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Characterization of Distinct Immunophenotypes across Pediatric Brain Tumor Types

Andrea M. Griesinger,∗† Diane K. Birks,‡∥ Andrew M. Donson,∗† Vladimir Amani,∗† Lindsey M. Hoffman,∗† Allen Waziri,§ Michael Wang,∗† Michael H. Handler,∗† and Nicholas K. Foreman∗†∥

Despite increasing evidence that antitumor immune control exists in the pediatric brain, these findings have yet to be exploited successfully in the clinic. A barrier to development of immunotherapeutic strategies in pediatric brain tumors is that the immunophenotype of these tumors’ microenvironment has not been defined. To address this, the current study used multicolor FACS of disaggregated tumor to systematically characterize the frequency and phenotype of infiltrating immune cells in the most common pediatric brain tumor types. The initial study cohort consisted of 7 pilocytic astrocytoma (PA), 19 ependymoma (EPN), 5 glioblastoma (GBM), 6 medulloblastoma (MED), and 5 nontumor brain (NT) control samples obtained from epilepsy surgery. Immune cell types analyzed included both myeloid and T cell lineages and respective markers of activated or suppressed functional phenotypes. Immune parameters that distinguished each of the tumor types were identified. PA and EPN demonstrated significantly higher infiltrating myeloid and lymphoid cells compared with GBM, MED, or NT. Additionally, PA and EPN conveyed a comparatively activated/classically activated myeloid cell–skewed functional phenotype denoted in particular by HLA-DR and CD64 expression. In contrast, GBM and MED contained progressively fewer infiltrating leukocytes and more muted functional phenotypes similar to that of NT. These findings were recapitulated using whole tumor expression of corresponding immune marker genes in a large gene expression microarray cohort of pediatric brain tumors. The results of this cross-tumor comparative analysis demonstrate that different pediatric brain tumor types exhibit distinct immunophenotypes, implying that specific immunotherapeutic approaches may be most effective for each tumor type.


Childhood brain tumors are now the leading cause of death from childhood cancers. In general, there has been no improvement in treatment options in the last two to three decades. Existing treatments often use radiation and chemotherapy, which carry with them damaging side effects. Immunotherapy is an appealing treatment modality because of the potential for tumor-specific cytotoxicity. Recent studies by our laboratory and others (1–3) have identified an association between host immunity and improved survival in children with brain tumors, specifically ependymoma (EPN), glioblastoma (GBM), and medulloblastoma (MED). The finding that host immunity may already impact survival in these tumor types suggests that they may be better candidates for immunotherapy.

Effective application of immunotherapy in brain tumors is thought to be hindered by the dampened immunity inherent to the CNS and also to brain tumor-mediated immunosuppression. These assumptions are largely based upon extensive brain tumor immunology research that has been conducted in the context of adult GBM. These numerous studies have demonstrated that GBM evades host immunity by a variety of mechanisms that include tumor-secreted and cell-surface immunosuppressive factors and tumor-induced immunosuppressive leukocyte populations (4–10). Whether this immunosuppressive phenotype extends to the types of brain tumor seen in childhood has not been clearly determined.

Polarization of the immune system into activated or suppressed phenotypes is thought to determine its antitumor activity. Classically activated Th cells (Th1) and myeloid cells (M1) can deliver a cytotoxic respiratory burst and secrete proinflammatory cytokines that play an important role in fighting intracellular pathogens and neoplastic cells (11–14). In contrast, alternatively activated Th cells (Th2) and myeloid cells (M2), characterized by low proinflammatory cytokine production, are involved in tissue repair and remodeling, angiogenesis, antiparasitic, and allergic reactions. Th2 T cells and M2 myeloid cells have been ascribed protumorigenic roles (15, 16).

