

TH1 predominance is associated with improved survival in pediatric medulloblastoma patients

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Abstract Medulloblastoma, a primitive neuro-ectodermal tumor that arises in the posterior fossa, is the most common malignant brain tumor occurring in childhood. Even though 60–70% of children with medulloblastoma will be cured with intensive multimodal therapy, including surgery, radiotherapy, and chemotherapy, a significant proportion of surviving patients may suffer from long-term treatment-related sequelae. Therapeutic success is limited especially in younger children by radiotherapy-induced neurocognitive longterm deficits. In order to avoid or delay craniospinal radiotherapy, high-dose chemotherapy followed by autologous stem cell transplantation (HSCT) has become an established treatment modality. Data on the host immunologic environment in medulloblastoma patients are rare, notably data on cytokine expression and immune reconstitution in patients with medulloblastoma undergoing HSCT are lacking. In this present study, we therefore decided to prospectively assess immune function following 24 consecutive autologous HSCT in 17 children with medulloblastoma treated according to the German-Austrian-Swiss HIT-2000-protocol. TH1 predominance was found to

be the most important factor for probability of survival. Already before HSCT, survivors showed higher IFN γ levels in sera as well as higher numbers of IFN γ -positive T-cells. After transplantation, this effect was even more pronounced. Patients with higher numbers of IFN γ - and TNF α -positive T-cells had a more favorable outcome at all analyzed time points. In addition, patients in complete remission (CR) before transplantation, known to have a better prognosis a priori, showed higher expression of IFN γ in T-cells. Taken together, this is the *first* report to demonstrate that high expression of IFN γ and TNF α in T-cells of medulloblastoma patients in the early post-transplant period correlates with a better prognosis. Our data point toward a potentially important influence of TH1-cytokine expression before and after transplantation on the survival of pediatric medulloblastoma patients.

Keywords Pediatric medulloblastoma · High-dose chemotherapy · T cell reconstitution · IFN γ and TNF α · TH1 predominance · Prognostic factors

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Introduction

Medulloblastoma represents the most common malignant central nervous system tumor constituting more than 20% of all pediatric brain tumors [1]. Even though 60–70% of children with non-metastasized medulloblastoma may be cured by aggressive multimodal therapy, including surgery, radiotherapy, and chemotherapy, a significant proportion of surviving patients may suffer from long-term treatment-related complications, especially from leukoencephalopathy and cognitive deficits [2, 3]. In particular, in younger children the increased susceptibility of the immature brain to radiotherapy-induced cognitive deficits represents a major

restriction for therapy. These deficits may even increase for years after radiotherapy [4]. This has set age limits on the use of radiotherapy especially in young children [5].

In order to delay or to even replace craniospinal radiotherapy, high-dose chemotherapy has been pioneered as a therapeutic option [6–8]. High-dose chemotherapy followed by autologous stem cell transplantation (HSCT) is designed to eliminate higher numbers of chemosensitive residual tumor cells. The efficacy of this treatment modality is based on the chemosensitivity of the malignant tumor cells with a steep dose–response curve as well as the choice of drugs for which bone marrow impairment is the dose-limiting toxicity [9]. To rescue the hematopoietic system, patients receive cryopreserved autologous hematopoietic stem cells. HSCT has become an established treatment for distinct pediatric tumors [10–13]. We and others have been able to demonstrate, that it can be effective [14, 15], even in relapsed patients with extraneural metastatic medulloblastoma [14].

However, a great number of metastatic or recurrent childhood medulloblastoma have become chemoresistant and results of the most recent relapse trial within the German-Austrian-Swiss HIT-brain tumor network (HIT-Rez-97) have been disappointing [16]. Relapse rates are known to be high during the first year after completion of therapy [17, 18]. These relapses have been attributed mainly to the failure of high-dose chemotherapy to eradicate minimal residual disease. Potential mechanisms include resistance to apoptosis [19], down-regulation of death receptors, presence of decoy receptors, and loss of downstream death-signaling pathway elements [20–22]. In medulloblastoma cell lines, resistance toward TRAIL-induced apoptosis was shown to correlate with loss of caspase-8 mRNA [21]. This loss of apoptosis induction could be reversed in vitro by IFN γ . IFN γ -mediated restoration of caspase-8 expression resulted in the restoration of sensitivity to TRAIL-induced apoptosis and in an increased response to chemotherapy and radiotherapy [12, 19, 23]. IFN γ , a pleiotropic cytokine, produced mainly by T- and NK-cells, is known to be involved in antiviral responses, immune surveillance, inhibiting cellular proliferation, and in tumor suppression [24–26]. However, in contrast to the in vitro findings, data on the potential impact of cytokine expression in medulloblastoma patients are rare.

