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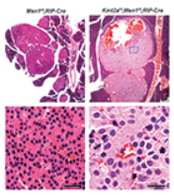

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RESEARCH PAPER

NKG2D ligand expression in pediatric brain tumors

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ABSTRACT

Adult brain tumors establish an immunosuppressive tumor microenvironment as a modality of immune escape, with several immunotherapies designed to overcome this barrier. However, the relationship between tumor cells and immune cells in pediatric brain tumor patients is not as well-defined. In this study, we sought to determine whether the model of immune escape observed in adult brain tumors is reflected in patients with pediatric brain tumors by evaluating NKG2D ligand expression on tissue microarrays created from patients with a variety of childhood brain tumor diagnoses, and infiltration of Natural Killer and myeloid cells. We noted a disparity between mRNA and protein expression for the 8 known NKG2D ligands. Surprisingly, high-grade gliomas did not have increased NKG2D ligand expression compared to normal adjacent brain tissue, nor did they have significant myeloid or NK cell infiltration. These data suggest that pediatric brain tumors have reduced NK cell-mediated immune surveillance, and a less immunosuppressive tumor microenvironment as compared to their adult counterparts. These data indicate that therapies aimed to improve NK cell trafficking and functions in pediatric brain tumors may have a greater impact on anti-tumor immune responses and patient survival, with fewer obstacles to overcome.

Abbreviations: AT/RT, atypical teratoid/rhaboid tumor; CNS, central nervous system; FBS, fetal bovine serum; GBM, glioblastoma; HGG, high-grade glioma; IHC, immunohistochemistry; LDH5, lactate dehydrogenase isoform 5; LGG, low-grade glioma; MIC, MHC class I chain; MIC A, MIC-related gene A; MIC B, MIC-related gene B; NK, Natural Killer cell; PBL, peripheral blood leukocyte; PNET, primitive neuroectodermal tumor; RBS, red blood cell; TMA, tissue microarray; ULBP, UL-16 binding proteins; WHO, World Health Organization

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Immune privilege; immune surveillance; immunosuppressive; NKG2D ligands; NK cells

Introduction

Tumors of the central nervous system (CNS) account for one fifth of childhood cancers, and are the most lethal solid tumors in children.[1,2] The most frequently occurring tumors are astrocytoma, medulloblastoma, and ependymoma, which collectively account for about 60% of all pediatric brain tumors[3], half of which are rapidly dividing high-grade tumors. High-grade pediatric tumors also include rarer or divergent diagnoses, such as primitive neuroectodermal tumor (PNET, WHO grade IV) and atypical teratoid/rhaboid tumor (AT/RT, WHO grade IV), accounting for <5% and 1% of all pediatric CNS tumors, respectively.[4,5] Despite the aggressive nature of high-grade pediatric brain tumors, many of these cancer types respond well to treatment.[6] Following standard of care therapy of tumor resection, chemotherapy, and radiation therapy, 70-80% of children diagnosed with average-risk medulloblastoma are disease-free 5 y after initial diagnosis.[7,8] The most common form of low-grade glioma (LGG), pilocytic astrocytoma, has a cure rate of 90% following surgical resection.[9] However, therapies that target rapidly dividing cells have a significant impact on developing organ systems in pediatric patients. In addition to damaging healthy tissues, the most successful treatment regimens are also associated with lasting side effects in 2-thirds of surviving patients, including neurocognitive damage, hearing loss, infertility, endocrinopathies, and a predisposition to secondary malignancies.[10,11] Therefore, the development of novel therapies with fewer negative side effects would be of considerable benefit to pediatric brain tumor patients. Highly specific and minimally invasive treatment regimens may also improve outcomes of patients with treatment-resistant, multifocal, or metastatic disease.

Studies in both mice and humans demonstrate the importance of an intact immune system in preventing tumor growth.[12-15] This balance between immune surveillance and tumor progression is known as immunoediting and consists of 3 phases: elimination, equilibrium, and escape.[16,17] During the elimination phase, innate and adaptive immune cells recognize

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aberrant surface protein expression on transformed cells and specifically eliminate them.[16,17] In adult patients with functioning immune systems, the elimination phase can last for decades and prevent the growth of a clinically significant tumor burden, making age the single greatest risk factor for cancer. However, it has been proposed that some tumor cells may evolve to avoid elimination, existing in equilibrium with the host immune system.[16,17] During this phase, small numbers of tumor cells may completely escape immune surveillance and initiate the creation of a suppressive tumor microenvironment.[18] This microenvironment is the product of changes in the expression of proteins that result in reduced immune cell activation, as well as the recruitment of cells that suppress immune cell functions,[19-21] allowing establishment of a clinically significant tumor burden.

Natural Killer (NK) cells are cytotoxic effector cells of the innate immune system that play a significant role in the elimination of transformed cells. Tumor-mediated impairment of NK cell function may be critical to the control of tumor growth, as NK cell function correlates with survival in patients with solid tumors.[22-24] Patients deficient in functional NK cells prematurely and rapidly progress through the stages of tumor development.[25,26] Activation of NK cells can be induced through ligation of activating surface receptors, such as NKG2D.[27-29] Cells undergoing stress, particularly viral infection or transformation, can express one or several of the ligands for NKG2D, which include MHC class I chain (MIC) related genes A and B (MIC A and MIC B) and the UL-16 binding proteins (ULBP) 1-6.[30-32] Interaction of NKG2D with any one of these ligands is sufficient for NK cell activation, resulting in the secretion of pro-inflammatory cytokines, and the directed release of granules containing perforin and granzyme for cytolysis of NKG2D ligand-expressing cells.[33,34]

Mechanisms of immune escape are common to the most aggressive adult brain tumors, and impair tumor cell lysis by cells of both the innate and adaptive immune systems, particularly affecting cells responsible for killing tumor cells, such as NK cells. Successful immune escape of tumor cells in adult patients include the production of soluble factors by tumor cells, such as lactate dehydrogenase isoform 5 (LDH5), and TGFβ, that prevent NK cell functions mediated by the activating receptor NKG2D.[35-41] Immune suppression by the tumor microenvironment is associated with poor survival and increased resistance to treatment,[42,43] indicating that restoration of a functional immune system may improve adult patient outcomes. The mechanisms that govern immune surveillance and suppression in pediatric brain tumor patients, however, are poorly defined.

In this study, we sought to determine whether the model of immune escape observed in adult brain tumors is reflected in patients with pediatric brain tumors by interrogating pediatric patient-derived brain tumor tissue microarrays for NKG2D ligand expression and immune cell infiltration. In contrast to what is observed in adult brain tumor patients, NKG2D ligand expression in high-grade glioma (HGG) did not significantly differ from normal adjacent tissue isolated from brain tumor patients. Interestingly, none of the tumor types evaluated had extensive NK cell or myeloid cell infiltration as compared to normal adjacent tissue. Our data suggest that the tumor microenvironment in pediatric brain tumor patients is both less complex and less immunosuppressive than that of adult patients. Our future studies will evaluate whether increasing NK cell infiltration of pediatric brain tumor microenvironment impacts either tumor growth or NK cell functions.

