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Review

Focus on TILs: Prognostic significance of tumor infiltrating lymphocytes in human glioma

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Significant work in animal models combined with compelling studies in human patients together have begun to provide a higher resolution picture of how the immune system regulates cancer development. Currently, this immune system-tumor interaction is represented by the concept of cancer immunoediting, which emphasizes that immunity may subserve either classical cancer immunosurveillance functions or promote the eventual outgrowth of immunoevasive cancer cells. One important line of evidence supporting an immunosurveillance process in humans has been the finding that the presence of distinct profiles of TILs may be correlated with improved clinical outcomes in a subset of human cancers. However, the contribution of TILs to the natural history of gliomas is less clear. Moreover, understanding the relationship between TILs and cancers of the brain is particularly challenging because our understanding of how immune responses develop in the central nervous systems is still evolving. In this review, we will first provide an overview of three important themes in the central nervous system anti-tumor immunity–i.e., antigen expression, how antigen may be presented, and lymphocyte trafficking–and subsequently discuss the extant work on TILs in glioma.

Keywords: human, glioma, tumor-infiltrating lymphocytes, prognosis, immunoediting

Introduction

As Old pointed out over 25 years ago (1), the study of cancer immunology has long been framed by the search for specificity–i.e., whether the immune system could truly distinguish between normal and malignant cells. Indeed, the cloning of the first human tumor antigen by Boon and colleagues (2) was the spectacular culmination of preceding work in the field (1) which had provided substantial evidence that immunologic discrimination between tumor and normal cells existed. Significant progress has been made since in identifying an expanding number of tumor-specific antigens recognized by the adaptive immune system (3-6), and it is now clear that innate immune cells also recognize distinct molecular structures on cancer cells [reviewed in (7-10)]. Thus, it is now well established that, in its relationship with cancer, immunity has the capacity to recognize-but does it have the capacity to protect?

A significant body of work has emerged over the last decade to provide substantial evidence that an "immunosurveillance" process exists in mice to protect against tumor formation; this process has since been codified into a broader view of the tumor immune-system interaction termed "cancer immunoediting" (8, 9, 11-13). Ongoing work has provided several lines of evidence to suggest that a parallel cancer immunoediting process exists in humans (9). Specifically, immunosuppressed transplant patients demonstrate an increased susceptibility to the development of tumors without clear viral etiology [reviewed in (9, 12)]. Moreover, cancer patients mount both cellular and humoral adaptive, as well as innate, immune responses to the tumors they harbor (14). In addition to these findings, some of the most compelling data pointing to the development of protective anti-tumor immune responses in humans have come from histological studies of the types of immune cells found within solid tumors and their correlations with clinical outcomes. Specifically, a growing number of studies have shown that the presence of tumor infiltrating lymphocytes (TILs) within a tumor can presage enhanced survival. The landmark studies on TIL analysis were conducted in melanoma (15-17) and have been followed by studies in a range of tumors including cancers of the ovary (18-20) and colon (21-24).

In this review, we will discuss the current data on TILs in gliomas, which represent the most common subset of primary intracranial neoplasms of the central nervous system (CNS). Historically, thinking on the relationship between immunity and the brain has been influenced heavily by the hypothesis that the central nervous system was an "immunologically privileged" site (25). However, this notion has been slowly overturned by the realization that the neuro-immunologic interface is, in fact, dynamic and not absolved of immunity's capacity to recognize danger. Thus, following a brief introduction of the clinical entity of gliomas, we will review current thinking on how immunity–and specifically, tumor immunity–may develop in the central nervous system by focusing on (a) brain tumor-specific antigenicity, (b) how these antigens may be presented, and (c) how lymphocytes may traffic to glioma sites in the brain. Finally, we will review the extant literature on the lymphocytic infiltration that occurs during the *in situ* immune response to gliomas and underline critical future directions in this important line of investigation.

Glioma: an overview

Gliomas comprise several types of tumors of glial cell origin and include astrocytomas, oligodendrogliomas, ependymomas, and other rare variants (26). Astrocytomas are subdivided into benign or malignant types: grade I tumors are rare in adults and relatively indolent, while grade II-IV tumors occupy a spectrum of increasing malignancy. Malignant gliomas, typically considered grade III and grade IV tumors, are the most common primary central nervous system cancers and kill 18,000 persons per year in the United States (27). The distinct pathologic grades of these tumors correlate with well-known biologic behaviors. Specifically, while grade I astrocytomas exhibit defined margins and therefore may be removed surgically, higher grade tumors invade the surrounding brain parenchyma. Thus, because these tumors extend beyond their visually identifiable borders (26), they cannot be excised

curatively. Tumor recurrence and/or progression to higher grade are inexorable, resulting in a predictable clinical course: patients diagnosed with the highest grade of malignant glioma, glioblastoma (GBM), have a 2-year survival rate of approximately 25% after diagnosis despite aggressive surgical resection combined with radiation and chemotherapy (28). As improvements in outcome over the past several decades have been modest (28-30), novel approaches–including gene therapy, molecularly targeted therapies such as monoclonal antibodies [e.g., Avastin (31)] or tyrosine kinase inhibitors [e.g., VEGF inhibitors (32), Iressa (33)], and immunotherapeutic approaches–may harbor greater potential for improved patient survival.

Recent work has begun to reveal the molecular underpinnings of GBM that may ultimately serve as targets for specific chemical or biologic therapies. Together, these studies have identified two distinct subtypes of GBM on the basis of clinical presentation and the unique genetic changes each tumor subtype manifests (34). The first GBM subtype, which presents in older patients as *de novo* GBM, displays deletions of the cell cycle-related genes $p16^{INK4A}$ and $p19/p14^{ARF}$ and gene amplifications of an epidermal growth factor receptor (EGFR) variant. The second subtype, which tends to arise in younger patients as a lower-grade glioma which progresses to GBM, is characterized by mutations in p53, amplifications of CDK4 or loss of Rb, and overexpression of platelet-derived growth factor (PDGF) (35). Despite progress in our understanding of the molecular basis of gliomagenesis, we still lack comprehensive knowledge of the genes whose activity is essential for the transforming events and maintenance of GBM, and which govern the lethal motile phenotype of glioma cells.

In addition to work that has led to the increased understanding of the genetic basis of glioma, a substantial body of work has also pointed to a growing recognition of the interplay between glioma and immunity. While the details of this relationship are elaborated elsewhere (36-39), data suggest that glioma cells both develop mechanisms to evade immune effector functions and promote the attenuation of the peripheral anti-glioma immune response. Thus, the glioma-immune system interaction may represent an example of cancer immunoediting in humans (Dunn and Curry, manuscript in preparation).

Anti-tumor immune responses in the brain: assessing the potential for TILs in glioma

Irrespective of the anatomic site of solid tumor origin, there are central principles which likely must exist for tumor-specific lymphocytes to infiltrate them effectively: tumors must express antigenic structures recognizable by immune cells, naïve immune cells must be activated in order to perform their effector functions, and activated effector cells must be able to enter the appropriate tissue parenchyma. Whereas the CNS has been considered "immunologically privileged" in the past (25, 40), detailed study of inflammatory conditions such as multiple sclerosis, experimental autoimmune encephalitis, and paraneoplastic syndromes (41, 42) has unraveled some of the characteristics of intracranial immunity. Rather than provide the type of broad discussion of neuroimmunology provided elsewhere in the literature (41, 43, 44), our goal in this section is to present a focused outline of these salient points that will frame our discussion on TILs in glioma.

