Differential Immune Microenvironments and Response to Immune Checkpoint Blockade among Molecular Subtypes of Murine Medulloblastoma

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Abstract

Purpose: Despite significant strides in the identification and characterization of potential therapeutic targets for medulloblastoma, the role of the immune system and its interplay with the tumor microenvironment within these tumors are poorly understood. To address this, we adapted two syngeneic animal models of human Sonic Hedgehog (SHH)-driven and group 3 medulloblastoma for preclinical evaluation in immunocompetent C57BL/6 mice.

Experimental Design and Results: Multicolor flow cytometric analyses were used to phenotype and characterize immune infiltrating cells within established cerebellar tumors. We observed significantly higher percentages of dendritic cells, infiltrating lymphocytes, myeloid-derived suppressor cells, and tumor-associated macrophages in murine SHH model tumors compared with group 3 tumors. However, murine group 3 tumors had higher percentages of CD8+ PD-1+ T cells within the CD3 population. PD-1 blockade conferred superior antitumor efficacy in animals bearing intracranial group 3 tumors compared with SHH group tumors, indicating that immunologic differences within the tumor microenvironment can be leveraged as potential targets to mediate antitumor efficacy. Further analysis of anti-PD-1 monoclonal antibody localization revealed binding to PD-1+ peripheral T cells, but not tumor infiltrating lymphocytes within the brain tumor microenvironment. Peripheral PD-1 blockade additionally resulted in a marked increase in CD3+ T cells within the tumor microenvironment.

Conclusions: This is the first immunologic characterization of preclinical models of molecular subtypes of medulloblastoma and demonstration that response to immune checkpoint blockade differs across subtype classification. Our findings also suggest that effective anti-PD-1 blockade does not require that systemically administered antibodies penetrate the brain tumor microenvironment. Clin Cancer Res; 22(3); 582–95. ©2015 AACR.

Introduction

Medulloblastoma, the most common malignant primary brain tumor of childhood, remains incurable in approximately one third of patients despite surgical resection, radiation therapy, and aggressive chemotherapy (1–3). Patients endure significant morbidities from such treatments, thus necessitating more targeted strategies that utilize accurate molecular subtype classification (1–3). Mediating consistent and safe treatment for patients with medulloblastoma represents the next goal in achieving an unmet need for the successful eradication of these malignancies (4).

Immunotherapy presents an effective approach that has shown considerable advances in generating sustained and robust antitumor responses in malignant gliomas (5). However, the exploration of immune-based strategies in pediatric brain tumors has been limited and to date, few successful applications have been reported for patients with medulloblastoma (6, 7). A considerable obstacle in the development of medulloblastoma immunotherapy has been a lack of understanding regarding the complex immunologic interactions that occur within the tumor microenvironment. Recent IHC and gene expression evidence has shed some light on the immunologic phenotype across medulloblastoma subtypes and shown that tumor-associated macrophage and inflammatory gene upregulation could additionally be used to stratify the different molecular subgroups (8). These observations demonstrate that medulloblastoma tumor subtypes contain highly distinct immune profiles, and further suggest that each subgroup may have different mechanisms of facilitating immune suppression and evasion. Despite a plethora of genetic and histopathologic information from patient samples, an absence of relevant preclinical animal models has also hindered the investigation of promising immunotherapeutic targeting strategies of medulloblastoma in vivo.
Medulloblastoma remains incurable in one third of patients despite aggressive multimodality standard therapies. The morbidities associated with such treatments make medulloblastoma an attractive candidate for immunotherapeutic intervention. However, little is known regarding the host immunologic interactions within the tumor microenvironment across molecular subtypes of medulloblastoma. Here, we demonstrate unique immune microenvironments and response to immune checkpoint blockade in syngeneic animal models of human SHH and group 3 medulloblastomas. We analyzed immune cell infiltration in established intracranial tumors and report higher frequencies of myeloid cells and lymphocytes in SHH group tumors compared with group 3 tumors. PD-1 expression was observed to be higher on lymphocytes within group 3 medulloblastomas, and blockade of PD-1 conferred a significant antitumor effect in group 3 tumors, but not in SHH tumors. Examination of receptor occupancy by anti-PD-1 monoclonal antibody demonstrated binding to T cells in the periphery but not to T cells within the tumor microenvironment. Systemic antibody treatment, however, resulted in a marked increase of PD-1 negative tumor-infiltrating lymphocytes after immune checkpoint blockade. Our work sheds light on the distinct immune profiles across molecular subtypes of medulloblastoma that may respond differentially to specific immunotherapeutic targeting strategies. These findings have relevant implications for the clinical development of immunotherapy targeting medulloblastoma and potentially other cancers.