Prognostic factors in childhood medulloblastoma have been defined based on clinical criteria, and only recently the knowledge of molecular markers has increased [6, 27]. Outcome of patients <4 years of age, with metastatic disease, brainstem, or fourth ventricular floor involvement is inferior [28]. Between 20 and 40% of patients who meet the current clinical standard risk criteria (non-metastatic disease, no post-operative residual tumor) will experience

tumor relapse, but cannot be identified in advance. Most recently, molecular markers and gene expression profiles have been investigated by our group and others [27, 29, 30]. Pfister et al. identified genomic aberrations such as genomic amplification of MYC/MYCN or the gain of 6q and 17q, as powerful independent markers for disease progression and reduced survival [27]. Furthermore, c-myc and trkC have been identified as promising markers in paraffin embedded tissue samples [20, 29]. However, the optimal combination of clinical and biologic markers for disease stratification has not been established yet.

For the further improvement of multimodal therapy, a better understanding of the biologic mechanisms of host tumor interaction in medulloblastoma is of utmost importance. In the present study, we therefore focused on one important aspect of the host environment; we *prospectively* analyzed immune function in 17 consecutive children with medulloblastoma after 24 autologous HSCT. All patients were treated according to the German-Austrian-Swiss HIT-2000-protocol and transplanted during the period from June 01, 2002 until December 31, 2006. As surrogate parameters of immune function, four-color flow cytometry, intracellular cytokine staining, and measurement of serum cytokine concentrations were performed. We asked whether distinct parameters of immune reconstitution, especially in cytokine expression, might be predictive for the prognosis in these high-risk children.

Our results confirmed for the *first* time that high IFN γ and TNF α production in T-cells at distinct time points in the immediate post-HSCT time period are in fact important predictive factors for long-term disease-free survival. These novel observations underline the importance of cytokine expression for tumor surveillance in childhood medulloblastoma.

Materials and methods

Patients

We prospectively studied T-/B- and NK-cell reconstitution as well as intracellular cytokine expression in T-cells in consecutive pediatric patients with medulloblastoma/supratentorial PNET or pineoblastoma undergoing autologous peripheral hematopoietic stem cell transplantation with unmanipulated grafts at the University Children Hospital of Wuerzburg between June 01, 2002 and December 31, 2006, at the following predefined time points: before stem cell transplantation, at the time of engraftment, and on days +30, +60, +100, +200, +365, 15 months and 2 years after transplantation. In total, 185 samples from 24 transplantations (17 patients) were analyzed. Pre-transplant chemotherapy and conditioning

regimes were *uniformly* administered according to the HIT 2000 protocol that *includes* pediatric patients of defined histology (*medulloblastoma* of all subtypes, including the rare supratentorial PNET or pineoblastoma) and age (<21 years). Patients who received different treatment schedules were excluded from this study. Following the second to fourth chemotherapy cycle, all patients underwent leukapheresis for peripheral blood stem cell collection. Stem cells were mobilized by G-CSF.

This study was approved by the Human Subjects Committee of the University of Wuerzburg (Study Nr. 133/04) and was registered at ClinicalTrials.gov as NCT00231712. Patients and guardians participating in this study gave informed consent according to institutional guidelines in accordance with the Declaration of Helsinki.

Antibodies and reagents

Antibodies to the surface epitopes CD3 (clone UCHT1), CD4 (RPA-T4), CD8 (RPA-T8), CD16 (3G8)/56 (B159), CD19 (HIB19), CD45RA (HI100), CD45RO (UCHL1), TGF β (TB21), TNF α (Mab11), IFN γ (B27), IL2 (MQ1-17H12), and IL4 (MP4-25D2) were all purchased from Becton–Dickinson (Heidelberg, Germany).

Unstimulated sera were measured for cytokines in Opteia-ELISA Kits (IL15, IL13, IFN γ , IL4; Becton–Dickinson: Heidelberg, Germany) and in Biosource ELISA kits (IL7, TGF β ; Biosource: Camarillo, USA) according to the manufacturer's instructions.

Intracellular cytokine staining

Initially, 1×10^6 leukocytes in heparinized peripheral blood (min. 100 μ l) were added to 1,000 μ l RPMI-1640 medium supplemented with 10% FCS, 2 mmol/l L-glutamine, 100 U/L penicillin, 1 mM/L sodium pyruvate, and 100 μ g/L streptomycin (Gibco, Eggenstein, Germany). Diluted peripheral blood cells were incubated with Phorbol-myristate acetate (PMA, 10 ng/ml) and ionomycin (1 nmol/ml) and cultured for 20 h at 37°C and 5% CO₂. In order to block cytokine secretion, Brefeldin (10 μ g/ml, Sigma, Taufkirchen, Germany) was added for at least 16 h. Intracellular cytokine staining was performed as described previously [31, 32]. After incubation, cells were stained for surface markers using standard procedures. Subsequently, cells were fixed with 4% paraformaldehyde, permeabilized with 0.3% saponin (Riedel–de–Haen, Deisenhofen, Germany), stained intracellularly, washed twice in 0.1% saponin, and analyzed on a FACS Calibur[®] using Cell-Quest[®] software (BD, Heidelberg, Germany).