A better understanding of the immunophenotype of pediatric brain tumors would give support to the development of effective immunotherapeutic approaches. To address this, the current study systematically measured the frequency and phenotype of tumor-infiltrating leukocytes in pilocytic astrocytoma (PA), EPN, MED, and GBM—the four most common pediatric brain tumor types.
Materials and Methods

Patient cohort

Surgical tumor samples were obtained from 42 patients who presented between 2009 and 2013 for treatment at Children's Hospital Colorado (Aurora, CO) who were diagnosed with PA (n = 7; median age 8 y), EPN (n = 19; 3 y), GBM (n = 5; 15 y), or MED (n = 6; 5 y) according to World Health Organization guidelines. All tumor samples used in this study were obtained at the time of initial resection and prior to therapy, apart from a single EPN sample that was obtained at second-look surgery of the initial presentation thus having received chemotherapy. Control nontumor brain (NT) tissue was obtained from patients with pediatric epilepsy (median age 13 y) at the time of surgical intervention. Five epilepsy samples of temporal lobe were excluded that had no neoplasm or frank immune cell infiltration. Patient collection was conducted in compliance with Institutional Review Board regulations (COMIRB 95-500 and 09-006).

Disaggregation of tissue samples

Samples were collected in Neurobasal A media (Life Technologies, Grand Island, NY) and immediately disaggregated as described previously (17). Briefly, resected tumor was disaggregated by finely mincing with a razor and further triturated by vigorous pipetting. A single-cell suspension was obtained by passing the sample through a 70-μm cell strainer that is of sufficiently large pore size to permit passage of all immune and tumor cells but not clumped tumor cells (BD Biosciences, Franklin Lakes, NJ). Due to differences in the relative cohesiveness of the tumor types in the study, there may be different levels of tumor cell retention in the 70-μm filters, resulting in a semiquantitative measurement of tumor cells by FACS. Heterogeneous distribution of infiltrating immune cells may be exist within the tumor margins, but this cannot be addressed by FACS once tissue samples have been disaggregated. Rather, FACS of disaggregated samples provides a net proportion of cellular subpopulations within the margins of each particular specimen. The majority of tumor samples processed measured at least 1 cm³ in total volume, providing a significant portion of the total tumor mass. Disaggregated cells were reliably frozen in standard freezing media containing 10% DMSO and stored in liquid nitrogen for subsequent analysis by FACS. Different immune cell populations may be more or less susceptible to cell death after freeze/thaw, but comparative analysis of data generated from identically processed samples minimizes the effect of this potential deficiency.

FACS Ab panel design

Myeloid cells, predominantly macrophage/microglial cells in the context of the CNS, were distinguished in disaggregated tumor specimens by co-expression of CD45 and CD11b. HLA-DR and FcγR CD64 were used as markers of an activated myeloid cells, CD64 also being a marker of the activation state of M1 polarized macrophages (18). These myeloid markers have been associated with improved outcome in microarray-based studies of EPN and GBM, providing further rationale for inclusion in this study (1, 2). Scavenger receptor CD163 and mannose receptor CD206 were used in the current study as markers of this suppressed inflammatory or alternatively activated M2 myeloid phenotype (19). CD50 (ICAM-3) is expressed by undifferentiated or immature myeloid cells (20, 21).

CD45 and CD3 coexpression was used to distinguish tumor-infiltrating lymphocytes. To reveal the phenotype of T cells infiltrating pediatric brain tumors, expression of CD4 and CD8 was used to distinguish Th cells and CTL, respectively. Effector memory T cells were identified by expression of CD45RO, a tumor-infiltrating T cell population that has been associated with a favorable outcome in a number of cancer types (22). Promyelocytic leukemia (PML) protein 1 (PD1-1; CD279) expression was also measured, being a clinically targetable marker of inhibitory T cell activity that has previously been identified as a mediator of immunosuppression in GBM (8, 23, 24). Details of Abs used are described in Supplemental Table 1.

FACS analysis

Viable frozen disaggregated cells were gently thawed and suspended in PBS supplemented with 10% human serum (The Jackson Laboratory, Bar Harbor, ME) as a blocking agent and DAPI (Fisher, Pittsburgh, PA) for live/dead cell identification. After blocking for 15 min at 4 °C, samples were resuspended in FACS buffer (PBS/10% FBS) and multicolor Ab panels or corresponding isotype controls. Samples were stained for 30 min at 4 °C and then washed with FACS buffer, fixed in 2.5% paraformaldehyde, and immediately analyzed by FACS. PD-1 staining was performed on cells that had been incubated for 4 h with 50 ng/ml PMA and 0.5 μg/ml ionomycin as previously described (25).