Here, we demonstrate the successful adaptation and immune characterization of two intracranial syngeneic animal models recapitulating human Sonic Hedgehog (SHH) and group 3 medulloblastoma. The SHH medulloblastoma group tumors recapitulate the phenotype of pathologically classified "classic" and "desmoplastic" human medulloblastomas, while the group 3 medulloblastoma group tumors comprise the more invasive and aggressive "anaplastic" human medulloblastomas (9). Among the first genetically engineered models of SHH-driven medulloblastoma, the Patched homolog 1 (Ptch1)-knockout mouse was generated through homologous recombination of the Ptch1 gene, causing a loss of PTCH1 protein expression and constitutive SHH pathway activation (10). Although homozygous mutations in the Ptch1 gene are embryonically lethal, heterozygotes (Ptch1+/−) are viable and form medulloblastoma tumors in 15% to 20% of mice at 16 to 25 weeks (10–12). In addition to the Ptch1 model of SHH-associated medulloblastoma (Ptch1 medulloblastoma), a model of MYC-amplified medulloblastoma or human group 3 medulloblastoma (hereafter referred to as neural stem cell medulloblastoma or NSC medulloblastoma) was originally created through the retroviral transduction of sorted cerebellar granule neuron precursor cells (CD133+ / lineage negative cells from the postnatal day 5 cerebellum) with genes encoding a stable form of Myc and a dominant negative form of p53 (13). Infected cells were implanted into the cerebella of immune deficient NOD/SCID IL2 receptor gamma knockout mice and formed tumors within 6 to 12 weeks (13). We adapted the aforementioned models for immunotherapeutic assessment through orthotopic transplantation of each tumor type into the cerebella of immune competent C57BL/6 hosts. After several passages, we produced a large stock of Ptch1 medulloblastoma and NSC medulloblastoma tumor cells that could be stereotactically implanted to generate large cohorts of uniformly tumor-bearing animals.

After successful adaptation and validation of these models, we characterized the tumor infiltrating immune cells in both the animal models of medulloblastoma. We analyzed both myeloid and T-cell populations and further investigated markers of activated and suppressive immune cell phenotypes. Ptch1 medulloblastoma tumors contained significantly increased frequencies of infiltrating dendritic cells (DC), T cells, and myeloid cells in comparison to NSC medulloblastoma tumors. However, higher percentages of CD8+ PD-1+ T cells of infiltrating CD3+ cells were identified in NSC medulloblastoma tumors. We show that in vivo blockade of the PD-1 expressing lymphocyte population showed a significant antitumor benefit in intracranial NSC medulloblastoma-bearing animals, but not in Ptch1 medulloblastoma animals. Further analysis of treated tumors in both the subtypes revealed anti-PD-1 monoclonal antibody to be only bound to PD-1+ T cells in peripheral lymphoid organs and absent on tumor infiltrating lymphocytes (TIL) in the brain. PD-1 blockade also resulted in significant increases in infiltrating PD-1-negative T cells within the tumor microenvironment. Our findings suggest that medulloblastoma subgroups have distinct immune profiles that may require different immunotherapeutic targeting strategies to mediate antitumor immunity.

Materials and Methods

Tumor cells

Ptch1+/− mutant mice and NSC medulloblastoma cells were provided in collaboration with Dr. Robert Wechsler-Reya (Sanford-Burnham Research Institute, La Jolla, CA, USA). The NSC medulloblastoma tumor was originally isolated from a tumor arising from implanted cerebellar stem cells that had been retrovirally transduced with mutations in p53 and c-myc (13). The Ptch1 medulloblastoma tumor was isolated from a spontaneously arising tumor in the Ptch1+/− mutant mouse on a C57BL/6 background (10). Both tumors were passaged in vivo six times in the C57BL/6 background. Explanted tumor cells from the sixth passage were frozen down and used for all subsequent optimization and efficacy experiments.

Mice

Five- to eight-week-old C57BL/6 mice were obtained from the Jackson Laboratory. The investigators adhered to the “Guide for the Care and Use of Laboratory Animals” as proposed by the committee on Care of Laboratory Animal Resources Commission on Life Sciences, National Research Council. The facilities at the Duke Cancer Center Isolation Facility and the University of Florida Biomedical Science Building are fully accredited by the American Association for Accreditation of Laboratory Animal Care, and all studies were approved by the Duke University and University of Florida Institutional Animal Care and Use Committee.

Intracranial tumor implantation

Frozen tumor cells were thawed and immediately washed twice with sterile PBS. For intracerebellar NSC medulloblastoma tumor implantation, cells were mixed 50/50 with 10% methylcellulose
in PBS and loaded into a 250 μl syringe (Hamilton) with an attached 25-gauge needle. For intracerebellar Ptc1 medulloblastoma tumor implantation, cells were mixed in PBS and loaded into a 5 μl syringe (Hamilton). Mice were positioned into a Kopf stereotactic frame and a half-inch incision was made at the midline of the scalp over the position of the cerebellum. Implantation into the cerebellum was measured at 1 mm lateral to the midline at a depth of 3 mm. Based on tumorigenicity studies, a minimum tumorigenic dose of 1 × 10^5 NSC medulloblastoma cells and 1.25 × 10^6 Ptc1 medulloblastoma cells were implanted in subsequent survival studies.

**Microarray analyses**

RNA was isolated from tumor cells using the RNeasy Mini Kit (Qiagen). RNA was labeled and hybridized to Affymetrix Mouse Genome 430 2.0 arrays. Microarray data were processed using robust multichip analysis in Partek Genomics Suite 6.5 (Partek, Inc.). Differentially expressed genes were identified based on a fold change cutoff (PANOVA. Genes were selected based on a fold change cutoff value with FDR <0.05.

