Review

Clinical Cancer Research

T-cell Dysfunction in Glioblastoma: Applying a New Framework

Karolina I. Woroniecka[1,2], Kristen E. Rhodin[1], Pakawat Chongsathidkiet[1,2], Kristin A. Keith[1], and Peter E. Fecci[1,2]

Abstract

A functional, replete T-cell repertoire is an integral component to adequate immune surveillance and to the initiation and maintenance of productive antitumor immune responses. Glioblastoma (GBM), however, is particularly adept at sabotaging antitumor immunity, eliciting severe T-cell dysfunction that is both qualitative and quantitative. Understanding and countering such dysfunction are among the keys to harnessing the otherwise stark potential of anticancer immune-based therapies. Although T-cell dysfunction in GBM has been long described, newer immunologic frameworks now exist for reclassifying T-cell deficits in a manner that better permits their study and reversal. Herein, we divide and discuss the various T-cell deficits elicited by GBM within the context of the five relevant categories: senescence, tolerance, anergy, exhaustion, and ignorance. Categorization is appropriately made according to the molecular bases of dysfunction. Likewise, we review the mechanisms by which GBM elicits each mode of T-cell dysfunction and discuss the emerging immunotherapeutic strategies designed to overcome them. *Clin Cancer Res; 24(16); 3792–802. ©2018 AACR.*

Introduction

For more than a century, many have advanced an intimate role for the immune system in restricting cancer development. As early as 1909, Paul Erlich stipulated the actuality of "immune surveillance," proposing that aberrant cells continuously arise during growth and development in a manner that would ultimately result in an enormous frequency of cancers if not for the host's immunologic defense mechanisms (1). Conversely, Erlich postulated that cancer instead emerges when these aberrant cells outstrip and escape normal immune-surveillance function, winning the metaphoric tug-of-war. More recently, the term "immunoediting" has been commonly applied to describe this delicate tug-of-war between tumor elimination and immune escape (2).

In order to promote tumor survival and favor immune escape, tumor cells frequently hijack a host's evolved immunoregulatory mechanisms. Glioblastoma (GBM), the most common primary malignant brain tumor, is a notoriously capable immune evader and is among the most immunosuppressive of solid tumors despite confinement to the intracranial compartment (2). GBM remains universally lethal, with a median survival of 15 to 17 months following diagnosis, and immunotherapies have demonstrated only limited success (3). Although the intracranial environment (4) certainly contributes restrictions to effective antitumor immunity, the tumor itself exhibits vast capacities for immune subterfuge, provoking severe cellular and humoral immune deficits that have been catalogued for more than 40 years (5). Immunosuppressive mechanisms run the gamut, affecting both local and systemic immunity, and are extensively reviewed (2, 6, 7). Ultimately, tumor-imposed immunosuppression is often aimed at crippling the effector arm of the cellular immune response, therefore conjuring various modes of T-cell dysfunction. The elicited insults of T-cell function have historically been categorized quite simply as either quantitative or qualitative deficiencies. In the context of this division, quantitative deficits (i.e., lymphopenia) have been appreciated in malignant gliomas dating back to 1977, albeit without a characterized source (8). Qualitative deficits, in turn, have also been highlighted since the 1970s, arising when patients with primary intracranial tumors were first recognized to have defects in rosette-forming T cells (9). Since these early landmark studies by Brooks and Roszman, a wide variety of T-cell deficiencies have been reported but have often been placed under the single, all-inclusive label of "anergy."

It is now clear, that the label of anergy is neither sufficient nor accurate for properly describing T-cell dysfunction in GBM, or more broadly, in cancer. Without an accurate description or understanding of the mechanisms underlying tumor-induced T-cell dysfunction, strategies for countering immune escape will be poorly informed and likely ill-fated. To date, many of the labels applied in the literature are frequently confused or incorrectly interchanged. The goal of this review, then, will be to reassign long-observed T-cell dysfunction in GBM into the appropriate categories: senescence, tolerance, anergy, exhaustion, and ignorance.

Senescence

T-cell senescence is a hypofunctional state resulting from shortened telomeres (Fig. 1). Excessive telomere erosion arises through two primary mechanisms: chronic proliferative activity (as seen in

[1]Duke Brain Tumor Immunotherapy Program, Department of Neurosurgery, Duke University Medical Center, Durham, North Carolina. [2]Department of Pathology, Duke University Medical Center, Durham, North Carolina.

Corresponding Author: Peter E. Fecci, Duke Brain Tumor Immunotherapy Program, Department of Neurosurgery, Duke University Medical Center, Box 3050, Durham, NC 27710. Phone: 919-684-8111; Fax: 919-684-9045; E-mail: peter.fecci@duke.edu

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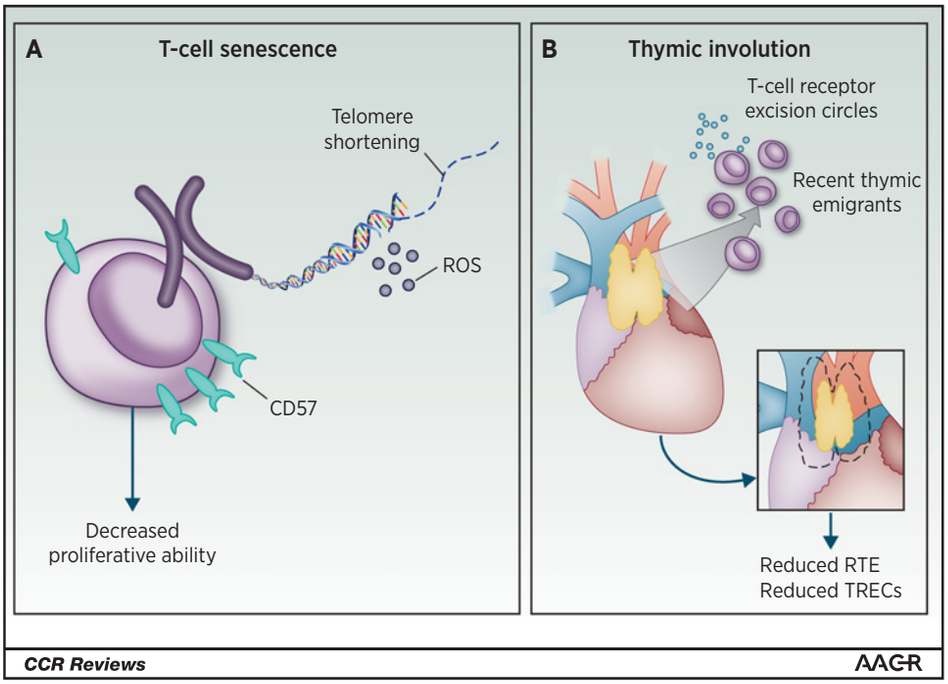

