kostianovsky and colleagues[62] further explored the potential influence exerted by glioma cells on microglia/macrophages, demonstrating that monocytes cultured with glioma cells are rendered tolerogenic for CD4+ T cells and cocultured monocytes.

More recently, a subset of monocytic cells, which have been consolidated under the rubric of the myeloid-derived suppressor cell (MDSC), have been studied as potent inactivators of CD4+ and CD8+ T cells and have been increasingly identified in several human cancers.[63] In addition to their expression of CD11b and variable expression of CD14, MDSC have been characterized to express CD33, CD34, and decreased levels of HLA-DR.[63] Patients with GBM harbor a relative peripheral monocytosis.[60,64,65] In addition, several studies have indicated that a high percentage of circulating monocytes in patients with GBM harbor a subset of functional abnormalities previously described as characteristic of MDSC. Flow cytometry-based analysis of the expression of relevant surface markers on peripheral blood monocytes from patients with GBM demonstrates decreased numbers of HLA-DR+ cells.[49,64] Ogden and colleagues[64] also noted that peripheral monocytes from these patients harbor significant deficits in the in vitro generation of mature dendritic cells compared with circulating monocytes from patients with other intracranial tumors. Further functional characterization of these abnormal monocytic populations in GBM patients is ongoing in the authors' laboratory and others.

From a systemic standpoint, monocytes have the potential to traffic into and out of areas of immunologic activity within the central nervous system, thereby providing a hypothetical bridge between the tumor microenvironment and circulating lymphocyte populations.[66] It is therefore possible that monocytes accessing the local tumor environment are encouraged by tumor-specific factors to adopt functional characteristics of MDSC, which subsequently spill back into the circulation to exert suppressive effects on peripherally located T cells. Although these data provide intriguing preliminary evidence, confirmation of the MDSC phenotype in GBM patients has yet to be completed. In addition, a potential mechanistic link between GBM and the development of cells with possible MDSC characteristics remains to be identified.

IDENTIFICATION AND CHARACTERIZATION OF T CELLS WITH REGULATORY CHARACTERISTICS IN GBM

Over the past decade, several newly described populations of T cells with potent regulatory properties have been studied in various immunologic systems, including malignant brain tumors. The presence of lymphocytes with suppressive effects

on activated T cells in patients with GBM was initially suggested decades ago.[59,67] However, recent experimentation in other immunologic systems, focusing on the identification and characterization of regulatory cells, has provided significant insight into new mechanisms for endogenous regulation of cellular immunity.

The most well-characterized class of relevant lymphocytes is the "T regulatory cell," or "Treg." Progressive analysis of these cells has provided detailed knowledge regarding their phenotype and functional activity. Tregs are a subset of CD4+ T cells, generally found at low numbers within circulating T-cell populations of normal individuals, which have been most definitively identified by their expression of the transcription factor FoxP3.[68] In addition to their expression of CD4 and FoxP3, Tregs are typified by increased expression of the high-affinity IL-2 receptor (also known as CD25). Although all T cells upregulate CD25 in response to activation, levels of CD25 on Tregs are distinctly higher than levels expressed by other activated T cells.[68] In addition to being CD25hi, Tregs have been shown to express CTLA-4, glucocorticoid-induced tumor necrosis factor receptor (GITR), and various other markers.[69] The powerful functional activity of Tregs in the suppression and regulation of activated T cells has been described in many immunologic systems. Studies involving depletion of Tregs in animal models have confirmed their central role in preventing autoimmunity.[70] In addition, depletion of Tregs provides improved immune responses to nonself antigens (ie, bacterial infection) and tumors in several animal models.[71,72] The mechanism by which Tregs are generated or recruited remains unclear; however, studies of T-cell receptor expression on Treg populations suggest that these cells may have increased affinity for self-antigen/MHC complexes.[70] As Tregs are believed to emerge from the thymus in a previously mature state, it is possible that T cells with significant self-reactivity during early selection may be shunted into the Treg population within the thymus itself.[70] Tregs are proposed to exert their suppressive effects through secretion of cytokines (TGF-β, IL-10), perforin/granzyme-mediated killing of responder T cells, or by modulation of antigen presenting cell (APC) function.[70] Tregs have also been shown to express the endonucleosidases CD39 and CD73, responsible for extracellular generation of adenosine, which has immunosuppressive properties.[73]