**IHC**

Tissue was fixed for 24 hours in a 10% formalin solution (Sigma) and then transferred to 70% ethanol before paraffin embedded. Tissue was sectioned at a thickness of 5 μm and was deparaffinized and rehydrated in an ethanol series. Sections were blocked with Background Sniper (Biocare Medical) for 15 minutes. Hematoxylin and eosin staining was performed according to common procedures (Sigma). Sections were stained with primary antibodies against synaptophysin (Invitrogen, 08-130, prediluted), SFRP1 (Abcam, ab4193, 1:1,000), or NPR3 (Abcam, 37617, 1:100) overnight at 4 degrees. Sections were then incubated with secondary antibodies at 1:200 for 30 minutes at room temperature. Staining was developed by DAB (Vector Laboratories) followed by counterstaining with hematoxylin (Sigma) and mounted with Cytoseal (Thermo Scientific). For PD-1 staining, slides were subjected to a heat retrieval process at 120 degrees for 4 minutes before stained with anti-PD-1 antibody (R&D, AF1021, 0.8 μg/ml). Goat-on-Rodent HRP polymer (Biocare Medical) was used to detect anti-PD-1. For PD-L1 staining, a proprietary generated clone from Merck (MEB077.6H4.181) was used with a similar heat retrieval method. Rat-on-Mouse HRP polymer (Biocare Medical) was used to detect anti-PD-L1. Microscopy was performed with Zeiss Axioplan 2 for IHC for subtype-specific markers and Olympus IX70 for Ki-67 and caspase-3 IHC. Positive cell counts and marker density measurements were quantified using Image-Pro Premier 9. Images were generated using QImaging QCapture Pro 6 Software (QImaging Corporation) for the aforementioned IHC and an Aperio Scanscope slide scanner was used to generate images for IHC of PD-1 and PD-L1 staining.

**Dissociation of tumor samples**

To measure tumor-infiltrating immune cells in the Ptc1 medulloblastoma and NSC medulloblastoma tumors, symptomatic mice were sacrificed and tumors were removed. Tumors were dissociated to a single-cell suspension following mechanical disruption and papain (Worthington) digestion. Cells were passed through a 70-μm nylon mesh strainer (BD Biosciences) to remove large pieces and washed in PBS (Gibco). Cells were resuspended in a PBS solution with 2% FBS (Seradigm) and stained for 30 minutes at 4°C with respective antibodies or isotype controls. Samples were fixed in 1% formalin and run on a BD FACS CALibur.

**Analysis of immune cell infiltrates**

DC were identified among dissociated tumor cells by coexpression of CD11c and I-A/I-E (MHC Class II). CD80 and CD40 were used as additional markers of activation on DC. CD3 expression was used to identify tumor-infiltrating lymphocytes and CD4 and CD8 were used to further distinguish the phenotype of the T cells. CTLA-4 and PD-1 expression were both measured as established markers of T-cell suppression (14). Myeloid-derived suppressor cells were identified by coexpression of CD11b and Gr-1 and tumor-associated macrophages were distinguished by coexpression of CD11b and F4/80 (15). Rat anti-mouse IgG1 antibody was used to detect the PD-1 monoclonal antibody based on the mouse IgG1 isotype of the PD-1 blocking antibody. Antibody information is supplied in Supplementary Table S2. All samples were analyzed using the Flowjo version 10 (Tree Star) and were gated on size and granularity, followed by omission of debris. For NSC medulloblastoma samples, unstained NSC medulloblastoma (GFP+ ) tumor cells were mixed with unstained Ptc1 medulloblastoma (GFP+) tumor cells to set up voltage settings and used as a standard fluorescence minus one (FMO) control for samples stained in the FL-1 GFP and FL-2 PE channels. Therefore, false positives were avoided by excluding the FMO control, which comprised of intensely expressing GFP+ tumor cells. CaliBRITE 4 Color Beads (BD Biosciences) were used to determine the compensation values. IgG controls were used to draw all gates. Total percentage values for infiltrating cells within the NSC medulloblastoma tumor samples were calculated by multiplying the non-GFP population by the percentage of positive cells determined within the non-GFP population. Calculations were confirmed by analysis of antigen-presenting cells (APC) markers on the live cell gate of both tumor subtype populations, of which the emission spectra does not overlap with GFP in the FL-1 channel (Supplementary Figs. S5–S7). For display purposes, the number of events included on representative histogram plots of Ptc1 medulloblastoma tumor infiltrates was increased to 2 × 10^4 events. Comparative analyses were conducted using Prism (GraphPad).

**Statistical analyses**

Analysis of data comparing immune cell frequencies between medulloblastoma subtypes was performed using the unpaired two-tailed t test to assess means and SD with a significant result limited to P values of less than 0.05. Survival curves were estimated for each group using the product-limit estimation of Kaplan and Meier. Primary comparative analysis of the curves for each group receiving various treatments was conducted using the log-rank test. All analyses were conducted using Prism (GraphPad).

**Ex vivo expansion of T cells**

DC were isolated from the bone marrow of C57BL/6 mice using a previously published protocol with few modifications (16). Briefly, femurs and tibias of C57BL/6 mice were harvested and bone marrow flushed with RPMI (Gibco) + 10% FBS. Red cells were lysed and mononuclear cells were re-suspended in Complete DC Media (RPMI-1640, 5% heat-inactivated FBS, 1 mol/L HEPES, 100 U/ml penicillin/streptomycin, 2 mmol/L L-glutamine, 10% heat-inactivated FBS, 10 mmol/L HEPES) and passed through a 70-μm nylon mesh strainer (BD Biosciences). Mice were positioned into a Kopf stereotactic frame and a half-inch incision was made at the midline at a depth of 3 mm. Based on tumorgenicity studies, a minimum tumorigenic dose of 1 × 10^5 NSC medulloblastoma cells and 1.25 × 10^6 Ptc1 medulloblastoma cells were implanted in subsequent survival studies.
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55 mmol/L β-mercaptoethanol, 100 mmol/L sodium pyruvate, 10 mmol/L nonessential amino acids, 200 mmol/L L-glutamine, 10 μg GM-CSF, 10 μg IL-4, 5.5 mL Penicillin/Streptomycin and plated into 6-well plates at a density of 10⁶ cells/mL. Nonadherent cells were discarded at day 3. At day 7, cells were re-plated onto 60-mm tissue culture dishes. Resulting cells were electroporated the following day with 25 μg of total RNA isolated from either Ptch1 medulloblastoma or NSC medulloblastoma. RNA-pulsed DCs were collected the following day and cocultured with splenocytes for ex vivo expansion. Splenocytes harvested from tumor-bearing animals were expanded ex vivo using primary DCs pulsed with total tumor RNA and grown in T-cell media (RPMI-1640, 10% heat-inactivated FBS, 55 mmol/L sodium pyruvate, 10 mmol/L nonessential amino acids, 200 mmol/L L-glutamine, 5.5 mL Penicillin/Streptomycin) and 100IU IL2 for 7 days before use in functional assays.