FACS was performed using a Beckman Coulter Gallios (Beckman Coulter, Brea, CA). All Abs were titrated for optimal Ab fluorescence intensity, and voltages were set using optimized volumes. Compensation was calculated using the autosetup-preprogrammed compensation on Gallios, and settings were saved and used as starting settings for subsequent experiments.

FACS files were analyzed using FlowJo version 9.5.2 (Tree Star, Ashland, OR). All samples were first gated on size and singularity, followed by dead cell exclusion. Gating strategies for myeloid and T cell populations and phenotype markers are shown in Supplemental Fig. 1. All gates were drawn using IgG controls for each sample (Supplemental Fig. 2). Population percentages were compiled for each sample to create a data matrix. Comparative and statistical analyses were performed on this matrix using Microsoft Excel (Microsoft, Redmond, WA) two-tailed Student t test and GraphPad Prism (version 5; GraphPad) Pearson’s correlation analysis.

Principal components analysis (PCA) and unsupervised clustering of flow data were performed using R (http://cran.r-project.org) using publicly available software packages from Bioconductor (http://www.bioconductor.org/).

Gene expression analysis

Microarray processing was performed for pediatric brain tumor and NT samples as described previously (26). The gene expression study sample dataset included 15 PA, 46 EPN, 20 GBM, 22 MED, and 13 NT brain samples. Tumors were obtained from pediatric patients at initial presentation and with no prior treatment. NT brain was collected from autopsy or epilepsy surgery. Sample collection was conducted in compliance with Institutional Review Board regulations (COMIRB 95-500 and 09-006).

Briefly, RNA was extracted from snap-frozen surgical samples, processed, and applied to HG-U133 Plus 2 GeneChip microarrays (Affymetrix). Scanned microarray data were background corrected and normalized using the guanine cytosine robust multiarray average algorithm resulting in log2 gene expression values (27). Gene expression data corresponding to myeloid and T cell markers were then extracted for further analysis. The probeset with the highest expression was selected in cases in which multiple probesets for the same gene existed. Expression levels of PD-1 were below the threshold for accurate detection (log2 normalized expression < −95) in some samples and thus excluded from this analysis. CD45RO is a splice form of CD45 and could therefore not be distinguished in this analysis. Gene expression levels associated with the more prevalent myeloid cells were accordingly higher, allowing all myeloid markers to be assessed by this gene expression analysis. PCA and unsupervised clustering of gene expression data were performed as described above. The microarray data discussed in this publication have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database and are publicly accessible under accession number GSE50161 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE50161).

Statistical analyses

Statistical analyses including those described above were performed using Microsoft Excel (Microsoft), GraphPad Prism (GraphPad), and Bioconductor.

Results

Extent of myeloid cell infiltration varies widely dependent on pediatric brain tumor type

The average percentage of endogenous myeloid cells in the different tumor tissues varied widely, the most myeloid cell–rich being PA (31.6%), followed by EPN (27.1%), GBM (7.6%), MED (4.1%), and then NT (0.4%) (Fig. 1A). The proportion of immune cells in patient tumor specimens was compared with that seen in control NT specimens to estimate the influence of malignancy on extent of myeloid cell infiltration. A large and significantly increased number of infiltrating myeloid cells was observed in both PA and EPN, more infiltrating myeloid cells were also observed in GBM compared with NT (17.2-fold), showing a trend toward significance (p = 0.074). MED exhibited the lowest levels in myeloid infiltration compared with NT (4.1-fold; p = 0.154).
FIGURE 1. Characterization of infiltrating myeloid cells in pediatric brain tumors. Multicolor FACS was used to measure myeloid cell infiltration and functional phenotype in disaggregated samples from NT, PA, EPN, GBM, and MED. Representative FACS data with gating are shown for each marker (left panel). Histograms of individual patient sample data (middle panel) and mean average values (right panel) are shown (error bars indicate SD). Mean average values represented by black bars are those tumor types with values that were significantly different compared NT ($p < 0.05$). (A) Percentage of infiltrating myeloid cells identified by coexpression of CD45 and CD11b (black arrow). Within the myeloid population, the percentage of HLA-DR– (B), CD64– (C), CD50– (D), CD163– (E), and CD206-positive (F) cells are shown. SSC, Side scatter.
The functional phenotype of tumor-infiltrating myeloid cells is skewed differently between different pediatric brain tumor types