Glioma-specific antigens

Adaptive cellular immunity

As is the case in other cancers, the identification of immunogenic human glioma tumor antigens capable of recognition by T cells demonstrates that there are unique antigenic substrates that could form the basis for spontaneous immune responses to CNS neoplasms. However, unlike cancers such as melanoma, relatively few antigens have actually been cloned; moreover, none have been identified using glioma-specific TILs. Nevertheless, those adaptive and innate antigens identified by either cellular or humoral approaches are catalogued in Table 1; regarding T cell antigens, only targets whose protein expression has been validated and whose presentation mediates CTL activity are listed.

Two subsets of glioma-specific antigens have been identified that can mediate CTL-directed recognition of human glioma cells. The first subset includes antigens whose expression appears to be restricted to transformed glial tissue, while the second subset includes antigens expressed in both tumor and normal tissue. In this section we will focus on select antigens in the former classification. From an immunotherapy standpoint, the optimal tumor antigen candidate is one that is selectively expressed in glioma and not in normal tissue. The cancer/testis (CT) antigens represent a group of proteins that meet this criterion in that they are expressed in tumors as well as germ cells and trophoblastic tissue but are absent from normal tissues (45). However, although several studies have examined expression of CT antigens–including SSX-1, -2, and -4, SCP-1, MAGE-1 and -3, and GAGE (46-48)–in gliomas by RT-PCR, few studies thus far have assessed either the protein expression or immunogenicity of CT antigens expressed in these tumors. However, Liu *et al.* (49) demonstrated that primary glioblastoma cells with high-level MAGE-1 or gp100 expression stimulated IFN-γ production by cognate antigen-specific CTL clones. Further work is thus necessary to assess the immunogenicity of CT antigens in glioma.

The EGFRvIII mutation of the EGF receptor gives rise to a tumor-specific mutant protein that is not expressed in normal cells which may also serve as a candidate tumor antigen. The EGFRvIII protein is formed by in-frame deletion of a portion of the extracellular region of the protein encoded by exons 2-7 (50). Whereas the EGF receptor is overexpressed in over 50% of gliomas, the EGFRvIII mutant was found to have variable expression in up to 25-100% of tumors analyzed (51-54). In a recent study, CTLs raised against an HLA-A*0201-restricted peptide lysed U87 glioma cells transfected with the EGFRvIII protein (55). Further studies will be necessary to determine the susceptibility of native EGFRvIII-expressing patient samples to lysis by CTLs raised against EGFRvIII. In vaccination studies, EGFRvIII peptide administration in patients with known EGFRvIII-expressing glioblastomas was safe in a phase II clinical trial (56). All tumors from which tissue was obtained at the time of recurrence had lost EGFRvIII expression, suggesting development of an antigen-loss variant.

Several other antigens demonstrate restricted protein expression. Specifically, studies of the SART3 antigen have yielded compelling data. The SART3 antigen was identified in 18/18 glioma cell lines and 31/34 brain tumor tissue samples in which 12/14 glioma specimens expressed SART3 (57); moreover, SART3 protein was not detected in 5 non-tumor samples. In addition, CTLs raised against SART3 peptides lysed SART3-expressing human glioma cell lines. IL13Rα2 represents

Table 1
Human glioma tumor antigens.

Antigen Type	Gene	HLA Allele	Expression in Glioma	Expression in Normal Tissue	CTL / Antibody Reactivity	References
MHC class I	TRP-2	A2	variable expression in 30/47 GBM samples by IHC	Yes; isoform-dependent expression in normal brain by RT-PCR	TRP-2-specific CTL lyse panel of TRP-2$^+$ GBM targets	54, 150
	EphA2	A2	variable in 19/19 astrocytomas and gliomas by IHC	Minimal expression in normal tissue by IHC	EphA2-specific CTL lyse EphA2$^+$ glioma targets	151, 152
	AIM-2	A1	Spliced and non-spliced forms expressed in 40/43 and 38/43 GBM lines by RT-PCR, respectively	Yes: spliced and non-spliced forms expressed in 6/12 and 6/12 normal brain samples, respectively	AIM-2-specific CTL lyse AIM-2$^+$ GBM targets	153
	$SART1_{259}$	A24	Expression in 13/18 (72%) glioma cell lines and 5/14 (35.7%) glioma tissue by Western blot	Unknown	SART1-specific CTL release IFN-γ on recognizing SART1$^+$ glioma cell lines	154
	SART3	A24	Expressed in 12/14 glioma specimens by Western blot	No; absent in 0/5 normal brain tissue by Western blot	SART3-specific CTL lyse SART3$^+$ glioma targets	57
	HER-2	A2	Expressed in 76% of 43 GBM lines by flow cytometry	Yes; detectable by RT-PCR in 4/6 normal brain tissue samples	HER-2$^+$-specific CTL lyse and release IFN-γ when incubated with HER-2$^+$-glioma targets	49, 152
	gp100	A2	Expressed in 8/47 (17%) GBM samples by IHC and in 45% of 43 GBM lines by flow cytometry	Detectable by RT-PCR in normal brain tissue	gp100-specific CTL lyse and release IFN-γ when incubated with gp100$^+$-glioma targets	47, 49, 54, 152
	MAGE-1	A1	Expressed in 38% of 43 GBM lines by flow cytometry and 12/14 glioma samples by Western blot	Not detected by RT-PCR in 6 and 11 normal brain tissue samples	MAGE1-specific CTL release IFN-γ when incubated with MAGE1$^+$ glioma targets	47, 49, 155
	IL13Rα2	A*0201	Detected in 47/47 GBM samples by IHC and in GBM tissue by IF	Detectable in normal astrocytes by IF, and in normal brain by real time RT-PCR	IL13Rα2-specific CTL lysed 3 HLA-A*0201$^+$, IL13Rα2$^+$ glioma cell lines	54, 59, 60
	ARF4L	A2	Detected in 10/10 glioma cell lines tested by RT-PCR	Detectable in all tissues tested by RT-PCR	ARF4L-specific GBM patient CTL lyse ARF4L$^+$, HLA-A2$^+$ glioma cell line	156
	GALT3	A2	Detected in 9/10 glioma cell lines tested by RT-PCR	Detectable in most normal tissues tested by RT-PCR	GALT3-specific GBM patient CTL lyse GALT3$^+$, HLA-A2$^+$ glioma cell line	157
	SOX2	A*0201	Overexpressed in 9/10 GBM by real time PCR; expressed in 2 GBM cell lines and variably expressed in 11 GBM tissue samples by IHC	Minimal expression detected in normal brain, skeletal muscle, and testes by real time RT-PCR	SOX2-specific CTL lyse SOX2$^+$- GBM cell lines and primary GBM cells	158
	EGFRvIII	A2	Detected in 25-100% of GBM samples by IHC	No expression detected by real time RT-PCR of normal brain tissue	Peptide-induced CTL produce IFN-γ and lyse EGFRvIII-transfected U87 glioma cells	51-55
SEREX	SOX6	NA	Detected in 18/18 glioma samples by IHC	Detected in few cells in normal cerebral cortex by IHC and in brain and testes by RT-PCR	Autoantibodies detected in 12/36 (33%) glioma patients, 1/54 (1.9%) patients with other cancers, 1/37 (2.7%) healthy controls	67, 159
	GLEA1	NA	Unknown	Unknown	Autoantibodies detected in 15/62 (24.2%) glioma patients	66
	GLEA2	NA	Detected in all glioma samples by RT-PCR and Northern blot analysis	Detected in all normal tissues by Northern blot analysis	Autoantibodies detected in 30/62 (48.4%) glioma patients that correlated with enhanced survival ($P = 0.012$); found in 2/22 (9%) patients either healthy or with non-glioma tumors	64, 66
	PHF3	NA	Detected in GBM samples by IHC; variable expression in glioma panel by Northern blot and RT-PCR analysis	Detected in placenta, stomach, and muscle by Western blot and weakly in normal brain by IHC; in all normal tissues tested by Northern blot	Autoantibodies detected in 35/62 (56.5%) glioma patients that correlated with enhanced survival ($P = 0.0031$) ; absent in 14 healthy control	65, 66, 160
"Innate"	NKG2D, NKp30, NKp44, NKp46 ligands	NA	Receptor fusion proteins stain 10-20% of CD133$^+$ and CD133$^-$ tumor cells; 3-5x increased in staining following stimulation with IFN-γ	See box below for NKG2D; NKp30/44/46 ligand expression/identity unclear	NK cells lyse CD133$^+$ and CD133$^-$ targets at least in part through NK2D/NKp30/NKp44/NKp46 and IFN-γ-dependent mechanism	72, 161
	MICA/B	NA	Increased expression in glioma by IHC with some variation by tumor grade	Scant expression in normal brain by IHC; expressed in intestinal epithelium and is induced in normal cells by select pathogens, oxidative/genotoxic/heat shock stress	Polyclonal NK cells lyse LNT-229 glioma cells transduced with TGF-B siRNA in MICA/ULBP-dependent manner	71, 73, 162 163
	ULBP1-3	NA	Increased expression in glioma by IHC with some variation by tumor grade	Scant expression in normal brain by IHC; upregulated in normal cells by genotoxic stress and select virus	Polyclonal NK cells lyse LNT-229 glioma cells transduced with TGF-B siRNA in MICA/ULBP-dependent manner	71, 73, 163

