Review

The Role of Regulatory T Cells in Malignant Glioma

ADAM M. SONABEND, CLEO E. ROLLE and MACIEJ S. LESNIAK

The Brain Tumor Center, Section of Neurosurgery, The University of Chicago Pritzker School of Medicine, 5841 South Maryland Ave, MC 3026, Chicago, IL 60637, U.S.A.

Abstract. *The aggressive nature of gliomas is closely related to their capacity to evade the anti-tumoral immune response. The mechanisms implicated in this phenomenon are only partially understood. A subset of T cells, termed CD4$^+$ CD25$^+$ regulatory T cells (Treg), have been shown to inhibit the actions of effector lymphocytes. These Tregs are increased in the blood and tumors of glioma patients and animals with experimental brain tumors. Moreover, tumor infiltration by Tregs correlates with tumor grade and in animal models, depletion of Tregs is associated with prolonged survival. This review focuses on the role of Tregs in the immune suppression exhibited by malignant gliomas. The biology of these cells is briefly described in this context and finally, potential therapeutic strategies related to Treg ablation are explored.*

High grade gliomas represent the most common primary malignant tumor of the adult central nervous system (CNS) and unfortunately, the one with the worst prognosis. The aggressive nature of this neoplasia is closely related to its complex pathophysiology. In particular, evasion of the immune system by gliomas limits an effective anti-tumoral response.

The immunodeficient status associated with gliomas was described more than twenty years ago. Initially, it was noted that the cellular immune response of patients and animals with gliomas is impaired (1-3). The first reports described a lack of proliferation of the peripheral T cells of these patients after exposure to phytohemagglutinin (1, 2). More recently, the immunodeficiency induced by these tumors has been partially explained by the secretion of TGF-beta (4-8) and prostaglandins by tumor cells (9, 10). Moreover, tumor-infiltrating microglia express immunosuppressive cytokines such as interleukin 10 (IL-10) (11). In addition, the decreased level of major histocompatibility complex (MHC) class I expression by gliomas (12, 13) and the expression of human leukocyte antigen (HLA)-G, a non-typical class I MHC molecule (14) appear to play a role in this immunosuppressant status (15). To date, multiple factors contributing to the evasion of immune response by gliomas have been described, albeit, the precise relation between these mechanisms and their relative roles remain unknown.

CD4$^+$ CD25$^+$ regulatory T cells (Tregs) have been recently shown to infiltrate gliomas and their fraction is increased in the blood of patients bearing these tumors. Current evidence suggests a major role in the evasion of immune rejection by these cells (16-24). In this review, the role of Tregs in immune evasion by glioma is explored. In addition, the biology and the mechanism of action of these cells are described.

Biology of Regulatory T Cells

Tregs are lymphocytes that have a physiological role in the modulation of the immune response. Specifically, these cells prevent autoimmunity by inhibiting autoreactive effector T lymphocytes. Systemic depletion of Tregs has been associated with a wide variety of autoimmune diseases in murine models as well as in humans (25-31). In the context of cancer, the presence of Tregs is believed to maintain a lack of immune rejection of neoplastic cells in many malignancies including colorectal (32, 33), esophageal (34), gastric (34), pancreatic (35), breast (36-39), lung (39-42) and ovarian (42) tumors. Therefore, the precise understanding of the modus operandi of Tregs has potential therapeutic implications that should be explored.

Essentially, Tregs are distinguished from other lymphocytes by several characteristics. First, instead of being induced *de novo* from naïve T cells upon antigen exposure, Treg development takes place in the thymus. These cells leave the

Correspondence to: Maciej S. Lesniak, MD, Section of Neurosurgery, The University of Chicago Pritzker School of Medicine, 5841 South Maryland Ave, MC 3026, Chicago, IL 60637, U.S.A. Tel: +773 834 4757, Fax: +773 834 2608, e-mail: mlesniak@surgery.bsd.uchicago.edu