In order to ensure optimal culture conditions, mitogen concentrations (PMA 1–50 ng/ml and ionomycin 0.1–5 nmol/ml) as well as culture periods (4, 6, 8, 10, 20, 24, 36, and

48 h) had been titrated with PBMCs from healthy individuals. Best results for all cytokines were obtained with 10 ng/ml PMA and 1 nmol/ml ionomycin after 20 h, which were used for all subsequent assays.

Statistical analysis

Student's paired t-test for mean differences was used to analyze data for levels of statistical significance among two groups (survival vs. non-survival), Kruskal–Wallis Test were used among three age groups (0–4; 5–12; >12 years). Correlation between surface isotope expression and cytokine production was assessed using the Pearson correlation coefficient. Kaplan–Meier Plots were used for survival probability. Statistical correlations between parameters were analyzed using a bivariate fit model (JMP 5.0 software, SAS, Cary, NC, USA). Additional multivariate analysis was performed against conventional outcome predictors (age, gender, disease status, and stem cell dosage) between multiple groups by using repeated measures analysis of variance (ANOVA) and Bonferroni's multiple comparison test. In all statistical applications, a *P* value of <0.05 was considered to indicate a statistical significant difference between the groups.

Results

Engraftment

Following high-dose chemotherapy, a median of 4.6×10^6 CD34⁺/kg body weight (range: 2–48 $\times 10^6$ CD34⁺) non-manipulated peripheral blood stem cells were transplanted. All patients received G-CSF until granulocyte recovery. All patients showed engraftment after transplantation with a median time to engraftment of 11 ± 5 days (range: 8–14 days).

The median patient age in this study was 6.9 years (range 12 months–16 years; 11 male, 6 female). Patients' follow-up was finally evaluated on May 01, 2010, with a median follow-up of 4.3 years (range 106–3,285 days). At this time point, 8 of 17 patients were still alive (Table 1). Four of these were in complete remission, four with stable disease. 9 patients died of disease progression. There was no transplant related mortality (0/24).

Cytokine production before transplantation: comparison of survivors vs. non-survivors

As a first step, immune parameters immediately before the start of the conditioning were investigated (Table 2). The obtained data were retrospectively analyzed for differences in survivors vs. non-survivors.

Table 1 Patient characteristics

No	sex	Age (y)	Histology	Protocol	Status at HSCT	Conditioning	CD 34 ⁺ cells x10 ⁶ /kgBW	Engraftment in days	Clinical Course and Outcome
1	f	7	Pineo	HIT2000	CR1	CPM, L-PAM	5	10	CR (follow-up 9 years)
2	m	16	Medullo	HIT2000	PR	CBGDA, VP-16	4.1	13	Recurrence day +118, 2.autoHSCT day +131
2	m	16	Medullo	HIT2000	PD	TTP, L-PAM	4.1	12	Recurrence day + 334, radiopetid and hyperthermy therapy, DOD+1327
3	m	9	Medullo	HIT2000	CR2	CBGDA, VP-16, TTP	6:16	12	CR (follow-up 7 years)
4	f	4	Medullo	HIT2000	PR	CBGDA, VP-16, TTP	15.5	9	2.autoHSCT day +256
4	f	4	Medullo	HIT2000	PR	TTP, L-PAM	15.5	10	SD w/o therapy (follow-up 7 years)
5	f	2	Medullo	HIT2000	PR	CPM, L-PAM	12.5	13	SD, on valproat (follow-up 6years)
6	m	5	Medullo	HIT2000	CR1	CBGDA, VP-16, TTP	48.8	9	Recurrence day +327, DOD day +410
7	m	3	Medullo	HIT2000	PR	CBGDA, VP-16, MTX ith	20.7	14	2.autoHSCT day +70
7	m	3	Medullo	HIT2000	PR	TTP, L-PAM, MTX ith	20.7	14	Recurrence day +163, Radiatio, DOD day +229
8	f	2	Medullo	HIT2000	PR	CBGDA, VP-16	3.8	12	Recurrence day +65, DOD day +200
9	m	15	Medullo	HIT2000	PR	TTP, L-PAM	7.3	9	Recurrence day +450, DOD day +507
10	m	4	Medullo	HIT2000	PR	CBGDA, VP-16	2.25	14	2.autoHSCT day +132
10	m	4	Medullo	HIT2000	PR	CPM, TTP	2.25	15	Oral temodal, radiation, VGPR (follow-up 3 years)
11	m	15	Medullo	HIT2000	SD	CBGDA, VP-16	2.5	11	2.autoHSCT day +102
11	m	15	Medullo	HIT2000	SD	TTP, L-PAM	2:15	11	DOD+442
12	f	14	Medullo	HIT2000	SD	CPM, L-PAM	2.91	11	Recurrence day + 689, DOD day +1398
13	m	5	Medullo	HIT2000	PR	CBGDA, VP-16	4.62	9	2.autoHSCT day +77
13	m	5	Medullo	HIT2000	PR	CMP, L-PAM	4.62	14	VGPR (follow-up 4 years)
14	f	1	PNET	HIT2000	CR1	CBGDA, VP-16	23.2	10	2.autoHSCT day +69
14	f	1	PNET	HIT2000	CR1	TTP, L-PAM	23.2	9	CR (follow-up 7 years)
15	m	2	Medullo	HIT2000	PR	TTP, L-PAM	8	12	Recurrence day +76, DOD day +106
16	m	12	Medullo	HIT2000	SD	CBGDA, VP-16, TTP	2.91	11	Recurrence day +787, Palliative chemotherapy , DOD day +1163
17	m	9	Medullo	HIT2000	PR	CBGDA, VP-16, TTP	9.5	8	Oral temozolomide, liposome cytarabine, CR (follow-up 5 years)
Median		6.9				4.6		11±5	

