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Preferential In Situ CD4\(^+\)CD56\(^+\) T Cell Activation and Expansion within Human Glioblastoma

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Recent evidence suggests that suppression of the cellular immune response is often attributable to populations of functionally distinct T cells that act to down-regulate Ag-specific effector T cells. Using flow cytometry, we evaluated tumor-infiltrating lymphocytes (TIL) from patients undergoing neurosurgical resection of glioblastoma multiforme (GBM), metastatic lung carcinoma, and meningioma for markers known to be expressed on immunoregulatory T cells. Ex vivo phenotypic characteristics, cellular proliferation, and cytokine expression patterns were compared between T cell subsets found in the PBMC and within TIL from fresh tumor samples. Interestingly, nearly half of all T cells infiltrating GBM specimens were CD56\(^+\) T cells, while much smaller percentages of similar cells were identified within metastatic lung tumors and meningiomas. CD56\(^+\) T cells identified within GBM were not canonical, or “invariant,” NKT cells, as they demonstrated diverse TCR expression, a primarily CD4/CD8 ratio (that is, 3- to 4-fold higher than the proportion of proliferating CD56\(^−\) T cells from these lesions. In addition, direct ex vivo analysis of cytokine expression by TIL from GBM demonstrated significant numbers of IL-4/IL-13 positive cells, cytokines that are integral in the cell-mediated repression of tumor immunity in experimental models. We propose that GBM has a unique capacity to recruit and activate CD4\(^+\)CD56\(^+\) T cells, a population that has not been previously described within human tumors. *The Journal of Immunology, 2008, 180: 7673–7680.

Escape from the cellular immune response is a hallmark of many malignant tumors. Perhaps most adept in this regard is glioblastoma multiforme (GBM),\(^2\) a highly infiltrative and aggressive cerebral neoplasm that has consistently resisted efforts to improve prognoses for patients with these lesions. Unfortunately, patients with GBM are known to mount extremely poor tumor-specific immune responses. Although these individuals are not systemically immunocompromised, reports have described global changes affecting the cellular arm of their immune response including increased CD4 to CD8 ratios, altered delayed-type hypersensitivity reactions, and blunted T cell proliferation in response to mitogenic stimulation (reviewed in Ref. 1, 2). Although locally secreted factors (TGF-\(\beta\), IL-10) and alterations in the expression of immunologically relevant surface markers (FasL, decreased MHC class I) have been implicated in the escape of GBM from tumor-specific CTLs, the source of the local immunosuppressive effect has not been reliably identified. In addition to the inhibition of endogenous tumor-specific immune responses, GBM-specific factors are likely to inactivate CTL generated or augmented by various tumor vaccine strategies. Therefore, elucidation of the in vivo factors responsible for facilitating tumor evasion from immune clearance will be essential in the development of effective immunotherapy against GBM.

Over the past decade, several subtypes of lymphocytes have been identified that exert a powerful regulatory or suppressive influence upon other cells of the immune system (3). These include CD4\(^+\)CD25\(^{high}\) regulatory T cells (“Treg”), NKT cells, and, more recently, CD56\(^{bright}\) NK cells. Although many studies have evaluated the tumor-specific expression of proteins with potential immunosuppressive activity, little data has been presented regarding the intratumoral presence of cells with known immunoregulatory functions in patients with GBM.

Using flow cytometry, we evaluated fresh ex vivo tumor infiltrating lymphocytes (TIL) from GBM, metastatic lung cancer, and meningioma for the frequency, phenotype, and activation status of T cells with known immunoregulatory properties. In addition, we performed direct ex vivo analysis of cytokine expression by TIL from the various tumors tested to evaluate these cells for the presence of a Th2 phenotype, which has been associated with cell-mediated tumor suppression in a number of experimental models.