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thymus as a mature and distinct population with a defined functional phenotype (43). Moreover, these cells express IL 2 receptor α chain (IL-2R α chain or CD25) and are dependent on stimulation with IL-2 for their expansion and function (25, 44, 45). Consistent with this fact, mice that are deficient in IL-2, IL-2R, or signal transducer and activator of transcription 5 (STAT5) have a marked reduction in Tregs and subsequently develop autoimmunity (44, 46-50). The majority of the IL-2 needed for the activation of these cells is secreted by other T cells. Thereby, the presence of effector T cells and the resultant IL-2 secretion activate Tregs that subsequently modulate the activity of reactive cells. This feedback mechanism has been proposed to be constantly limiting the autoimmune response (reviewed by Sakaguchi in (51)). Tregs specifically express forkhead box P3 (FoxP3), a transcription factor that plays a key role in the definition of their phenotype (52-54). FoxP3 appears to be distinctive for Tregs as it is expressed in $CD4^+$ $CD25^+$ T cells and $CD4^+$ $CD25^+$ CD8- thymocytes whereas it is not found in other thymic cells, T cells, B cells, natural killer or natural killer T cells (52, 53). FoxP3-deficient mice fail to develop Tregs and die from inflammatory diseases that can be abrogated by the transfer of these cells from naïve mice. Similarly, in the case of humans, a syndrome characterized by immune dysregulation, diabetes mellitus type I, thyroiditis, inflammatory bowel disease, and allergies is associated with mutations on the FoxP3 gene (55-57). Finally, Tregs are capable of suppressing the proliferation and action of other T cells. FoxP3 and CD25 are reliable and constant markers that have served to isolate and characterize Tregs.

Recent studies have described the existence of other populations of T regulatory lymphocytes which unlike classic $CD4^+$ $CD25^+$ Tregs, are induced in the periphery *via* T cell receptor (TCR)-MHC/peptide stimulation. At least three populations of peripherally induced $CD4^+$ regulatory T cells have been described, Tr1 cells, Th3 cells, and iTregs, which differ in their genesis, their suppressive mechanisms, and their respective FoxP3 expression. Tr1 cells are induced in the periphery in a process that is dependent on IL-10 (58) and interactions with immature dendritic cells (DC) (which lack the expression of co-stimulatory molecules). Tr1 cells are characterized by the expression of $CD4^+$ $CD25^{int/high}$ and mediate suppression by secreting large amounts of IL-10. In contrast to Tregs, FoxP3 is not constitutively expressed in Tr1 cells (59). Th3 cells are induced in the periphery through a TGF-beta dependent process, and these cells require IL-10 for expansion. Th3 cells suppress *via* the secretion of transforming growth factor beta (TGF-beta) (60). Both Tr1 and Th3 cells are implicated in oral tolerance (61). With respect to their role in neoplasia, these cells constitute part of the tumor infiltrating lymphocytes from gastric cancer patients (34, 62). Lastly, iTreg are induced from $CD4^+$ $CD25^-$ T cells upon the exposure to the suppressive cytokine milieu at the tumor site. These cells express high levels of FoxP3 and can suppress *via* both cell-cell contact and soluble factors. iTreg can also be induced *via* interactions with Tregs (63).

While there are multiple populations of regulatory T cells with distinct suppressive mechanisms, this review will focus on Tregs, characterized by co-expression of CD4, CD25 and FoxP3. The precise means by which Tregs inhibit effector T cells has not been fully elucidated. Some evidence has suggested the implication of cell-cell contact, with a significant contribution of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (64-67) and membrane-bound TGF-beta (68, 69). In addition, heme oxygenase-1 (HO-1), a rate-limiting enzyme in heme catabolism, seems to play a role in the immune suppressive phenotype of Tregs. HO-1 is constitutively expressed in human $CD4^+$ $CD25^+$ Tregs (70) and its expression is induced by FoxP3 (71). HO-1 suppresses T cell proliferation through the production of carbon monoxide (72, 73).

Regulatory T Cells in Glioma

Our group has found tumor infiltration of Tregs in patients with glioblastoma multiforme (GBM) (17). The expression of $CD4^+$ $CD25^+$ $FoxP3^+$ T cells was significantly higher in patients with GBM than in controls, with a mean of 24.7% of Tregs among the glioma-infiltrating lymphocytes, whereas these cells were absent from control brain specimens ($p<0.01$). Moreover, higher levels of FoxP3 expression in the $CD4^+$ $CD25^+$ cells were observed in regulatory T cells isolated from the tumor tissue (55.1%) in comparison to autologous patient blood (33.4%) and blood from control individuals (15.6%) ($p<0.01$). In an *in vitro* suppression assay Tregs inhibited T cell proliferation in a dose-dependent manner. Among various markers analyzed, the expression of CD62L and CTLA-4 was elevated in the glioma Tregs in comparison to that of the controls.

