Published in final edited form as:

Cancer Immunol Immunother. 2017 December ; 66(12): 1589–1595. doi:10.1007/s00262-017-2051-6.

Regulatory T cell subsets in patients with medulloblastoma at diagnosis and during standard irradiation and chemotherapy (PBTC N-11)

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Abstract

BACKGROUND—We evaluated circulating levels of immunosuppressive regulatory T cells (T_{regs}) and other lymphocyte subsets in patients with newly-diagnosed medulloblastoma (MBL) undergoing surgery compared to a control cohort of patients undergo craniectomy for correction of Chiari malformation (CM) and further determined the impact of standard irradiation and chemotherapy on this cell population.

METHODS—Eligibility criteria for this biologic study included age 4 – 21 years, patients with CM undergoing craniectomy (as non-malignant surgical controls) and receiving dexamethasone for prevention of post - operative nausea, and those with newly-diagnosed posterior fossa tumors (PFT) undergoing surgical resection and receiving dexamethasone as an anti-edema measure.

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Presented in part in abstract form at the International Society of Pediatric Neuro-Oncology Meeting at Liverpool, U.K. June 13–15, 2016[1]

Conflict of interest: The authors declare that they have no conflict of interest

Patients with confirmed MBL were also followed for longitudinal blood collection and analysis during radiotherapy and chemotherapy.

RESULTS—A total of 54 subjects were enrolled on the study [22- CM, 18- MBL, 14-PFT]. Absolute number and percentage T_{regs} (defined as CD4+CD25+FoxP3+CD127^{low/-}) at baseline were decreased in MBL and PFT compared to CM [$p=0.0016$ and 0.001 respectively]. Patients with MBL and PFT had significantly reduced overall CD4+ T cell count ($p=0.0014$ and 0.0054 respectively) compared to those with CM. Radiation and chemotherapy treatment in patients with MBL reduced overall lymphocyte counts, however, within the CD4+ T cell compartment, T_{regs} increased during treatment but gradually declined post therapy.

CONCLUSIONS—Our results demonstrate that patients with MBL and PFT exhibit overall reduced CD4+ T cell counts at diagnosis but not an elevated proportion of T_{regs} . Standard treatment exacerbates lymphopenia in those with MBL while enriching for immunosuppressive T_{regs} over time.

Keywords

Medulloblastoma; immunotherapy; regulatory T cells; CD4; posterior fossa tumors

INTRODUCTION

Medulloblastoma (MBL) is the most common malignant brain tumor in children [2]. While significant advances have been made in treating children with newly diagnosed disease and survival is excellent with surgery followed by irradiation and chemotherapy[3], the outcome for patients with recurrent tumor is dismal[4]. The cytotoxic therapies for this tumor also result in severe late sequelae. Immunotherapy has the potential to target tumor cells with minimal side effects and is a novel approach to treating brain tumors [5]. However, malignant brain tumors often engage various pathways to evade immune recognition and produce immune suppression [6]. We and others have previously shown that T-regulatory cells (T_{regs}) in patients with GBM and other cancers induces a severe state of immunosuppression and is likely one of the factors responsible for immune evasion [7]. These cells are a subset of CD4+ T lymphocytes, express high levels of CD25 [IL-2 α receptor (IL-2R α)] and Foxp3 transcription factor, and have a unique dependence on IL-2R α signaling for survival [7]. As immunotherapy is emerging as a potential strategy to treat MBL, we wanted to evaluate the immunologic profile (including T_{regs}) in newly diagnosed patients with MBL and other posterior fossa tumors (PFT) just prior to surgical resection as compared to a control population of Chiari malformation (CM) patients undergoing a similar intradural posterior fossa surgical procedure and also receiving perioperative steroids (in the case of CM for control of anesthesia- related nausea and vomiting). We also wanted to evaluate the effect of cytotoxic therapy (irradiation and chemotherapy) on T_{regs} and other lymphocyte subsets.