Materials and Methods

Tissue acquisition and ex vivo tumor sample preparation

Fresh specimens of glioblastoma multiforme (\(n = 14\)), metastatic lung cancer (\(n = 5\)), and meningioma (\(n = 9\)), along with corresponding arterial blood samples, were obtained intraoperatively from patients undergoing neurosurgical procedures at Columbia-Presbyterian Medical Center. All tissues were collected under the auspices of Columbia University Institutional Review Board Protocols AAAA4666 and AAAA9471. Tumor samples were placed into sterile DMEM and were immediately transported to the research laboratory for preparation and analysis. PBMCs were isolated from fresh arterial blood samples by Ficoll-Hypaque centrifugation using standard protocols (Sigma-Aldrich). Tumor samples were prepared for single-cell ex vivo analysis as we have previously described (4). In brief, freshly resected tumor specimens (~50–100 mg each) were physically dissociated with razor blades and then sharply minced. Selected samples

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2 Abbreviations used in this paper: GBM, glioblastoma multiforme; Treg, regulatory T cell; TIL, tumor infiltrating lymphocyte; aGalCer, α-galactosylceramide; NCAM, neural cell adhesion molecule.

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were enzymatically treated with DNAseI and collagenase IV (Sigma-Aldrich) for 20 min at 37°C to facilitate further mechanical disruption; previous studies demonstrated no difference in surface expression of relevant markers between treated and untreated samples. The resulting cell suspensions were passed through 70-micron filters and washed several times with sterile PBS for subsequent use in flow cytometry.

**Immunohistochemistry**

Representative samples of GBM were paraffin embedded and sliced for immunohistochemical staining with mouse α-CD3 (1/200; DakoCytomation), mouse α-CD4 (1/80; Biogenex), or mouse α-CD8 (1/50, DakoCytomation). Slides were developed using the EnVision-HRP kit (DakoCytomation).

**Ab staining and flow cytometry**

PBMCs (10^6 cells/tube) and ex vivo tumor preparations (1–2 × 10^6 cells/tube) were stained with saturating concentrations of fluorophor-conjugated Abs and the appropriate isotypes (αCD3-allophycocyanin, αCD4-FITC, αCD4-CyC, αCD8-CyC, αCD25-PE, αCD45RA-PE, αCD56-IFITC, αeK-67-FITC, α6B11-PE, BD Pharmingen/BD Biosciences; αVα24-FITC, Coulter Immunotech). Subsets of samples were fixed and permeablized after initial Ab binding to allow for subsequent intracellular staining with αKi-67 (BD Cytofix/Cytoperm Kit, BD Pharmingen). Staining samples were run on a FACS caliber scanner equipped for four-color analysis (BD Biosciences). Resulting data was analyzed using the FlowJo software package (Tree Star). Statistical analysis was performed using the GraphPad InStat software package.

**Staining of GBM TILs with CD1d tetramer**

CD1d tetramers labeled with Alexa 488 (a gift of Dr. Manfred Brigl, Brigham and Women’s Hospital, Boston, MA) were loaded with α-galactosylceramide (αGalCer; KRN7000, Kirin Brewery) as previously described (5–7). Cells were stained with CD56-Chromeo, Vα24-PE, CD4, or CD8-FITC mAbs (BD Biosciences) and analyzed on a FACSCaliber (BD Biosciences).

**Reactivity of GBM TIL populations with CD1d**

A C1R cell line transfected with CD1 isotypes and anti-CD1d Ab (clone 6B11-PE; BD Pharmingen/BD Biosciences) were loaded with α-galactosylceramide and anti-CD1d Ab staining and flow cytometry.

**Intracellular cytokine staining**

PBMCs (5 × 10^5) and ex vivo preps (1 × 10^6 cells) were plated into 24-well flat-bottom plates and cultured in RPMI 1640/10% FCS in the absence or presence of 50 ng/ml PMA/1 μM ionomycin for 6 h. Monensin was added after 1 h of culture as per kit instructions at 4 μl per ml of culture (GolgiStop, BD Pharmingen) to allow for the accumulation of intracellular cytokines. Cells were harvested and initially stained for extracellular CD3, then were fixed and permeablized as above for staining with αIFN-γ-allophycocyanin, αIL-4-allophycocyanin, αIL-13-allophycocyanin, αIL-10-allophycocyanin, or αIL-2-allophycocyanin. Additional αCD3-allophycocyanin was added after fixation/permeablization to increase the signal-to-noise ratio of T cells to tumor, as decreased CD3 fluorescence intensity was noted after stimulation and subsequent processing. Flow cytometry and data analysis was performed as described above.