At the same time, Fecci *et al*., (20) found that whereas the absolute counts of both $CD4^+$ T cells and $CD4^+$ $CD25^+$ $FoxP3^+$ $CD45RO^+$ Tregs were greatly diminished in the peripheral pool of patients with malignant glioma, the Treg fraction was increased in the remaining CD4 compartment in 5 out of the 8 patients evaluated (Figure 1). The proportion of Tregs in the peripheral blood of patients with GBM was 2.63 times higher than that found in the blood of normal volunteers ($p=0.004$). Interestingly, their experiments suggested that despite the reduction in their total number, the increased Treg fraction ($p=0.0003$ *versus* controls) was sufficient to elicit the characteristic impairment of T-cell responsiveness *in vitro* (Figure 2). The patients with an elevated Treg fraction showed significant $CD4^+$ T cell proliferative dysfunction ($p<0.0001$), whereas the patients without Treg elevation possessed $CD4^+$ T cells that proliferated at normal levels. After Treg depletion *in vitro*, T cells from the patients bearing malignant gliomas regained their function and proliferated to levels equivalent to those of normal controls (20).

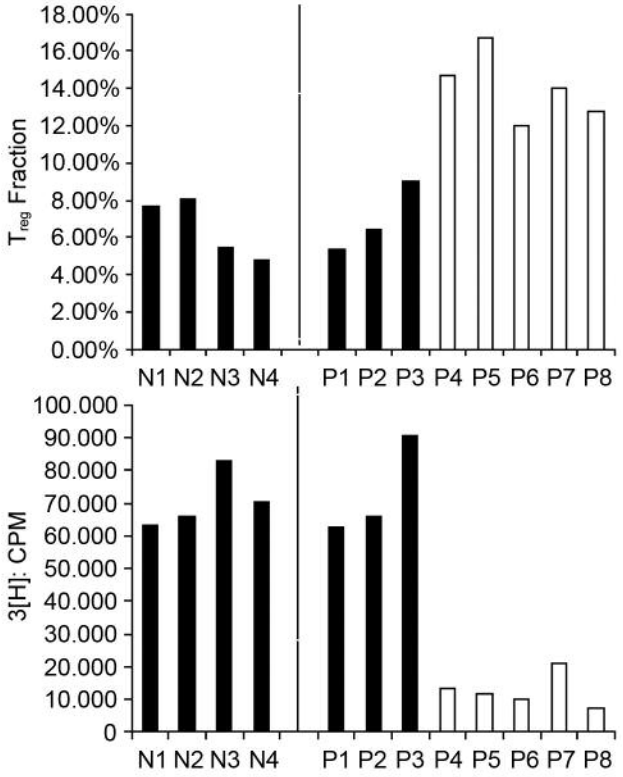

Figure 1. *The fraction of Tregs (top) and the proliferation levels of CD4+ cells (bottom) in eight patients with GBM (P1 to P8) and four healthy individuals (N1 to N4) who underwent leukophoresis. Of note, the 5 patients with an increased Treg fraction had a poor CD4+ lymphocyte proliferation (white columns). 3[H]: CPM (counts per minute). Reprinted with permission from (20).*

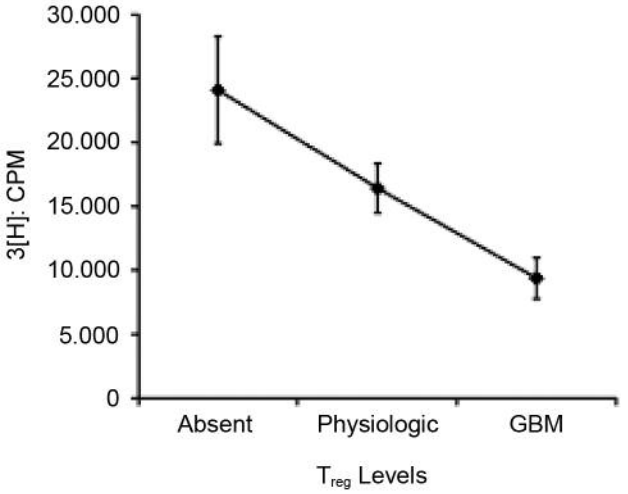

Figure 2. *The increased Treg fraction in 5 out of the 8 patients with GBM is associated with a proliferative impairment of CD4+ lymphocytes from healthy human donors. For this experiment Tregs from GBM bearing patients and CD4+ cells from healthy controls were isolated and mixed to a final fraction of an absence of Tregs (0%), physiological fraction of Tregs (8%), or the Treg fraction observed in patients with GBM (16%). The cells were stimulated with anti-CD3. Increases in the Treg fraction to those levels found in patients with GBM were sufficient to produce significant deficits in the ability of T cells to proliferate. Reprinted with permission from (20).*