Functional stimulation assay
To determine antitumor T-cell function, effector T cells were cocultured with target cells overnight and supernatant was collected 48 hours later. A Cytometric Bead Array kit was used to determine mouse Th1/Th2 cytokine release in the supernatant. We carried out the assay using primary DCs pulsed with total tumor RNA and grown in T-cell media (RPMI-1640, 10% heat-inactivated FBS, 55 mmol/L sodium pyruvate, 10 mmol/L nonessential amino acids, 200 mmol/L L-glutamine, 5.5 mL Penicillin/Streptomycin) and 100IU IL2 for 7 days before use in functional assays.

Reagents and treatments
For immune checkpoint blockade experiments, anti-CTLA-4 and anti-PD-1 antibodies were first administered 5 days following intracranial tumor implantation. For CTLA-4 blockade, an initial dose of 100 μg anti-CTLA-4 (clone 9H10, BioXCell) was administered intraperitoneally, followed by three 50 μg maintenance doses every 3 days. For PD-1 blockade, mice were administered anti-PD-1 (mDX-400; Merck) intraperitoneally at a dose of 10 mg/kg every 5 days for a total of 4 doses.

Results
Generation and validation of immunocompetent animal models of SHH and group 3 medulloblastoma
We adapted two existing animal medulloblastoma models recapitulating human SHH (Ptch1 medulloblastoma) and group 3 medulloblastoma (NSC medulloblastoma) tumors for immunotherapeutic preclinical evaluation. We stereotactically implanted tumors cells arising from the conventional Ptch1⁺/⁻ model (17) and the MYC-driven model established by Pei and colleagues (13) into the cerebella of C57BL/6 mice (Supplementary Fig. S1A). After six in vivo passages, we generated a large stock of explanted tumor cells for subsequent preclinical studies. We observed that orthotopic transplantation of tumor cells from this frozen stock were not rejected by the host immune system. In order to determine a minimum tumorigenic dose required for uniform lethality within one month, animals were implanted with decreasing doses of tumor cells and measured for survival (Supplementary Fig. S1B and S1C). We used the Affymetrix Mouse Genome 430 2.0 array to assess tumor mRNA expression for subtype-specific genes previously identified in the literature to ensure that in vivo passage of both tumor lines in the C57BL/6 background did not change the identity of the tumor (9). We confirmed that both subtypes maintained expression of key subtype-specific genes, including Sfrp1 in the Ptch1 medulloblastoma tumor-bearing animals (P = 2.37 × 10⁻⁴) and Npr3 in NSC medulloblastoma tumor-bearing animals (P = 6.12 × 10⁻⁴; Supplementary Fig. S2A and Supplementary Table S1). IHC for these markers also confirmed specific SFRP1 and NPR3 immunoreactivity. Both tumor subtypes were additionally stained with the neuronal marker synaptophysin, which was more diffusely expressed in the NSC medulloblastoma subtype compared with the Ptch1 medulloblastoma subtype. Transplanted Ptch1 medulloblastoma tumors maintained histology associated with the original Ptch1⁺/⁻ model, displaying large areas of uniform cells with high nuclear to cytoplasmic ratio and round, hyperchromatic nuclei. Transplanted NSC medulloblastoma tumors also conserved large cell anaplastic histology, consistent with the original MYC-amplified animal model of human group 3 tumors (Supplementary Fig. S2B). Medulloblastoma identification was also confirmed by a board-certified neuropathologist.

Characterization of immune cell infiltrates within tumor microenvironment
To analyze the endogenous infiltration of lymphocyte and myeloid cell populations, we measured proportions of immune cells from dissociated tumor tissue and compared them with normal cerebellar control samples (see Materials and Methods). We first evaluated the infiltration of APCs via coexpression of costimulatory molecules CD80 and CD40 and the expression of the MHC Class II molecule I-A/I-E on CD11c DC. Expression of CD80 on CD11c cells was not significantly different between the two medulloblastoma subtypes (Fig. 1A, P = 0.1799); however, the Ptch1 medulloblastoma subtype had significantly increased CD40 expression on CD11c cells (Fig. 1B, P = 0.04) and CD11c I-A/I-E expression on CD11c cells (Fig. 1C, P = 0.03).

Endogenous T-cell infiltration was determined using combined expression of CD3 and CD4 or CD8. Percentages of both infiltrating CD4 T cells (Fig. 2A) and CD8 T cells (Fig. 2B) were significantly higher within the Ptch1 medulloblastoma tumor microenvironment compared with the NSC subtype (P = 0.0034 and 0.0088, respectively). Both CD4 and CD8 T-cell infiltration strongly correlated with DC infiltration into the Ptch1 medulloblastoma tumor microenvironment (R² = 0.9847 and 0.9986, respectively; Supplementary Fig. S3). No significant correlation between T-cell infiltration and DC infiltration was observed in the NSC subtype, showing a negative trend (R² = 0.4048 and 0.1761 for CD4 and CD8, respectively) between the T-cell subtypes and DC infiltration (Supplementary Fig. S4).