Results

Expression of NKG2D ligand transcripts on frozen pediatric brain tumor samples

Pediatric LGG and HGG cases collectively account for roughly 40% of all childhood CNS tumors.[1,2] In adult patients with gliomas, tumor grade is associated with increased NKG2D ligand expression,[44,45] increased immune cell infiltration into the tumor,[46,47] and the accumulation of redundant mechanisms of immune suppression.[48-50] To determine the possible correlation between the grade of pediatric gliomas and expression of NKG2D ligands, we evaluated 5 LGG and 5 HGG human pediatric frozen tumor tissue samples for mRNA expression of NKG2D ligands (Fig. 1), and found transcriptional expression of all 8 NKG2D ligands in all samples analyzed. Similar to adult glioblastoma (GBM) frozen tumor samples,[25] we found HGG samples to have an average 2.86-fold increase in ULBP-1 (Fig. 1A) and an average 1.71-fold increase in MIC B (Fig. 1H) transcripts as compared to LGG. Additionally, transcript expression of ULBP-2 was significantly increased in HGG relative to LGG tumor samples (average 5.60-fold increase) (Fig. 1B). In contrast, ULBP-4 transcript levels were significantly higher in LGG samples as compared to HGG (average 1.32-fold increase) (Fig. 1D). These data initially suggest a positive correlation between pediatric brain tumor grade and NKG2D ligand mRNA expression.

Expression of NKG2D ligands on pediatric brain tumor TMA

Our data regarding the expression of NKG2D ligand mRNA is limited as it is based off of a small number of LGG and HGG patient samples (Fig. 1). Therefore, to evaluate NKG2D ligand protein expression in a larger patient cohort, we created 5 pediatric tissue microarrays (TMA) with the most common pediatric brain tumor diagnoses: ependymoma (TMA1); medulloblastoma (TMA2); HGG (WHO grade III and IV) (TMA3); AT/RT and PNET (TMA4); and LGG (WHO grade I) (TMA5) (see Figs. S1 and S2 for representative H&E staining; clinical sample details in Tables 2-6). In addition to addressing differences between transcript and protein expression in patient samples, TMA analysis of pediatric tumor tissue can reveal if NKG2D ligands are localized at the cell membrane, where they may interact with NKG2D expressed on immune cells, or in the extracellular space, suggesting ligand shedding or secretion. This information could expose similarities with adult brain tumors, which have been shown to impair NK cell activation through the downregulation of NKG2D by providing either a constitutive and desensitizing signal, or shedding NKG2D ligands into the tumor microenvironment.[51-53]

To determine the expression of soluble and membrane-associated expression of NKG2D ligands, TMAs were stained for expression of NKG2D ligands and analyzed using

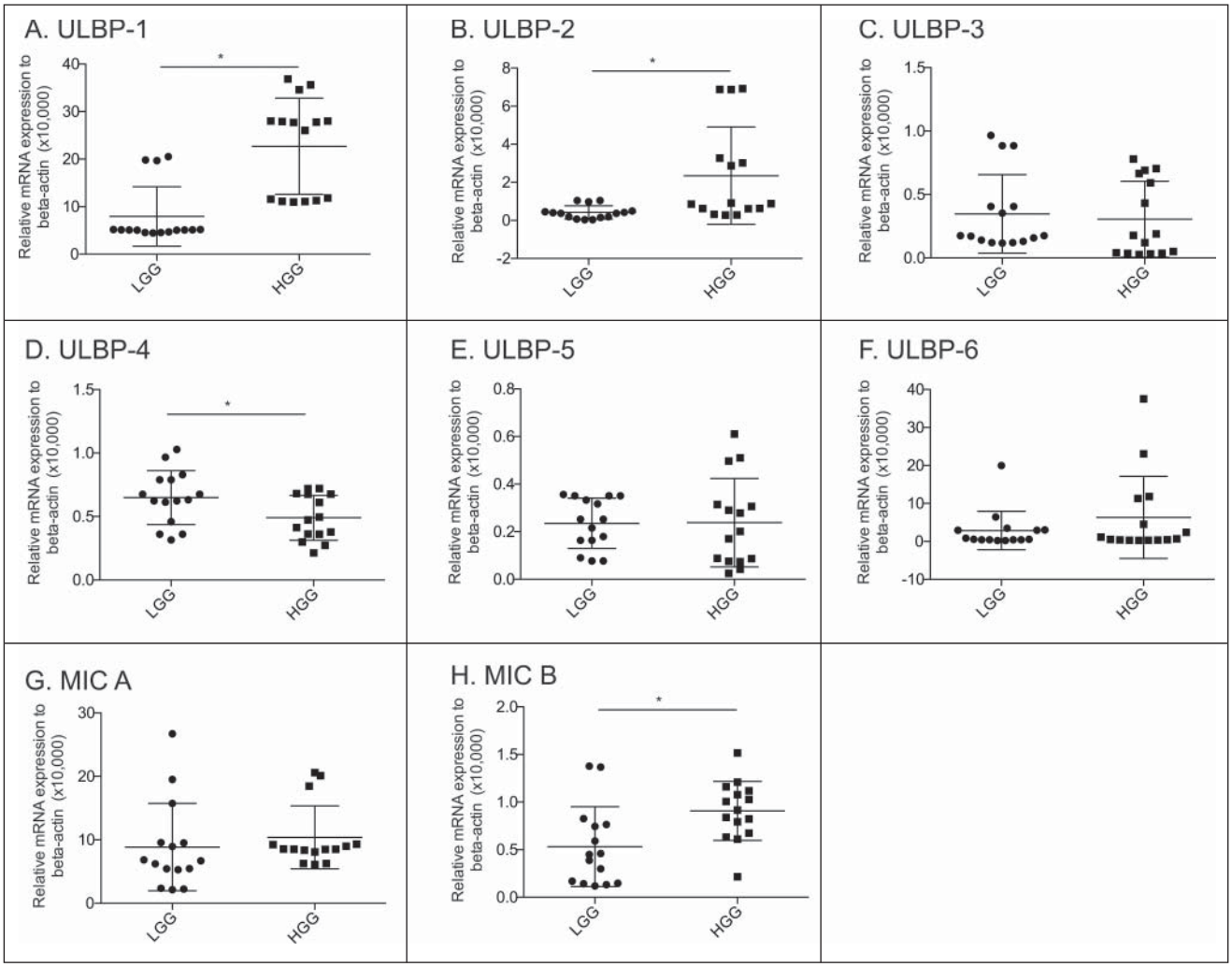

Figure 1. NKG2D ligand transcript expression in pediatric low-grade and high-grade glioma samples. Frozen low-grade and high-grade pediatric tumor samples were analyzed for NKG2D ligand transcript expression (A-H). Tumor tissue samples were homogenized with mortar and pestle. mRNA was extracted from cell pellets using RNeasy Mini Kit (Miltenyi). RT-PCR was performed for cDNA synthesis, and qPCR was performed on cDNA samples 3 times in triplicate. Shown as mean +/− SD. Statistical analysis via unpaired t-test, * = $p < 0.05$.

Table 1. Primers used in this study.