More recently, our group has found a correlation between glioma Treg infiltration and tumor grade (Figure 3) (74). In this study, the correlation between FoxP3 and HO-1 was investigated. The highest level of FoxP3 expression was found in patients with grade IV tumors (11.54%) *versus* grade III (6.74%) or grade II (2.53%) ($p<0.05$). Moreover, in grade IV tumors, the frequency of HO-1 mRNA expression in $CD4^+$ $CD25^+$ cells was 11.8±2.45% *vs.* 7.42±0.31% in grade III and 2.33±0.12% in grade II. HO-1 has been shown to accumulate during glioma progression, and apparently, it plays a role in FoxP3 mediated immune suppression. Tumor infiltrating Tregs stained positively with anti-HO-1 antibody and the expression of HO-1 correlated with $CD4^+$ $CD25^+$ $FoxP3^+$ infiltration (r=0.966). These results suggested that the induction of HO-1 mRNA expression is linked to the expression of FoxP3 in $CD4^+$ $CD25^+$ glioma infiltrating Tregs. Collectively, this data supports the notion that HO-1 is a key suppressive factor for regulatory T-cells during the growth of malignant brain tumors (74).

Chemokines: Mediators of Tumor Infiltration by Tregs

Chemokines are a series of soluble peptides that have been implicated in various processes including angiogenesis and CNS development. Of utmost interest, chemokines play a central role within the immune system, as the secretion of these molecules leads to "chemotactic" migration of leucocytes (75-77). The vast variety of known chemokines are classified according to their cystin motifs (C), and accordingly, different families for these receptors such CXC, CC, C and CX3C have been described (78). Binding of specific chemokines to their cognate receptors, which are coupled to G proteins, promotes distinct chemotactic effects depending on the leucocyte population and chemokine receptor expression patterns. Chemokines appear to play a significant role in various human diseases including cancer (79-82). Since it is known that chronic inflammation can predispose to cancer formation and progression, it is suspected that the expression of chemokines could contribute to this process (reviewed by Rollins in (79)). On the other hand, chemokines might elicit an intrinsic effect on tumor cells. For instance, multiple human cancers including leukemias, lymphomas, gliomas and various epithelial carcinomas express CXC receptor 4 (CXCR4) and respond to its