Figure 1.
Senescence. A, T-cell senescence results from telomere shortening as a result of T-cell proliferation/activation or through DNA damage, for example, exposure to reactive oxygen species (ROS). CD57 serves as a marker for senescent T cells. **B,** Thymic involution, or thymic shrinkage, occurs with age and is prominent in GBM, as evidenced by reduced recent thymic emigrants (RTE) and T-cell receptor excision circles (TREC). Redrawn from an illustration by Megan Llewellyn, MSMI; copyright Duke University with permission under a CC-BY 4.0 license.

chronic inflammatory states and malignancy) and DNA damage resulting from increased production of reactive oxygen species (ROS; ref. 10). Leonard Hayflick initially described cell senescence in 1961, when he demonstrated that fetal cells are limited to between 40 and 60 cellular divisions before entering a state of terminal nondivision (11). The phenomenon of senescence reflects the "end replication problem" (12), or the shortening of telomeres with each cell division. Once telomeres shorten beyond a threshold, further cell replication is prohibited. Some cells, however, express telomerase, an enzyme capable of reforming or extending telomeres. Telomerase activity becomes quite pertinent in the context of malignancy, as cancer cells (including GBM cells) may upregulate telomerase, thereby permitting tumor cells to specifically resist senescence (13, 14). Immune cells, however, have no such capacity, and may instead be predisposed to more rapid telomere shortening and a senescent state in the context of tumor-induced inflammation.

In human $CD4^+$ and $CD8^+$ T cells, telomere shortening appears to be the consequence of T-cell stimulation. This is perhaps best illustrated in young patients with X-linked lymphoproliferative syndrome (XLP), a disease hallmarked by excessive T-cell stimulation, in which young patients demonstrate shortened telomere lengths traditionally seen at a more advanced age (15). Telomere shortening is also seen in the states of chronic infection, such as HIV, (16) and in chronic inflammatory states (17), as often seen with cancer. Whether T cells in patients with GBM demonstrate decreased telomere lengths and corresponding senescent states remains an active area of investigation.

Phenotypic indicators of T-cell senescence include CD57, a well-known marker of terminal differentiation in human T cells (18), as well as loss of the costimulatory molecules CD27 and CD28 (19). These changes correlate with critical telomere shortening and loss of telomerase activity. $CD57^+CD27^+$ T cells have recently been categorized as incompletely differentiated tumor-infiltrating lymphocytes (TIL), which maintain the ability to proliferate after T-cell receptor (TCR) stimulation but become senescent with further antigenic exposure (20). In GBM, immunosenescence of the $CD4^+$ compartment has been correlated with poor prognosis: Overall survival is significantly shorter in GBM patients with higher levels of $CD4^+CD28^-CD57^+$ T cells (21).

Immunosenescence, albeit not specifically T-cell senescence, is perhaps also reflected in thymic senescence, a mode of dysfunction characterized by involution of the thymus. Thymic involution is a natural byproduct of aging but also accompanies states of chronic inflammation such as those seen with obesity, viral infection, and malignancy (22). It inevitably leads to a decrease in the output of immature T cells, also termed "recent thymic emigrants" (RTE; ref. 23). Decreases in $CD8^+$ RTE may be a component to the well-recognized, negative association between patient age and GBM prognosis (24). One study advancing this association quantified RTE by measuring T-cell receptor excision circles (TREC) in the peripheral blood of 24 newly diagnosed and 18 recurrent GBM patients. TRECs are circular DNA molecules generated during TCR rearrangement in the thymus. The presence of TRECs in blood is a clear indicator that TCR rearrangement has occurred and is, therefore, considered a reliable tool for tracking and quantifying RTE as a surrogate of thymic activity (25). As an extension, the absence of detectable TREC can serve as a marker of thymic senescence. The aforementioned study showed that within patients with GBM (comparisons to controls were never made), TREC levels correlated with the clinical outcome of GBM better than did patient age, with lower TREC levels predicting poorer clinical outcomes. In addition, numbers of RTE maintained a stronger correlation with predicted clinical outcomes in vaccinated GBM patients than did immunologic parameters, such as IFNγ production (24). These findings were corroborated preclinically in studies that demonstrated decreased thymic function and decreased output of RTEs in murine models of intracranial glioma (26). In these murine models of glioma, thymic atrophy appeared to be secondary to increased Notch-1 and Jagged-1 signaling, resulting in the induction of apoptosis of thymocytes (27).

Overall, however, T-cell senescence remains the most poorly studied mode of T-cell dysfunction in GBM. The contribution of T-cell telomere length as well as detailed studies into the restrictions for immunotherapy posed by thymic senescence in an already aged population remain areas ripe for further investigative advances. Continued characterization and opportunities for therapeutic intervention are desired.