**Results**

**Intratumoral localization of CD3+ T cells within GBM**

The ability of immunoregulatory cells to interact with infiltrating CTLs in a physiologically relevant manner likely depends on their close proximity within the tumor microenvironment. To demonstrate the intratumoral localization of T cell infiltrates by immunohistochemistry, serial sections from several GBMs were stained with Abs to CD3, CD4, and CD8 (Fig. 1). T cell infiltrates were predominantly limited to and closely clustered within the perivascular spaces, with infrequent T cells found interspersed more deeply within the tumor parenchyma. As a proportion of the total cellularity of the tumor, estimates of the number of T cells identified by immunohistochemistry were congruent with the proportion of ex vivo CD3+ cells subsequently identified by flow cytometry.

**A significant proportion of TIL within GBM are CD4 single-positive CD3+CD56+ T cells**

We evaluated the expression patterns of a number of receptors known to be potentially indicative of an immunoregulatory or immunosuppressive T cell functional phenotype on TIL from a range of intracranial neoplasms. Demographic data were similar for patients with all tumors in regards to age (56.6 ± 3.9 years, 55.5 ± 3.8 years, and 59.0 ± 4.2 years for GBM, meningioma, and lung metastases, respectively) and male to female ratio of the cohort (data not shown). The proportion of cells that were CD3+ within the total ex vivo population ranged from 0.1 to 3.2% for GBM (mean 0.7 ± 0.3%), 0.1 to 0.4% for lung metastases (mean 0.2 ± 0.1%), and 0.1 to 6.0% for meningiomas (mean 1.7 ± 0.7%) (data not shown). Interestingly, nearly half of all CD3+ cells present in the TIL population from GBM coexpressed CD56 (48.2 ± 4.1%) (Fig. 2). This phenomenon was not seen among CD3+ cells present in the TIL population from other nonglial intracranial tumors, including meningioma (26.2 ± 3.3%) and metastatic nonsmall cell lung cancer (23.8 ± 6.7%) (Fig. 2). As expected, comparison of PBMCs for all tumor types indicated that only a minority of CD3+ peripheral lymphocytes expressed CD56 (14.2 ± 2.2%, 16.0 ± 3.5%, and 13.4 ± 3.2% for patients with GBM, meningioma, and metastasis, respectively).

To further characterize the phenotypic characteristics of the CD56+ T cells identified within GBM, we compared the expression of CD4 and CD8 on these cells in PBMC and within TIL. Although CD56 expression has been shown to correlate with activation or cytolytic function of CD8+ T cells (9), it has not been previously reported to be expressed in a similar fashion upon activated CD4+ T cells. Consistent with these findings, the vast majority of CD3+CD56+ cells found in the periphery of patients with GBM were CD8 single positive (73.6 ± 4.4%), while far fewer were CD4 single positive (7.4 ± 2.3%), CD4/CD8 double positive (3.1 ± 0.7%), or CD4/CD8 double negative (16.1 ± 4.1%) (data not shown). In sharp contrast, nearly half of CD3+CD56+ TIL from

**FIGURE 1.** Localization of T cells within glioblastoma. Representative immunohistochemical staining of a glioblastoma specimen for CD3 (A, D), CD4 (B, E), and CD8 (C, F). Low (upper panels) and high (lower panels) power magnification demonstrate scant parenchymal lymphocytes and focal perivascular collections (arrows).

![GBM TIL staining](https://example.com/image1.png)
GBM were CD4 single positive (47.1 ± 4.0%) while fewer were CD8 single positive (38.7 ± 4.1%), CD4/CD8 double positive (9.8 ± 2.1%), or CD4/CD8 double negative (4.8 ± 1.0%) (Fig. 3).

**CD3⁺CD56⁺ T cells within GBM are not canonical “invariant” NKT cells**

Two major dividing lines in the subdifferentiation of NKT cells depend upon restriction to interaction with the nonclassical HLA-like CD1d molecule vs more traditional HLA interactions, and expression of an invariant TCR (Va14-Jα18-Vβ8.2 in the mouse and Va24-Jα18-vβ11 in the human) vs a variable set of TCR subunits. To determine whether the CD3⁺CD4⁺CD56⁺ T cells present within GBMs were CD1d restricted, we stained TILs present in ex vivo GBM specimens with Abs directed against Va24. These Abs consistently failed to stain any of the CD3⁺CD4⁺CD56⁺ subsets of TIL from GBM (Fig. 4, A and B).