Table depicts the data of the investigated patients: age at transplantation, histology, chemotherapy induction protocol, disease status at transplant, type of conditioning regime, number of CD34-positive stem cells contained in the graft/kg body weight, day of engraftment after transplant and current disease status. Seven patients received two consecutive transplants. Out of 17 patients, 8 are currently alive; of these 4 patients are in CR, 4 are alive with disease; these patients are shaded in gray. 9 patients died of progressive disease. There was no transplant related mortality. CPM cyclophosphamid, CBGDA carboplatin, L-PAM melphalan, TTP thiotepa, VP-16 Etoposid, autoHSCT autologous stem cell transplantation, CR complete remission, PR partial response, VGPR very good partial response, SD stable disease, PD progressive disease, DOD dead of disease, ith intrathecal, engraftment first of three consecutive days with absolute neutrophil count of >500/μl

Table 2 Data obtained by FACS analysis after intracellular cytokine staining before high-dose chemotherapy

Lymphoid subpopulation	Before transplantation in % (alive vs. death)	<i>P</i> values
CD3 IFN γ	30 \pm 5 vs. 17 \pm 3	NS (0.05)
CD8 IFN γ	17 \pm 5 vs. 3 \pm 2	0.02
CD3 CD45RO IFN γ	19 \pm 3 vs. 5 \pm 1	0.007

Patients who were alive on May 01, 2010 were compared to patients who had died of progressive disease. The data depict the percentage of cytokine-positive cells in the respective T-cell subpopulation. The survivors show higher expressions of IFN γ , especially in CD45RO-positive cells, than non-survivors measured at the time point *before* transplantation

Patients with a favorable outcome (survivors) showed higher production of IFN γ in distinct T-cell subsets: we could demonstrate that CD8⁺ cytotoxic T-cells (17 \pm 5 vs. 3 \pm 2% IFN γ ⁺/CD8⁺, *P* = 0.02) and even more pronounced memory T-cells (19 \pm 3 vs. 5 \pm 1% IFN γ ⁺/CD45RO⁺, *P* = 0.007) showed increased IFN γ production (Table 2). The described correlations could be confirmed by analyzing both relative percentages of IFN γ -positive cells and absolute counts of IFN γ -positive cells within the above-mentioned T-cell subpopulation. Importantly, age-dependent influences of cytokine expression profiles could be excluded in our cohort (data not shown). In contrast, analysis of additional intracellular cytokines (IL2, IL4, IL5, IL10 as well as TNF α) before transplantation did not show any statistical significant differences between survivors and non-survivors (data not shown).

With respect to cytokine secretion in unstimulated sera before transplantation, we found a tendency toward higher IFN γ levels in surviving children (*P* = 0.04, supplementary online Figure S1B).

Cytokine production after transplantation

All patients: immunoreconstitution after autologous SCT

We analyzed the cytokine expression at predefined time points after autologous stem cell transplantations (engraftment, days +30, +60, +100, +200, +365 and 15 months). Here, we observed that IL4, TGF β and IFN γ secretion in unstimulated sera showed a peak at the time of engraftment and thereafter returned to pre-transplant levels during the first-year post-transplantation (data not shown).

Using intracellular cytokine staining, we found *increasing* values of IFN γ and TNF α expression in all T-cell subpopulations until day +200 (Fig. 1 depicts a representative example (patient # 3). Thereafter, IFN γ and TNF α declined and returned to pre-transplant levels (Table 3). In contrast, TH2-cytokine expression (IL4, IL5 and IL10) remained *stable* during the observation period. IL2 production in T-cells peaked at engraftment and returned to pre-transplant levels at one-year post-SCT.