Tolerance

Immune tolerance is the physiologic mechanism for preventing aberrant autoimmunity through the programmed induction of T-cell unresponsiveness (28). Malignancies such as GBM, which overwhelmingly consist of misexpressed self-antigens, can usurp physiologic tolerizing mechanisms to circumvent the antitumor immune response. Physiologic tolerance occurs and is enforced either centrally or peripherally. Central tolerance encompasses the process of negative selection during T-cell development prior to the final maturation and circulation of T cells. Negative selection occurs in the thymus, where developing T cells expressing TCRs with overly high affinity for self-antigen/MHC complexes are necessarily eliminated (29). The process is not exhaustive, however, and self-reactive T cells, particularly those possessing specificity for organ-specific antigens not presented in the thymus, have the potential to elude elimination and gain access to the peripheral circulation (30). As a result, numerous mechanisms for the peripheral enforcement of tolerance have evolved to prevent continuous T-cell self-reactivity and autoimmunity (Fig. 2). Modes of peripheral T-cell tolerance include peripheral deletion (31), suppression by regulatory T cells (Treg; ref. 32), and the activation of imprinted programs forcing T cells into a hyporesponsive state (33, 34). GBM exhibits the noteworthy capacity to usurp each of these tolerizing mechanisms, preventing an effective antitumor response. Understanding the molecular mechanisms underlying such subterfuge will permit future strategies for breaking T-cell tolerance to cancer antigens while avoiding concomitant autoimmune damage.

Peripheral T-cell deletion. The most obvious method for evading T cells is perhaps to eliminate them, a capacity recognized in GBM dating to the late 1990s. First described in melanoma, this mechanism for eliciting T-cell apoptosis involves a FasL-mediated deletion of invading lymphocytes (35), which has subsequently been described in GBM (36, 37). Both $CD4^+$ and $CD8^+$ T cells demonstrate increased susceptibility to apoptosis in patients with GBM, with those T cells expressing FasL having significantly increased susceptibility (38). Indeed, one study revealed that 22.6% of GBM TILs are in the early stages of apoptosis, and less than 50% of the TILs present are even viable. It is important to note that deletion is also the final stage of T-cell exhaustion (39), and, therefore, exhaustion (discussed later) may have unwittingly contributed significantly to the observations of early apoptosis in this study.

Tregs. Tregs contribute substantially to peripheral tolerance by suppressing T-cell antigen–specific responses. Tregs are a subset of $CD4^+$ T cells expressing the transcription factor Foxp3 (40). In a tumor setting, Tregs potently suppress antitumor responses and promote tolerance through secretion of the Th2-polarizing immunoregulatory cytokines TGFβ and IL10 (41). These, in turn, limit T-cell IL2 and IFNγ production (42), resulting in impotence and even cytolysis of the effector cells necessary for the control and limitation of tumor growth (43). Patients with GBM demonstrate increased proportions of Tregs among $CD4^+$ cells, both systemically and at the tumor site, contributing to the decreased cellular immunity observed in these patients (44, 45). Likewise, countering or depleting Tregs has proven capable of restoring much of the T-cell dysfunction that has been reported for decades in patients (44, 46). This makes strategies to inhibit Tregs attractive in GBM and identifies tumor-induced tolerance as a key mode of T-cell dysfunction in the context of these tumors.

Broadly, there are two classifications of Tregs: natural Tregs (nTreg) and induced Tregs (iTreg). nTregs are selected in the thymus for their moderate affinity for self-antigen in the context

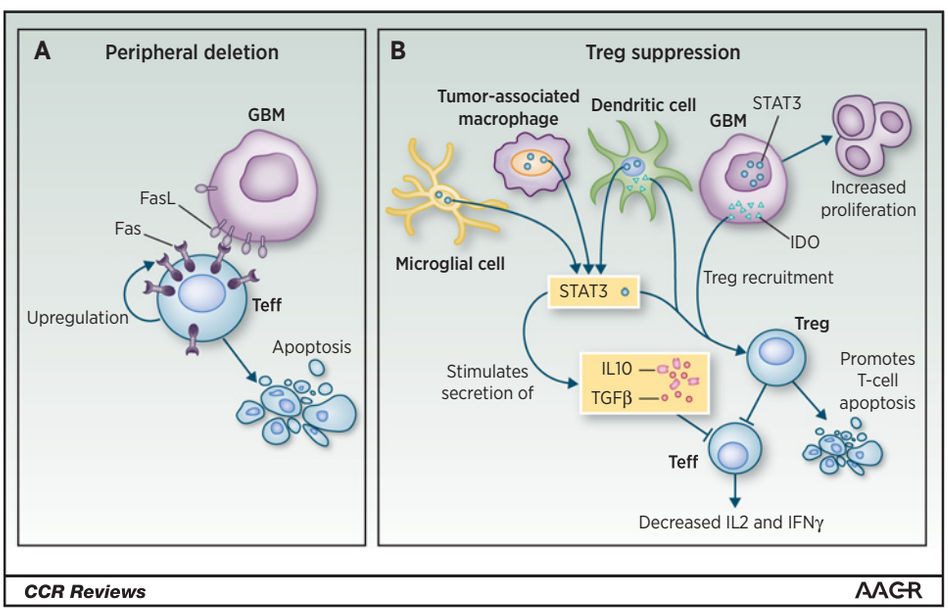

Figure 2.
Tolerance. A, Peripheral deletion is a form of peripheral tolerance. Peripheral deletion in GBM is accomplished through FasL-mediated apoptosis. **B,** Regulatory T cells (Treg) induce immunosuppressive effects both peripherally and at the tumor site in GBM. STAT3 and IDO both modulate Treg function, resulting in further immunosuppression. Redrawn from an illustration by Megan Llewellyn, MSMI; copyright Duke University (Durham, NC) with permission under a CC-BY 4.0 license. Teff, effector T cell.

of MHC class II, and they play a pivotal role in maintaining immune homeostasis. iTregs are otherwise responder $CD4^+$ T cells that acquire both CD25 and Foxp3 expression outside of the thymus in subimmunogenic contexts of inflammation, autoimmunity, transplantation, or malignancy (47). Studies have shown nTregs to be the predominant population responsible for immunotherapeutic failure, as thymectomized mice have significantly decreased numbers of tumor-infiltrating Tregs (48). In addition, the transcription factor *Helios* is expressed in nTregs, but not iTregs (49). *Helios*$^+$ Tregs have been shown to have a higher suppressive capability and predominate in human GBM (48).