another antigen whose expression and immunogenicity has recently been assessed. Although detectable in 47/47 glioma samples analyzed (54), studies of its expression in normal tissue are equivocal; IL13Rα2 expression has been shown to be limited to testes in normal tissues (58) but also expressed in astrocytes by immunofluorescence (59) and in normal brain by real time RT-PCR (54). Nevertheless, CTLs raised against a peptide derived from IL13Rα2 specifically killed IL13Rα2-expressing glioma cell lines (60). Targeting the IL-13 receptor with conjugated toxins has been shown to be a safe method to deliver cytotoxic agents to glioma cells *in vivo* (61).

Additional antigens including AIM-2, Trp-2, ARF4L, SOX2, and GALT3 demonstrate a broader tissue expression and are summarized in Table 1. Continued study is needed to evaluate glioma-specific tumor antigens that are not expressed in normal tissue. This parsimony of expression is critical to the design of immunotherapeutic approaches to cancers of the brain, as collateral autoimmune damage to normal brain leading to long-term functional deficits would represent prohibitively dangerous side effects to vaccination approaches. Nevertheless, the studies described in this section show that gliomas *can*, in fact, be recognized by the naturally-occuring immune response; the pivotal next step in the study of antigen-specific, anti-glioma immunity will surely be the identification of those antigens that glioma-infiltrating lymphocytes actually *do* recognize during the endogenous anti-tumor immune response.

Adaptive humoral immunity

Studies have also documented tumor-specific humoral immune responses to several glioma antigens. These analyses build upon work by Old, who established the technique of autologous serological typing to demonstrate the existence of specific tumor-specific antigens on astrocytomas (62). Subsequent investigations employed the technique of serological identification of recombinantly-expressed tumor antigens (SEREX), the methodology pioneered by Pfreundschuh and colleagues (63). SEREX analysis of serum from glioblastoma patients identified antibodies to the GLEA1, GLEA2, and PHF3 antigens (64, 65). These autoantibodies were present in 15%, 24%, and 57% of 62 adult glioblastoma patients, respectively, and additional epidemiologic analysis subsequently revealed that the presence of autoantibodies to GLEA2 and PHF3 correlated significantly with enhanced survival following surgical resection (66). A separate study identified the antigen SOX6 by SEREX analysis; antibodies to SOX6 were detected in 12/36 glioma patients (67). Due to its near-selective expression in transformed glioma tissue and testis, SOX6 likely represents a CT antigen. Several additional antigens have also been identified using this technology but did not elicit autoantibody responses in the majority of patients analyzed (68). Thus, while serological approaches have complemented cellular strategies in glioma antigen identification, additional work is needed to determine whether the naturally-occuring humoral immune response to glioma antigens is integrated with a TIL response.

Innate immunity

In concert with adaptive immune recognition, recent provocative data suggest that the innate immune system also discriminates between tumor cells and normal cells. The seminal studies of Spies and colleagues first identified that the MHC class I chain-related proteins A and B (MICA and MICB) were expressed almost exclusively on tumor cells (69). Expression of these NKG2D ligands has been demonstrated in cancers of the lung, kidney, prostate, ovary, and colon (69), as well as in melanoma (70). Recently, these findings have been extended to glioma. Eisele *et al.* (71) showed that human glioma cells express MICA/B as well as ULBP1-3 and that the expression of these ligands in glioma may be influenced by TGF-β. Moreover, Wu *et al.* (72) provided evidence that tumor-initiating cells within gliomas–also referred to as glioma stem cells and characterized by their CD133 cell surface marker expression–express NKG2D and NKp30/44/46 ligands. In this study, up to 60% of both CD133+ and CD133- tumor cells stained positively with NK receptor fusion proteins when treated with IFN-γ; however, further work is necessary to delineate which NK receptor ligands were expressed and how their expression is regulated by IFN-γ. In addition, it will be important to determine whether the induction of the NKG2D ligands is dependent on intracellular events during transformation such as genotoxic stress (73). Nevertheless, these data show that glioma cells express antigenic substrates that can be recognized by NK receptor-expressing innate cells–a finding that will be particular important if TILs in glioma include NK cells, especially in light of recent observations suggesting that NK cells may play a prominent role in effective intracranial anti-tumor immunity after dendritic cell vaccination (74). Taken together, these data underscore the growing understanding of glioma antigenicity and highlight the further studies necessary to understand the adaptive and innate contributions to the glioma immunome.

Initiation of the anti-glioma immune response: antigen presentation in the CNS

The mechanism behind the generation of antigen-specific immune responses in peripheral tissues is well-characterized in the literature (75). However, these details are less clear for immune responses to antigens in the CNS. *A priori*, the biology of immune responses within the CNS would appear to be obligately distinct due to the lack of secondary lymphoid tissues within the brain parenchyma, as well as to the possible obstacles presented by the blood-brain barrier. Principally, the antigens discussed in the previous section must be acquired and presented by antigen-presenting cells (APCs), which in turn must activate the tumor-specific lymphocytes that may ultimately infiltrate brain neoplasms. In this section, we will address both the potential mechanisms of antigen presentation as well as the identities of APCs in the CNS. Prior to this discussion, however, it is important to clarify that (a) the identification of physiologically-relevant APCs in the CNS is an active, ongoing area of investigation and (b) much of the work in this area has not employed tumor models.