Gene	Forward primer	Reverse primer
β Actin	GCCGACAGGATGCAGAAGGAG	AAGCATTTGCGGTGGACGATG
MIC A	ACAATGCCCCAGTCCTCCAGA	ATTTTAGATATCGCCGTAGTTCCT
MIC B	TGAGCCCCACAGTCTTCGTTA	CCTGCGTTTCTGCCTGTCATA
ULBP-1	TGCAGGCCAGGATGTCTTGT	CATCCCTGTTCTTCTCCCACTTC
ULBP-2	CCGCTACCAAGATCCTTCTGT	CGTGGTCCAGGTCTGAACTT
ULBP-3	CTCGCGATTCTTCCGTACCT	TCTGGACCTCACACCACTGT
ULBP-4	AGGGAATTCTTAGGGCACTGG	ATCGAGGACCACAGGTGAACA
ULBP-5	TGTCCCCTGCGATCCAACTC	ATCCACCTGGCCTTGAACC
ULBP-6	ATTTCATCTTCCAGGATCCACC	GGTCTGAACTTAGGGATGACGG

immunohistochemical (IHC) staining (Table 7; see Fig. S3 for representative staining). Quantification of the resulting images revealed LGG tissue expressed 2.54-fold more soluble, and 10.09-fold more membrane-associated ULBP-2/5/6 as compared to normal adjacent brain tissue control samples (Fig. 2A). Similarly, we found LGG to express 1.22-fold more soluble and 2.24-fold more membrane-associated ULBP-4 compared to control samples (Fig. 2B). Increased expression of NKG2D ligands by LGG tumors suggests that this grade may be the most likely to engage NKG2D and be the most visible to a patient's immune system, which is consistent with a good

Table 2. List of IHC antibodies used in this study.

Antibody	Company	Number	Staining mode	Pre-treatment	Dilution	Incubation time	Incubation temp	Other conditions
NCR1	Abcam	Ab14823	Ventana	CC1	1:200	32 min	37°C	A/B block
CD163	Leica	NCL-L-CD163	Ventana	CC1	1:200	32 min	37°C	
MICA/B	Abcam	Ab54413	Ventana	CC1	1:50	64 min	37°C	
ULBP-1	Atlas	HPA007547	Ventana	CC1	1:100	32 min	37°C	
ULBP-2/5/6	R&D	AF1298	hand stain	1X citrate, 25 min	1:50	Overnight	Room temp	
ULBP-3	Biorbyt	orb5602	Ventana	CC1	1:200	32 min	37°C	
ULBP-4	R&D	MAB6285	Ventana	CC1	1:1000	32 min	37°C	A/B block
LDHA	ProteinTech	19987-1-AP	Ventana	CC1	1:2000	32 min	37°C	A/B block

Table 3. Ependymoma pediatric TMA.

Patient	Cores per TMA	Diagnosis	Specific Diagnosis	WHO Grade
1	2	Ependymoma	Ependymoma	II
2	2	Control	Control	N/A
3	2	Ependymoma	Ependymoma	II
4	2	Ependymoma	Ependymoma	III
5	2	Ependymoma	Ependymoma	II
6	2	Ependymoma	Ependymoma	II
7	2	Ependymoma	Ependymoma	II
8	2	Ependymoma	Ependymoma with vascular proliferation and high Ki-67	II
9	2	Ependymoma	Myxopapillary ependymoma	I
10	2	Ependymoma	Ependymoma	II
11	2	Ependymoma	Ependymoma	II
12	2	Ependymoma	Ependymoma	II
13	2	Ependymoma	Anaplastic ependymoma	III
14	2	Ependymoma	Anaplastic ependymoma	III
15	2	Ependymoma	Ependymoma	II
16	2	Ependymoma	Anaplastic ependymoma	III
17	2	Ependymoma	Ependymoma	II
18	2	Ependymoma	Ependymoma	II
19	2	Ependymoma	Ependymoma	II
20	2	Ependymoma	Ependymoma	II
21	2	Ependymoma	Ependymoma with vascular proliferation	II-III
22	2	Ependymoma	Ependymoma	II-III
23	2	Ependymoma	Ependymoma	II
24	2	Ependymoma	Ependymoma	II
25	2	Ependymoma	Ependymoma	II

prognosis, resulting in over 90% 10-year survival for those patients following surgical resection of tumors.[9]

Interestingly, medulloblastoma (12.22 +/− 20.07%), and AT/RT and PNET (7.63 +/− 12.60%) high grade tumors expressed significantly less membrane-associated ULBP-4 compared to control tissue (37.01 +/− 27.28%) (Fig. 2B). In contrast to frozen patient sample transcript analysis, TMA analysis of ULBP-1, ULBP-3, and MIC A/B protein expression did not yield significant differences between any tumor type and control tissue (see Fig. S4 for transcript analysis). This is not likely due to antibody insensitivity, as specific staining in some samples from each TMA was readily detectable for other antibodies (Fig. 2; see Fig. S4 for transcript analysis).

Collectively, these data suggest that LGG are the most likely to bind NKG2D on tumor-infiltrating NK cells as compared to other diagnoses investigated, which express lower amounts of NKG2D ligand proteins. Given the disparities between mRNA expression (Fig. 1) and protein expression (Fig. 2) in patient samples with various brain tumor diagnoses, future studies will determine molecular mechanisms that prevent robust NKG2D ligand expression. We hypothesize that higher-graded pediatric brain tumors have intrinsic mechanisms to evade NK cell detection by preventing NKG2D ligand protein expression, allowing tumor growth to be invisible to the immune system. Importantly, these features would allow tumors to grow unchecked without the need to establish the immunosuppressive cellular compartment of the tumor microenvironment that is so well studied in adult brain tumor patients.

Table 4. Medulloblastoma pediatric TMA.

Patient	Cores per TMA	Diagnosis	Specific Diagnosis	WHO Grade
1	3	Medulloblastoma	Desmoplastic medulloblastoma	IV
1	1	Control	Control	N/A
2	4	Medulloblastoma	Medulloblastoma, desmoplastic/nodular type	IV
3	4	Medulloblastoma	Medulloblastoma, large cell, anaplastic	IV
4	3	Medulloblastoma	Medulloblastoma	IV
4	1	Control	Control	N/A
5	4	Medulloblastoma	Large cell medulloblastoma	IV
6	2	Medulloblastoma	Medulloblastoma	IV
7	2	Medulloblastoma	Medulloblastoma, desmoplastic/nodular type	IV
8	2	Medulloblastoma	Anaplastic medulloblastoma	IV
9	4	Medulloblastoma	Medulloblastoma with extensive nodularity and anaplasia	IV
10	4	Medulloblastoma	Medulloblastoma, desmoplastic/nodular type	IV
11	2	Medulloblastoma	Medulloblastoma	IV
12	2	Medulloblastoma	Large cell medulloblastoma	IV
13	2	Medulloblastoma	Medulloblastoma	IV
14	2	Medulloblastoma	Desmoplastic medulloblastoma	IV
15	2	Medulloblastoma	Medulloblastoma	IV
16	2	Medulloblastoma	Medulloblastoma	IV
17	2	Medulloblastoma	Medulloblastoma	IV
18	2	Medulloblastoma	Medulloblastoma	IV
19	2	Medulloblastoma	Medulloblastoma	IV
20	2	Medulloblastoma	Medulloblastoma	IV
21	2	Medulloblastoma	Medulloblastoma	IV
22	2	Medulloblastoma	Medulloblastoma with myogenic differentiation	IV
23	2	Medulloblastoma	Anaplastic medulloblastoma	IV
24	2	Medulloblastoma	Medulloblastoma, large cell/anaplastic variant	IV
25	2	Medulloblastoma	Desmoplastic medulloblastoma	IV
26	2	Medulloblastoma	Medulloblastoma	IV
27	2	Medulloblastoma	Medulloblastoma, desmoplastic/nodular type	IV
28	2	Medulloblastoma	Medulloblastoma	IV

Table 5. High grade glioma pediatric TMA.