GBM promotes expansion of Treg representation and function through a variety of mechanisms. GBM-conditioned media induces the *in vitro* expansion of Tregs (50), suggesting a direct role for tumor-elaborated factors. Less directly, the expression of indoleamine 2,3-dioxygenase (IDO; discussed further below) by dendritic cells (DC) in tumor-draining lymph nodes has been implicated in inducing antitumor tolerance via the induction and recruitment of Tregs (51). The upregulation of CCL-2, a Treg chemokine, is also commonly seen in patients with GBM (52). Likewise, T-cell immunoglobulin and mucin domain–containing molecule 4 (TIM4), a molecule with a newly described role in immune regulation, is expressed in GBM-derived macrophages. TIM4-expressing macrophages phagocytose tumor-specific T cells expressing phosphatidylserine (PS) and develop tolerogenic expression of aldehyde dehydrogenase and TGFβ, resulting in the induction of Tregs within the tumor microenvironment (53). The pleiotropy of the systemic and infiltrating Treg population found within GBM suggests similar variability in the mechanisms employed by GBM to expand Treg-tolerizing capacities. Likewise, treatments designed to counter Treg activity will need to be equally varied in their mechanistic targets.

Simple depletion of Tregs has been the most straightforward and frequently attempted counter to Treg activity in tumors. It has been accomplished to date through several means, including anti-CD25–denileukin diftitox (an engineered protein combining IL2 with diphtheria toxin), anti–CTLA-4, and anti-GITR. The high-affinity IL2 receptor (IL2R or CD25) has been a classic target due to its constitutive expression on Tregs. Studies in mice have shown success inhibiting Treg function with anti-CD25 (46) as well as prolonging survival in murine models of glioma (46, 54). Clinical trials of patients with metastatic melanoma have demonstrated efficacy for the anti-IL2R mAb daclizumab (55), and thus far in patients with GBM, a placebo-controlled pilot study (NCT00626015) has shown that administration of daclizumab with a peptide vaccine against EGFRvIII and with temozolomide selectively depletes Tregs (56).

Glucocorticoid-induced TNFR-related protein (GITR) is a receptor enriched on Tregs, which when activated, inhibits Tregs (57). Intracranial delivery of an agonistic anti-GITR antibody results in a significant increase in survival in mice bearing GL261 gliomas, whereas peripheral administration of the antibody has only a modest effect (58). Although peripheral administration of anti-GITR significantly decreased granzyme B expression by Tregs, intratumoral administration results in selective depletion of Tregs via FcγR-mediated destruction (58).

Cytotoxic T lymphocyte associated molecule 4 (CTLA-4) is an immune checkpoint (discussed at length below) that additionally contributes to the suppressor function of Tregs (59). CTLA-4 loss or inhibition on Tregs results in reduced Treg function and may be a benefit associated with anti–CTLA-4 treatment. Indeed, recent studies showed that anti–CTLA-4 mAb results in loss of intratumoral Tregs, along with expansion of $CD8^+$ effector T cells (Teff), leading to an enhanced Teff:Treg ratio (60).

Ultimately, targeting Tregs through the above mechanisms has proven successful in small trials as a means of reversing T-cell tolerance and licensing the antitumor immune response. Developing future methods for inhibiting Treg function in GBM may play a key role in targeted immunotherapy.

STAT3. STAT3 is a transcription factor that plays a significant and pleiotropic role in both oncogenesis and immunosuppression in GBM. It is often upregulated in tumor cells and is a recognized negative prognostic factor (61). Within GBM cells, increased STAT3 expression or activity promotes tumor survival, proliferation, and invasion (62, 63).

Ultimately, STAT3 activation proves crucial for tumor-induced immune tolerance and immune evasion within the GBM microenvironment (64, 65). For instance, IL2-mediated STAT3 activity expands tumor-associated Tregs, enhancing the expression of Foxp3 in $CD4^+CD25^+$ T cells (66). STAT3 expression in antigen-presenting cells (APC), such as tumor-associated macrophages or microglia, results in suppression of antitumor mechanisms and tolerance to tumor antigens. STAT3 has been shown to skew effective Th1 responses toward suppressive Th17 responses (67). Inhibiting STAT3 via conditional knockout (66) or via the miRNA miR-124 (68) decreases Treg prevalence while enhancing T-cell mediated clearance of murine glioma. STAT3 inhibition also promotes TIL accumulation at the tumor site in the humanized U87 glioma model (69). Small-molecule STAT3 pathway inhibitors, such as WP1066, demonstrate enhanced activation of T cells and APCs in murine models, with accompanying increases to production of immune-stimulatory cytokines and to T-cell proliferation (70). Given these preclinical data, targeting the STAT3 pathway may present a therapeutic opportunity. A phase I trial investigating WP1066 in patients with either recurrent brain tumors or with melanoma metastatic to the brain is set to begin in the spring of 2018 (NCT01904123).

IDO. IDO, an enzyme produced in response to IFNγ, is involved in the metabolism of tryptophan into kynurenine. IDO-mediated tryptophan degradation and/or kynurenine accumulation has multiple immunosuppressive effects, including inhibition of T-cell proliferation, promotion of T-cell apoptosis, and the induction of Tregs (71). It is highly expressed in human GBM tissue (as compared with low-grade gliomas; ref. 72), and patients with GBM likewise have decreased serum tryptophan levels compared with patients who do not have GBM (65). Furthermore, the increased IDO expression observed in GBM negatively correlates with patient survival (73).

Preclinical studies have further elucidated the role of IDO in GBM, including the role of GBM tumor cell and host IDO expression. GBM-expressed IDO increases Treg accumulation and negatively impacts overall survival in murine glioma models (73) in a manner independent of its canonical enzymatic role (74). Conversely, IDO inhibition in murine GBM cells through siRNA knockdown significantly extends survival (75). In seeming contrast, however, enzymatically active host IDO1 appears instead pivotal for maximal response to immune checkpoint blockade (76). These findings potentially highlight the need for an IDO-

targeting therapeutic that inhibits noncanonical IDO1 activity in GBM cells, without disrupting host IDO1 activity. Multiple IDO1 enzyme or pathway inhibitors are being tested in clinical trials, including epacadostat (Incyte), GDC-0919 (Genentech), PF-06840003 (Pfizer), and indoximod (D1-MT; New Link Genetics), but their ultimate efficacy in GBM remains to be demonstrated.