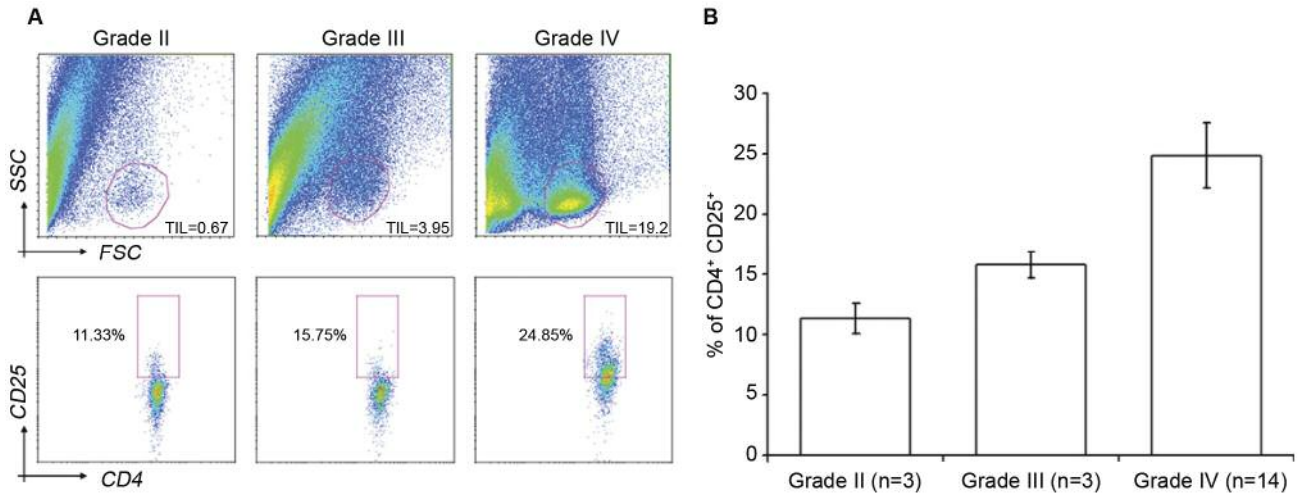

Figure 3. *Frequency of tumor infiltrating lymphocytes in patients with astrocytoma (grade II–IV). (A) Tregs infiltration in human astrocytoma was examined by flow cytometry analysis of CD4 and CD25 expression of electronically gated lymphocytes. (B) shows the frequency of Tregs with grade of disease. The mean frequency of regulatory T-cells was 11.33±1.52% (range: 10.46 –13.5%) in grade II, 15.75±1.5% (range: 14.88–17.5%) in grade III, and 24.85±2.67% (range: 20–28.4%) in grade IV glioma tumors ($p<0.02$) in total $CD3^+$ T-cells. TIL (tumor infiltrating lymphocytes). Reprinted with permission from (74). SSC (Site scatter), FSC (Forward scatter).*

ligand CXC ligand 12 (CXCL12). This ligand-receptor interaction promotes the migration and metastatic establishment of tumor cells (82).

With regards to the migration of Tregs into tumors, there is data suggesting that cancers express a series of chemokines that promote the infiltration by these regulatory lymphocytes. For instance, chemokine CCL22 promotes the migration of Tregs into prostate and ovarian carcinomas (83, 84). Human gliomas express chemokines CCL2 and CCL22, and secrete CCL2. This has been investigated in the human glioma cell lines D-54, U-87, U-251, and LN-229 as well as in tumor cells from eight patients with GBM. Interestingly, the Tregs from these brain tumor patients had significantly higher expression of the CCL2 receptor CCR4 than the Tregs from healthy controls. Migration experiments have suggested that Treg migration is mediated by CCL2 and CCL22. Moreover, this migration was blocked by antibodies to the chemokine receptors CCR2 and CCR4 (85).

Future Perspectives: Therapeutic Ablation of Tregs

Ideally, the ablation of Tregs could lead to an effective immune response against gliomas. This rational is supported by two facts. Firstly, effective anti-tumoral responses documented in anti-glioma immunotherapies suggest an important role for T cells. Secondly, the main targets of Tregs's suppressive features are also T cells.

Anti-CD25 antibodies. Different alternatives to neutralize Tregs are being explored. The use of an anti-CD25 antibody is an illustrative example of this principle (16, 21, 22). Consistent with findings in human patients with gliomas (86), in addition to CD4 lymphopenia, the Treg fraction is increased in glioma-bearing mice (16, 87). To evaluate the role of Tregs in tumor development, our group has tested an anti-CD25 antibody in a murine model for glioma, where tumors were established by intracranial implantation of the cell line GL261. The tumor-infiltrating lymphocytes isolated from mice with GL261 tumors were found to have a significant increase in Tregs compared with the control animals ($p<0.05$). The animals injected with anti-CD25 antibody exhibited a decrease in Tregs ($CD4^+$ $CD25^+$) and lived significantly longer than the untreated tumor-bearing control animals (Figure 4) ($p<0.05$).

Fecci *et al*., have also found that the anti-CD25 antibody is beneficial for the treatment of experimental brain tumors (21). Consistent with their findings in human patients with gliomas, in addition to CD4 lymphopenia, the Treg fraction was increased in glioma-bearing mice, but systemic anti-CD25 administration failed to completely eliminate Tregs, reducing their number only moderately. Nonetheless, the suppressive function of the Tregs decreased leading to enhanced lymphocyte proliferative and interferon gamma (IFN-gamma) responses and up to 80% specific lysis of glioma cell targets *in vitro* (21).