**FIGURE 2.** Frequency of CD3⁺CD56⁺ cells within PBMC and TIL. Representative flow cytometric analysis of PBMC (upper panels) and TIL (lower panels) from patients with GBM (A, D), meningioma (B, E), and lung mets (C, F). Cells were initially selected by scatter properties consistent with lymphocytes, then were gated upon CD3 (x-axis) and CD56 (y-axis). G, Mean percentage of CD3⁺ cells that were simultaneously CD56⁺ for the three tumor types in PBMC and TIL. There was a significant enrichment for CD56⁺ T cells within GBM TIL (D, upper right panel) when compared with matched PBMC or TIL from meningioma (E) or lung mets (F) (p = 0.0003). Tumor cells from GBM express considerably more CD56 (D, upper left panel) than either meningioma (E) or lung mets (F).

**FIGURE 3.** CD4 and CD8 expression patterns on CD56⁺ T cells within PBMC and TIL from glioblastoma. Graphical representation of CD4 and CD8 expression on CD3⁺CD56⁺ cells within PBMC (upper row) and TIL (lower row) from all 14 GBM specimens tested. There is a significant shift from a predominantly CD8 single-positive phenotype in the periphery to a CD4 single-positive phenotype within the tumors. In addition, CD4/CD8 double-negative cells were rare within TIL.
were similarly elevated within GBM. Although CD25 is expressed
cers (10); therefore, we investigated that possibility that Treg cells
inhibitory effects on activated CTLs in a variety of systemic can-
munoregulatory lymphocytes previously shown to exert profound
expression of IFN-γ stimulation with cells expressing the CD1d isoform by upregu-
ating expression of IFN-γ, neither of the CD3\(^+\)CD56\(^{low}\) T cells within the various CD1 subtypes. Whereas
differing in expression levels of CD56, in experiments using
CD\(^+\)CD56\(^{low}\) and CD\(^+\)CD56\(^{high}\) GBM TIL populations stained with Vα24 mAb nor the aGalCer loaded CD1d tetramer. Populations of GBM TILs were tested for functional
recognition of CD1d, C1R cells transfected with isoforms of CD1 were fixed and
analyzed (\(D–F\)). Neither the CD56\(^{low}\) or CD56\(^{high}\) GBM TIL populations responded to the CD1d transfected C1R cells. All
the NK cell clone responded to plate bound anti-CD1 with IFN-γ secretion: CD56\(^{low}\) cells, 3,703 ± 112 pg/ml; CD56\(^{high}\) cells, 2,329 ± 27 pg/ml; and invariant NKT cell clone, 6,312 ± 307 pg/ml.

Moreover, analysis of TCRβ expression demonstrated that the
CD\(^+\)CD56\(^{low}\) cells in this tumor were polyclonal, as there was no
overrepresentation of any of the Vß subunits tested (data not
shown).

To further confirm that the CD\(^+\)CD4\(^-\)CD56\(^{+}\) T cells did not represent classical invariant NKT cells, we established T
cell lines from an ex vivo GBM tumor specimen using mito-
genic stimulation/IL-2 expansion in vitro. We identified several
lines that overwhelmingly expressed CD3 and CD56 but failed to
express the Vα24 chain of the TCR that is associated with
invariant NKT cells (Fig. 4D). Further staining of these T cell
lines indicated that CD3\(^+\)CD56\(^{+}\) T cells were present that ex-
pressed CD4 or CD8 surface receptors, consistent with our ex
vivo analyses of TILs. Staining these subsets of CD3\(^+\)CD56\(^{+}\) T cells failed to demonstrate any substantial staining with human
CD1d tetramer loaded with \(\alpha\)-Gal-Cer, a ligand for invariant
NKT cells (Fig. 4, E and F). To further confirm the lack of
CD1d reactivity, we stimulated CD3\(^+\)CD56\(^{+}\) T cells from TIL,
differing in expression levels of CD56, in experiments using
C1R cells transfected with the various CD1 subtypes. Whereas
a traditional, invariant NKT cell clone readily responded to
stimulation with cells expressing the CD1d isoform by upregu-
landing expression of IFN-γ, neither of the CD3\(^+\)CD56\(^{+}\) T cell populations within TIL from GBM responded to any CD1 mol-
ecules (Fig. 4G).