Anergy

As alluded to earlier, T-cell anergy has been a frequently misapplied term, often serving as a black box for T-cell dysfunction in the GBM and other cancer literature. Broadly, T-cell anergy describes a mechanism by which lymphocytes become perpetually inactive following an antigen encounter (Fig. 3). Anergy was initially described in 1908, when Von Pirquet noted the loss of delayed-type hypersensitivity (DTH) responses to tuberculin in individuals infected with measles (77). The same observation was made later in 1972 in patients with GBM, when they failed to respond to dinitrochlorobenzene (78). Alternatively, in the 1980s, anergy was used to describe the functional inactivation of B cells after tolerance induction with repeated antigen administration (79). Here, the term "clonal anergy" emerged due to the nature of the lost antigen-specific response. Currently, the term anergy is used to describe two separate phenomena: clonal or "*in vitro*" anergy and adaptive tolerance or "*in vivo*" anergy (34). Although different modes of dysfunction, both terms encompass impairments to IL2 production and T-cell proliferation. Ultimately, however, clonal anergy and adaptive tolerance are distinct biochemical states: clonal anergy results primarily from defective costimulation resulting in RAS/MAPK dysfunction (80), whereas adaptive tolerance results from continuous low levels of antigen exposure and deficient Zap70 kinase activity, promoting impaired mobilization of calcium and NF-kB. It is important to note that anergy has primarily been studied in $CD4^+$ T cells. Therefore, although many of its features may overlap with those of tolerance and exhaustion, these latter programs have been studied in more detail in $CD8^+$ T cells, as will be discussed further.

Clonal T-cell anergy has been shown to be a long-lived defect in cell-cycle progression and effector function and, although predominately irreversible, some have reported a degree of correction with strong stimuli. More specifically, anergic T cells produce negligible amounts of IL2, which is crucial for clonal expansion; however, addition of high levels of exogenous IL2 can sometimes reverse the phenotype (81). The source of decreased IL2 production is decreased IL2 transcription, secondary to further defects in the upstream mitogen-activated protein kinase (MAPK) family (82). Diminished IL2 production in patients with GBM was first noted when peripheral blood lymphocytes (PBL) were found to have fewer phytohemagglutinin (PHA)-responsive cells, and these PHA-activated cells produced significantly lower levels of IL2 as compared with healthy controls (83). Subsequently, however, this phenomenon was attributed at least in part to increased Treg activity in patients with GBM (thereby making it more reversible) and is, therefore, perhaps more closely associated with T-cell tolerance than with anergy, the latter term likely then having been misapplied in the study (by the same authors as this review; ref. 44). Although decreased transcription of IL2 in T cells does not appear to be solely related to the program of clonal anergy (as it had been so far perhaps incorrectly defined in GBM), mechanisms for inducing clonal anergy may still be relevant. For instance, tumor-induced CTLA-4 upregulation on T cells results in

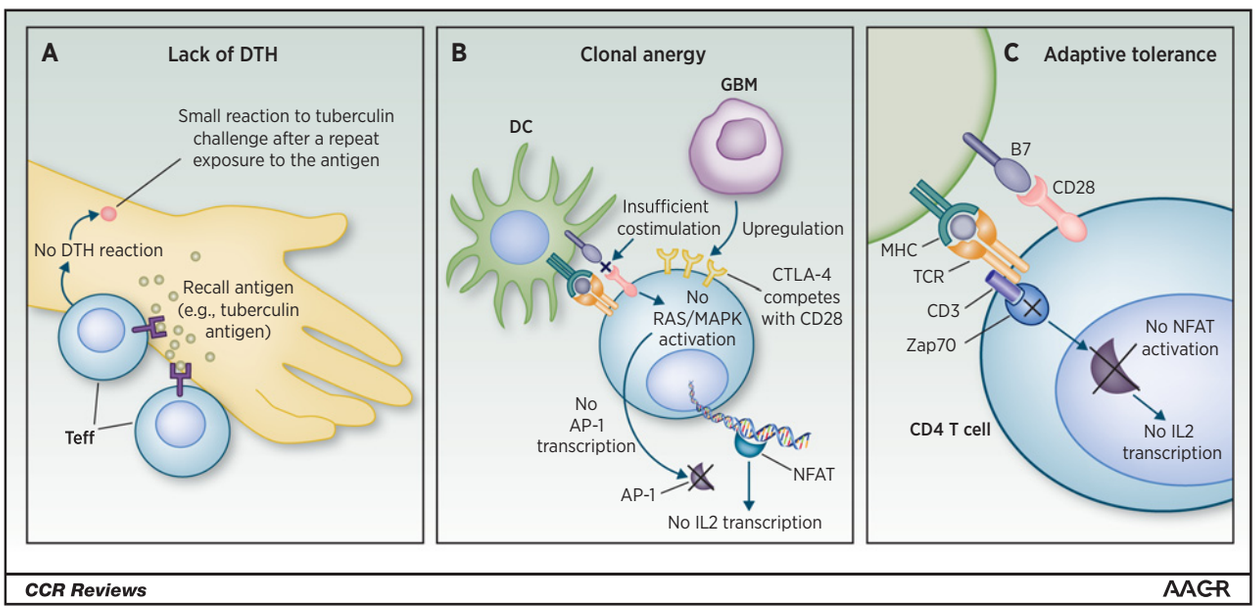

Figure 3.