To assess the immunosuppressive state of tumor-infiltrating T cells, CTLA-4 expression on CD4 T cells and PD-1 expression on CD8 T cells were analyzed. No significant differences were observed for the CD4⁺ CTLA-4⁺ T cells between the two subtypes (Fig. 2C, P = 0.64). Interestingly, there was a significantly higher proportion of CD8⁺ PD-1⁺ cells observed in the NSC medulloblastoma microenvironment compared with Ptch1 medulloblastoma (Fig. 2D, P = 0.004) suggesting a more profound immunologic suppression or exhaustion phenotype on T cells in these mice.

Due to significant differences observed in the proportions of suppressive CD8⁺ PD-1⁺ T cells, we evaluated levels of surface PD-L1 expression on both tumors. Overall PD-L1 expression in both tumor subtypes showed elevated expression compared with normal cerebellar controls, with the Ptch1 medulloblastoma tumor expressing significantly more compared with the NSC medulloblastoma tumor (Fig. 3A, P = 0.04). Because overall PD-L1 expression was relatively low in both subtypes compared with normal cerebellar controls, we evaluated the impact of Ptch1 medulloblastoma on PD-L1 expression on DCs in vivo as well as T-cell immune function. We showed that Ptch1 medulloblastoma tumors significantly decreased PD-L1 expression compared with NSC tumors (Supplementary Fig. S4).

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with well-studied peripheral tumors, such as B16 melanoma (18), we hypothesized that the predominant source of PD-L1 originated from myeloid infiltrating cells. We evaluated the expression of PD-L1 on myeloid cells and observed significantly more CD11b⁺PD-L1⁺ myeloid cells in the Ptch1 medulloblastoma compared with NSC medulloblastoma (Fig. 3B, \(P = 0.03\)). Myeloid cell infiltration was determined through the measurement of both myeloid-derived suppressor cells (MDSC) and tumor-associated macrophages (TAM). Combined expression of CD11b⁺Gr-1⁺ (MDSCs) or CD11b⁺F4/80⁺ (TAMs) were also shown to be significantly higher in the Ptch1 medulloblastoma tumor (Fig. 3C and D, \(P = 0.01\) and 0.03, respectively).

Differences in functional capacity of immune cells and response to immune checkpoint inhibitors

Because significant differences were observed in the infiltration of both CD4⁺ and CD8⁺ T cells and MDSCs and TAM cells, we hypothesized that the phenotypically undifferentiated nature of the NSC medulloblastoma tumor contributed to a more immunologically “silent” profile compared with the more infiltrative
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Ptc1 medulloblastoma tumor, and therefore lacked presentation of immunogenic antigens capable of eliciting reactivity from the immune system. To test the immunogenicity of antigens expressed by both medulloblastoma subtypes, we harvested spleenocytes from animals bearing intracranial NSC medulloblastoma or Ptc1 medulloblastoma and expanded them with primary DC electroporated with total tumor RNA from each subtype. The resulting effector T cells were tested for functionality and tumor specificity through a restimulation assay measuring specific TNFα and IFNγ release upon encounter with tumor target cells (Fig. 4A). Despite differences in T-cell and myeloid cell proportions, medulloblastoma tumors expressed antigens capable of eliciting specific inflammatory immune responses. T cells generated from both the Ptc1 medulloblastoma and NSC medulloblastoma tumor-bearing animal showed significant Th1 cytokine release upon coculture with tumor target cells compared with negative control cells pulsed with irrelevant antigen ovalbumin RNA (Fig. 4B and C). Although the antigenic profile of both medulloblastoma subtypes did not differentially affect their immunogenic potential, we next assessed each subtype’s response to immunotherapeutic intervention aimed on modulating the tumor microenvironment. Because the two subtypes contained significant differences in the CD8+ PD-1+ T-cell population and no differences in the CD4+ CTLA-4- T-cell population, we used PD-1 and CTLA-4 blocking antibodies to determine if the differences in immunosuppressive T-cell subsets could be leveraged to mediate antitumor efficacy in vivo (Fig. 5A). Ptc1 medulloblastoma tumor-bearing animals treated with either anti-CTLA-4 alone, anti-PD-1 alone, or in combination, saw no treatment benefit over untreated tumor-only controls (Fig. 5B). However, in NSC medulloblastoma tumor-bearing animals, animals treated with anti-PD-1 alone or in combination with anti-CTLA-4 showed a significant survival benefit over untreated controls (Fig. 5C, P = 0.02 and 0.009, respectively). Treatment in this model with anti-CTLA-4 alone showed no benefit, thus suggesting PD-1 expressing T cells as a key axis of immune suppression and tumor outgrowth in the NSC medulloblastoma subtype, but not in the Ptc1 medulloblastoma subtype.

To evaluate the effect of PD-1 blockade on both medulloblastoma subtypes, we used IHC to assess expression of PD-1 and PD-L1 in untreated and anti-PD-1–treated tumors. IHC for PD-1 and PD-L1 revealed PD-L1 reactivity to mainly arise from infiltrating cells and cells consistent with blood vessel endothelial cells. These findings corroborate our observations by flow cytometry, in that the predominant source of PD-L1 staining derived from nontumor cell populations. In anti-PD-1–treated tumors, both subtypes demonstrated moderate increases in PD-1 and PD-L1 reactivity (Fig. 5D). To determine the effect of PD-1 blockade on proliferation and apoptosis within medulloblastoma tumors, we used IHC to assess reactivity to Ki-67 and cleaved caspase-3. Both tumor subtypes exhibited a decrease in Ki-67-positive cells with the NSC medulloblastoma subtype demonstrating a significant decrease (P = 0.02). No significant changes were observed in caspase-3 reactivity between untreated and anti-PD-1–treated tumors in either medulloblastoma subtype (Fig. 5E).