CD\(^+\)CD25\(^{high}\) Treg cells demonstrate only a modest proportional increase within GBM

CD\(^+\)CD25\(^{high}\) Treg cells represent another major class of im-
munoregulatory lymphocytes previously shown to exert profound
inhibitory effects on activated CTLs in a variety of systemic can-
cers (10); therefore, we investigated that possibility that Treg cells
were similarly elevated within GBM. Although CD25 is expressed

on virtually all activated T cells, our group, as well as others, have
previously demonstrated that human FoxP3-high Treg cells can be
discriminated from other activated CD\(^+\) T cells by their compar-
avely high expression of CD25 (11). Fecci et al. have recently
reported that CD\(^+\)CD25\(^{high}\) Treg cells represent an increased fraction of circulating/ peripheral CD\(^+\) T cells in patients with GBM when
compared with normal controls (12) and that selective reduction
of these cells may improve antitumor immunity in a murine glioma
model (13). In parallel, Andaloussi and Lesniak (14) suggested
that Treg frequency, as a proportion of all TIL, increases with
tumor grade in patients with glioma; however, their comparison
was based upon data detailing the frequency of CD\(^+\) TIL that
were CD25\(^+\) (which includes the population of activated CD\(^+\) T cells, characterized as CD25\(^{low}\), that are not Tregs), rather than the
specific identification of CD25\(^{high}\) CD\(^+\) T cells within these
lesions.

Therefore, to determine whether Tregs are found in significantly
increased proportions within GBM, we gated upon CD25\(^{high}\)
CD\(^+\) T cells within PBMC and TIL from the various tumors
tested. We did not find a significant difference in the percentage of peripheral CD\(^+\) T cells with a Treg phenotype between GBM,
metastases, or meningiomas (1.1 ± 0.5, 0.8 ± 0.6, and 0.4 ±
0.1%, respectively) (Fig. 5). Although there was a modest expan-
sion of Tregs within TIL from GBM (4.4 ± 1.5%) when compared
with matched PBMC samples or TIL from meningiomas (0.6 ±
0.2%), the total proportion of Tregs within TIL from GBM was
vastly lower than the percentage of CD56\(^{low}\) T cells found within
these lesions. In contrast, Tregs within TIL from metastatic tumors
were greater than 12-fold more prevalent (10.3 ± 4.7%) than
Tregs identified in corresponding PBMC samples, suggesting that
these cells may play an increased role in the suppression of the
cellular immune response in metastatic lesions.

![FIGURE 4](http://www.jimmunol.org/DownloadedFrom/)
CD56+ T cells within GBM are phenotypically and functionally activated in situ

A widely accepted indicator for the progression of a naive T cell to a “memory” phenotype is the loss of CD45RA expression, generally associated with a concomitant increase in the CD45RO isoform, which is induced by antigenic exposure and T cell activation (15). Although the majority of peripheral CD56+ T cells from patients with GBM were CD45RA positive (72.2 ± 9.6%), only 21.0 ± 5.3% of CD56+ T cells found within TIL from GBM retained CD45RA on the cell surface. This shift was more highly reflected within the CD4+CD56+ population (49.8 ± 16.8 in the periphery vs 9.8 ± 6.1% within TIL) than in the CD8+CD56+ population (76.6 ± 8.4% in the periphery vs 28.4 ± 6.4% within TIL) (Fig. 6).

To evaluate functional activation of TIL from GBM with as little in vitro manipulation as possible, intracellular staining for Ki-67 (an Ag strictly expressed by proliferating cells) was used as a surrogate marker for in vivo proliferative activity. Percentages of proliferating cells within PBMC were similar for CD3+CD56+ cells and CD3+CD56+ cells in both CD4+ (2.4 ± 0.5% vs 8.1 ± 2.4%, respectively) and CD8+ (2.6 ± 0.9% vs 2.1 ± 0.9%, respectively) fractions. However, CD3+CD56+ TIL from GBM demonstrated 3- to 4-fold higher percentages of proliferating cells than the CD3+CD56+ TIL in both CD4+ (17.3 ± 4.4% vs 5.7 ± 2.0%, respectively) and CD8+ (18.3 ± 4.4% vs 5.0 ± 2.3%, respectively) populations (Fig. 7).