Patient	Cores per TMA	Diagnosis	Specific Diagnosis	WHO Grade
1	2	Glioblastoma multiforme	Glioblastoma multiforme	IV
2	2	Glioblastoma multiforme	Glioblastoma multiforme	IV
3	2	Glioblastoma multiforme	Glioblastoma multiforme	IV
4	2	Glioblastoma multiforme	Glioblastoma multiforme	IV
5	3	Anaplastic astrocytoma	Anaplastic astrocytoma	III
6	2	Glioblastoma multiforme	Glioblastoma multiforme	IV
7	2	Glioblastoma multiforme	Glioblastoma multiforme	IV
8	2	Anaplastic astrocytoma	Anaplastic astrocytoma	III
9	2	Anaplastic astrocytoma	Anaplastic astrocytoma with desmoplastic features	III
10	2	Anaplastic astrocytoma	Anaplastic astrocytoma with desmoplastic features	III
11	2	Glioblastoma multiforme	Astrocytoma (glioblastoma multiforme)	IV
12	2	Glioblastoma multiforme	Glioblastoma multiforme	IV
13	2	Anaplastic astrocytoma	Anaplastic astrocytoma	III
14	2	Anaplastic astrocytoma	Ganglioma with focal transformation to anaplastic astrocytoma	III
15	2	Glioblastoma multiforme	Glioblastoma multiforme	IV
16	2	Glioblastoma multiforme	Glioblastoma multiforme	IV
17	2	Glioblastoma multiforme	Glioblastoma multiforme	IV
18	2	Anaplastic astrocytoma	Anaplastic astrocytoma	III
19	2	Glioblastoma multiforme	Glioblastoma multiforme	IV
20	2	Anaplastic astrocytoma	Anaplastic astrocytoma	III
21	2	Anaplastic astrocytoma	Anaplastic astrocytoma	III
22	2	Glioblastoma multiforme	Glioblastoma multiforme with mixed astrocytic and oligodendroglial features	IV
23	2	Glioblastoma multiforme	Glioblastoma multiforme	IV
24	2	Glioblastoma multiforme	Morphological glioblastoma multiforme	IV
25	2	Anaplastic astrocytoma	Anaplastic astrocytoma	III
26	4	Glioblastoma multiforme	Glioblastoma multiforme	IV
27	2	Anaplastic astrocytoma	Anaplastic astrocytoma	III

Table 6. AT/RT and PNET pediatric TMA.

Patient	Cores per TMA	Diagnosis	Specific Diagnosis	WHO Grade
1	2	PNET	Primitive neuroectodermal tumor	IV
2	2	PNET	Malignant neoplasm with PNET-like features	IV
3	2	PNET	Primitive neuroectodermal tumor	IV
4	4	PNET	Primitive neuroectodermal tumor	IV
5	2	PNET	Supratentorial primitive neuroectodermal differentiation	IV
6	1	PNET	High grade anaplastic tumor	IV
7	3	PNET	High grade anaplastic tumor	IV
8	2	PNET	Primitive neuroectodermal tumor with dyshistogenic brain	IV
9	1	PNET	Primitive neuroectodermal tumor	IV
10	1	PNET	Primitive neuroectodermal tumor	IV
11	2	PNET	Primitive neuroectodermal tumor	IV
12	2	PNET	High grade primitive tumor	IV
13	2	PNET	Primitive neuroectodermal tumor with divergent differentiation	IV
14	4	ATRT	Malignant rhabdoid tumor	IV
15	2	ATRT	Atypical teratoid/rhabdoid tumor	IV
16	2	ATRT	Atypical teratoid/rhabdoid tumor	IV
17	2	ATRT	Residual atypical teratoid/ rhabdoid tumor	IV
18	2	ATRT	Recurrent/residual atypical teratoid/rhabdoid tumor	IV
19	0	ATRT	Residual atypical teratoid/ rhabdoid tumor	IV
19	2	Control	Control	N/A
20	2	ATRT	Residual atypical teratoid/ rhabdoid tumor	IV
21	2	ATRT	Atypical teratoid/rhabdoid tumor	IV
22	3	ATRT	Atypical teratoid/rhabdoid tumor	IV
23	2	ATRT	High grade tumor with malignant rhabdoid phenotype	IV
24	3	ATRT	Atypical teratoid/rhabdoid tumor	IV
24	1	Control	Control	N/A
25	1	ATRT	Rhabdoid tumor	IV
25	3	Control	Control	N/A
26	2	ATRT	Atypical teratoid/rhabdoid tumor	IV
27	2	ATRT	High grade malignant neoplasm	IV

Characterizing immune cell infiltration of pediatric brain tumor samples

It has recently been demonstrated that the central nervous system, once believed to be immune privileged, regularly interacts with the immune system through a distinct lymphatic structure.[54] Demonstration of lymphatic system in the CNS clarified the mechanism described in many earlier studies showing rapid recruitment of immune cells during brain injury and infection.[55-57] To determine whether pediatric brain tumors recruit immune cells for either surveillance or to promote an immunosuppressive tumor microenvironment, we investigated the infiltration of NK and myeloid cells. Interestingly, we found no significant NK cell or myeloid cell infiltration in any tumor tissue type as compared to control tissue (Fig. 3). In contrast to studies of adult brain tumor patients, these data suggest that pediatric brain tumors do not result in an infiltration of NK cells; indeed, it appears that tumor diagnosis, grade, and prognosis are independent of both suppressive myeloid cell recruitment and of impaired infiltrating NK cell immune surveillance.

Table 7. Low grade glioma pediatric TMA.

Patient	Cores per TMA	Diagnosis	Specific Diagnosis	WHO Grade
1	2	Low grade glial	Glioma	I
2	4	Low grade glial	Glioma	I
3	4	Low grade glial	Glioma	I
4	2	Low grade glial	Glioma	I
5	2	Low grade glial	Glioma	I
6	2	Low grade glial	Glioma	I
7	2	Low grade glial	Glioma	I
8	2	Low grade glial	Glioma	I
9	2	Low grade glial	Glioma	I
10	2	Low grade glial	Glioma	I
11	4	Low grade glial	Glioma	I
12	2	Low grade glial	Glioma	I
13	2	Low grade glial	Glioma	I
14	4	Low grade glial	Glioma	I
15	2	Low grade glial	Glioma	I
16	2	Low grade glial	Glioma	I
17	2	Low grade glial	Glioma	I
18	2	Low grade glial	Glioma	I
19	2	Low grade glial	Glioma	I
20	2	Low grade glial	Glioma	I
21	2	Low grade glial	Glioma	I
22	2	Low grade glial	Glioma	I
23	2	Low grade glial	Glioma	I
24	2	Low grade glial	Glioma	I
25	2	Low grade glial	Glioma	I
26	2	Low grade glial	Glioma	I
27	2	Low grade glial	Glioma	I

To better understand the immune cells that infiltrate pediatric brain tumors, we characterized the phenotype of circulating and tumor-infiltrating immune cells isolated from freshly resected tumor tissue and matched blood of pediatric brain tumor patients using flow cytometry (see Fig. S5 for gating strategy). Following segregation of live immune cells from tumor cells by way of CD45 expression in concert with a Live/Dead dye, immune cells were delineated into myeloid cells, NK cells, and T cells using antibodies directed against CD14 and CD3 (see Fig. S5 for gating strategy). In a separate panel, myeloid cells were evaluated with antibodies directed against CD14 and CD33 (CD14+CD33+), and subclassified using antibodies directed against CD11b (an integrin commonly expressed on monocytes and macrophages) and HLA-DR (MHC class II surface receptor).[58] (see Fig. S5 for gating strategy) Finally, we used the neural adhesion marker CD56, the carbohydrate epitope CD57, and the activating receptor NKG2D to characterize NK cell phenotypes (CD14-CD3-).[59,60] (see Fig. S5 for gating strategy).