PD-1 blockade occurs in the periphery and leads to increase in infiltrating lymphocytes

To elucidate whether the differential response to PD-1 blockade across medulloblastoma subtypes was resultant of limitations in PD-1 antibody penetration of the blood brain barrier, we used flow cytometry to measure PD-1 monoclonal antibody (mAb) receptor occupancy following in vivo administration. As the PD-1 mAb is a mouse IgG1 isotype, we used a rat anti-mIgG1 antibody to detect the PD-1 mAb on infiltrating lymphocytes within the tumors, spleens, cervical lymph nodes (CLN) and inguinal lymph nodes (ILN) of both medulloblastoma subtypes (Fig. 6A). Significant PD-1 mAb was detected bound to the surface of T cells in the spleen, CLNs, and ILNs of treated animals of both medulloblastoma subtypes (Fig. 6B). Of the PD-1 mAb detected in the spleen, CLN, and ILN, the majority of antibody was bound to CD4 T cells compared with CD8 T cells (data not shown). Interestingly, there was no receptor occupancy (surface-bound anti-PD-1 antibody) observed within TILs, demonstrating that anti-PD-1 antibodies were not detectable at appreciable levels in either tumor model. In contrast, systemic administration of anti-PD-1 antibodies led to a significant increase in the percentage of CD8+ T cells within the tumor microenvironment of treated animals in both the Ptc1 and NSC medulloblastoma tumors (Fig. 6C, P = 0.002 and 0.01, respectively). Further analysis of the infiltrating T cells revealed that these T cells represented a marked influx or expansion of PD-1-negative T cells within the tumor microenvironment (Fig. 6D, P = 0.01 for Ptc1 medulloblastoma, P = 0.02 for NSC medulloblastoma). Similarly, evaluation of CD8+ revealed significantly higher frequencies in treated animals of both medulloblastoma subtypes (Fig. 6E, P = 0.004 for Ptc1 medulloblastoma, P = 0.01 for NSC medulloblastoma). Evaluation of CD4+ T cells in PD-1–treated animals revealed an increase in both medulloblastoma subtypes, however, only the NSC medulloblastoma subtype demonstrated a significant change in this compartment (Fig. 6F, P = 0.009). The results demonstrate that the efficacy of PD-1 blockade within the NSC medulloblastoma model was not due to differential accessibility of the tumor to monoclonal antibody penetration. In fact, tumor penetration by the monoclonal antibody either directly or carried by infiltrating T cells is not observed within the context of efficacious immune checkpoint blockade.

Discussion

Immunotherapy has gained significant credence as an effective strategy at redirecting the cytotoxic power of the immune system to target a multitude of malignancies (19–24). However, the examination of immune-based strategies in pediatric brain tumors has been marginal and requires a better understanding of the interplay between the immune system and the tumor microenvironment. This understanding promises to yield targeted immune-based therapies that can re-program distinct immune microenvironmental pathways across medulloblastoma subgroups as well as enhance current antigen-targeted strategies.

Based on genetic profiling and IHC analyses of patient samples, a recent study by Margol and colleagues demonstrated a higher presence of tumor-associated macrophages in the SHH-driven group of medulloblastoma (8). Our study not only corroborates these findings, but is also the first to characterize immunologic differences within molecular subtypes of murine medulloblastoma and demonstrate differential responses to immunotherapy in relevant preclinical models. These responses are likely secondary to the unique immune phenotypes among medulloblastoma subgroups and our results suggest that strategies targeting the dominant immunologic phenotype within medulloblastomas
and stratification of responses by molecular subtype should be considered. We show that Ptch1 medulloblastoma tumors contain higher percentages of infiltrating CD4+ T cells and CD8+ T cells. Interestingly, NSC medulloblastoma tumors contain a higher proportion of CD8+ PD-1+ expression within the CD3+ population. Differences in T-cell proportions within the Ptch1 medulloblastoma or NSC medulloblastoma tumor microenvironment did not hinder the recognition of both subtypes by antigen-specific T cells and did not diminish Th1 cytokine responses in vitro. However, differential response to anti-PD-1 blockade in tumor-bearing animals suggests that the PD-1/PD-L1 axis is a key immunoregulatory pathway in group 3 medulloblastomas that may unlock the potency of immunotherapeutic intervention. A previous study by Zeng and colleagues demonstrated the synergistic interaction of anti-PD-1 blockade in combination with stereotactic whole brain irradiation through the influx of CD8+ T cells in treating intracranial malignant glioma (GL261) tumors (25), suggesting a CD8 requirement for abrogating tumor growth with a PD-1 blocking strategy. Depletion of the CD8+ T cells resulted in the loss of efficacy, although the depletion of CD4+ T cells still yielded some benefit (25). In our experiments, the blockade of PD-1 pathway using anti-PD-1 monoclonal antibody as a monotherapy was sufficient to extend the median survival of treated animals in the NSC medulloblastoma subtype but not SHH subtype despite clear evidence of a biologic response to PD-1 blockade and changes within the tumor microenvironment in both tumor models. It is possible that changes in the ratio of lymphocytes to other immunosuppressive cell populations within the tumor microenvironment may explain the refractory nature of SHH tumors in these experiments, as the greater baseline proportions of myeloid cells may require greater lymphocyte expansion to result in effective tumor rejections. Alternatively, the elevated PD-1 expression on lymphocytes in the group 3 model may be a marker more indicative of tumor where the PD-1/PD-L1 axis is a more relevant pathway for immune intervention.