PA exhibited the most activated myeloid phenotype of pediatric tumor types in the current study. Myeloid activation markers HLA-DR and CD64 were both significantly higher ($p < 0.05$) in PA compared with NT (3.5- and 2.5-fold, respectively). Markers for undifferentiated and immune-suppressed myeloid cells, CD50 and CD163, respectively, were significantly lower (3.7- and 3.2-fold, respectively) in PA than NT. Myeloid suppression marker CD206 was also lower than NT (2.3-fold), showing a trend toward significance ($p = 0.053$).

As observed in PA, EPN significantly overexpressed both myeloid HLA-DR (3.0-fold) and CD64 (2.3-fold) and underepressed all three nonactivated myeloid markers, CD50, CD163, and CD206, compared with NT (1.72-, 2.4-, and 2.3-fold less, respectively; all trending toward significance ($p < 0.1$)).

GBM showed evidence of a suppressive M2 phenotype. Unlike PA and EPN, nonactivated myeloid phenotype markers CD50, CD163, and CD206 in GBM were not significantly different from NT. M2 markers CD163 and CD206 were, respectively, 5.0- and 2.5-fold higher in GBMs than PA and similarly overexpressed when compared with EPN. Despite the relatively elevated levels of nonactivated myeloid markers, the level of activation markers in GBM was comparable to PA and EPN (HLA-DR, 3.7-fold; CD64, 2.9-fold) when compared with NT.

Compared to other tumor types, including GBM, MED contained a myeloid population that was more clearly skewed toward a suppressive M2 phenotype. MED were distinct from other brain tumors studied in that CD64, indicative of a classically activated M1 phenotype, showed no increase compared with NT. In addition, alternatively activated M2 marker CD206 was significantly higher in MED than both PA and EPN and showed a trend to significance when compared with NT. CD50 and CD163 were not different from NT.

*T cell infiltration is correlated with, but significantly lower than myeloid cell infiltration across all tumor types*

Total T cell infiltration was determined using combined expression of CD45, CD3, and CD8 or CD4. The average number of infiltrating T cells correlated strongly with myeloid cell infiltration in corresponding tumor and NT (Pearson $R = 0.96$; $p = 0.0088$). However, T cells were consistently less common (~10%) than myeloid cells in each tumor type and NT. The average percentage of endogenous T cells in the different tumor types varied widely but was in all cases higher than NT (0.02%), the most T cell rich being PA (4.05%) followed by EPN (2.27%), GBM (0.79%), and MED (0.40%).

*CD8$^+$ and CD4$^+$ T cell infiltration varies significantly between tumor types and NT*

Compared to NT and other tumor types, PA contained on average the highest number of infiltrating CD8 T cells (3.28%), ~300-fold more frequent than NT (Fig. 2A). EPN, GBM, and MED exhibited progressively lower CD8 T cell infiltrations than PA, but all were higher than NT: 125-, 46-, and 23-fold higher, respectively. CD4 T cell infiltration in EPN (1.40%) was higher than NT (83-fold) and all other tumor types (Fig. 2B). PA, GBM, and MED exhibited progressively lower CD4 T cell infiltration than EPN but that was again higher than NT: 73-, 26-, and 13-fold, respectively; although GBM and MED were not statistically different from NT.