Analysis of circulating immune cells suggests that there may be significant differences not only between pediatric patients with low and high-grade brain tumors, specifically with ganglioglioma and anaplastic medulloblastoma, but also between pediatric and adult patients with the highest grade malignancies

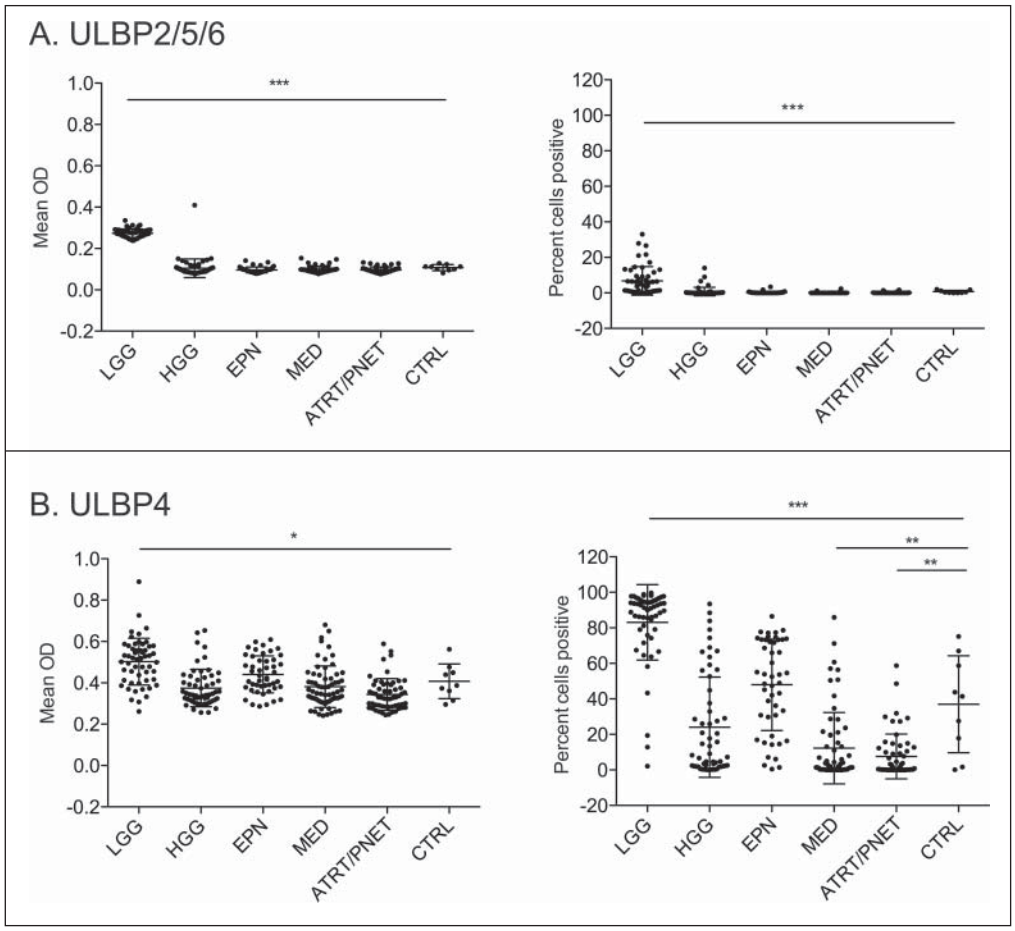

Figure 2. Quantification of pediatric TMA IHC NKG2D ligand analysis. TMAs from 5 types of pediatric brain tumors were constructed and verified for validity by pathologist-reviewed H&E staining. TMAs were then stained for expression of the 8 NKG2D ligands (ULBP 1-6, MIC A, MIC B), and analyzed via immunohistochemistry (IHC). Each tissue core was captured by Nuance camera at 10X and analyzed using inForm software (Perkin Elmer). Control sample is normal adjacent brain tissue. (A) Mean OD of positive area and percent cells positive for ULBP-2/5/6. (B) Mean OD of positive area and percent cells positive for ULBP-4. Shown as mean +/− SD. Data was analyzed for statistical significance using One-way ANOVA in conjunction with Dunnet's post-test. $^{*} = p < 0.05$; $^{**} = p < 0.01$; $^{***} = p < 0.001$.

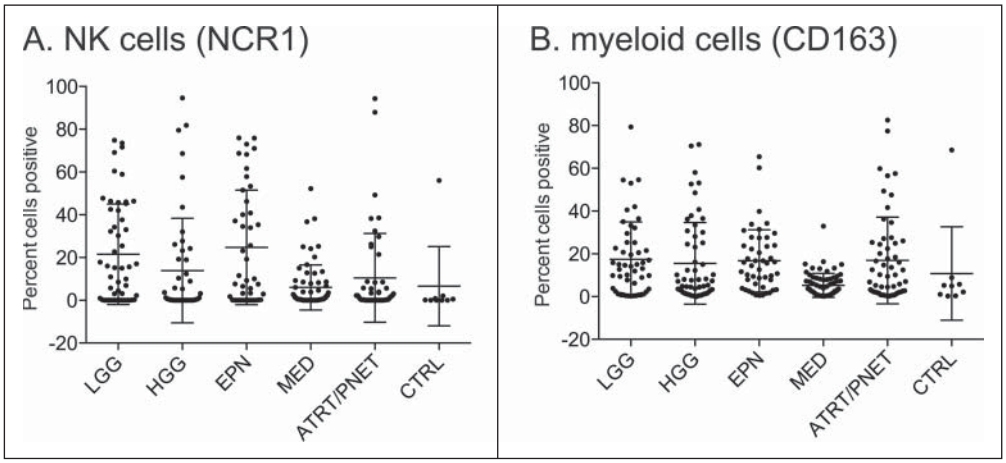

Figure 3. Quantitative analysis of infiltrating immune cells in pediatric TMAs by IHC. TMAs from 5 types of pediatric brain tumors were constructed and verified for validity by pathologist-reviewed H&E staining. TMAs were stained for the presence of immune infiltrating cells using antibodies directed against NCR1 (NK cells) and CD163 (myeloid cells), and then analyzed via IHC. Each tissue core was captured by Nuance camera at 10X and analyzed using inForm software (Perkin Elmer). Control samples are normal adjacent brain tissue. (A) Percent cells positive for NCR1 (NK cells). (B) Percent cells positive for CD163 (myeloid cells). Shown as mean +/− SD. Data was analyzed for statistical significance using One-way ANOVA in conjunction with Dunnet's post-test. $^{*} = p < 0.05$; $^{**} = p < 0.01$; $^{***} = p < 0.001$.

(Fig. 4). Although circulating NK cells in all patients were mostly CD56lo, a hallmark of increased cytotoxic capacity,[61] the patient with Grade I ganglioglioma had a larger CD56hi population, which produces pro-inflammatory cytokines,[62] as compared to both pediatric and adult Grade IV tumor patients (Fig. 4D). Also noteworthy is the decrease in CD11b and HLA-DR expression on myeloid cells found in the pediatric patient with Grade IV anaplastic medulloblastoma (Fig. 4F).