METHODS

The primary objectives of the study were (1) to evaluate the fraction of peripherally circulating T_{regs} in patients with MBL and those with PFT as compared to levels in a control

group of patients undergoing craniectomy for CM and (2) the longitudinal effects of standard cytotoxic therapy on the overall population of lymphocytes and fraction of T_{regs} in MBL patients. The study was approved by the Institutional Review Boards of the participating institutions and informed consent was obtained from patients or parents prior to surgery by one of the designated study personnel.

Inclusion criteria for this study included newly diagnosed patients (over 3 years of age) with posterior fossa tumors (who routinely receive dexamethasone for edema control) scheduled to undergo sub occipital craniotomy for tumor resection and patients with known CM scheduled to have a craniectomy and receive steroids peri-operatively to decrease risk of post anesthesia nausea and emesis. Those with unexplained febrile illness, active infections, autoimmune or immunosuppressive disorders were excluded.

Blood sample procurement and processing

Blood samples in patients with MBL were obtained pre-and post-operatively, prior to RT, post RT (+/- 1 week), prior to (or within 72 hours) and 3 weeks (or +/- 72 hours) after the first cycle of standard chemotherapy following RT. In patients with CM, blood samples were obtained pre-and post-operatively only. Those with PFT had a pre-operative blood sample only. Samples were processed immediately (within an hour) upon arrival in lab. Samples were divided into 100 μL aliquots and surface stained at room temperature for 15 minutes with the following antibodies: CD4-FITC (RPA-T4), CD127-PE (hIL-7R-M21), CD39-PE (TU66), CD49d-PE (9F10), HLA-DR-PE (L243), CD8-PE (RPA-T8), CD45RO-PE (UCHL-1), CD25-APC (M-A251), and msIgG-PE (MOPC-21). Samples were fixed for 15 minutes in the dark with 1 ml Fix/Permeabilization (eBioscience, 00-5523 kit). After addition of 3ml PBS, samples were centrifuged at $600 \times g$ for 5 minutes at 10°C and carefully decanted and 2 ml permeabilizing buffer (eBioscience, 00-5523 kit) added. Tubes were centrifuged at $800 \times g$ for 5 minutes followed by careful decantation. Fox-p3-PerCPcy5.5 (eBioscience, PCH101), ratIgG2a-PerCPcy5.5 (R35-95), Ki67-PE (B56), and msIgG-PE were added to appropriate tubes, and incubated in the dark for 15 minutes. After washing twice with Permeabilization buffer, samples were re-suspended in 300 μL PBS containing 0.5% formalin and analyzed by flow cytometry on a BD FAC Calibur.

Statistical considerations

Evaluable patients were those who had obtained all samples as required by protocol. Based on the report from Fecci et al., on T_{regs} in GBM as compared to healthy controls [8], a total of 30 patients were to be enrolled in the MBL and CM groups respectively to detect a difference of $> 6\%$ in T_{reg} fraction between the two groups. Enrollment in the PFT group would continue until 30 patients were enrolled into the MBL group. Only baseline T_{regs} levels were analyzed in patients with PFT. Due to slow accrual in the MBL and CM cohort, a decision was made to close the study before planned enrollment was completed. Descriptive statistics were provided for all protocol variables. To compare the distribution of a given marker across multiple subgroups of patients, we used Kruskal-Wallis test, which is a non-parametric counterpart of One-Way ANOVA. To investigate the significance of change in a given marker between two time points, we used Wilcoxon-Signed-Rank test, which is a

non-parametric counterpart of the paired-t-test. We modeled the change of a given marker overtime through Random Coefficients Models to assess linear and quadratic change overtime; the models assumed different intercept and slope for each patient. The p-values reported in the Results section were not adjusted for multiplicity.