Expression of PD-L1 on tumor cells has been demonstrated as an important prognostic indicator of response to anti-PD-1/PD-L1 therapy. In a phase I trial testing anti-PD-1 in solid tumors, analyses of pretreatment biopsies showed that the presence of membranous PD-L1 expression correlated to regressions in tumor growth following anti-PD-1 therapy (26). In our studies, we observed marginal surface PD-L1 expression on tumors of both medulloblastoma tumor subtypes by both flow cytometry and IHC, of which was largely expressed by nontumor cell populations, but differences in PD-L1+ myeloid cells did not correlate with therapeutic benefit. Few studies have evaluated the prognostic value of PD-1 expressing TIL distribution at the tumor site (27). An examination of PD-1 expressing TILs in pretreated solid tumor samples significantly correlated with PD-L1 expression on both tumor cells and immune cell infiltrates (28). In the same study, overall frequencies of TILs and PD-L1 expression on immune cell infiltrates did not significantly correlate to an objective clinical response to anti-PD-1 therapy; however, assessment of PD-L1 expression on TILs was borderline associated with clinical response (28). Interestingly, our studies corroborate these observations and demonstrate that despite higher percentages of both myeloid and lymphocyte infiltration as well as increased frequencies of PD-L1 expressing myeloid infiltrating cells in Ptch1 medulloblastoma tumor-bearing animals, there was no response to PD-1 blockade in this subtype. PD-1 therapy did show efficacy in extending the median survival in NSC medulloblastoma tumor-bearing animals, suggesting that proportions of CD8+ PD-1+ expressing lymphocytes may serve as an indicator of response to PD-1 blockade and should be further evaluated in pretreatment and posttreatment patient tumor samples.

We show that the Ptch1 medulloblastoma tumor contains higher percentages of infiltrating MDSCs and TAMs, contributing to an increased immunologically suppressive tumor microenvironment. MDSCs and TAMs have long been characterized for their notable ability to negatively regulate innate and adaptive immune responses (15). The expansion and activation of myeloid infiltrating cells rely heavily on cytokines produced by tumor cells and activated T cells, directly leading to suppressive signaling pathways driven by the transcriptional factor STAT3 in MDSCs (29). Abad and colleagues observed similar increases in MDSC activity in spontaneously arising tumors in the Smo transgenic mouse, a murine model of the SHH-driven medulloblastoma. Conditional knockout of STAT3 in medulloblastoma cells led to greater CD4 and CD8 infiltration with reduction in regulatory T cells and MDSCs (30). Despite higher proportions of lymphocyte subsets, our studies demonstrate that the Ptch1 medulloblastoma model was less responsive to immune checkpoint inhibitors, and suggests that selective blockade of macrophage and myeloid-derived suppressor populations may unlock intratumoral effector cells in SHH-driven medulloblastoma (8). Additionally, as suggested by Margol and colleagues, increased presence of myeloid and macrophage populations may be used to clinically stratify treatment and immunotherapeutic strategy. Examination of tumor-infiltrating lymphocytes to myeloid cell infiltrate ratios using proportions and absolute counts would be an interesting stratification between molecular subtypes and within responding versus nonresponding tumors to perhaps provide further insights into the influences of tumor microenvironment on response to anti-PD-1 treatment.

Our detection of PD-1+ mAb bound to the surface of T cells found only in the peripheral lymphoid organs, but not at the tumor site in anti-PD-1-treated animals suggests a systemic mechanism by which PD-1 blockade mediates antitumor efficacy within the brain. Our studies are the first to suggest that the physical presence of PD-1 antibody may not be required at the tumor site, specifically the brain, and potentially has important implications in PD-1 blocking strategies in other solid cancers. However, although no antibody was detected on the surface of tumor infiltrating T cells of sacrificed animals, we cannot rule out that the antibody may gain entry to the tumor site at different time points throughout tumor treatment than those examined in this study.
Figure 3.
Evaluation of PD-L1 expression in Ptch1 medulloblastoma (MB) and NSC medulloblastoma. Freshly dissociated tumor from moribund tumor-bearing animals was stained for PD-L1 expression. Representative histograms NSC medulloblastoma (left) and Ptch1 medulloblastoma (right). A, percentage of PD-L1 expression on total cells was significantly higher in the Ptch1 medulloblastoma subtype compared with the NSC medulloblastoma subtype ($P = 0.04$ by unpaired t test). B, PD-L1 coexpression on infiltrating myeloid cells was also significantly higher in the Ptch1 medulloblastoma subtype ($P = 0.03$ by unpaired t test). C, myeloid-derived suppressor cells and, D, tumor-associated macrophages infiltration frequencies were significantly higher in the Ptch1 medulloblastoma subtype compared with the NSC medulloblastoma subtype ($P = 0.01$ and 0.03 by an unpaired t test, respectively).
study. Alternatively, T cells may rapidly internalize bound antibody, clearing it from the surface within the tumor microenvironment. We also acknowledge that PD-1 mAb binding to receptors on T cells within the tumor microenvironment could occur at levels below the limit of appreciable detection of our assay, thus necessitating further studies to confirm PD-1 receptor occupancy by PD-1 mAb within brain tumors. We observed, however, significant influxes in PD-1-negative T cells in treated animals,