When evaluating tumor-infiltrating leukocytes from these same patients, very few CD45+ cells were found to infiltrate anaplastic medulloblastoma tissue, whereas a substantial CD45+ population was found in low-grade ganglioglioma tissue (Fig. 5A). Analysis of ganglioglioma CD45+ cells showed the majority to be myeloid cells (96%), with small percentages of NK cells (2.12%) and T cells (1.26%) (Fig. 5B). Further delineation of these myeloid cells revealed that nearly all were CD11b+HLA-DR+ (97.6%, Fig. 5F). Despite expression of CD56 and CD57, NK cells were nearly devoid of NKG2D expression (4.13% and 10.9%, respectively) (Fig. 5C and D), suggesting that NK cells infiltrating pediatric tumors may not respond to NKG2D ligand protein expression. This is especially noteworthy when taken with the expectation that nearly 100% of circulating NK cells express NKG2D[63], and the loss of NKG2D receptor expression in mouse models of cancer predisposes those animals to a variety of spontaneously arising tumors.[64]

These findings for pediatric WHO grade I tissue are in contrast to what we observe in adult glioblastoma WHO grade IV tissue (Fig. 5). Specifically, in adult GBM we find a small population of infiltrating CD45+ leukocytes (3.91%) (Fig. 5A), of which a large percentage of myeloid cells are CD11b+HLA-DR+ (72%) (Fig. 5F). NK cells present in adult glioblastoma tissue express low levels of NKG2D in both CD57+ and CD56+ subsets (33.4% and 28.2%, respectively) (Fig. 5C and D), which has been shown to significantly increase following surgical resection.[25] While it is recognized that additional patient samples must be evaluated, these data not only highlight the differences in immune response to pediatric and adult brain tumors, but may also provide insight into experimental therapies aiming to promote immune cell activation in pediatric patients.

Collectively, these data reveal a complex regulation of NKG2D ligands by pediatric brain tumors. The lack of increased NKG2D ligand expression and minimal infiltration of NK cells in high-grade tumors, and a notable absence of NKG2D expression on NK cells infiltrating even low-grade gliomas suggest that NK cells may be an ideal population to evaluate for immune-based therapies for these patients. This is in contrast to the selective outgrowth of NKG2D ligand-deficient cells following NK cell infiltration and surveillance observed in adult brain tumors.[65] Future experiments will determine the regulation of protein expression in these tumors, properties that govern which NKG2D ligands are expressed, and the impact of improving NK cell infiltration into tumors as a method to support traditional therapies. By improving NK cell functions and infiltration in aggressive pediatric brain tumors, we hypothesize that we can improve immune cell recognition of tumor cells, and improve the outcomes of patients undergoing standard of care therapies.

Discussion

Despite fundamental differences between adult and pediatric brain tumors,[66-68] children often receive the same treatment modality as adults, which most frequently includes maximal surgical resection, radiation, steroids to reduce inflammation, and chemotherapy.[69] One reason for this is the assumption that pediatric brain tumors establish a microenvironment that supports tumor immune escape. Our group has demonstrated that peripheral and tumor-infiltrating NK cells from adult glioblastoma (GBM, WHO grade IV) patients have decreased expression of NKG2D, and impaired cytotoxicity, compared to benign meningioma samples, despite the expression of NKG2D ligands on tumor cells.[25] In these patients, tumor resection restores NKG2D expression on CD8 T cells and NK cells, as well as NKG2D-mediated tumor cell lysis.[25] Subsequently, we and others demonstrated that in addition to glioblastoma

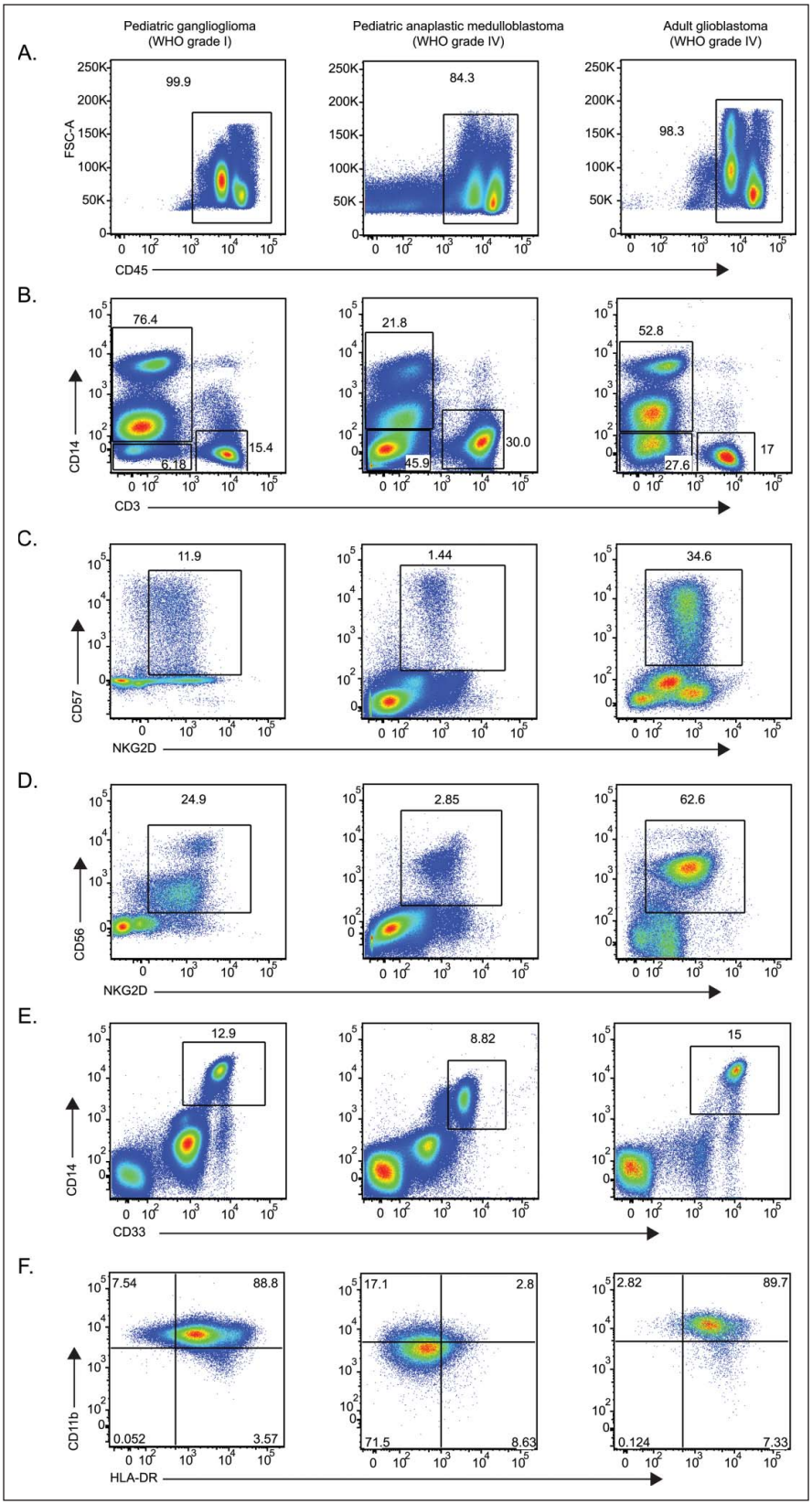

Figure 4. Phenotypic comparison of peripheral blood lymphocytes between pediatric and adult patients. The phenotype of circulating lymphocytes from pediatric ganglioglioma (WHO grade I), pediatric anaplastic medulloblastoma (WHO grade IV), and adult glioblastoma (WHO grade IV) patients was assessed by flow cytometry. Representative plots showing (A) Percent CD45+ lymphocytes; (B) Delineation of myeloid cells, NK cells, and T cells using antibodies directed against CD14 and CD3; (C) Subgrouping of NK cells using antibodies directed against CD57 and NKG2D; (D) Subgrouping of NK cells using antibodies directed against CD56 and NKG2D; (E) Delineation of myeloid cells using antibodies directed against CD14 and CD33; and (F) Subgrouping of myeloid cells from CD14+CD33+ population using antibodies directed against CD11b and HLA-DR. Percent of population is listed for each gate.