The ratio of CD8 to CD4 T cells has been identified as a prognostic factor in FACS studies of certain non-CNS tumors (28). In the current study, CD8/CD4 T cell ratios were higher in all tumor types than NT. The average ratio of CD8 to CD4 cells in PA was 3.91, significantly higher ($p = 0.0011$) than the ratio in NT, in which CD8 and CD4 T cells were present in roughly equal numbers (average ratio CD8/CD4, 0.83). The average ratio of CD8/CD4 in EPN, GBM, and MED was progressively lower (3.59, 2.83, and 2.78, respectively). The higher CD8/CD4 ratios in tumor compared with NT brain suggest a preferential recruitment of CD8 T cells to the tumor microenvironment rather than a nonspecific migration of CD8 and CD4 T cells from adjacent normal brain. Activated/memory T cell marker CD45RO was significantly higher ($p < 0.05$) in PA and GBM than NT (1.9- and 2.1-fold, respectively) (Fig. 2C). EPN also demonstrated CD45RO expression on CD8 T cells that was elevated above NT, and this difference showed a trend toward significance ($p = 0.077$). CD8 T cell CD45RO was not significantly elevated in MED compared with NT. CD4 T cells in all tumor types exhibited modestly elevated CD45RO compared with NT (Fig. 2D).

Evidence for an active phenotype in the majority of pediatric brain tumor–infiltrating T cells was presented by analysis of PD-1 expression, a marker of T cell inactivation (Fig. 2E, 2F). CD8 and CD4 T cells in NT were predominantly expressed PD-1 (72.4 and 81.9%, respectively). Compared to NT, all tumor types exhibited significantly ($p < 0.05$) lower PD-1 expression on both CD8 and CD4 T cells, apart from GBM, which trended toward significance with average expression of PD-1 on CD8 and CD4 T cells 1.9- and 1.5-fold lower than NT ($p < 0.1$) (Fig. 2E, 2F). Again, PA exhibited the most activated immune cell phenotype, exhibiting the lowest PD-1 expression in both CD8 and CD4 T cells of any tumor analyzed.

Unbiased clustering analyses of immune markers emphasizes distinct tumor type–specific immunophenotypes

The multiple immune markers measured in this study were combined to create a data matrix that was subjected to unbiased clustering analyses. PCA demonstrated that based on these immune markers, partial grouping of samples occurs depending on tissue type (Fig. 3A). The MED group overlapped with the NT group, with GBM, EPN, and PA groups moving progressively farther away from the NT group. GBM, EPN, and PA clusters showed some overlap with each other, demonstrating varying levels of heterogeneity within groups.

Unbiased hierarchical clustering of the 12 myeloid and T cell immune markers was performed to identify relationships between expression patterns of immune markers across all samples (Fig. 3B). This analysis identified two distinct groups that corresponded to markers of activated and suppressed phenotypes, respectively, and not according to whether markers were myeloid or T cell specific. This analysis also identified strong correlation between some markers, most notably HLA-DR and CD64, which showed the closest grouping of any marker used in the current study. The data matrix was further examined to directly measure correlations between myeloid markers HLA-DR, CD64, CD50, CD163, and CD206 across all samples. This analysis showed that HLA-DR and CD64 had the strongest correlation of any myeloid marker (Pearson’s $R = 0.79$; $p < 0.0001$), in accord with the clustering analysis. This analysis also demonstrated an inverse correlation of HLA-DR with CD206 ($R = -0.33; p = 0.032$) and a trend to inverse correlation of HLA-DR with CD206 ($R = -0.28; p = 0.078$). CD50 positively correlated with CD163 ($R = 0.31; p = 0.045$) and CD206 ($R = 0.31; p = 0.044$). The positive and inverse correlations observed between myeloid markers prompted reanalysis of FACS data to identify whether those markers identified distinct subpopulations of cells within the myeloid cell population. This analysis demonstrated that none
FIGURE 2. Characterization of infiltrating CD8 and CD4 T cells in pediatric brain tumors. Multicolor FACS was used to measure T cell infiltration and functional phenotype in disaggregated samples from NT, PA, EPN, GBM, and MED. Representative FACS data with gating are shown for each marker (left panel). Histograms of individual patient sample data (middle panel) and mean average values (right panel) are shown (error bars indicate SD). Mean average values represented by black bars are those tumor types with values that were significantly different compared NT ($p < 0.05$). (A) Percentage of infiltrating T cells was identified by coexpression of CD45 and CD3. Percentage of CD8 T cell infiltration was then determined by expression of CD8 on T cells. (B) Percentage of CD4 T cell infiltration was determined similarly by expression of CD4 on CD45$^+$CD3$^+$ T cells. Activated/memory T cells were identified by expression of CD45RO in CD8 (C) and CD4 (D) T cells. T cell inactivation was measured by expression of PD-1 in CD8 (E) and CD4 (F) T cells after PMA/ionomycin stimulation. SSC, Side scatter.
of the studied markers was expressed exclusively. HLA-DR and CD64 showed the strongest coexpression, consistent with clustering analysis; however, CD50-, CD163-, and CD206-expressing populations each showed expression of HLA-DR and CD64 (data not shown). This is consistent with previous studies that demonstrated overlapping marker expression across polarized myeloid phenotypes (18).