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Frequency of CD4+CD25high Treg cells within PBMC and TIL. Representative flow analysis (A–F) and mean percentages (G, H) of Treg cells within the CD4+ population from PBMC and TIL. CD3+ cells within PBMC (A–C) and TIL (D–F) from GBM (A, D), meningioma (B, E), and metastasis (C, F) were subsequently differentiated by CD4 (y-axis) and CD25 (x-axis) staining; Treg cells were identified by gating upon cells with exceedingly high levels of CD25 expression as we have previously described. Scatter plots demonstrate similar frequencies for Tregs observed within the CD4+ population from PBMC (G) for all tumors tested. Tregs were identified at a significant but modestly increased frequency within TIL from GBM when compared with matched PBMC (H); however, Tregs within TIL from metastatic lesions were significantly increased when compared with TIL from other tumors or matched PBMC (ANOVA, \( p = 0.0019 \)).

![Figure 6](http://www.jimmunol.org/)

**FIGURE 6.** CD45RA expression on CD56+ T cells within PBMC and TIL from glioblastoma. Representative (A and B) and mean (C) data from flow cytometric analysis for CD45RA expression. CD3+CD4+ or CD3+CD8+ cells were pregated and compared for CD56 (x-axis) and CD45RA (y-axis) expression within PBMC (A) and TIL (B) from GBM. There was a significant decrease from a CD45RA+ to CD45RA− phenotype in both CD3+CD56+CD4+ (\( p = 0.0016 \)) and CD3+CD56+CD8+ cells (\( p < 0.001 \)) from PBMC to TIL.
Production of Th2 cytokines by TIL from GBM

To evaluate the functional bias of TIL from GBM with as little manipulation as possible, we used direct ex vivo intracellular cytokine detection to determine the cytokine expression patterns of TIL found within the various tumors (Fig. 8). We hypothesized that T cells activated and productive of cytokines within the tumor environment would be observed at detectable frequencies ex vivo without the need for exogenous activation, and that avoidance of nonspecific stimulation in vitro would allow for more accurate representation of T cells activated by a physiologically relevant stimulus. In addition, it has been shown that CD56\textsuperscript{+}/NK and T cells are biased toward a cytolytic or Th1 cytokine-secreting phenotype when exposed to nonspecific mitogenic agents in vitro, conditions which are not likely indicative of in situ stimulation (16, 17). PBMC controls were used in both unstimulated (representative of background cytokine production) and stimulated (as internal positive controls) conditions. As expected, there was little variation in baseline cytokine production by unstimulated PBMCs from patients with any tumor type. In contrast, we found that TIL from GBM harbored increased numbers of cytokine-positive cells in the unstimulated condition relative to either matched PBMC samples or TIL from other tumors, although the differences were not statistically significant for IFN-\(\gamma\), IL-2, or IL-10. However, there was a significant difference in the number of cells positive for IL-4 or IL-13 expression within TIL from GBM (3.2 ± 0.4%)
when compared with either unstimulated PBMC from GBM (1.4 ± 0.4%) or TIL from either meningioma (0.9 ± 0.3%) or metastases (0.9 ± 0.3%) (ANOVA, p = 0.034). The IL-4/IFN-13 positive population was markedly expanded after stimulation with PMA/ionomycin in several GBMs, although there was considerable variation in the group (10.6 ± 6.4%).