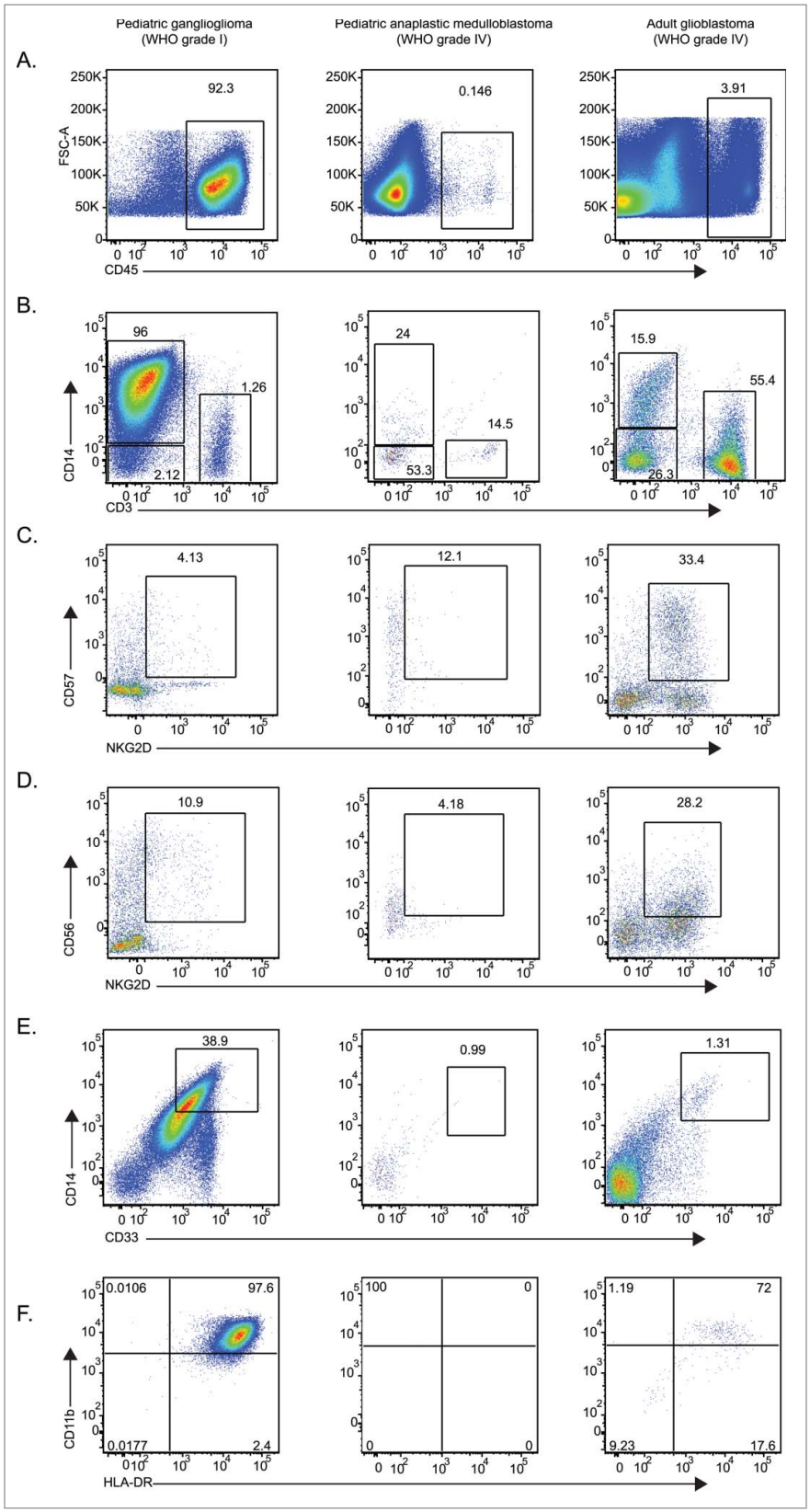

Figure 5. Phenotypic comparison of tumor-infiltrating lymphocytes between pediatric and adult patients. The phenotype of tumor-infiltrating lymphocytes from pediatric ganglioglioma (WHO grade I), pediatric anaplastic medulloblastoma (WHO grade IV), and adult glioblastoma (WHO grade IV) patients was assessed by flow cytometry. Representative plots showing (A) Percent CD45+ lymphocytes; (B) Delineation of myeloid cells, NK cells, and T cells using antibodies directed against CD14 and CD3; (C) Subgrouping of NK cells using antibodies directed against CD57 and NKG2D; (D) Subgrouping of NK cells using antibodies directed against CD56 and NKG2D; (E) Delineation of myeloid cells using antibodies directed against CD14 and CD33; and (F) Subgrouping of myeloid cells from CD14+CD33+ population using antibodies directed against CD11b and HLA-DR. Percent of population is listed for each gate.

tumor cells, myeloid cells recruited to the tumor microenvironment express NKG2D ligands in response to extracellular LDH5, resulting in decreased NK cell antitumor immunity through the depletion of perforin and granzyme, which are required for effective tumor cell lysis.[45,70] Together, these studies indicate that reversal of NK cell suppression may improve anti-tumor immunity and reduce tumor burden. Recent clinical trials for patients with brain tumors indicate that activation of the immune system may provide some clinical benefit, although the mechanisms for this are poorly understood.[71]

Until recently, the CNS was classified as immune privileged, as a necessity to strictly regulate the infiltration and local activation of immune cells that may cause irreparable damage in response to immunological insults.[72] However, in a striking study, Louveau and colleagues recently demonstrated novel lymphatic structures in the CNS.[54] Their data show that circulating immune cells penetrate the blood brain barrier to perform routine immune surveillance of healthy tissue.[54] This immune surveillance by circulating immune cells, particularly NK cells, can eliminate transformed cells as they arise, delaying the establishment of a tumor burden and the associated immunosuppressive tumor microenvironment.[73,74] This study suggests that routine surveillance would prevent tumor growth in the absence of a suppressive tumor microenvironment, although this lymphatic structure has not yet been evaluated in the developing CNS of children.

Although we have shown both increased infiltration of NK cells and myeloid cells into the tumors of adult glioblastoma patients compared to control tissue,[25] we found no significant difference in the frequency of NK cells or myeloid cells in the pediatric tumors that we evaluated. Other groups have also shown that there is no statistical difference in myeloid cells in pediatric glioblastoma and medulloblastoma brain tumors compared to non-tumor controls.[75] Together, these data suggest that even aggressive pediatric brain tumors may not recruit suppressive immune cells. If the brain tumor microenvironment in pediatric patients is not as actively immunosuppressive as their adult counterparts, immune-based therapies for pediatric brain tumor patients may have fewer obstacles to overcome in order to successful eliminate tumors. Additional evaluation of unfixed patient tumor samples with a variety of diagnoses will determine whether persistence of malignant pediatric brain tumors is the result of poor trafficking of immune cells to the tumor site, and thus lack of immune surveillance.