As observed by PCA, clustering analysis of FACS data (Fig. 3B) illustrated the varying activation phenotype between sample types, the distinction between PA (high activation) and NT (low activation) being particularly strong. Again, EPN, GBM, and MED exhibit an immune phenotype that is progressively less distinct from NT, with GBM expressing markers of both activated and nonactivated phenotypes.

Gene expression analysis distinguishes pediatric brain tumor–specific immunophenotypes

Gene expression microarray data were mined to determine whether immunophenotypes identified by FACS were reflected by corresponding immune marker gene expression in microarray data expression profiles of a larger cohort of pediatric brain tumor samples.

PCA analysis of immune marker gene expression correlated with results of the same analysis using FACS data, the biggest difference being that NT samples were less distinct from other brain tumor types (Fig. 4A). The strongest separation was between MED and PA, as was seen with FACS immune markers. EPN and GBM clusters exhibited a more spread-out pattern that overlapped with each other and to some extent with PA, but was centered between PA and MED. Hierarchical clustering demonstrated that activation (HLA-DR and CD64) and suppression markers (CD50, CD163, and CD206) clustered separately into two groups, as was seen with FACS data (Fig. 4B). As was seen with clustering analysis of FACS data, gene expression of activation markers between PA and NT was distinct, with EPN, GBM, and MED expressing progressively lower levels of activation marker genes. Thus, the tumor type–specific immunophenotypes observed by FACS analysis were recapitulated by gene expression analysis of a larger cohort of tumor samples.

To determine if gene expression of specific immune markers can predict immune environment within a tumor, myeloid gene expression from the microarray database was correlated with corresponding population percentages obtained by FACS. Linearized gene expression and FACS data were averaged for each tissue type and correlated using Pearson’s correlation. CD11b gene expression (ITGAM; Affymetrix probeset ID 205786_s_at) was positively correlated with FACS data ($R = 0.83; p = 0.044$). With respect to the functional markers, significant correlation was observed with immune activation markers but not those associated with immune suppression. CD64 (FCGR1A/FCGR1C; 216950_s_at) had the best correlation ($R = 0.91; p = 0.057$) for gene expression by microarray and expression level by FACS. Although not exclusively expressed by immune cells, HLA-DR (210982_s_at) also...
had a positive statistically significant correlation with FACS data 
\( R = 0.84; p = 0.037 \). In summary, myeloid activation marker 
gene expression microarray data reiterated FACS results, demon-
strating that different brain tumor types confer distinct infil-
trating myeloid cell phenotypes (Fig. 4C). Based on these results, 
extent of myeloid cell infiltration and activation phenotype can be 
estimated by expression of these key markers in independent gene 
expression microarray datasets. No significant correlation between 
gene expression and FACS data for T cell markers CD8 or CD4 
was observed, likely due to the relative rarity of infiltrating T cells.

The use of control tissue from lateral temporal and frontal lobes 
the FACS study described above means that there is no location 
matched control NT for those tumors arising in the cerebellum, 
namely MED and \( \sim 50\% \) of PA, due to the rare surgical availability 
of such tissue. This potential pitfall was partly addressed by es-
timation of myeloid infiltration and activation between different 
compartments of normal brain, specifically cerebrum, cerebellum, 
and brainstem/thalamus using samples available in the gene expres-
sion microarray database. No significant difference in expression 
of immune markers CD11b, HLA-DR, and CD64 was observed 
among cerebrum (\( n = 8 \)), cerebellum (\( n = 2 \)), or brainstem/thalamus 
(\( n = 3 \)) (Supplemental Fig. 3).