**Discussion**

Although the concept of the brain as an “immunoprivileged” organ has been debated, it is clear that activated T cells traffic behind the blood-brain barrier and that brain-derived Ags can induce effective immune responses both in vitro and in vivo (18). Even though reliable tumor Ags have been identified for a range of human malignancies, and Ag-specific CTL can be generated that exhibit potent in vitro killing of tumor cells, clinical vaccine trials and cell transfer experiments have been globally unsuccessful (19). Given recent evidence that immunoregulatory T cells may be the primary modifiers of the cellular immune response, inhibition of tumor-specific CTLs within GBM is likely attributable to the activity of immunosuppressive cells either recruited to or expanded by factors in the local tumor environment. We have confirmed that the majority of infiltrating T cells within GBM are closely restricted to the perivascular spaces, supporting the notion that regulatory cells operate within the immediately accessible tumor environment and locally prevent expansion or infiltration by cytotoxic effector T cells.

Previous analysis of tumor-specific cellular immune responses within GBM has been limited, due to a rarity of infiltrating lymphocytes and the restricted set of interpretations that can be drawn from immunohistochemical analysis. Although cultured TIL can be generated from within GBM samples, the nonphysiologic conditions required to expand these cells in vitro inevitably results in phenotypic drift (of both T cells and tumor cells) and preferential clonal bias or expansion (4, 20). Similarly, the bulk of the available literature indicates that immunoregulatory T cells are extremely sensitive to variations in culture conditions for their ultimate functional activity. As there was no long-term manipulation or culturing of tumor specimens between the time of resection and flow analysis in our experimental paradigm (between 4 and 6 h), we believe our data offer the most accurate representation of the phenotypic and functional characteristics of TIL in vivo.

We have shown that there is a significant expansion of CD56+ T cells within human GBM, most notably within the CD4+ compartment, when compared with PBMC from these patients. This phenomenon was not similarly observed within the other intracranial tumors included in our analysis, implicating a GBM-specific factor. We hypothesize that CD4+ CD56+ T cells within GBM are recruited and activated in a tumor-specific fashion, and subsequently proliferate within the tumor microenvironment, for several reasons as follows: 1) the shift from a peripheral CD45RA+ to a CD45RA− phenotype of CD4+ CD56+ T cells within GBM indicates that the increased prevalence of these cells is not simply attributable to nonspecific T cell infiltration; 2) the expression of a noninnovariant array of TCR subunits, in conjunction with loss of CD45RA expression, by CD56− T cells within GBM suggests that these cells are activated in situ in an Ag-specific manner via traditional TCR-HLA interactions; and 3) evidence for in situ proliferation, demonstrated by an increased proportion of Ki-67+ CD4+ CD56+ TIL when compared with matched PBMC, suggests that these cells are activated and proliferate locally.

The functional relevance of the expanded and activated population of CD4+ CD56+ T cells within GBM remains unclear. Although CD8+ CD56+ T cells within TIL from GBM likely represent activated cytolytic effector cells, the expression and potential functional role of CD56 expression by CD4+ T cells within human tumors has not been previously described. However, it is possible that parallels exist between the CD4+ CD56+ T cells described herein and CD4+ immunoregulatory T cells described in other systems.

NKT cells are a diverse population that express CD3 and a monomorphic NK cell markers. Canonical, or “invariant,” type I NKT cells express Va14-Ja18-Vb8.2 in the mouse and Va24-Ja18-vb1 in the human and are restricted to interaction with the nonclassical HLA-like CD1d molecule. Type I NKT cells are distinguished in their ability to rapidly secrete high levels of either Th1 or Th2 cytokines upon activation, including IFN-γ, IL-4, IL-10, and IL-13, depending on the type of stimulus and experimental conditions. Studies have demonstrated that in vitro stimulation of type I NKT cells with aGalCer (a glycosphingolipid found to be a potent activator of invariant NKT cells when presented in the context of CD1d) results in the production of high levels of both Th1 and Th2 cytokines. The physiologically relevant analog of aGalCer is not known. However, coculture systems using human dendritic cells as stimulators of NKT cells in the absence of aGalCer resulted in the isolated production of IL-13, suggesting that in a more physiologically relevant environment human NKT cells are biased toward the Th2 phenotype (21).