Comparison of survivors vs. non-survivors

When comparing cytokine production after transplantation in patients who survived vs. patients who died, survivors—

Fig. 1 Increasing TH1-cytokine expressions until day \pm 200 in one representative patient (# 3). Figure 1 depicts representative results obtained by FACS analysis of PBMC from patient number # 3. Cells were stimulated with PMA and ionomycin, as described in the section of “Materials and methods”. Gating was performed on lymphocytes by scatter characteristics and CD3⁺ cells. The expression of IFN γ (upper lane) and TNF α (lower lane) in CD3-positive T-cells before (*left column*), 60 days (*middle column*) and 200 days (*right column*) post-transplant is shown

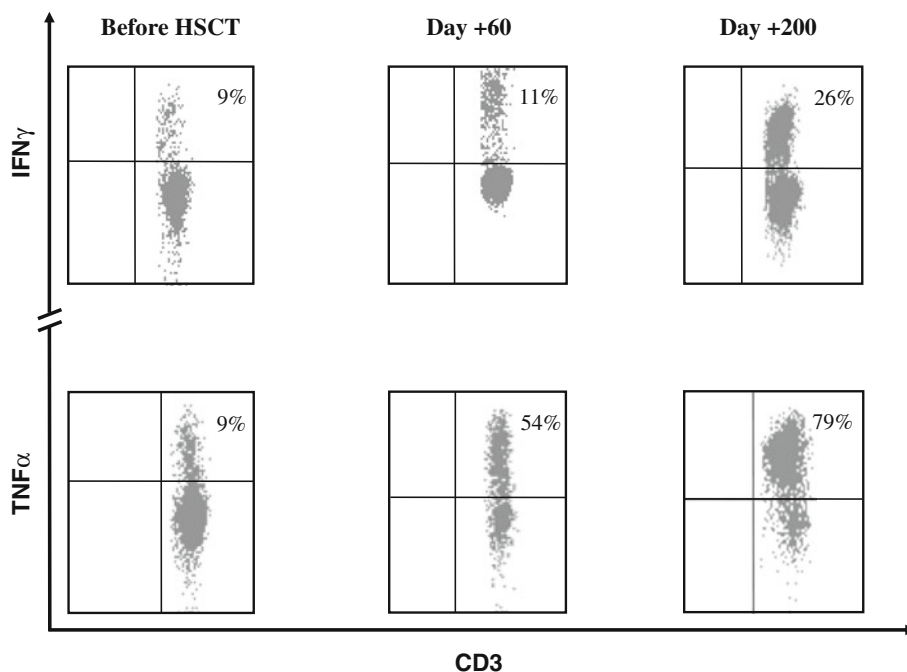


Table 3 Immune reconstitution after autologous stem cell transplantation

Mean values	Before autoHSCT	Engraftment	Day +30	Day +60	Day +100	Day +365	>2 years	Normal range
CD16/56/ μ l	160 \pm 125	100 \pm 70	232 \pm 250	197 \pm 78	172 \pm 75	152 \pm 100	260 \pm 73	300–600
CD3/ μ l	845 \pm 498	354 \pm 427	550 \pm 253	456 \pm 278	387 \pm 142	837 \pm 24	1,147 \pm 335	1,800–3,600
CD4/ μ l	507 \pm 352	175 \pm 279	224 \pm 183	249 \pm 112	135 \pm 90	422 \pm 124	620 \pm 156	800–1,800
CD8/ μ l	290 \pm 140	89 \pm 124	185 \pm 81	224 \pm 96	234 \pm 112	322 \pm 43	348 \pm 43	800–1,500
CD3CD45RA%	52 \pm 11	29 \pm 16	28 \pm 9	36 \pm 8	36 \pm 7	45 \pm 8	44 \pm 8	60–70
CD3CD45RO%	33 \pm 12	53 \pm 15	61 \pm 10	53 \pm 13	53 \pm 7	43 \pm 9	38 \pm 8	21–40
CD19/ μ l	278 \pm 194	62 \pm 86	101 \pm 124	146 \pm 113	199 \pm 129	148 \pm 48	462 \pm 144	500–1,300
IgG mg/dl	203 \pm 64	193 \pm 35	186 \pm 25	197 \pm 53	127 \pm 88	200 \pm 78	250 \pm 134	570–1,500
CD3 ⁺ IL2%	21 \pm 9	50 \pm 20	35 \pm 9	35 \pm 5	30 \pm 4	20 \pm 10	27 \pm 9	33–52
CD3 ⁺ IFN γ %	23 \pm 6	21 \pm 10	47 \pm 13	52 \pm 14	60 \pm 5	60 \pm 15	26 \pm 5	15–30
CD3 ⁺ TNF α %	23 \pm 9	25 \pm 5	38 \pm 11	37 \pm 12	55 \pm 6	40 \pm 15	31 \pm 11	16–29
CD3 ⁺ IL4%	0.5 \pm 0.5	2 \pm 0.5	0.5 \pm 0.3	1 \pm 0.5	1 \pm 0.5	0.6 \pm 0.4	1 \pm 0	0.5–2