Cervical lymphatic drainage of the brain

Although the brain parenchyma does not harbor conventional lymphatic channels, several studies in animals have demonstrated that antigens nevertheless can drain to the cervical lymph nodes. Using radiolabeled serum albumin, Cserr and colleagues showed that labeled antigen transferred to a range of intracerebral locations in rabbits–including the midbrain, caudate nucleus, internal capsule, forebrain, and cerebrospinal fluid (CSF)–drained at varying rates to the cervical lymph nodes (76, 77). Up to 18-47% of injected antigen was ultimately recovered in the draining cervical nodes. Further work showed that intracerebrally-injected antigens track through the subarachnoid space along cranial nerves and cross the cribiform plate to the nasal mucosa where they access lymphatic drainage basins (78). Corroborating data have been obtained in both experimental models of multiples sclerosis (MS) and in MS patients. In animal models of experimental autoimmune encephalitis (EAE) in which monkeys and marmosets were immunized with either myelin basic protein (MBP) or myelin oligodendrocyte glycoprotein (MOG), a statistically-significant number of myelin antigen-containing cells were identified in the cervical lymph nodes of animals with EAE compared to control animals (79). Moreover, patients with MS harbored myelin-containing APCs in the cervical lymph nodes. While additional studies will be necessary to determine the relevance of these potential antigen-draining lymphatic pathways to humans bearing CNS neoplasms, these data reveal a nexus between the nervous system and peripheral lymphoid tissue that underscores one potential starting point for the initiation of brain tumor-specific immune responses.

The search for the CNS APCs

Complementary studies have searched for the identity of the APCs that may stimulate anti-CNS immune responses. While a broad range of cell types–including vascular endothelial cells, smooth muscle cells, astrocytes, microglia, perivascular macrophages, choroid plexus epithelial cells, neurons, and dendritic cells [reviewed in (80, 81)]–have been implicated as potential CNS APCs, it remains unclear which cell types effect physiologically-relevant APC functions *in vivo*. However, recent work has begun to point strongly to the dendritic cell (DC) as the critical APC (82), and thus several recent studies centering

on DCs in animal models will be highlighted in detail in this section.

Data obtained from several laboratories on the cellular dynamics of the CNS immune responses together point to a model in which CNS antigen-specific T cells are primed by antigen-loaded, immigrant APCs within the lymph nodes that drain the CNS parenchyma before trafficking into the brain. Specifically, Karman *et al.* (83) showed that ova-carrying DCs injected into the brain traffic to the cervical lymph nodes. Moreover, anti-ova CTLs were identified in both the spleen and ultimately in the brain, demonstrating the development of systemic immunity to intracranial antigen. Ehtesham *et al.* (84) made similar observations; in a rat glioma immunotherapy model, intratumorally-injected DCs enhanced host survival via a mechanism in which injected DCs were identified in the draining lymph nodes and increased percentages of CD4+ and CD8+ T cells were identified within tumors. Additional studies demonstrated that CD8+ CTLs specific for intracerebral antigens could be activated via cross-presentation. Calzascia *et al.* (85) presented one of the few studies of antigen cross-presentation in the setting of antitumor immunity. In this work, $H2^b$-expressing, $H2^d$-deficient MT gliomas were transfected with HLA-CW3 and transplanted into a tolerant $H2^b$ x $H2^d$ F_1 recipient. When recipient mice were transplanted intracranially with the CW3-expressing MT tumors cells, cross-primed $H2^d$-restricted anti-CW3 CD8+ CTLs were identified within the brain tumors. Walter *et al.* (86) also demonstrated that intracranial CD8+ CTLs could be primed via cross-presentation; adoptively-transferred anti-ova OT-1 T cells trafficked to the brain after documented cell division in the cervical lymph nodes following intracerebral challenge with ova-expressing splenocytes. While the identities of the physiologically relevant APCs in this model were not determined, it was shown that CD40 was required for cross-priming to occur and that this process also required CD4+ T cells.

Recent work by Greter *et al.* (87) provided strong evidence in support of the physiologic relevance of the DCs in CNS antigen presentation. In this elegant study, the cellular requirements for *in vivo* antigen presentation in the EAE mouse model of multiple sclerosis were examined using bone marrow chimera and transgenic approaches. Autoimmune disease is manifested in this model following the adoptive transfer of *in vitro*-primed, anti-myelin protein encephalitogenic CD4+ T cells into naïve mice; importantly, the anti-MOG T cells must be re-stimulated *in vivo* in order to cause disease. The authors took advantage of the radioresistance of CNS resident cells to assess whether APCs inside or outside of the CNS were critical for T cell-mediated disease development. Using congenic mice, it was first shown that, unlike cells such as microglia, bone marrow-derived cells were largely excluded from the CNS parenchyma in non-inflammatory states. Interestingly, APCs outside the CNS were required for disease development, as encephalitogenic T cells did not cause disease when transferred into lethally-irradiated recipient mice reconstituted with MHC class II-deficient bone marrow. The authors then provided evidence that the DC could represent a candidate for this critical radiosensitive APC. By driving expression of MHC class II under the control of the CD11c promoter in an MHC class II-deficient background, MHC class II expression was limited to DCs. Strikingly, the restricted expression of MHC class II to DCs was sufficient to permit anti-MOG T cell-mediated disease development. These data thus demonstrated that DCs could represent physiologically-relevant APCs in an animal model of CD4+ T cell-dependent autoimmune disease.

Taken together, these data in animal models suggest that the initiation of immunity in the brain shares substantial similarities with the process in other tissues in that lymphocyte activation by immigrant DCs appears to occur in draining secondary lymphoid tissue prior to trafficking to sites of antigen challenge in the brain. Subsequent restimulation by either migrating or resident brain APCs may also be required for effector functions to be carried out. Ultimately, although DCs may indeed be the primary APCs in driving intracranial immunity, more study is necessary to validate this principle in brain tumor models.

Trafficking to the CNS: how lymphocytes could infiltrate gliomas

In the last step for TIL manifestation, antigen-specific activated TILs must be able to move to relevant tumor sites in the brain. Given the anatomical complexity of the CNS, it has been proposed that there are three methods by which immune cells may access the CNS: (i) from blood to the CSF via the choroid plexus, (ii) from blood to the subarachnoid space, and (iii) from blood to parenchyma (88). In this section, we will review the blood-brain barrier (BBB) and its integrity in the setting of glioma before addressing how lymphocytes may traffic from the blood to tumor sites in the CNS parenchyma.