**Discussion**

In the current study, comparative analysis of tumor-infiltrating leu-
kocytes identified distinct immunophenotypes for PA, EPN, GBM, 
and MED, the four most common pediatric brain tumor types. 
Although the nature of this study is descriptive, the use of well-
established functional immune markers allows for inference of 
the functional status of tumor-infiltrating immune cells. The vast 
majority of existing research in the immunobiology of brain tumors 
has been conducted in adult GBM, from which there is a consensus
that GBM exhibit an immunosuppressed or actively immunosuppressive phenotype. The present study provides context to this body of knowledge, finding that the GBM-specific immunosuppressed phenotype does not necessarily extend to all other brain tumor types. In brief, it identified that PA and EPN, by comparison with GBM, MED, and NT, have more leukocyte infiltration and that these cells express markers of a more activated phenotype. Conversely, MED are less infiltrated with leukocytes and express markers that suggest a more immunosuppressed phenotype than GBM.

PA and EPN exhibited high myeloid and lymphocyte infiltration levels that were accompanied by high activation marker expression. Few published studies of tumor-infiltrating leukocytes in EPN exist. In a previous gene expression microarray study by our laboratory (1), the predominant factor associated with improved survival in EPN was overexpression of immune function genes. Expression of the majority of those genes was found to be restricted to tumor-infiltrating leukocytes and that the abundance of tumor-infiltrating leukocytes, specifically myeloid and CD4 T cells, was associated with improved survival, corresponded to gene expression data. Based on these data, it was inferred that the host immune system plays an antitumor role in EPN patients. In the current study, the identification of an activated EPN tumor-infiltrating leukocyte phenotype supports this hypothesis. As in EPN, PA is similarly little studied with respect to immune characteristics. In a recent histology-based tumor-infiltrating leukocytes study, PA were used as a control against which to compare GBM (29). This semiquantitative analysis concluded that significant differences existed in immunophenotype between diagnoses, consistent with the findings of the current study. An earlier gene expression microarray study of PA identified immune gene expression as a distinguishing factor in comparison with higher-grade astrocytomas and normal brain, again consistent with the current study (30). As in EPN, it can be inferred that infiltrating immune cells in PA may also be exerting significant control of tumor growth, consistent with regression of minimal residual tumor in PA that are cured by surgical debulking alone.

Although fewer in number than PA or EPN, the majority of GBM-infiltrating myeloid cells were activated, expressing key mediators of cellular and humoral adaptive immune responses, HLA-DR and CD64, respectively. However, activity of these cells may be attenuated by coexpression of CD163 and CD206. Consistent with previously documented T cell immunosuppression in GBM, the current study found that CD8 and CD4 T cell infiltration was relatively infrequent. Additionally PD-1 expression, a marker of T cell inactivation, was higher in GBM than all other tumor types studied.

Infiltrating myeloid cells in MED were rarer and potentially more immunosuppressed phenotype than GBM in the present analysis. MED exhibited a trend to an alternatively activated M2 phenotype, having lower levels of M1 marker CD64 and higher M2 marker CD206 than any other tumor or NT. As with PA and EPN, minimal published data exist with respect to the immune status of MED. A single immunohistochemistry study identified tumor-infiltrating leukocytes in MED, but did not identify any difference in frequency of infiltration between MED and other brain tumor types including astrocytoma (31). This lack of distinction between diagnoses is likely a function of the less quantitative nature of histology versus FACS.

Tumor-infiltrating leukocytes represent a source of endogenous effector cells that can potentially be exploited therapeutically. The present study has implications for each of the three main categories of immunotherapeutic approach—passive, adoptive, and active. Passive immunotherapy approaches are currently dominated by the use of therapeutic Abs. Ab-dependent cell-mediated cytotoxicity is considered to be the dominant mechanism of the in vivo activity of Abs against tumors and is particularly dependent on engagement of Ab with FcγRs expressed by immune effector cells (32). The results of the current study are particularly pertinent in this respect, identifying abundant expression of FcγR CD64 on the majority of tumor-infiltrating myeloid cells in PA, EPN, and GBM. Furthermore, CD64 has previously been implicated in brain tumor antitumor activity by its association with improved outcome in a gene expression microarray study of EPN (1). The extent of CD64 expression in brain tumor-infiltrating immune cells had not previously been appreciated partly because of technical limitations; specifically, that CD64 cannot be measured by immunohistochemistry in formalin-fixed paraffin-embedded material. The finding that PA-, EPN-, and GBM-infiltrating myeloid cells are potentially primed for Ab-mediated tumor killing is, therefore, novel and suggests that these tumor types might respond favorably to Ab-mediated immunotherapy.