Anergy. A, Historically, anergy described the lack of delayed-type hypersensitivity (DTH) responses when patients with GBM failed to react to recall antigens. **B,** Clonal or *in vitro* anergy describes a mostly unresponsive state elicited by insufficient costimulation resulting in defective RAS/MAPK activation. Defective RAS/MAPK activation results in decreased AP-1 transcription, preventing T-cell activation. **C,** Adaptive tolerance or *in vivo* anergy results from continuous low levels of antigen exposure, leading to impairments in IL2 production and T-cell proliferation through deficits in Zap70 kinase activity. Defective Zap70 activation results in impaired mobilization of calcium and NF-κB. Redrawn from an illustration by Megan Llewellyn, MSMI; copyright Duke University (Durham, NC) with permission under a CC-BY 4.0 license. NFAT, nuclear factor of activated T cells.

decreased costimulatory signals through CD28. CD28 expression on T cells typically leads to a costimulatory signal upon interaction with APC-expressed CD80 and CD86, resulting in activation and initiation of effector function. High levels of CTLA-4 expression on T cells, however, creates competition with CD28 and results in insufficient costimulation, resulting in loss of T-cell proliferation and function. It remains to be seen whether other mechanisms of clonal anergy play roles in GBM or in other solid tumors, or how these mechanisms might be countered through more appropriate immunotherapeutic design.

The role of adaptive tolerance in GBM and other cancers is less clear at this time, and it may be difficult to truly distinguish from other modes of dysfunction. Defects in the Zap70 kinase have been found to be a key instigator of adaptive tolerance in T cells (80); this same mechanism, however, has also been implicated in T-cell exhaustion (84), which similarly results from continuous low levels of antigen exposure. Likewise, the transcription factor nuclear factor of activated T cells (NFAT), downstream of Zap70, plays a primary role in both exhaustion and adaptive tolerance (85). In a B16 melanoma model, one study showed that T cells from NFAT-1–deficient mice were resistant to tumor-induced anergy, resulting in delayed tumor appearance and slowed tumor growth (86). We propose that the terms "*in vivo* anergy" and "adaptive tolerance" might best be absorbed into the definition of T-cell exhaustion, as the mechanisms of these processes appear to be the same in both $CD4^+$ and $CD8^+$ T cells. Using one term to describe the phenomenon of chronic and, perhaps, suboptimal antigen exposure leading to defects in calcium mobilization via NF-κB and NFAT will facilitate discussion and reviews on this topic, as well as effective targeting strategies.

Exhaustion

T-cell exhaustion is a hyporesponsive (not unresponsive) state resulting from repeated antigenic exposure under suboptimal conditions (Fig. 4; ref. 87). It was initially discovered in $CD8^+$ T cells in the setting of chronic viral infection (39, 87) that the exhausted state serves as an adaptive "stalemate" between host and pathogen meant to limit collateral autoimmune destruction under chronic inflammatory conditions. Cancers, however, have now also been shown to disrupt T-cell function and elicit similar modes of T-cell exhaustion, which assuredly are tumor adaptive (88).

Exhaustion represents a specific transcriptional program in T cells, resulting in a hierarchical loss of effector functions following their initial acquisition in the context of antigenic exposure. Many transcription factors have been implicated in programmed T-cell exhaustion, including T-bet, Eomesodermin (Eomes), and NFAT. T-bet and Eomes are related transcription factors that regulate the process of memory T-cell formation, and the classic LCMV-induced T-cell exhaustion signature reveals inverse functions for T-bet and Eomes (89). Exhausted T cells contain high levels of Eomes and low levels of T-bet, with these two transcription factors differentially regulating T-cell exhaustion in part through direct modulation of inhibitory receptors, such as PD-1 (90). The transcription factor NFAT is involved in both $CD8^+$ T-cell activation and exhaustion. When bound to AP-1, the complex results in differentiation into Teff; however, in the absence of AP-1, NFAT binds regulatory regions and results in the transcription of genes associated with an exhausted state (91). Apart from reduced effector function, this unique transcriptional program and metabolic state is characterized by the increased surface expression of multiple classical, as well as more newly characterized, coinhibitory immune checkpoints (89). The term "immune checkpoint" refers to specific molecular interactions at the interface between T cells and APCs, resulting in restrictions to the proliferative capacity of T cells. Certain T-cell inhibitory receptors, such as PD-1 and CTLA-4 (the so called "classical" immune checkpoints), serve to inhibit clonal T-cell proliferation and are a recognized component to physiologic immune autoregulation. Cancer cells, however, are

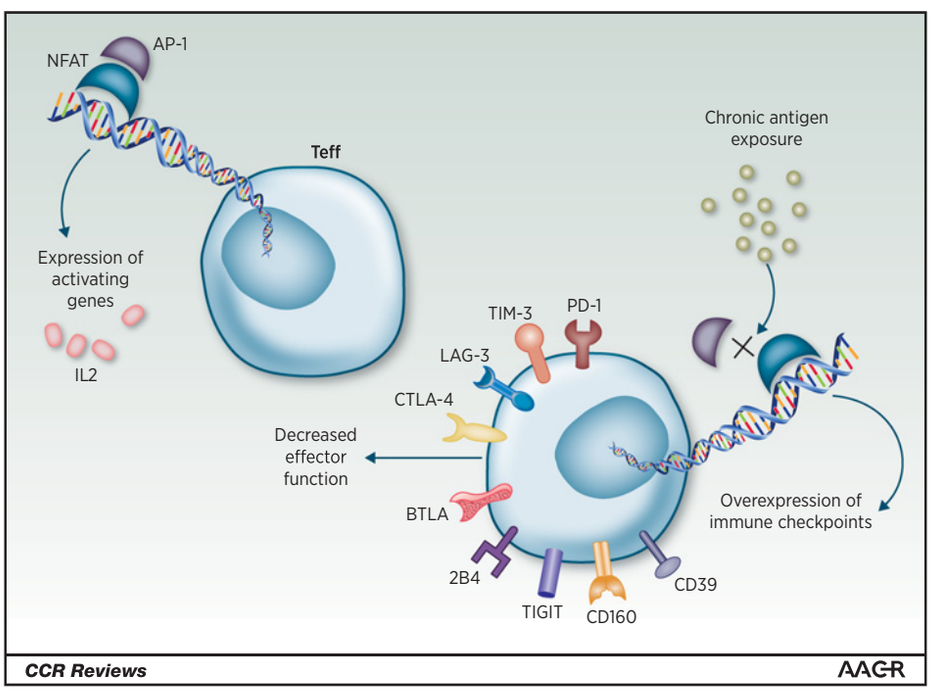

Figure 4.