Although the majority of NKT cells in mice are of this invariant subtype, there are significant differences between mice and humans, as well as between different tissues within each species, in regards to TCR expression and functional activity in either in vitro or in vivo systems (22, 23). Recently, increasing attention has focused upon another population of physiologically active NKT cells (“type II”) that demonstrate CD1d restriction yet express a variable array of TCRs. Although type I NKT cells have been associated with both antitumor and immunoregulatory capabilities, recent studies have implicated type II NKT cells, and more specifically the CD4 single positive population, in the suppression of antitumor or autoimmune responses (24, 25). Jahng et al. (26) identified a population of type II NKT cells, expanded after vaccination of rats with a myelin-related glycolipid, that secrete high levels of both IFN-γ and IL-4 upon stimulation and exert strong protective effects against the development of experimental autoimmune encephalomyelitis in vaccinated animals. These results support the notion that certain brain-derived Ags may encourage the expansion of regulatory T cells that protect against CNS-specific immune responses. Terabe et al. (27, 28) confirmed that CD4-single-positive type II NKT cell production of IL-13 is uniquely responsible for suppressing native antitumor immunity in a fibrosarcoma mouse model, and that removal or absence of NKT cells could restore effective immunological clearance of implanted tumors. They went on to demonstrate that IL-13 produced by CD4 single-positive NKT cells was directly responsible for inducing GM-CSF secretion by myeloid suppressor cells, which were subsequently shown to be responsible for the direct inhibition of antitumor cytolytic activity (29). Our data demonstrating a significant expansion of the CD4+ CD56+ T cell subset within TIL from GBM, combined with a prevalence of cells found to be positive for IL-13 expression directly ex vivo, suggest that suppression of CTLs within the microenvironment of GBM may be mediated in a similar fashion. We have generated correlational data identifying increased numbers of circulating myeloid suppressor cells in human subjects with GBM; these patients harbor a significant overrepresentation of circulating HLA-DR negative mononuclear cells and exhibit profound deficits in their ability to generate mature dendritic cells from PBMC, when compared with normal controls or patients with metastatic brain tumors (30).
A mechanism for the specific recruitment and activation of CD4\(^+\)CD56\(^+\) T cells by human glioblastoma remains to be determined. CD56, also known as neural cell adhesion molecule (NCAM), has been shown to form homotypic interactions that lead to activation of a number of potent downstream regulatory pathways. CD56/NCAM is a crucial molecule involved in cellular adhesion, migration, neuronal proliferation, differentiation, and survival, in addition to playing an active role in synaptic plasticity. We observed that a significant proportion of GBM tumor cells express high levels of CD56/NCAM, while meningiomas and lung metastases did not (Fig. 1). The capacity for astrocytes, also known to express NCAM, to induce the generation of regulatory T cells in coculture systems has also been described (31). We hypothesize that specific recruitment or induction of CD4\(^+\)CD56\(^+\) T cells within GBM may be attributable to CD56/NCAM interactions between GBM tumor cells and infiltrating T cells.

Further functional characterization of tumor-infiltrating CD4\(^+\)CD56\(^+\) T cells has been technically difficult, primarily due to the scarcity of CD3\(^+\) lymphocytes within GBM. Given the possibility that CD56/NCAM expression by tumor cells is responsible for the biased expansion and activation of CD4\(^+\)CD56\(^+\) T cells within GBM, we are exploring in vitro strategies involving T cell activation in the context of CD56/NCAM costimulation that may more accurately recapitulate the immunological microenvironment of GBM. Ultimately, further functional characterization of this unique cell population may provide additional insight into factors contributing to the local suppression of the cellular immune response in patients with GBM.

Disclosures

The authors have no financial conflict of interest.

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2. Walker, P. R., T. Calzascia, N. de Tribolet, and P. Y. Dietrich. 2003. T-cell response in patients with GBM. A unique cell population may provide additional insight into factors of GBM. Ultimately, further functional characterization of tumor-infiltrating CD4\(^+\)CD56\(^+\) T cells may be technically difficult, primarily due to the scarcity of CD3\(^+\) lymphocytes within GBM. Given the possibility that CD56/NCAM expression by tumor cells is responsible for the biased expansion and activation of CD4\(^+\)CD56\(^+\) T cells within GBM, we are exploring in vitro strategies involving T cell activation in the context of CD56/NCAM costimulation that may more accurately recapitulate the immunological microenvironment of GBM. Ultimately, further functional characterization of this unique cell population may provide additional insight into factors contributing to the local suppression of the cellular immune response in patients with GBM.


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