RESULTS

Between April 2010 and August 2014, 54 evaluable patients from nine participating centers were enrolled on the study (22- CM, 18- MBL, 14- PFT, Table 1). We could not obtain baseline samples from one patient each with CM and PFT respectively. Hence evaluation of samples at baseline was performed in 21, 18, and 13 patients in CM, MBL, and PFT cohorts respectively. Absolute number [median 48 vs. 254] ($p=0.0016$) and percentage [median 0.18% vs. 0.73%] ($p=0.0015$) of baseline activated T_{regs} ($CD4+CD25+FoxP3+CD127^{lo/-}$) (were decreased in MBL as compared to CM (Table 2) (Figures 1 and 2d). Similarly, both absolute number [median 43 vs. 254] ($p=0.001$) and percentage [median 0.17% vs. 0.73%] ($p=0.0015$) of baseline activated T_{regs} ($CD4+CD25+FoxP3+CD127^{lo/-}$) were decreased in PFT as compared to CM (Table 2, Figure 2, panel d). Those with MBL and PFT were also characterized by having a significantly reduced overall $CD4+$ T cell count compared to patients with CM [median 6347.5 and 6073 vs. 11114] ($p=0.0014$ and 0.0054 for MBL and PFT respectively) (Table 2, Figure 2, panel a), which was also observed for the non- memory and memory T-lymphocytes ($CD45RO-$ and $CD45RO+ CD4+$) (Table 2, Figure 2, panels b and c). However, the percentage of baseline non-memory $CD8+$ T lymphocytes ($CD45RO- CD8+$) but not absolute numbers were elevated in MBL and PFT as compared to CM [median 57.8% and 61.5% vs. 44.0%] ($p < 0.0001$). Since absolute numbers of these cells were not significantly different, this increased proportion of $CD8+$ T cells is likely explained by the lower $CD4+$ T cell count in these patients. Radiation and chemotherapy treatment reduced overall lymphocyte counts (Figure 3, panels a and b). In contrast, however, within the $CD4+$ T cell compartment, T_{regs} increased during therapy (Figure 3, panels c and d, $p=0.0628$ and 0.0070 , respectively). There was also a trend toward increased numbers of actively dividing T_{regs} during therapy (Figure 3, panel e). The absolute and percentage central memory $CD8+$ T- cell population ($CD45RO+CD8$ lymphocytes) decreased during therapy but rebounded soon thereafter (Figure 3, panel f).

DISCUSSION

Immune evasion is one of the hallmarks of cancer and can be due to active or passive conditions of tolerance that can inhibit natural immune responses against tumors [8,9]. Passive mechanisms include downregulation of MHC class I expression on tumor cells, low antigenicity caused by immune editing and selective tumor growth, or depletion of tryptophan by indolamine 2,3-dioxygenase and inhibition of immune cells [8,9]. Active mechanisms include suppression of anti-tumor responses by infiltrating T_{regs} , myeloid derived suppressor cells, or tumor cells expressing inhibitory ligands and cytokines [9]. T_{regs} are a subgroup of $CD4+$ T lymphocytes and can be easily identified through co-expression of $CD25$ (IL-2 α receptor)(surface) and $FoxP3$ antigens (intracellular) [9]. Additional specificity is conferred by low or absent expression of the IL-7 receptor, $CD127$ ($CD4+CD25+FoxP3CD127^{lo/-}$) [10]. These cells have been identified as one of the most