Exhaustion. Physiologic coupling of NFAT and AP-1 results in expression of activating genes (i.e., IL2). In the course of chronic antigen exposure, failure of NFAT to form a complex with AP-1 leads to expression of inhibitory checkpoints. Redrawn from an illustration by Megan Llewellyn, MSMI; copyright Duke University (Durham, NC) with permission under a CC-BY 4.0 license.

able to usurp this physiologic mechanism and either upregulate or bind inhibitory immune checkpoints on T cells, resulting in their dysfunction. Antibodies blocking immune checkpoints perpetuate the activity of T cells and can, in some instances, even reverse their exhausted phenotype and functional defects (89). Blockade of the classical immune checkpoints, CTLA-4 and PD-1, constitutes an FDA-approved strategy in many solid tumors, whereas clinical trials remain ongoing in GBM (NCT03367715, NCT02311920, NCT02017717, and NCT03233152).

Although PD-1 and CTLA-4 represent the classical immune checkpoints, newer characterized checkpoints with implications for T-cell exhaustion include TIM-3, LAG-3, BTLA, 2B4, CD160, TIGIT, and CD39, among others (92). TIM-3 mediates immune suppression via binding of its ligands, including galectin 9 and CEACAM. Increased TIM-3 expression levels are associated with higher tumor grades and lower Karnofsky Performance Status (KPS) scores in patients with glioma (93). The frequent coexpression of TIM-3 and PD-1 represents a "deeply" exhausted state (94). Human GBM TILs that coexpress PD-1, TIM-3, and LAG-3 are nonfunctional (95). TIM-3 has been targeted preclinically in glioma, generally in combinatorial strategies. One such study blocked TIM-3 alone and in combination with a blocking agent to CEACAM-1, achieving 80% long-term survival in the combinatorial group in the GL261 glioma model (96). Likewise, combinatorial therapy with anti–PD-1, anti–TIM-3, and focal radiation resulted in regression of murine GL261 gliomas (97). A phase I trial is currently underway testing anti–TIM-3 mAb alone and in combination with anti–PD-1 in patients with advanced solid tumors (NCT02817633).

LAG-3 is expressed on activated T cells (98). LAG-3 shares approximately 20% homology with the CD4 costimulatory molecule and, when present, competes with CD4 for MHC II, conferring instead a negative regulatory function upon binding (99). In addition, LAG-3 enhances the immunomodulatory function of Tregs through cytokine- and contact-dependent mechanisms within the tumor (100). Tregs can acquire MHC class II through the process of trogocytosis, where T cells complexed with APCs develop the ability to express different cell-surface molecules (101). Tregs expressing MHC class II are then able to engage LAG-3 on Teff and mediate suppression (102). Despite the homology with CD4, activated $CD8^+$ T cells also demonstrate increased LAG-3 expression. LAG-3 is exclusively expressed in conjunction with PD-1 on human GBM TILs (95). A phase I clinical trial is currently employing anti–LAG-3 antibody alone and in combination with anti–PD-1 in recurrent GBM (NCT02658981).

TIGIT is a member of the CD28 family. TIGIT inhibits T-cell immune responses in both cell-extrinsic and cell-intrinsic manners. TIGIT competes with the stimulatory ligand CD226 (DNAM-1) for binding with the receptors CD155 (PVR) and CD112 (PVRL2). Cell-extrinsic ligand competition for PVR and PVRL2 results in TIGIT phosphorylation and recruitment of SHP1, which inhibits signaling through the MAPK and Akt pathways (103). Intrinsic T-cell TIGIT engagement inhibits their proliferation and cytokine production (103). TIGIT has recently been shown to be expressed on human GBM TILs (95).

CD39 is an ectonucleotidase present on various cell types, including both GBM (104) and its infiltrating immune cells. CD39 promotes an immunosuppressive state via conversion of extracellular ATP to adenosine, which binds a variety of receptors to exert a constraining influence over immune cells (105). Adenosine enhances the immunosuppressive functions of Tregs and macrophages in addition to inhibiting the effector function of both T cells and natural killer (NK) cells (105). In the context of GBM, a study has shown increased expression of CD39 on Tregs, correlating with shortened survival (104). CD39 is also found on nonfunctional human GBM $CD8^+$ TILs (95).

In addition to restricting the proliferation of T cells, some immune checkpoints contribute to exhaustion via their influence on the metabolic functions of both T cells and tumor cells. Within the tumor microenvironment, tumor cells compete with neurons, glia, and TILs for glucose. GBM cells express high levels of GLUT1, allowing them to exceed normal brain tissue glucose uptake (106). T cells express GLUT1 and are highly dependent on glucose to support their glycolytic metabolism and cellular demands (107). Studies have demonstrated that PD-1 signals decrease GLUT1 expression on T cells, subsequently lowering glucose uptake (108). In addition, PD-1 alters glycolytic pathways and T-cell glucose utilization (108). On tumor cells, PD-1 and PD-L1 expression has been observed to promote glycolysis (109). By expressing PD-1 and PD-L1, GBM not only inhibits T-cell proliferation and induces T-cell exhaustion, but also suppresses T-cell glucose access and utilization while promoting its own. CTLA-4 expression on T cells also results in inhibited expression of GLUT1, thereby inhibiting T-cell metabolism (108).

Ignorance

T-cell ignorance results from competent T lymphocytes failing to mount a productive immune response despite the presence of antigen, due to either anatomical barriers sequestering the antigen from immune surveillance (i.e., immune privileged location) or to antigen expression levels being at insufficient concentrations (Fig. 5; ref. 30). In contrast to tolerant T-cells, ignorant T cells are fully functional, though antigen inexperienced and naïve. If ignorant T cells become exposed to antigen or activated by external stimuli, ignorance can in theory be easily overcome.