Although the majority of adoptive cell and vaccine clinical trials in brain tumors are directed at adult GBM, a growing number of pediatric trials are being performed that include tumors other than GBM (33–35). Based on the findings of the current study, the less immunosuppressed PA and EPN would in theory provide a more permissive tumor microenvironment for such immunotherapeutic approaches. Conversely, MED would present a less favorable immunophenotype, an inference that is supported by preliminary results of a pediatric dendritic cell vaccine trial in which glial tumors responded more favorably than MED and primitive neuroectodermal tumors (33). The use of ex vivo–expanded tumor-infiltrating autologous T cells as adoptive T cell therapy has been most effectively applied to malignant melanoma and treatment of advanced-stage melanoma has demonstrated a 50% objective response rate (36). The more abundant T cell infiltrations found in PA and EPN would facilitate adoptive T cell immunotherapy for these tumor types, more so than for GBM or MED.

The distinct immunophenotypes across pediatric brain tumor types should be considered when developing immunotherapy approaches. Recently, a number of strategies that improve the immunogenicity of Ab-, cell-, and vaccine-based immunotherapies have resulted in improved survival in refractory tumor clinical trials. Recent successes include the use of Ab-mediated depletion of immunosuppressive CTLA-4 cells in melanoma (37), chimeric Ag receptor–modified T cell therapy in lymphoid leukemia (38), and tumor Ag/GM-CSF fusion protein–primed dendritic cell vaccine in prostate cancer (39). The novel data generated by this study will aid in adapting new immunotherapeutic approaches to the treatment of pediatric brain tumors and advancing these to clinical trials.

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Disclosures
The authors have no financial conflicts of interest.

References


**Supplementary Figure 1: Gating strategy for multicolor flow cytometry analysis.**

(A) Myeloid cell characterization strategy. Cells were first gated on size and singularity followed by DAPI exclusion to identify live cells for further analysis. Live cells were gated on co-expression of CD45 and CD11b to identify myeloid cells. Finally, myeloid cells were gated for expression of immune phenotype markers CD50, CD64, CD163, CD206 and HLA-DR. (B) T cell characterization strategy. Cells were first gated on size and singularity and viability as described above. Live cells were then gated on co-expression of CD45 and CD3 to identify T-cells. T-cells were then further subgrouped into CD4+ and CD8+ T-cells. Finally, CD45RO and PD-1 were measured in both CD4+ and CD8+ T-cells.

**Supplementary Figure 2: Isotype controls for gating strategy.**

(A) Myeloid isotype controls for all antibodies used in the characterization of the myeloid cells. (B) T-cell isotype controls for all antibodies used in the characterization of T-cell cells. Dotplots demonstrate scatter and live cell gating followed by isotypes (red) and test Abs (blue) overlays. List of isotypes used for each antibody is in Supplemental Table I.

**Supplementary Figure 3: Immune marker genes CD11b, HLA-DR and CD64 are not differentially expressed between cerebrum, cerebellum and brainstem/thalamus.**

Average normalized gene expression analysis for immune markers CD11b (affymetrix probeset 205786_s_at), HLA-DR (210982_s_at) and CD64 (216950_s_at) in cerebrum (n=8), cerebellum (n=2) and brainstem/thalamus (n=3)(error bars = SD).
### Myeloid Characteristic flow panel

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Fluorophore</th>
<th>Isotype Control</th>
<th>Volume (µl)</th>
<th>Catalogue number (Becton Dickinson)</th>
</tr>
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### T-cell Characteristic flow panel

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<th>Isotype Control</th>
<th>Volume (µl)</th>
<th>Catalogue number (Becton Dickinson)</th>
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### Functional T-cell flow panel

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