The blood-brain barrier

The "blood-brain barrier" refers to the vascular structure in the brain which acts as a barrier to the passive transit of molecules between the CNS parenchyma and the systemic circulation. Its existence was first suggested by studies in the late 19th century by Ehrlich, who demonstrated that intravascularly-injected dyes did not stain the brain and spinal cord but did label other organs (89). Subsequent work has revealed the molecular basis for this phenomenon. The barrier itself exists primarily at the level of the intracerebral capillaries and is formed by the tight junctions of the capillary endothelial cells. These apposed junctions, which are composed of multi-component protein complexes (90), create a tight seal which severely limits the paracellular transport of molecules and thus separates the capillary lumen from the interstitial space. Moreover, relatively decreased pinocytotic activity combined with selective cellular transport mechanisms significantly reduces promiscuous transcellular transport (90, 91). More than 90% of the capillary endothelium is also ensheathed by astrocyte foot processes that form the "glia limitans" (91). The capillaries of the circumventricular organs of the brain represent areas in which there is no BBB: the neurohypophysis, median eminence, vascular organ of the lamina terminalis, subfornical organ, pineal gland, subcommisural organ, choroid plexus, and the area postrema are thus sites that more freely permit transport of molecules across vascular endothelial cells (92). The "blood-CSF barrier" also restricts free movement of cells and molecules between the CSF and the capillaries of the choroid plexus (88).

Within the glioma microenvironment, the vascular landscape is substantially altered and structures such as the BBB, which serve to protect the CNS in the non-transformed state, are significantly compromised. Therefore, the "blood-tumor barrier" is more porous than the intact blood-brain barrier. Long (93) identified many alterations of intra-glioma capillaries at the ultrastructural level, with the most extensive variability noted at the level of the endothelial cells, tight junctions, and basement membranes. Abnormal endothelial cells were observed to be hypervesicular, swollen, display villous processes, and harbor hyperchromatic nuclei with sometimes little cytoplasm. In many

areas, there appeared to be either grossly-dilated or absent tight junctions, as paracellular regions were patent and allowed passive transport of tracers. Additionally, some areas of the basement membrane were attenuated, dilated, or absent, and the investing glial sheath was almost uniformly absent. Additional studies have documented similar observations that glial tumor vasculature is composed of tortuous and chaotic sinusoidal structures which may develop gaps between adjacent endothelial cells owing to the downregulation of critical tight junction proteins (94), thus disrupting the blood-brain barrier. It is not clear whether increased permeability would lead to increased immune cell infiltration through passive migration in the absence of appropriate, inflammation- and/or tumor-induced chemokine stimuli. Moreover, permeable intra-tumoral capillaries may be comprised of a heterogeneous "mosaic"; several studies have documented that tumor cells themselves may line the lumen of dysregulated tumor capillaries alongside true endothelial cells (95, 96), possibly disrupting molecules that are critical for cellular homing (88). While further work is necessary to assess this possibility in human glioma, these data together suggest that the vascular microenvironment within tumor tissue is severely dysregulated and may contain poor target substrates with which to attract TILs. Thus, although intratumoral capillary permeability may increase within tumors, efficient lymphocyte trafficking may be significantly compromised.

Lymphocyte-trafficking to glioma

Whereas prior work has demonstrated the ability of T cells to traffic to the CNS in autoimmune diseases (97, 98), currently we have a limited understanding of how lymphocytes traffic to developing CNS neoplasms. While the process of lymphocyte movement has been reported extensively in disease models such as EAE (99), the possibility remains that these molecular details may not be relevant to the developing anti-tumor immune response. Nevertheless, one working model that explains how lymphocytes transit to the brain parenchyma is the "multi-step" model of migration (100), characterized by the "rolling, sticking, and migrating" paradigm. In this schema, T cells homing to the brain first slow down by gradually tethering to capillary endothelium in a "rolling" step mediated by interactions between endothelial cell E- or P-selectins and P-selectin glycoprotein ligand-1 (PSGL-1) (101). Subsequently, lymphocyte integrin molecules become "activated" when chemokines, likely present on the vascular endothelium, engage cognate G protein-coupled chemokine receptors on lymphocytes. Enhanced adhesion is likely mediated through interactions between integrin: ligand pairs α4,β1/7: VCAM-1 and LFA: ICAM-1 expressed on lymphocytes and endothelium, respectively (101). Lymphocytes ultimately transmigrate to the CNS parenchyma in the "diapedesis" step, which may occur via trans- or para-cellular endothelial transport (102).

A recent study by Calzascia *et al.* (103) provided provocative evidence that brain tumor antigen presenting APCs may "imprint" the CNS-homing phenotype of tumor-specific CTLs primed in the draining cervical lymph nodes. This particular study in mice monitored the activation of adoptively transferred transgenic anti-GP T cells following host challenge with the fibrosarcoma MC57 transduced with the LCMV GP protein. Interestingly, adoptively-transferred T cells were primed in deep and superficial cervical lymph nodes, as well as in lumbar lymph nodes. Furthermore, CNS-homing T cells expressed α4β1 integrins, and their ability to home to the CNS appeared to be influenced by the anatomic location from which the draining lymph node APCs were derived. Thus, this study suggests a link between the origin of APCs and downstream manifestation of TILs.

Identification of brain-tumor specific chemokines mediating chemotaxis during the anti-tumor immune response will be vital to better understanding how lymphocytes may infiltrate cancers of the brain. In EAE models, chemokines CCL2-5, CCL-7, CXCL-8, CXCL-10, and CXCL-12, as well as receptors CXCR3, CXCR4, CCR1 and CCR2, have been implicated in lymphocyte homing (88, 101), although studies employing knockout mice will be necessary to validate their requirements for trafficking. Several of these proteins have been studied in tumors. Specifically, Bajetto *et al.* assessed the expression in 31 glioma samples of a number of chemokines and their receptors using RT-PCR (104). The receptors CXCR1-5 were expressed in 60-90% of tumors examined, whereas cognate chemokines IL-8, GRO 1-3, IP-10, MIG, SDF1, and BCA-1 were identified in 16-42% of tumors. Because this analysis was conducted using mRNA from whole tissue, it is not clear whether expression was correlated with the presence of lymphocytes. Further experiments in this study focused on CXCR4 and its ligand SDF1, which was expressed in 28/31 (90%) and 13/31 (42%) of tumors, respectively, thus corroborating previous findings (105-107). Immunohistochemistry revealed that CXCR4 and SDF1 were expressed in both tumor and endothelial cells (104, 108). The expression of SDF-1 (also termed CXCL12) may be clinically relevant; in a study of 50 patients with low-grade glioma, expression of CXCL12 was associated with a statistically-significant decrease in time to disease progression (109). Because much of the functional data on CXCR4 centers on non-immunologic effects of its interaction with SDF-1, including tumor cell migration and proliferation (110), further work will be necessary to determine whether its expression on immune cells is relevant to glioma biology *in vivo*. Additional chemokines expressed in glioma tissues include MCP-1 (111) and also IL-8 (112). Although we are at an early stage in studying the contributions of specific chemokines in recruiting glioma-specific TILs, understanding TIL chemokine and chemokine receptor expression will be critical in clarifying the molecular underpinnings of lymphocyte trafficking to human gliomas.

TILs in glioma: *in situ* immunity in the CNS

Over the last 4 decades, a substantial number of studies have focused on the presence and type of immune cells that infiltrate various neoplasms of the brain. However, unlike the study of TILs in melanoma (15-17) as well as in ovarian carcinoma (18-20) and colorectal cancers (21-24), analyses of TILs in glioma have not yielded consistent correlation with clinical outcome. Thus, our aims in the following section and conclusion are two-fold: (i) to review the extant literature on TILs in glioma and (ii) to highlight the critical future directions that must be undertaken in this line of investigation.