Several groups have explored a potential role for Tregs in the suppression of cellular immunity in patients with GBM. Fecci and colleagues[74] identified an increased percentage of cells expressing high levels of CD25 and CD45RO within the circulating compartment of CD4+ T cells compared with normal volunteers. Further experiments using sorted populations of CD4+ T cells that were depleted of CD25+ cells demonstrated that observed deficiencies in mitogen-induced proliferation could be recovered with this strategy. Finally, using a murine intracranial glioma model, these investigators demonstrated that depletion of Treg populations using an anti-CD25 antibody resulted in improved survival. Parallel analysis of the frequency of putative Tregs within PBMC and TIL from patients with GBM was performed by El Andaloussi and colleagues,[75] who suggested that nearly 25% of all CD4+ cells within TIL were Tregs. However, it is unclear whether these cells were all truly Tregs as the gating strategy identified all CD25+ cells, a fraction which is known to contain not only Tregs but also traditional activated T cells. A series of correlative experiments were performed by Heimberger and colleagues,[76] who used single-color IHC to quantify the number of FoxP3+ cells within gliomas of varying grade and histologic subtype. They found an association between increased numbers of FoxP3+ cells with advanced tumor grade, and noted that the presence of FoxP3+ cells within GBM was associated with poorer prognosis. A caveat inherent to their analysis was a lack of phenotypic or functional confirmation of a true Treg phenotype, caused by the use of single-color IHC. This finding is particularly relevant in light of data recently presented by Ebert and colleagues,[77] who used 2-color IHC to demonstrate that FoxP3 can be expressed by ex vivo melanoma cells and a wide range of cancer cell lines, including GBM. The authors have used direct ex vivo multicolor flow cytometric analysis to quantify the frequency of Tregs within PBMC and TIL from patients with a range of intracranial tumors. Frequencies of CD4+CD25hi Tregs were similar within PBMC from patients with GBM, metastatic tumors, and meningioma. Although there was an increase in the proportion of Tregs within the CD4+ compartment of TIL from GBM, there was a nearly 10-fold increase in the number of similar cells in patients with metastatic lesions.[15] Recent data suggest that Tregs may play a role in glioma-associated immunosuppression. However, factors involved with the recruitment of these cells, and the contribution they may provide in blunting tumor-specific immune responses, remain to be determined.

A second class of lymphocytes with immunoregulatory properties is the natural killer T (NKT) cell, a diverse population that expresses an array of traditional NK cell markers (such as CD56) in

addition to the T-cell receptor and the coactivation receptor CD3. In contrast to canonical T lymphocytes, NKT cells are believed to be restricted to interaction with the nonclassic HLA-like CD1d molecule, suggesting a role as a regulatory bridge between the innate and adaptive immune systems.[78] NKT cells have attracted interest because of their capacity for rapid expression of high levels of Th1 or Th2 cytokines following stimulation, supporting their role as key early regulators in the cellular immune response. The traditional, or "type I," NKT cell expresses an invariant T-cell receptor and has known responsiveness to the glycolipid α-galactosylceramide (aGalCer). In experimental stimulation paradigms, type I NKT cells have been shown to produce Th1 and Th2 cytokines, and their ability to confer potent tumor killing in animal models has encouraged the study of this subset as a possible avenue for tumor immunotherapy. More recently, a second subset of NKT cells has been described. These "type II" NKT cells remain restricted to CD1d interaction but express a variable T-cell receptor. Recent studies have implicated type II NKT cells, and more specifically the CD4 single-positive population, in the suppression of antitumor immunity and the prevention of autoimmunity.[79–81] Terabe and colleagues[82] recently elucidated the mechanism of type II NKT cell suppression of the antitumor immune response using a mouse fibrosarcoma model, in which they demonstrated that IL-13 expression by CD4+ NKT cells specifically induced the activity of CD11b+Gr-1+ myeloid suppressor cells (MDSC, discussed earlier), which were in turn directly responsible for suppression of tumor-specific cytotoxic T cells.

The authors have recently published data documenting the presence of a novel population of CD4+ NKT cells found within TIL from direct ex vivo GBM specimens. Using comparative flow cytometric analysis of fresh intracranial tumor samples, the authors demonstrated that a significant percentage of TIL from GBM were CD4+CD56+ NKT cells. Although the percentage of peripheral NKT cells was similar between patients with GBM, metastatic tumors, and meningiomas, the increased proportion of NKT cells within TIL was unique to GBM (**Fig. 1**). NKT cells within GBM demonstrated evidence for antigenic activation and intratumoral proliferation, and direct ex vivo comparative analysis of cytokine expression patterns in matched peripheral T cells and TIL from GBM, using intracellular cytokine detection, demonstrated a significant increase in the percentage of T cells expressing IL-13 within GBM specimens.[15] Studies are ongoing to provide further functional relevance for NKT cells in patients with GBM and to identify potential sources of recruitment or intratumoral stimulation of these cells. Although preliminary, these studies suggest intriguing parallels between the NKT cells found within GBM and those described in animal models associated with the NKT-MSC immunosuppressive circuit.