significant inhibitors of anti-tumor immunity through suppressing T cell activation and proliferation, downregulating IL-2 production, and stimulating increase in T_H2 cytokine secretion in the target cells that can in turn increase T_{regs} expansion [8,11]. T_{regs} infiltrate the tumor mass in its early stages and inhibit local immune responses allowing tumor to continue to grow. Elevated fraction of peripheral T_{regs} have also been demonstrated in the peripheral blood in a wide variety of cancers including GBM, ovarian, and breast carcinomas [8]. Facets of the immunologic profile in patients with MBL have been previously described [12], including decreased class I HLA expression [13], increase in circulating T_H-17 lymphocytes [14], PD-L1 up-regulation (via CDK5) [15], and expression of CD1d (target for NK cells) [16]. Our current study is the first to evaluate the immunologic profile in children with newly diagnosed MBL and PFT with a focus on circulating T_{regs} . We found significantly lower levels of CD4+ T cell count and T_{regs} (absolute counts and percentage of CD4 T cells) compared to a control population of patients with CM who also received perioperative corticosteroids. In contrast, a similar study in adults with GBM revealed that while absolute CD4+ T cell and T_{regs} counts were decreased compared to controls, the T_{regs} fraction was significantly elevated. These observations suggest that T_{regs} might play a greater role in the immunosuppressive phenotype in patients with GBM as compared to other primary CNS malignancies [8,17]. The reason(s) for lower T_{reg} fraction in children with medulloblastoma compared to the control cohort is unclear. A limitation of this study is that we did not evaluate the degree of T_{regs} infiltration in the corresponding tumor samples from enrolled patients. It is possible that markedly different T_{regs} proportions are represented within the tumor-infiltrating lymphocytes of MBL. However, MBLs are histologically characterized by rather sparse lymphocytic infiltrates overall, and a close correlation between circulating T_{regs} and that in the tumor tissue in adult patients with GBM has been observed, suggesting peripheral blood levels may be indicative of intratumoral T_{regs} [8]. In addition, T_{regs} originate in the thymus and can migrate to lymph nodes and spleen in both healthy subjects and those with systemic cancers [18,19]. It is possible that cells in these sites along with peripheral blood T_{regs} might also be instrumental in causing immunosuppression.

As almost all newly diagnosed patients with brain tumors are routinely treated with dexamethasone (a glucocorticoid) prior to surgical resection, it is important to control for the impact of steroids on T cell subsets [20]. While our control patients with CM also received a single dose of dexamethasone pre-operatively, we were not able to control for differences in steroid doses and duration of exposure prior to surgery in the study population. However, high-dose methylprednisolone has been shown to actually increase both the naïve and effector T_{regs} several fold within 48 hours after treatment possibly related to increased transcription of Foxp3 following glucocorticoid receptor stimulation, that return to baseline in a week [21]. It is therefore unlikely that both the reduced number and fraction of T_{regs} in our patients with MBL and PFT are due to higher doses of steroid exposure. Similarly, Fecci et al., did not observe differences in T_{regs} fractions between non-GBM brain tumor patients receiving perioperative steroids and healthy controls [8]. As expected, cytotoxic therapy in our patients caused further lymphopenia, but the T_{regs} fraction increased during such treatment and slowly declined thereafter, suggesting resistance of these cells to chemo/radiotherapy and a more rapid recovery phase as previously reported [22,23]. This elevated

T_{regs} fraction during standard therapy may impact potential immunotherapy approaches for these patients and methods to decrease this subpopulation of CD4⁺ lymphocytes might be warranted in conjunction with immunotherapy protocols [9,17]. Overall, this study suggests that patients with MBL, similar to adult patients with GBM, are characterized at diagnosis by CD4 T cell lymphopenia, however, they do not reflect elevated peripheral blood T_{regs} as part of a spectrum of tumor-associated immunosuppression. Further functional analysis of immune deficits in this patient population warrants focus on other immunoregulatory populations of cells such as myeloid-derived suppressor cells and axis of immune checkpoint receptor-ligand interactions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Funding: This work was supported by Pediatric Brain Tumor Consortium grant U01CA81457, National Center for Research Resources grant M01RR00188, and the American Lebanese Syrian Associated Charities.

Abbreviations

CM	Chiari malformation
GBM	Glioblastoma Multiforme
MBL	Medulloblastoma
PFT	Posterior fossa tumors other than medulloblastoma
RT	Radiotherapy
T_H	Helper T cells
T_{regs}	Regulatory T-cells

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Figure 1.
T_{reg} lymphocyte population (CD4+CD25+Foxp3+CD127_{low}) identified by flow cytometry

Figure 2.