Targeting by TLR ligands. Toll-like receptors (TLR) are interesting molecules in the context of tumor immunity. Specifically, stimulation of TLR9 by DNA containing CpG sequences has been shown to elicit an effective anti-tumor

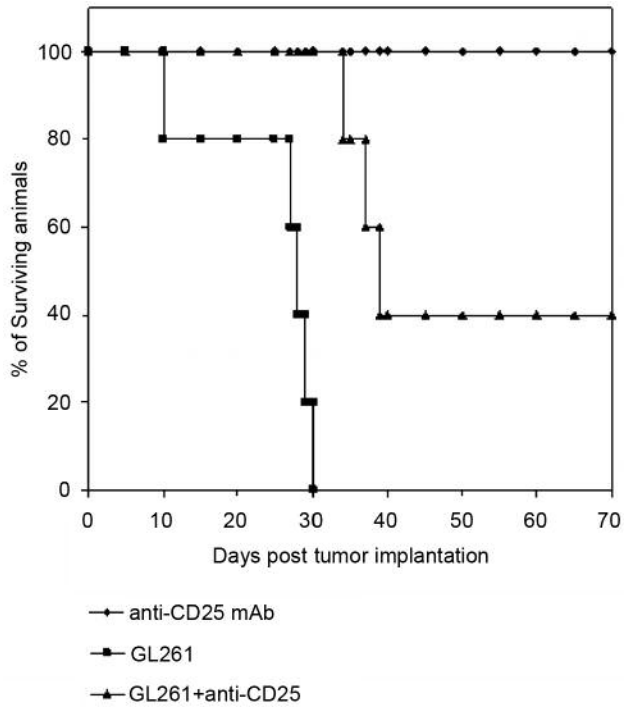

Figure 4. *Prolongation of survival of mice with experimental GL261 gliomas after treatment with anti CD25 antibody is represented on the Kaplan-Meier plot. Median survival was increased from 27 to 40 days. 40% of the mice treated with the anti CD25 antibody remained long-term survivors (>70 days) (p<0.005). Reprinted with permission from (16).*

response for various neoplasias including experimental brain tumors (19, 88-92). With regard to Tregs, some evidence suggests the possibility of neutralizing their effects by stimulating TLRs. For instance, one study has described the reversal of Treg suppressive function by stimulation with synthetic or natural ligands for human TLR8 (93). Interestingly, in this case, the effect was independent of DC, but required functional TLR8, MyD88 and IRAK4 (molecules implicated in the intracellular signaling pathway for various TLRs) signaling in the Treg cells. Most importantly, the transfer of TLR8 ligand-stimulated Tregs cells into tumor-bearing mice led to the enhancement of anti-tumor immunity (93).

Systemic treatment with TLR9 ligands has a deleterious effect on Tregs. We have described apoptosis induction in experimental brain tumors and the prolongation of survival following stimulation of TLR9 with CpGs. Interestingly, in addition to apoptosis, CpG stimulation of murine gliomas enhanced the antigen presenting capacity of microglia, shifted the immune response toward $CD8^+$ T cells, and decreased the number of infiltrating Tregs (19). The mechanism for this phenomenon is not clear, and in this study the extent to which Treg decrease contributed to the therapeutic benefit of CpG was not explored. Nevertheless, this finding supports the possibility of antagonizing Tregs by stimulation of TLRs, an illustrative example of modulation of lymphocyte function by the innate immunity.

Summary

Tregs contribute to the evasion of the immune response required for the development of malignant gliomas. These cells are found infiltrating tumors, and in the blood of patients and animals with gliomas. Compelling evidence suggests that tumoral infiltration by Tregs leads to the suppression of effector T cells, which would otherwise be capable of mounting an immune response against gliomas.

The causative relation of Tregs tumor infiltration and the progression of malignant gliomas is not clearly defined. Nevertheless, the possibility of Treg suppression of the anti-tumor immunity contributing to the development of gliomas is suggested by the correlation of Treg tumor infiltration and tumor grade, and by the fact that these cells are capable of suppressing tumor immunity.

In contrast to the development of other lymphocyte populations, naturally occuring Tregs seem to mature in the thymus rather than in the periphery. An interesting question that remains unanswered is the means by which gliomas can promote the generation of Tregs in the thymus or in the tumor site.

The role of Tregs in the tumor biology of gliomas might be of interest for its potential therapeutic implications. Indeed, animal models have shown evidence of antitumor effects derived from the ablation of Tregs and some preliminary studies suggest than these cells can be targeted by various means. Neutralization with anti-CD25 antibodies or DNA oligonucleotides that stimulate TLR ligands are two examples of such a principle. Further research is needed to investigate the best way to limit the activity of these regulatory cells. The modulation of anti-tumor immunity is a rapidly evolving field within brain tumor biology. This area of research warrants close attention by the professionals who treat patients with such a devastating disease, since a thorough understanding of this process might lead to interesting therapeutic implications in the near future.

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