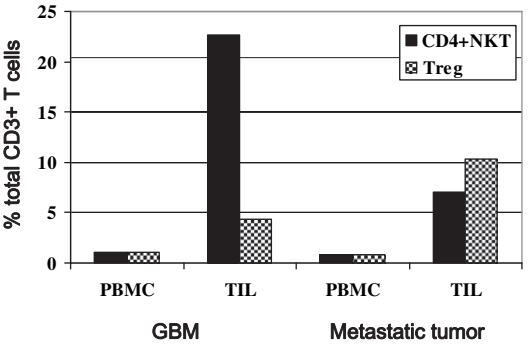

Fig. 1. Relative frequency of immunoregulatory T cells within PBMC and TIL from patients with GBM and metastatic lung tumors. Plots represent percentage of the total CD3+ fraction, determined by flow cytometry, that are either CD4+CD56+ NKT cells or CD4+CD25hi Treg cells.

The role that immunoregulatory lymphocytes play in glioma-associated suppression of cellular immunity, and potential tumor-specific pathways responsible for their generation or recruitment, is unclear. However, the regulatory T-cell hypothesis is attractive for several reasons. First, these cells have been increasingly identified as the primary regulators of the cellular immune response in many immunologic systems. The likelihood that these cells play a similar role in GBM offers an explanation. Second, the presence of these cells within the peripheral circulation allows for an explanation of system-wide aberrations in cellular immunity, which is otherwise difficult to explain through the action of a locally expressed factor. Finally, potential biologic relevance of these cells can be studied in animal models in a detailed and mechanistic fashion, allowing for the development of potential therapeutic strategies aimed at exclusion or functional disruption of the suppressive element.

SUMMARY

Although glioma-associated suppression of cellular immunity has been discussed for several decades, little clinically relevant progress in the understanding of this phenomenon has occurred. The continued failure of immunotherapeutic strategies, in conjunction with exciting new insights into the endogenous regulators of cellular

immunity, warrants renewed effort towards elucidation of factors involved with immunosuppression in GBM and the pathways associated with their generation.

A continuing theoretic conundrum in the study of glioma-related immunosuppression is that locally induced factors exert powerful effects on a more systemic level. It is possible that genetic mutation or otherwise altered expression of regulatory factors by tumor cells, driven by selective immunologic pressure, may derive the eventual escape from antitumor immunity. However, in contrast to the more sinister hypotheses focusing on de-novo, glioma-specific expression of immunosuppressive factors, observed defects in affected patients may simply represent tumor cooptation of endogenous, naturally regulated systems that function to shape cellular immunity. As understanding of the significant complexity of endogenous immunoregulatory networks grows, it becomes increasingly unlikely that a single factor, whether cell-associated or secreted, is responsible for the wide-ranging immunosuppressive effects seen in patients with GBM. More likely, identification of a series of cognate regulatory events, best elucidated at the systems level, will provide a binding hypothesis for the failure of antitumor cellular immunity.

Further studies using glioma cell lines, nonphysiologic culture systems, or poorly congruent animal models are not likely to provide meaningful insights into the nature of glioma-associated immunosuppression. Use of ex vivo human-tissue samples and newly developed animal models, in conjunction with correlative analysis from other immunologic systems, are more likely to provide relevant data for ongoing research. The continued participation and collaborative role of neurosurgeons in providing and studying fresh human tissues will continue to be an essential element of these research efforts, and of extending results into the clinical realm.

Suppression of cellular immune responses in patients with GBM remains poorly understood. Tumor-associated factors that function to suppress endogenous cellular immunity are likely to affect the efficacy of immunotherapeutic strategies similarly, therefore mandating the application of renewed effort towards the elucidation and therapeutic targeting of these factors. Recent observations regarding endogenous systems of immune control, and initial insights into mechanisms by which these systems are activated and regulated, allow for potential advances in improving antitumor immunity and the development of new therapeutic options for patients with GBM.

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