Characterizing TILs in glioma

Bertrand and Mannen (113) are credited as the first to analyze gliomas for the presence of TILs. In their study, 63/172 (36.6%) astrocytomas harbored perivascular lymphocytes, although the majority of these infiltrates were characterized as "weak". Moreover, no correlation to clinical outcome was established. Subsequently, Ridley and Cavanagh (114) explored the frequency and degree of lymphocytic infiltration in human gliomas. In their study, 91 glioma specimens taken at autopsy were analyzed for the presence of lymphocytes. In their analysis,

28/91 (31%) of tumors harbored a "definite" infiltration, 26/91 (29%) harbored a "slight" infiltration, and 37/91 (40%) tumors displayed an "absent" infiltration. In those tumors displaying a definite infiltration, the most frequently-observed pattern was perivascular rather than diffuse lymphocyte infiltration. However, because this study was a post-mortem analysis, it was not possible to correlate histologic patterns with clinical outcome. Takeuchi and Barnard (115) extended these data by evaluating lymphocyte infiltration in astrocytoma biopsy specimens rather than post-mortem specimens. In this study, central perivascular lymphocyte cuffing was observed in 32/114 (28%) of specimens. Thus, these early studies determined that a subset of gliomas harbored infiltrating lymphocytes, although no correlation with clinical prognosis was established.

Lymphocytes

Additional studies have focused on characterizing the phenotypes of immune cell infiltrates observed in glioma. Von Hanwehr *et al.* (116) evaluated the lymphoid cell subsets in 6 astrocytoma or glioblastoma specimens. In this study, lymphocytes were either rare or undetectable in 2 tumors, and the other 4 tumors demonstrated slight to moderate CD4+ infiltrates and absent, slight, or markedly intense CD8+ infiltrates. Hitchcock and Morris (117) assessed the presence of TILs in 5 low grade and 15 high grade astrocytomas. Using immunohistochemistry (IHC) of "imprints" of non-necrotic tumor areas, the authors demonstrated that both low- and high-grade tumors harbored an average of 4% and 10% CD4+ and CD8+ cells, respectively, and that macrophages represented over 20% of cells analyzed. In another study, Farmer *et al.* (118) demonstrated that CD8+ and CD4+ cells comprised 41% and 42%, respectively, of TILs in 9 high-grade gliomas. Regarding NK cells, Stevens *et al.* (119) demonstrated scant lymphocytic infiltration in 18 gliomas analyzed. Several other groups have demonstrated a variable percentage of lymphocytes within TILs (120-124). Overall, the variability in these data likely stems from differing methodologies, as there is inconsistency between reports with regard to assessing TIL incidence, degree of infiltration, and location.

Several groups have examined TIL function in glioma. Kuppner *et al.* (125) assessed the phenotypes and function of TILs from 7 glioblastoma specimens. Overall, few lymphocytes were present; 2 specimens demonstrated markedly intense CD3 staining, 1 demonstrated moderate CD3 staining, and 4 demonstrated slight to no staining. CD4 expression was moderate in 2 specimens and absent to rare in 5 specimens, while CD8 expression was markedly intense in 1 specimen, moderate in 2 specimens, and absent to rare in 4 specimens. When TIL function was assessed, lymphocytes from 1/5 TIL panels appeared to selectively lyse autologous tumor, 1 TIL panel lysed only the allogeneic target, and 1 panel lysed both, possibly underscoring the heterogeneity of TILs in glioma. Sawamura *et al.* (126) subsequently assessed the infiltration and function of lymphocytes in 24 malignant glioma specimens. The authors noted marked perivascular lymphocyte cuffing in 2/24 tumors, rare to mild lymphocyte perivascular cuffing in 15/24 tumors, and no lymphocytes in 7/24 tumors. Phenotypically, most TILs cultured from gliomas were comprised of a mixture of CD4+ and CD8+ T cells that killed autologous tumor, as well as several allogeneic targets. Further work is needed to explore the potential for enhanced autologous tumor specificity.

Several recent studies have investigated the presence of regulatory T cells (Tregs) within glioblastoma. El Andaloussi and Lesniak (127) studied TILs in 10 glioblastoma specimens and 6 control brain specimens. Flow cytometry analysis of single cell tumor suspensions revealed that lymphocytes represented approximately 17% (12-21%) of cells whereas less than 1% were found in control brain. Of the 25% of lymphocytes that were CD3+, approximately 6% were CD4+CD25+. Of the CD4+ cells in glioma TILs, 50-60% expressed FoxP3, a Treg-specific protein critical to regulatory T cell development and function (128). Finally, the authors showed that glioma Tregs displayed a suppressor function, as sorted CD4+CD25+ glioma TILs suppressed the proliferation of CD4+CD25- glioma TILs *in vitro*. In the study of Hussain *et al.* (129), the authors demonstrated a higher percentage of Tregs amongst glioma TILs. Of the CD3+ cells within glioma tissue, 20% were CD4+; strikingly, 96% of the CD4+ population also expressed FoxP3 although only 29% expressed CD25. Together, these data show that, as is found in other cancers (19, 20, 130), Tregs are present within glioma TILs and suggest that further TIL analysis of this tumor type must evaluate the presence of this population.

Macrophages

Intraglioma macrophages have also been a focus of intense study. Wood and Morantz (131) evaluated the immune cell infiltration of 45 CNS tumors, 9 of which were glioblastomas. In their analysis, cellular suspensions of minced tumors were assessed for composition. Of the 9 glioblastomas analyzed, lymphocytes represented 4-11% of total cells. However, a large percentage of macrophages was observed; 7/9 tumors harbored between 31-78% macrophages. In another report by the same group, an additional two tumors also harbored between 40-90% macrophages (132). Together, these studies were the first to point to a potential role for macrophages in the tumor microenvironment. However, it is unclear whether these tumor-associated macrophages are associated with antitumor immunity or are immunosuppressive.

Rossi *et al.* (133) used a panel of monoclonal antibodies to evaluate the infiltrating immune cell profile of up to 65 glioma biopsy samples. Both number and location of immune cell infiltrates were evaluated. Macrophages, profiled using antibodies to CD68, CD14, RFD7, or the CR3 receptor, were observed throughout the tumor bed; they were found at varying densities within the viable parenchyma, surrounding pericytes and blood vessels, and within necrotic tissue regions. Among CD68+ cells, 2 different antibodies stained macrophages in 88-97% of viable perivascular regions, in 100% of viable intraparenchymal areas, and in 96-100% of necrotic regions. Additional studies have also corroborated the substantial infiltration of glial tumors by macrophages (111, 134-136).

Two recent studies have examined the presence and functions of glioblastoma-infiltrating microglial cells (129, 137). In an analysis of 50 glioblastoma specimens, CD45+, CD11b+, CD11c+ microglia/macrophages comprised an average of 0.825% of glioblastoma tissue in contrast to 0.007% of normal brain tissue (129). There was no difference in cytokine expression, TLR expression, or MHC expression between cells isolated from glioblastoma vs. normal brain. Furthermore, glioblastoma-infiltrating microglia/macrophages did not activate alloreactive T cells in an MLR reaction in contrast with peripheral blood APCs, although it is not clear whether this lack of reactivity was due to an intrinsic inability of microglia/macrophages to prime alloreactive T cells or whether the function of these cells in glioblastoma is impaired. Additionally, microglia/macrophages from glioblastoma lysed the U87 glioblastoma cell line, although normal microglia/macrophages were more effective (124). Further analysis will hopefully shed

Table 2
Correlation between the presence of TILs and survival in human glioma.