Developers of immune-based therapies for pediatric brain tumor patients must also consider the immunosuppressive effects of standard of care therapies, including corticosteroids, radiation, and chemotherapy. Data are presented in this study with the caveat that a majority of the patients evaluated were on concomitant therapy that may impact immune cell populations. Therefore, immune based-therapies will ideally be administered in the absence of immunosuppressive treatments.

Interestingly, examination of pediatric glioma samples shows that transcriptional expression of NKG2D ligands only partially predicts protein abundance, suggesting intrinsic molecular mechanisms of the tumor cell that reduce NKG2D ligand expression, and therefore visibility to infiltrating NK cells. Previous studies demonstrate that highly expressed genes in cancer cells can have shorter 3'UTRs or fewer miRNA-binding sites, resulting in increased protein expression.[76] Conversely, reduced protein expression may be the result of targeting of NKG2D ligand proteins for rapid degradation or restricted mRNA translation.[76] Our future studies will confirm NKG2D ligand protein analysis in a larger cohort of patients and dissect the molecular mechanisms that reduce NKG2D mediated recognition of pediatric brain tumor cells.

Our findings support a model of immune privilege of the CNS in pediatric cancer patients that restricts immune cell infiltration in patients with high-grade malignancies, as opposed to a compromised blood brain barrier in adult patients that supports the creation of a suppressive tumor microenvironment. Future studies will dissect the mechanisms of immune cell infiltration into pediatric brain tumors, and the molecular mechanisms that govern tumor cell crosstalk with the developing immune system in pediatric patients. These studies will ultimately identify the most effective uses of patient immune cells for the treatment of brain tumors in children.

Materials and Methods

Acquisition and processing of human brain tissue and matched blood

Human tumor tissue and blood was acquired in accordance with guidelines set forth by Seattle Children's Research Institute Institutional Review Board (IRB #14412, approved 6/30/15). Tumor tissue was removed from the patient under sterile conditions in the operating room and placed on a saline-moistened Telfa pad in a sterile specimen cup that contained 40mL RPMI supplemented with 10% FBS. All tissues remained on ice until processed with < 2 hour ischemic time. Tissues were dissociated and tumor-infiltrating lymphocytes (TIL) were extracted using the Miltenyi Biotec Brain Tumor Dissociation Kit (P) according to the manufacturer's protocol (Miltenyi Biotec). The resulting cell pellet was resuspended in PBS, and aliquoted into a 96 well plate for phenotype analysis by flow cytometry.

Patient blood was obtained during surgery. Specifically, 3-6 mL of whole peripheral blood was drawn into BD Vacutainer Sodium Citrate Tubes or BD Vacutainer K_2EDTA Tubes (Becton Dickinson, BD) through an intravenous line. Tubes were centrifuged at 750 xg for 5 minutes at room temperature; plasma was then removed and frozen at −80°C. The remaining blood product was placed in 1X Red Blood Cell (RBC) Lysis Buffer (eBioscience) (10 ml RBC Lysis Buffer per 1 ml blood product) for 10 minutes at room temperature. RBC lysis reaction was stopped with 20 ml 1x Phosphate Buffered Saline (PBS) and centrifuged at 750 xg for 5 minutes. The supernatant was removed and remaining peripheral blood leukocyte (PBL) pellet was resuspended in PBS, and aliquoted into a 96 well plate for phenotype analysis by flow cytometry.

RNA extraction and reverse transcriptase quantitative PCR

mRNA was extracted from frozen tissue (5 low-grade glioma, 5 high-grade glioma) using the RNeasy Mini Kit (Qiagen). Less than 30mg of sample was cut from the tumor over dry ice, and was kept frozen until homogenized using a mortar and pestle.

cDNA was synthesized from total mRNA by oligo dT priming and SuperScript III reverse transcriptase (Life Technologies). PCR amplification was performed with Power SYBR Green PCR Master Mix (Life Technologies). Quantitative polymerase chain reactions were performed in triplicate on 3 separate days. Cq values were normalized to β-actin transcript expression and presented as relative expression units x 10,000. Data were collected and analyzed using the CFX96 Real Time System (Bio-Rad Laboratories). Validated primers used are listed in Table 1.

Tissue microarrays

Tissue microarrays (TMA) were constructed from formalin-fixed paraffin-embedded brain tumors (11 primitive neuroectodermal tumors, 13 atypical teratoid/rhabdoid tumors, 27 medulloblastomas, 25 ependymomas, 25 high-grade gliomas, 25 low-grade gliomas) using 1.5 mm cores (Arraymold Kit B, Thermo Fisher Scientific). Two to 4 separate samples of tumor were included from each case and resulting quantification was averaged; uninvolved brain was included if available. Hematoxylin and eosin–stained sections from all tissue blocks were reviewed by a pathologist to confirm the reported histologic subtype and to define representative tumor areas to be included in the array.

Immunohistochemistry

Samples were stained according to the protocols listed in Table 2. A single image of each core of each TMA was captured using a Nikon Eclipse Ci (10x, 0.45NA) with a Nuance Multispectral Imaging System (Perkin Elmer) every 20nm from 420-720nm. Multispectral images were analyzed using InForm software (version 2.0.584, Perkin Elmer). Spectra for hematoxylin and 3,3' Diaminobenzidine (DAB) were selected from positive control tissue (spleen). Configuration tools utilized were "Trainable tissue segmentation" and "Object segmentation." Trainable tissue segmentation was used to exclude white space in the field of view, and thus was excluded from analysis and percent area calculation. The trainable tissue segmenter used a large pattern scale with extra fine segmentation resolution, and successful training of the tool was >99%. Object segmentation parameters were set to identify objects in the tissue category using an object-based approach, which included a fixed scale optical density (OD) on DAB component (minimum = 0.110) and a minimum size of the object at 1 pixel with no maximum size. No cleanup metrics or edge rules were utilized in analyses.

Percent positive cells/field was calculated based on DAB positivity threshold set from positive control tissue, and hematoxylin staining. InForm software identified cell nuclei based on hematoxylin spectra, then calculated the number of cells above the DAB positivity threshold. Mean OD was calculated from only the area above the DAB positivity threshold set from positive control tissue. Negative tissue area was not averaged into the mean OD for the sample.

Phenotypic analysis of human tumor-infiltrating immune cells

Cells isolated from human brain tumor tissue and matched blood were surface stained with antibodies directed against CD45, CD3, CD33, CD56, CD57, CD11b, CD14, and NKG2D (all from BioLegend). LIVE/DEAD fixable dye (Thermo Fisher Scientific) was included to ensure analysis was only on viable cells. Myeloid cells were designated as CD45+CD14+CD3-, but were further delineated from a CD45+CD14+CD33+ population by CD11b and HLA-DR expression. NK cells were designated as CD45+CD14-CD3-, and further delineated by CD56, CD57, and NKG2D expression. Cells were fixed with 2% paraformaldehyde, and analyzed using the LSRFortessa (Becton Dickinson) and FlowJo software (Treestar).

Statistical analysis

Immunohistochemical stains were analyzed using a post hoc one-way ANOVA, after which each TMA was compared to the control samples only with a Dunnett's multiple comparison test. Transcript expression of NKG2D ligands in both pediatric cell lines and pediatric frozen tissue samples was analyzed via unpaired t-test. Statistical analyses were completed using Prism software (GraphPad Software).

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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