a–d: Dot plots representing total CD4 (panel **a**), non-memory (CD45RO⁻CD4⁺) (panel **b**), memory (CD45RO⁺CD4⁺) (panel **c**), and regulatory (CD4⁺CD25⁺FoxP3⁺CD127^{lo}) T-cells (panel **d**) in patients with Chiari Malformation (CM), medulloblastoma (MBL), and other posterior fossa tumors (PFT) demonstrating significantly lower counts of these lymphocyte subsets in patients with posterior fossa tumors as compared to control CM patients (p values are from Kruskal-Wallis test)

Figure 3.

a–f: Total lymphocyte count and subsets (absolute or percentage) prior to surgery, post-surgery, prior to RT (~ 30 days), post RT (~ 72 days), pre-chemotherapy (~ 114 days), and 3 weeks post chemotherapy in 18 patients with medulloblastoma. **Panels a and b** show total lymphocyte and CD4 counts decreasing as expected following cytotoxic therapy. **Panels c and d** show absolute and percentage regulatory T-cells (CD4CD25FoxP3CD127^{lo}) increasing during cytotoxic therapy only to decline thereafter. **Panel e** demonstrates CD4CD25FoxP3Ki-67 counts showing a corresponding increase suggesting that increase in regulatory T-cells during this period is due to actual proliferation of T_{regs}. **Panel f** shows decrease in effector T cells during cytotoxic chemotherapy with a slow rise thereafter. Dark lines in each panel represent the best model fit.

Table 1
Demographics of 54 patients with either Chiari Malformation or posterior fossa tumors

	Chiari Malformation (N=22)		Posterior Fossa Tumor: Medulloblastoma (N=18)		Posterior Fossa Tumor: Non-Medulloblastoma (N=14)	
	N	%	N	%	N	%
SEX						
Males	7	31.8	13	72.2	7	50.0
Females	15	68.2	5	27.8	7	50.0
ETHNICITY						
Hispanic or Latino	1	4.5	3	16.7	3	21.4
Non-Hispanic	20	90.9	15	83.3	9	64.3
Unknown	1	4.5	0	0.0	2	14.3
RACE						
Black	2	9.1	5	27.8	1	7.1
More than one race	1	4.5	0	0.0	0	0.0
Unknown	0	0.0	1	5.6	2	14.3
White, Non-Hispanic	19	86.4	12	66.7	11	78.6

Table 2
Lymphocyte subsets in patients with Chiari malformation and posterior fossa tumors

MARKER	GROUP	N	ABSOLUTE NO. (%) Median	RANGE (%) [*]	p-VALUE
Total Lymphocytes	CM	21	29876	21246–128913	
	MBL	18	29775	9594–37085	
	PFT	13	28456	3002–50537	
CD4	CM	21	11114	2917–20658	0.0014 (CM vs. MBL) 0.0054 (CM vs. PFT)
	MBL	18	6347.5	2416–12747	
	PFT	13	6073	433–14346	
CD45RO ⁺ CD4 ⁺	CM	21	5999 (19.9)	1090–9230 (6.14–27.2)	0.0079 (CM vs. MBL) 0.0085 (CM vs. PFT)
	MBL	18	1677 (9.4)	358–8002 (3.03–27.24)	
	PFT	13	2942 (8.2)	55–7257 (2.89–22.65)	
CD45RO ⁺ CD4 ⁺	CM	21	4873 (19.1)	1771–10516 (7.71–25.57)	0.0069 (CM vs. MBL) 0.038 (CM vs. PFT)
	MBL	18	2794 (13.5)	1570–6063 (6.55–26.38)	
	PFT	13	3477 (13.4)	195–6861 (7.17–24.37)	
CD4+CD25+FoxP3+CD127 ^{lo}	CM	21	254 (0.73)	1–752 (0–12.4)	0.0016 (CM vs. MBL) 0.001 (CM vs. PFT)
	MBL	18	48 (0.18)	11–385 (0.03–3.9)	
	PFT	13	43 (0.17)	3–165 (0.01–0.9)	

CM- Chiari malformation; MBL – Medulloblastoma; PFT- Posterior fossa tumors

* as a percentage of total lymphocyte count