TIL Correlation with Survival	Study Type	Tumor Types	TIL Data	Macrophage Data	Clinical outcome	Reference
Positive	Histologic analysis of biopsy specimens	GBM: 85 Astrocytoma: 43 Mixed glioma: 5 Ependymoma: 5 Oligodendroglioma: 2 Total: 140	64/141 (45.3%) lymphocyte perivascular infiltration; 77/141 (55%) absent lymphocytes	NA	Perivascular lymphocyte infiltration correlated with 2-4 month increase in survival	138
	Histologic analysis of resection specimens	Glioblastoma: 200	23/200 (11.5%) "definite" TILs, 46/200 (23%) "slight" TILs, 131/200 "absent" TILs	NA	"Definite" TIL presence correlated with longer survival ($P < 0.01$)	139
	Histologic analysis of resection specimens	Glioblastoma: 199	145/199 (72.6%) harbored some degree of infiltration	NA	Of 139 patients still alive 2 months after surgery, average survival without TIL was 5.6 months vs. 8.1 months with TIL ($P < 0.01$)	140
None	Histologic analysis of resection specimens	Glioblastoma: 269 Anaplastic astrocytoma: 55 Total: 324	Approximately 50% of 246 tumors harbored TILs	NA	In 246 patients assessed, TIL presence did not correlate with survival	141
	Histologic analysis of resection specimens	Astrocytoma: 68 (grades III/IV)	$CD8^+$ T cells in 56/68 (82%) of tumors—48/68 (70%) in parenchyma with mean cellularity 0.71, 41/64 (64%) in perivasculature with mean cellularity 0.76; $CD4^+$ T cells in 22/68 (32%) of tumors with mean cellularity 0.31	Macrophages found in parenchyma in 68/68 (mean cellularity 2.5) cases and in perivascular areas in 58/63 (92%) of tumors (mean cellularity 1.9)	Expression of CD4, CD8, presence of macrophages, or lymphocyte cuffing did not correlate with survival	142
Negative	Histologic analysis of resection specimens	Astrocytoma: 342 (grades III-IV)	35% harbored lymphocytes within tumor tissue, 29% harbored lymphocyte in peripheral tumor tissue	NA	Presence of TIL associated with average survival time of 8.4 months vs. 11.9 months in the absence of TIL ($P < 0.02$)	144

light on what function(s) this abundant tumor-infiltrating cell population may be subserving in glioma.

Glioma TILs and clinical outcome

Several studies in the literature sought to determine a correlation between the presence of glioma TILs and survival and have yielded incongruent results (Table 2). Three studies demonstrated a positive correlation between lymphocyte infiltration and survival. Brooks *et al.* (138) examined hospital records and histopathologic analysis of biopsy specimens from 149 patients from 1962-1976. Of those patients studied, 141 harbored gliomas, of which over 75% were either glioblastoma multiforme or anaplastic astrocytoma. Overall, 64/141 (45.3%) of glioma specimens displayed some degree of perivascular lymphocyte infiltration, whereas 77/140 (55%) of tumors displayed no lymphocyte infiltration along vascular structures. These findings correlated with survival in a subset of patients; in patients with glioblastoma multiforme or anaplastic astrocytoma, the presence of any degree of perivascular lymphocyte infiltration–and, notably, *not* diffuse infiltration–was correlated with a statistically significant 2-4 month increase in survival over patients without perivascular lymphocyte cuffing. Furthermore, survival increased as the degree of lymphocyte infiltration increased. While this study did not address which subsets of lymphocytes were present at the tumor site, it was the first to suggest that the presence as well as local organization of lymphocyte infiltration may represent positive prognostic indicators in patients with glioma.

The study of Brooks *et al.* (138) was followed closely by the report of Palma *et al.* (139). In this study, the authors assessed glioblastoma specimens resected from 200 patients between 1952 and 1973 for lymphocyte infiltration. Specifically, 23/200 (11.5%) of cases demonstrated a "definite" perivascular lymphocyte infiltrate, 46/200 (23%) demonstrated a "slight" perivascular lymphocyte infiltrate, and 131/200 (65.5%) demonstrated an "absent" lymphocyte infiltrate. The patients displaying a definite infiltrate were found to demonstrate enhanced survival that was statistically significant ($P < 0.01$); 5 patients were still alive 6 years after surgery as opposed to no survivors in the two other groups, although the longer pre-operative period in this group suggests that these particular patients may have harbored slower growing tumors. Nevertheless, these data point to the possibility that lymphocyte infiltration correlated with prolonged post-operative survival in these patients.

Böker *et al.* (140) also demonstrated a positive correlation between lymphocyte infiltration and survival. In this study, TILs were identified in 145/199 (72.6%) of samples, an incidence substantially higher than in previous studies. Further analysis revealed a positive correlation between TIL presence and survival irrespective of post-operative treatment; of the 139 patients still alive beyond a 2-month postoperative window, average survival was 8.1 months in 104 patients harboring varying numbers of TILs compared to 5.6 months in 35 patients without TILs ($P < 0.01$). However, subanalyses of TIL location or degree of infiltration and survival were not undertaken.

Two additional studies demonstrated no correlation between lymphocyte presence and survival. Specifically, Schiffer *et al.* (141) correlated the presence of TILs with the survival of 324 patients with malignant glioma. Of these cases, 269 patients harbored either primary or secondary glioblastoma and 55 harbored anaplastic astrocytoma. When the authors assessed survival in 246 patients of whom approximately 50% harbored TILs and 50% did not, there was no correlation between TIL presence and survival. In another study, Rossi *et al.* (142) examined the TILs of 68 grades III and IV astrocytomas. CD8+ T cells were detected in 56/68 (82%) tumor specimens; 48/68 (70%) harbored intraparenchymal lymphocytes and 41/64 (64%) harbored perivascular lymphocytes. In contrast, CD4+ lymphocytes were found in only 32% of samples. Macrophages were abundant within tumor specimens, as 100% of specimens displayed significant intraparenchymal infiltration and 92% displayed perivascular macrophage infiltration. However, neither the presence of CD8+ cells, CD4+ cells, nor macrophages correlated with enhanced clinical survival.

Finally, in the largest of these studies, Safdari *et al.* (143, 144) assessed the prognostic value of infiltrating lymphocytes in 342 patients diagnosed with grades III-IV astrocytoma and showed that there was a negative correlation between TIL presence and survival. Specific tissue areas were evaluated for the presence of immune cells: tumor, peripheral tissue, hypervascular areas, necrotic areas, and normal tissue. In these regions, 35% of patients harbored lymphocytes within the tumor and 29% displayed peripheral tissue lymphocytes. In contrast, lymphocytes were observed in only 4% of normal tissue. The presence of lymphocytes was associated with a shorter mean survival time; patients with TILs survived an average of 8.4 months, whereas patients without TILs survived an average of 11.9 months ($P < 0.02$). Further analysis showed that this statistically significant negative correlation was also observed when TILs were found specifically within the tumor as well as in necrotic or hypervascular areas. Moreover, TILs were associated with a statistically-significant decrease in survival time regardless of postoperative therapy. In patients undergoing surgery and radiation therapy, the presence of lymphocytes was associated with an average 4.6 month decrease in survival ($P < 0.05$). Thus, these data suggest that the presence of lymphocytes within malignant glioma is a negative prognostic indicator.