Lymphocyte reconstitution and cytokine expression in CD3⁺ T-cells before transplantation, at engraftment, on days +30, +100, +365 and >2 years post-transplantation. Cytokine production was determined by FACS after stimulation with PMA and ionomycin as described above. Percentages of cytokine-positive cells in CD3⁺ T-cells as mean \pm SD are shown

as in pre-transplant analysis—showed higher intracellular TH1 cytokine expression, especially high numbers of IFN γ -positive T-cells (day +100: CD3⁺/IFN γ ⁺: 69 \pm 9 vs. 31 \pm 8%; $P < 0.0001$, Table 4 and supplementary online Figure S1A) and TNF α -positive T-cells (day +100: CD3⁺/TNF α ⁺: 62 \pm 5 vs. 29 \pm 8; $P < 0.001$, Table 4) were found to be linked with a favorable outcome at all measured time points post-transplantation. As has been shown in the pre-transplant setting, the main contributing subpopulation for high production of IFN γ and TNF α in T-cells were cytotoxic (CD8⁺) and of IFN γ in memory T-cells (CD45RO⁺) (Table 4). Findings could be confirmed by similar correlations of the absolute counts of

IFN γ -positive cells (data not shown). Most of the patients showed a consistency of IFN γ expression prior to and after transplantation.

Besides the impact of cytokine expression in T-cells, additional parameters of immune reconstitution were analyzed. In the group of surviving medulloblastoma patients, higher T- and B-cells counts (day +100: CD45RO⁺: $P < 0.02$, CD8⁺: $P < 0.004$ and CD19⁺: $P < 0.03$) were found at all analyzed post-transplant time points (data not shown). Furthermore, we analyzed T-cell receptor diversity as previously described by our group [33] and could not find any significant differences (data not shown). In addition, analysis of thymic output post-transplant (TREC analysis) showed well-known age-dependent expression [34], but no correlation with prognosis (data not shown).

Table 4 Data obtained by FACS analysis after intracellular cytokine staining on day +100 post-transplant

Lymphoid subpopulation	Day +100 in % (alive vs. death)	P values
CD3 IFN γ	69 \pm 9 vs. 31 \pm 8	0.0004
CD8 IFN γ	50 \pm 7 vs. 14 \pm 5	<0.0001
CD3 CD45RO IFN γ	89 \pm 10 vs. 34 \pm 6	0.001
CD3 CD45RA IFN γ	76 \pm 12 vs. 27 \pm 5	0.04
CD3 TNF α	62 \pm 5 vs. 29 \pm 8	0.001
CD8 TNF α	45 \pm 16 vs. 15 \pm 8	0.004
CD3 CD45RO TNF α	62 \pm 6 vs. 40 \pm 11	NS (0.1)
CD3 CD45RA TNF α	29 \pm 9 vs. 7 \pm 5	0.008

Patients who were alive on May 01, 2010 to patients who had died of progressive disease were compared. The data depict the percentage of cytokine-positive cells in the respective T-cell subpopulation. The survivors showed higher expressions of IFN γ and TNF α , especially in CD8-positive and CD45RO-positive cells than non-survivors on day +100 post-transplantation

Impact of remission status on cytokine expression

We then analyzed the potential impact of remission status before SCT on cytokine expression after HSCT. Patients in CR before transplantation are known to have a better prognosis a priori [17, 35]. No differences were found in post-transplant cytokine sera levels between CR and Non-CR patients. However, looking at intracellular cytokine expression of T-cells after transplantation, higher IFN γ expression (especially in CD8⁺IFN γ ⁺ T-cells: $P < 0.02$; day + 100 and in CD3⁺ CD45RO⁺ IFN γ ⁺: $P < 0.007$ day + 100; Table 5) was found in CR patients at the time of transplantation and at all predefined time points until day +200 when compared to non-CR patients. All other cytokines tested, namely IL2, IL4, IL5, IL10 and TNF α , did not show any correlation with outcome.