Figure 4. Immunologic differences within tumor microenvironment do not affect immunogenicity of total tumor antigens of both medulloblastoma (MB) subtypes. Animals were implanted with the minimum tumorigenic dose of either NSC medulloblastoma or Ptch1 medulloblastoma tumor cells. A, splenocytes from tumor-bearing animals were harvested at day 16 (NSC medulloblastoma) or day 21 (Ptch1 medulloblastoma) and expanded with primary DC pulsed with total tumor RNA (ttRNA). B, splenocytes expanded with DCs pulsed with Ptch1 medulloblastoma ttRNA or, C, NSC medulloblastoma ttRNA showed specific Th1 responses upon restimulation with tumor targets.
Figure 5.
Anti-PD-1 blockade confers superior antitumor treatment effect in NSC medulloblastoma (MB) tumor-bearing animals. A, animals were implanted with the minimum tumorigenic dose of either NSC medulloblastoma or Ptch1 medulloblastoma tumor cells and administered CTLA-4 and PD-1 blocking antibodies, both alone and in combination. B, Ptch1 medulloblastoma tumor-bearing animals did not respond to either CTLA-4 or PD-1 blockade, whereas (C) NSC medulloblastoma tumor-bearing animals treated with anti-PD-1 alone and in combination with CTLA-4 blockade showed significant extension in median survival ($P = 0.02$ and 0.009, respectively, by log-rank test, $N = 7$ per group). D, explanted NSC medulloblastoma and Ptch1 medulloblastoma tumors from untreated and anti-PD-1–treated animals were stained for PD-1 and PD-L1 IHC. E, explanted NSC medulloblastoma and Ptch1 medulloblastoma tumors from untreated and anti-PD-1–treated animals were evaluated for differences in Ki67 and cleaved caspase-3 staining by IHC, with a significant decrease in Ki67-positive cells in anti-PD-1–treated NSC medulloblastoma tumor-bearing mice (top right, $P = 0.02$ by unpaired t test), but no significant differences in caspase-3 reactivity in both medulloblastoma subtypes (bottom right).
Figure 6. Anti-PD-1 blockade acts systemically and results in increased population of CD3 T cells within the tumor microenvironment. A, representative flow cytometric analyses of anti-PD-1 mAb staining via mIgG1 detection on CD3^+^ cells within the tumor, spleen, cervical lymph node (CLN), and inguinal lymph node (ILN). A rat anti-mouse IgG1 antibody was used to detect the anti-PD-1 mAb because it is a mouse IgG1 isotype. B, quantification of anti-PD-1 mAb-positive CD3^+^ T cells within the tumor and lymphoid organs of symptomatic tumor-bearing NSC and Ptch1 medulloblastoma (MB) untreated controls and anti-PD-1-treated animals. C, PD-1 blockade in both Ptch1 medulloblastoma and NSC medulloblastoma treated animals resulted in significant increases in CD3 infiltration (P = 0.002 and 0.01 by unpaired t test, respectively). D, representative flow cytometric analyses (left) and histogram (right) of significantly increased CD3^+^ PD-1^-^ infiltration in anti-PD-1-treated animals in both the Ptch1 and NSC medulloblastoma subtypes (P = 0.01 and 0.02 by unpaired t test, respectively). E, PD-1 blockade in both Ptch1 medulloblastoma and NSC medulloblastoma treated animals resulted in increases in CD8 infiltration, with a significant change in the NSC medulloblastoma subtype (P = 0.009, unpaired t test).
with significant increases in the CD8 compartment. Although treated animals of both tumor subtypes demonstrated increases in the CD4 infiltration, only the NSC medulloblastoma subtype exhibited a significant increase. Although this observation alone cannot explain why Ptch+ medulloblastoma tumors are refractory to PD-1 blockade, the significant increase in CD4 may provide another clue as to why NSC medulloblastoma responds to the PD-1 blockade. The mutational burden of a tumor has been demonstrated to be a predictor of response to PD-1/PD-L1 blocking strategies in non–small cell lung cancer (31, 32). In medulloblastoma, human group 3 tumors have been characterized by amplifications of MYC and OTX2 as well as alterations in numerous chromatin binding proteins. Such vast chromosomal aberrations and epigenetic changes, more numerous in the group 3 compared with the SHH group tumors, may contribute to the differential response observed by the two medulloblastoma subtypes due to the presence of mutations and neoepitopes in the NSC medulloblastoma tumor (4, 33). Recent findings have also identified predominant drivers of immune response to be linked to tumor-specific mutations recognized by CD4+ cells (34). The influx of CD4+ cells observed in the NSC medulloblastoma microenvironment following anti-PD-1 treatment may represent the antigen-specific T cells responsible for driving the antitumor response.

In summary, this immunologic characterization of the two medulloblastoma animal models yields potential avenues to exploit functional intratumoral immune subsets that may induce antitumor immunity. Physiologic responses observed following PD-1 blockade despite the absence of PD-1 mAb within the tumor microenvironment demonstrate the efficacy of PD-1 blocking strategies in intracranial tumors by which the PD-1/PD-L1 axis is the main mechanism of immune suppression. Other strategies may be necessary in tumors such as the SHH medulloblastoma subtype that exhibit alternative mechanisms of immune evasion. A better understanding of the immunologic subtypes utilizing accurate patient molecular subtype classification will allow for novel targeted therapies stratified toward disease subsets, bypassing the morbid effects of current strategies.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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