Taken together, these studies–which, to our knowledge, represent all available reports on glioma TILs and clinical outcome in the literature–do not provide support for an unequivocal correlation, either positive or negative, between the presence of TILs and survival. Indeed, 3 studies reveal a positive correlation, 2 studies reveal no correlation, and 1 study suggests that TIL presence is a negative prognostic indicator. There may be several explanations for these discordant results. First, it may be important to assess TILs within specific grades/types of tumor. While "glioma" comprises a broad range of glial-derived neoplasms, there may be disparities in immunogenicity between tumor types. Second, these clinical studies were conducted prior to the emergence of Tregs as pivotal players in human anti-tumor immunity (19, 20, 130). The previous studies may, in fact, have assessed tissue samples that may have displayed a wide range of Treg infiltrates that may have influenced clinical outcome. Third, future studies should establish consistency in TIL parameters. For instance, all studies should assess lymphocyte *profile*, *location*, and infiltration *intensity*. Finally, careful attention should be paid to the post-operative treatment patients receive, and meticulous multivariate analyses will be vital to distilling the impact of TILs on outcome in the setting of heterogeneous patient populations. However, until further studies are undertaken, these studies show that glioma infiltration by lymphocytes in the absence of immunotherapy is not currently a clinically informative parameter.

Glioma TILs in immunotherapy

Recent studies have provided provocative results using rapidly-expanded TILs in adoptive immunotherapy approaches to non-CNS cancers (145). Quattrocchi *et al.* (146) reported the results of a pilot study employing this approach in patients with recurrent malignant glioma. In this study, 6 patients who had undergone surgery for recurrent glioma received 1×10^9 TILs on days 1 and 14 following surgery, as well as IL-2 three times weekly for 1 month; cells and cytokine were administered through an Ommaya reservoir. TIL profiling showed that most cells were predominantly CD3+, and TIL cultures ultimately grew out mostly CD4+ or CD8+ cells. TILs that were mostly CD8+ lysed autologous tumor at a significantly higher incidence than allogeneic cell lines, whereas autologous target lysis by CD4 positive-dominated cultures was at background levels. After long-term follow-up, 3 patients died within 1.5 years after infusion, 1 patient had a complete response after almost 2 years, and 2 patients demonstrated a partial response after 2 years. Due to the sample size and variable prior and concurrent adjuvant treatments, it is difficult to extrapolate clinical benefit from this study although the protocol employed appeared safe to patients receiving TILs and IL-2. However, the study raises several points. First, it is not clear what percentage of TILs infused into the ventricles will traffic through the brain-CSF barrier to the tumor site. Second, the studies of El Andaloussi and Lesniak (127) and Hussain *et al.* (129) demonstrated that TILs may harbor variable levels of Tregs. Indeed, in the study of Quattrochi *et al.* (146), infused TILs harbored between 12-96% CD25+ cells, of which some were likely activated T cells and some may have represented Tregs. As it would be critical to avoid adoptive transfer of suppressing cell populations such as Tregs, it will be important to carefully profile and sort TILs prior to future transfer approaches.

Several studies have also documented the presence of TILs following DC-based immunotherapies. Yu *et al.* (147) reported the results of a Phase I trial in which 7 patients with GBM received vaccinations with DCs pulsed with peptides eluted from autologous resected tumor cells. Of the 7 vaccinated patients, 4 demonstrated a peripheral cytolytic T cell anti-tumor response. Furthermore, of 4 patients who underwent surgery for recurrent tumor after vaccination, 2 harbored a brisk CD8+ TIL profile that was absent in the initially-resected tumor specimens prior to vaccination. A subsequent study by the same group (148) provided similar evidence that DC-vaccinated glioma patients may harbor TILs. In this Phase I study, 14 patients with grade III-IV astrocytoma were vaccinated with DCs pulsed with the cell lysate of autologous tumor obtained from surgical resection. Of 6 patients undergoing surgery for disease progression, 3 harbored brisk CD8+ TILs that were absent in initially-resected tumor specimens. Yamanaka *et al.* (149) also demonstrated glioma TILs in a subset of glioma patients vaccinated with autologous lysate-pulsed DCs. Importantly, the studies of Yu *et al.* (147, 148) both provided preliminary data to suggest that DC vaccination may prolong survival. Further study is clearly warranted to determine optimal anti-glioma vaccination approaches and to determine the molecular basis for disease progression despite vaccination-induced TIL accumulation.

Conclusion and future directions

The study by Takeuchi and Barnard (115) concluded with the following sober assessment: "The therapeutic application of immuno-therapy to gliomas has yielded disappointing results to date". Three decades later, it is clear that this statement was perhaps unfair at the time but unquestionably prescient–we have not made clinically significant progress in harnessing the potent specificity of immunity into the development of anti-glioma therapies. Arguably, an enhanced understanding of the spontaneous, naturally-occuring immune responses to glioma would facilitate the rational development of immunologically-based treatments. To this end, while recognizing that there exist unique anatomical features in the CNS, conceptually it will be useful to continue to move away from over-emphasizing the unique features of immune responses to the brain in order to focus instead on the immunobiology shared with immune responses to other tissues. Indeed, in the study of brain tumor

immunology the term "immunologically-privileged" should be discarded and perhaps replaced with "immunodistinct" in order to highlight the viability of an intracranial immune response while conceding potential differences with peripheral immunity in mechanisms of antigen presentation and cellular trafficking. Further work is necessary to elucidate the themes discussed herein that are likely central to the manifestation of TILs within gliomas–namely, (a) the presence of distinct glioma-specific antigens, (b) how these antigens are presented to the immune system to drive the initiation of the immune response, and (c) how primed anti-tumor lymphocytes traffic to glioma sites.

Additional study is necessary to determine whether there is any clinical relevance to the presence of TILs in glioma, and there are clear lines of investigation that must be pursued to address this possibility. As we outlined previously, these additional research efforts should carefully account for the following parameters: (a) tumor grade/type, (b) careful immunophenotyping, (c) lymphocyte location and intensity, and (d) multivariate analysis to clarify the significance of potential clinical correlations. In addition, potential candidates for vaccine therapy approaches may benefit from determining which glioma antigens are recognized by endogenous or therapy-induced TILs. Finally, our studies on immune cells must be coupled with ongoing work into the molecular events underlying glioma-driven immune escape mechanisms in what may well be a dynamic example of human cancer immunoediting (Dunn and Curry, manuscript in preparation).

There remains significant potential in the study of the molecular and cellular immunobiology of the immune response to cancers of the brain. At the very least, the evolution of our understanding of TILs in glioma may provide us with new prognostic information for patients harboring these tumors. However, it is our hope that further study of the *in situ* anti-glioma response–both during the development of naturally-occurring immune responses, as well as during the administration of clinical immunotherapies–will continue to shed light on how we may augment anti-glioma immunity and why endogenous anti-glioma immune responses may often be attenuated *in vivo*. Ultimately, the more we understand the fundamentals of the cancer immunology of the nervous system, the closer we will be to meeting our greatest responsibility: bringing the promise of immunotherapy to bear on the treatment of patients with gliomas.

Abbreviations

CNS, central nervous system; CSF, cerebrospinal fluid; GBM, glioblastoma

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