Table 5 Data obtained by FACS analysis after intracellular cytokine staining at day +100 post-transplant

Lymphoid subpopulation	Day +100 in % (CR vs. Non-CR before HSCT)	<i>P</i> values
CD3 IFN γ	60 \pm 7 vs. 40 \pm 5	NS (0.05)
CD8 IFN γ	46 \pm 7 vs. 20 \pm 6	0.02
CD3 CD45RO IFN γ	82 \pm 8 vs. 40 \pm 5	0.007

IFN γ production in T-cells in patients who were in CR pre-SCT vs. patients who had detectable disease before transplantation was compared. The data depict the percentage of cytokine-positive cells in the respective T-cell subpopulation. Patients who were in CR pre-transplantation showed higher expressions of IFN γ , especially in CD8-positive and CD45RO-positive cells, on day +100 post-transplantation

Comparison of IFN γ high and low producers

Finally, we performed a Kaplan–Meier analysis for patients with high IFN γ production (>40% IFN γ ⁺/CD3⁺ cells) vs. patients with low IFN γ production (<40% IFN γ ⁺/CD3⁺) in T-cells measured at 60 days after SCT. This cutoff was selected based on previous data in adult patients [36, 37]. High production of IFN γ correlated significantly with a better survival post-transplant (55% for high producers vs. 15% for low producers, *P* < 0.0001; Fig. 2). Similar results were obtained in relation to the IFN γ production prior to transplantation and at day +30 post-HSCT (data not shown). We excluded patient age as a confounding factor by multivariate analysis (data not shown).

Discussion

High-dose chemotherapy followed by autologous hematopoietic stem cell transplantation (auto HSCT) has become

an effective and established treatment for certain malignant diseases [10, 12, 38]. Historically, neurosurgical operation, radiation therapy, and adjuvant chemotherapy have been established as standard therapy for medulloblastoma in childhood. Cooperative multicenter trials have improved prognosis over the last 15 years [6]. In an attempt to delay or to even substitute craniospinal irradiation notably in young children, therapy protocols have been amended to include HSCT in favor of a better overall survival with less neurocognitive deficiencies when compared to conventional craniospinal radiation [8].

While immune function following *allogeneic* transplantation has been extensively studied in recent years, few data on immune reconstitution following *autologous* transplantation for solid pediatric tumors exist. Most published studies on this issue comprised mixed cohorts of children with different underlying diseases, prior therapies and various conditioning regimes [37, 39–42]. In this prospective study, we therefore decided to analyze a homogeneous pediatric cohort of 17 medulloblastoma/PNET patients with uniform stratified treatment according to the HIT-2000 protocol.

Besides the reconstitution of adequate lymphocyte counts, a key element in the recovery of immune competence is an appropriate T-cell function as measured by cytokine production. Recent data underline the importance of cytokine expression in tumor surveillance in different tumor entities including lymphoma, solid tumors, and brain tumors [43]. To the best of the authors' knowledge, this is the *first* report to demonstrate data on cytokine expression after HSCT for pediatric medulloblastoma. Most importantly, we could demonstrate for the first time that children with favorable clinical course showed TH1 predominance in cytokine production. Interestingly, already before HSCT higher IFN γ production was found in the group of

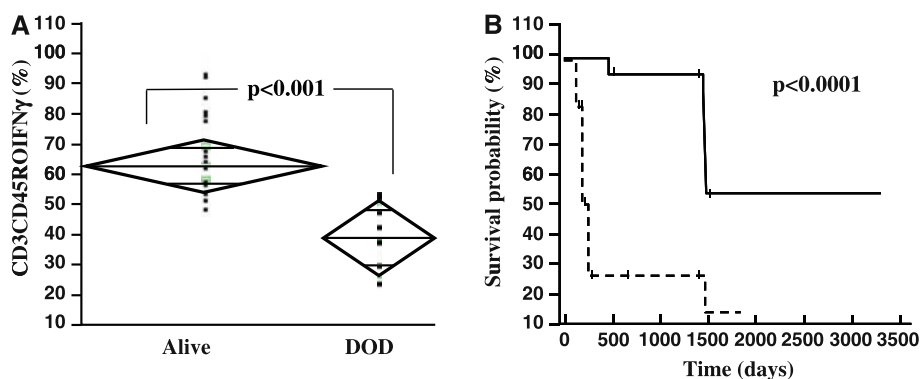


Fig. 2 IFN γ expression and survival. **a** Data are given as *boxplots* of percentage of IFN γ ⁺ cells in the CD45RO⁺CD3⁺-fraction on day +200. Survivors showed higher percentages of IFN γ producing memory T-cells. DOD death of disease. **b** Kaplan–Maier analysis for patients with higher vs. lower production of IFN γ in T-cells. At day + 60 post-transplantation, PBMC of patients (*n* = 17) were

stimulated and determination of IFN γ -positive cells was done by FACS analysis. Results were defined as IFN γ high producers (9/17) if more than 40% of T-cells showed IFN γ expression and as low producers (8/17) if less than 40% of T-cells were IFN γ positive. 1,500 days post-transplantation survival was 55% in IFN γ high producers vs. 15% in IFN γ low producers (*P* < 0.0001)

survivors. On follow-up post-transplant, the differences in IFN γ production between non-survivors and survivors were found to be even more pronounced. High absolute IFN γ levels as well as high relative expression of IFN γ in different T-cells were found in patients with favorable outcome (Table 4). As IFN γ expression is known to increase with age [44–46], we investigated potential age-dependent influences and could exclude patient age as a confounding factor. Furthermore, other conventional outcome predictors, such as gender, disease status, and stem cell dose, could be excluded by multivariate analysis (data not shown). The relatively small patient number certainly represents one of the limitations of the present study; therefore, results will have to be confirmed in a larger multicenter cohort in the future.