T-cell ignorance in GBM would at first glance appear to be quite relevant, given historic notions of immune privilege within the central nervous system (CNS). The concept of the "immunologic privilege" bestowed upon the brain had its first origins in the 1923, when Medawar's experiments showed that foreign homologous tissues grafted to the brain do not provoke an immune response (110). The lack of immunogenicity was believed due to the absence of a brain lymphatic drainage system, the presence of the blood–brain barrier (BBB), and the lack of resident specialized APCs within the CNS. With a variety of newer studies highlighting that CNS immune access is not quite so precluded (111, 112), it is now more widely accepted that the brain is more immunologically "distinct" than "privileged," (113), and the contribution of an immunologically distinct CNS to perceived T-cell ignorance is less clear. Likewise, while GBM is not a heavily T-cell–infiltrated tumor, activated T cells clearly traffic into GBM to represent anywhere from 4% to 40% of immune cells present (reviewed in ref. 114), with such infiltration potentially associated with greater overall survival (115).

Although the contributory role of anatomic barriers, such as the BBB, to a conceptual T-cell ignorance remains somewhat indeterminate, one very novel contributing mechanism newly discovered in GBM is that of T-cell sequestration. Our group has recently found that large numbers of mature T cells become trapped in the bone marrow of patients and mice with GBM, a phenomenon that appears to characterize tumors of the intracranial compartment

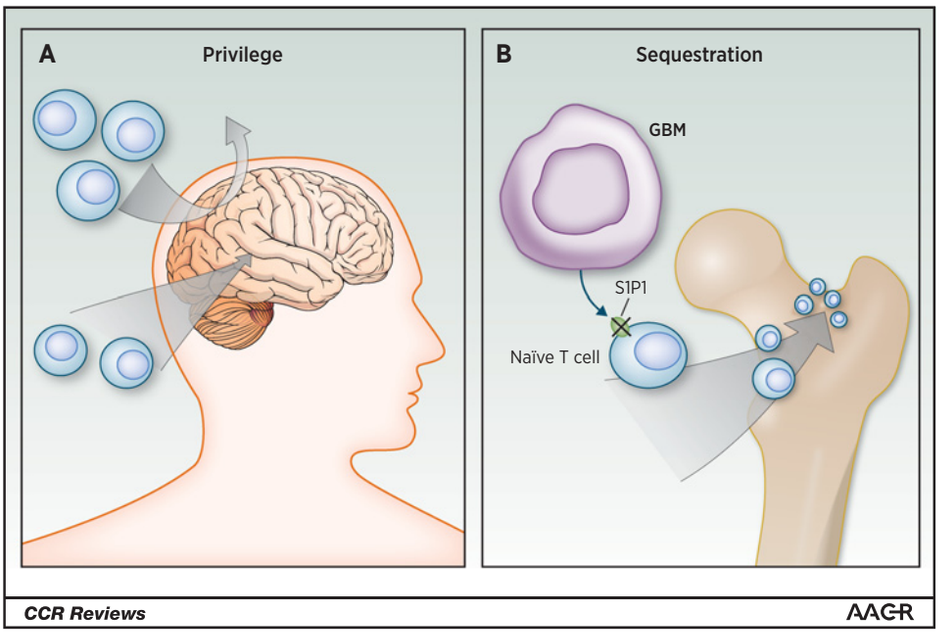

Figure 5.
Ignorance. A, The brain has long been characterized as immunologically "privileged," with limited ability of the immune system to infiltrate. Today, the brain is recognized as "immunologically distinct," where immune cells can and do infiltrate, yet the microenvironment may result in unique forms of immunosuppression. **B,** Clinically significant lymphopenia in patients with GBM, in part due to bone marrow T-cell sequestration as a result of S1P1 loss. Redrawn from an illustration by Megan Llewellyn, MSMI; copyright Duke University (Durham, NC) with permission under a CC-BY 4.0 license.

exclusively. The observed sequestration exists in the context of significant pretreatment lymphopenia and newly described hyposplenism in the GBM patient population. According to our analysis of 300 patients with treatment-naïve GBM, substantial reductions in spleen size (>50%) and severe T-cell lymphopenia are found with an approximate 25% to 30% frequency. Both $CD4^+$ and $CD8^+$ T-cell counts significantly drop in peripheral blood, and approximately 15% to 20% of patients present with AIDS-level $CD4^+$ counts (<200/μL; ref. 116). Multiple mouse glioma models recapitulate these findings. Importantly, sequestration of T cells in bone marrow appears to result from loss of the sphingosine-1-phosphate receptor 1 (S1P1) from the T-cell surface in the tumor-bearing state, precluding marrow egress. Conversely, stabilization of S1P1 genetically on T cells extricates them from the bone marrow, obviates sequestration-imposed ignorance, and licenses T-cell activating therapies in murine models of GBM (116). Thus, T-cell sequestration appears to be a new mode of T-cell dysfunction in GBM, most appropriately categorized as T-cell ignorance.

Conclusions

The immune system is increasingly recognized for its role in preventing the development and restraining the progress of cancer. Failure of the immune system to function adequately, whether by immunodeficiency or autoimmunity, is associated with increased prevalence of cancer. Furthermore, many tumors themselves can leave patients in an immune-deficient state, more likely to succumb to infections or other illnesses. Among these is GBM, the most common and the most lethal primary brain tumor. GBM is capable of expertly inhibiting the immune system, eliciting the full array of T-cell dysfunction, including senescence, anergy, tolerance, exhaustion, and ignorance. Each of these states reflects several shared features of T-cell dysfunction, ultimately converging upon decreased proliferative capacity and effector function. To date, these shared features have often resulted in confusion of the relevant terms for dysfunction within the literature. In this review, we clarify the unique molecular mechanisms and transcriptional programs underlying each mode of GBM-induced T-cell dysfunction, with the hope that a better understanding may enable the development of targeted and preventative therapeutics, thus improving the efficacy of immunotherapies for GBM.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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