In other tumor entities, TH1 predominance has been characterized as a favorable prognostic indicator, for example in adult lymphoma, where TH1-cytokine predominance (mainly IFN expression) has been associated with good prognosis before and during therapy [47]. No data exist so far for pediatric brain tumors. Even though we did not find higher levels of TH2-cytokines and could therefore not directly demonstrate a TH2 shift [47], we could clearly demonstrate significantly lower pre- and post-transplant IFN γ (and TNF α) values in patients who later developed relapse (Fig. 2/Table 4).

In recent years, the importance of IFN γ in tumor surveillance has been intensively studied. IFN γ has been shown to enhance antigen presentation by up-regulation of MHC expression [48, 49]. In addition, IFN γ is responsible for the induction of costimulatory molecules on antigen-presenting cells [49]. Furthermore, IFN γ promotes TH1 development, in part by enhancing IL12 secretion by macrophages and partly by up-regulation of functional IL12-R on CD4⁺T-cells, rendering them to be more responsive to IL12. There is increasing evidence that IL12 production of antigen-presenting cells and IFN γ production of T-cells are correlated with the induction of anti-tumor immunity [48, 49]. However, in the current study we did not analyze IL12 expression. This specific question will be the subject of future studies.

Besides its essential role for the induction of anti-tumor responses, it has been suggested that IFN γ may mediate a direct physiological effect on the development of the central nervous system [50, 51]. Transgenic mice with ectopic expression of IFN γ in the CNS showed hypomyelination and abnormal cerebellar development [52, 53]. More recently, Lin et al. demonstrated in a mouse model that IFN γ expression in a very narrow developmental window of perinatal period can induce medulloblastoma as well as Atypical Teratoid Rhabdoid Tumors [54]. Interestingly, once the tumor was established, further expression of IFN γ induced host immunologic response with lymphocytic

infiltration and tumor apoptosis [54]. Finally, in medulloblastoma tissue itself it could be demonstrated that mRNA expression of IFN γ -R2 is significantly lower compared to normal brain tissue. Therefore, reduced IFN γ signaling, induced by either receptor down-regulation or reduced IFN γ expression, seems to play an important role in medulloblastoma pathobiology [19].

Furthermore, in medulloblastoma cell lines, IFN γ is known to increase chemosensitivity, cytotoxicity and induction of apoptosis [20, 23]. In this context, caspase-8 activity has to be discussed as an important element in the biologic mechanisms. Demethylating agents (for example valproic acid) and additional IFN γ are discussed to be contributing factors for increasing caspase-8 activity by transcriptional activation through the Stat-1/IRF1-dependent pathway. Sensitization to death receptor-induced apoptosis as well as increased sensitivity to chemotherapy and radiotherapy is a result of higher caspase-8 levels in in vitro experiments [19, 23]. Inactivation of caspase-8 to escape apoptosis might therefore be an immunologic cancer escape mechanism, which has already been described for certain tumors, including neuroblastoma, medulloblastoma, Ewing sarcoma, and melanoma [20, 23]. In this context, it is important to note that loss of caspase-8 activity correlates with inferior survival outcome in medulloblastoma [55], suggesting that up-regulation of caspase-8 activity may represent an interesting approach for future therapy options. Taken together, biologically based new treatment approaches, especially focusing on IFN γ , may turn out to become critical contributing factors for improving the prognosis of high-risk medulloblastoma patients.

The focus of the present study was the analysis of cytokine expression and secretion as an important factor of tumor–host interaction during the course of HSCT. Future studies will analyze the cytokine expression and receptor status in situ within tumor tissues to address the question whether the tumor itself may influence immunologic surveillance and IFN γ expression. Alternatively, genetic predispositions, such as polymorphisms of cytokine genes or cytokine receptors, as has been demonstrated in certain malignancies [56–58], have to be discussed and will be the focus of future studies.

In summary, this is the *first* report to demonstrate that IFN γ expression in T-cells is associated with prognosis in pediatric medulloblastoma patients. This interesting finding may be of importance for future risk stratification as well as for novel immunotherapy approaches in pediatric medulloblastoma. IFN γ may represent a novel promising window of opportunity for medulloblastoma patients in an effort to overcome primary or secondary therapy resistance.

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