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Phenotypical analysis, relation to malignancy and prognostic relevance of ICOS + T regulatory and dendritic cells in patients with gliomas

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ABSTRACT

We determined circulating T helper, T regulatory and ICOS + T regulatory as well as DC cell counts in 29 patients with cerebral gliomas. Samples from patients with gliomas vs. healthy controls and from patients with glioblastomas vs. patients with glioma WHO grades I–III contained significantly ($p < 0.05$) decreased numbers of total as well as mature, i.e. myeloid and plasmacytoid DCs. Patients with glioblastomas demonstrated significantly lower values of CD4+ as well as an increased fraction of ICOS + T regulatory/CD4+ cells. Higher CD4+ cell counts (≥225 cells/μl, median) were associated with improved survival in glioblastomas.

1. Introduction

Reduced numbers of CD4+ cells and an increased fraction of inhibitory T regulatory cells (T regs)/CD4+ cells (Fecci et al., 2006; Gousias et al., 2010) contribute significantly to the well documented impairment of cellular immunity in patients with malignant gliomas (Tada and de Tribolet, 1996; Dix et al., 1999; Zisakis et al., 2007). T regs are physiologically responsible for the immune homeostasis and the maintenance of self-tolerance through inhibition of autoreactive responses (Erdman and Poutahidis, 2010; Mougiakakos et al., 2010; Sakaguchi et al., 2010; Wan, 2010; Wilke et al., 2010). Systemic malfunction of T regs may induce severe autoimmunity, such as type 1 diabetes, inflammatory diseases and severe allergy (Nishikawa and Sakaguchi, 2010) whereas increased fractions of T regs/CD4+ cells and therefore hyperfunction may downmodulate numerous immune responses, leading among others to tumor immune escape (Vocanson et al., 2010). T regs may also elicit tolerance to tumor associated antigens (Nishikawa and Sakaguchi, 2010), many of which are self-antigens (Kawakami and Rosenberg, 1997; Scanlan et al., 2004; Boon et al., 2006).

The prognostic significance of circulating and/or tumor infiltrating T regs in patients with gliomas has been intensely studied in the past years, however, so far with inconclusive results (Heimberger et al., 2008; Humphries et al., 2010; Jacobs et al., 2010). Noteworthy, El Andaloussi et al. showed a prolonged survival of mice with experimental brain tumors after depletion of T regs (El Andaloussi et al., 2006). T regs expressing the inducible costimulator (ICOS) suppress both dendritic cell (DC) and T cell function (Ito et al., 2008). Recently, Vocanson et al. reported that the ICOS + subset of natural T regs play a central role in controlling skin inflammation in a mouse model of contact hypersensitivity to haptens (Vocanson et al., 2010). Subsequent studies showed that ICOS gene polymorphisms may affect the risk of breast cancer (Xu et al., 2011) and identified ICOS + populations of T regs in the microenvironment of epithelial ovarian cancer as strong predictors of disease progression (Conrad et al., 2012). The phenotype as well as the prognostic relevance of ICOS + T regs has not been studied in patients with gliomas.

The prognosis of malignant gliomas remains poor. Autologous dendritic cell (DC)-based immunotherapy has emerged as a promising novel treatment (Cho et al., 2009; Van Gool et al., 2009; Ardon et al., 2012). DCs are the most potent antigen-presenting cell (APC) population mediating antitumor responses (Banchereau et al., 2000; Fong and Engleman, 2000). After recognizing and capturing antigens/tumor cells they undergo maturation and induce an antigen specific T cell response. Thus, vaccination therapies using in vitro generated autologous tumor antigen-specific DCs may serve as an alternative and safe adjuvant therapy, since they act with precision on tumor cells while sparing healthy brain tissue.

Some evidence of alterations of the relative frequency of DC subpopulations has been obtained in peripheral blood samples of patients with renal, breast and squamous cell carcinoma of head and neck (Troy et al., 2000; Hoffmann et al., 2002; Della Bella et al., 2003). Pinzon-Charry et al. reported an increased ratio of immature/total DCs in the peripheral

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blood of patients with different types of cancer (Pinzon-Charry et al., 2005). Several tumor derived factors such as interleukins (ILs), prostanoids and vascular endothelial growth factor (VEGF) have been shown to suppress the maturation and differentiation of DCs (Gabrilovich et al., 1997; Menetrier-Caux et al., 1998; Almand et al., 2000; Sombroek et al., 2002). Little is known concerning the phenotype of DCs in patients with gliomas. In particular, reports using more recent classifications, i.e. distinguishing between myeloid (mDCs) with their subpopulations and plasmacytoid (pDCs) DCs, are lacking.

In the present study we analyze the preoperative immunological profile (i.e. the phenotype of CD4+ cells, T regs, ICOS + T regs and DCs subsets, and their relative proportions) in patients with glioma WHO grades I–IV and explore potential correlations with the WHO histological grade and the patients' prognosis.

2. Material and methods

2.1. Patients and clinical data

We examined preoperatively collected fasting morning EDTA stabilized blood from 29 consecutive patients >18 years with subsequently histologically confirmed de novo gliomas (WHO grade I: 3, grade II: 6, grade III: 3, and grade IV: 17) operated at the Department of Neurosurgery of the University Hospital of Bonn between November of 2010 and February of 2011. Since a circadian rhythm of the immunological parameters is known (Dimitrov et al., 2009) all our fasting blood samples were collected between 8:00 and 9:00 am prior to the administration of the morning dexamethasone dose (see below). Samples were processed within 30 min after collection in order to ensure optimal flow cytometry analysis.

The series was 58% male. Median age was 53 years. 23 (82%) patients presented with a Karnofsky Index (KPI) ≥ 90%. The patients' samples were collected after their informed consent was obtained in accordance with the tenets of the declaration of Helsinki and after approval of the study by the Ethics Committee of the Medical Faculty of the University of Bonn. Patients with a history of a previous brain tumor or other cancer, or of an immunological or hematological disease were excluded from the study. Six (20.6%) patients had a biopsy only due to the eloquent location of the tumor. 43% of resections were gross total. Chemo- and radiotherapy were administered to all patients with malignant gliomas (high grade gliomas [HGG]). One patient diagnosed with a low grade glioma (LGG) received radiotherapy after a biopsy. The demographics of our study population are shown in Table 1. All patients were prospectively followed through the Department's outpatient clinic and telephone interviews as required. Median follow-up was 14 months.

Twelve patients (1 patient diagnosed with an astrocytoma WHO grade II, 1 patient with an astrocytoma WHO grade III, and 10 pts. with glioblastomas) were treated with dexamethasone at the time of blood collection, 9 of which (7 pts. with glioblastomas) received the medication for >3 days. Median duration of dexamethasone therapy prior to blood collection was 10 days. Median total dose of dexamethasone (defined as days of administration * dose per day) was 120 mg.

Overall Survival (OS) and Progression Free Survival (PFS) were defined as the time from initial surgery to death or tumor recurrence, respectively. Tumor recurrence was diagnosed by the treating neurosurgeon and neuroradiologist using follow up MRI scan performed routinely or because of clinical deterioration. Routine follow up MRI scans were obtained within 3 days after surgery and then every 3, 4, 6 and 12 months in patients with WHO grade IV, II, II and I tumors, respectively. The statistical analysis followed standard procedures (comparison of medians, linear and cox regression, Kaplan–Meier estimates, Pearson test). P values < 0.05 were considered to be statistically significant.

Table 1
Study group demographics.

Variable		WHO grade I	WHO grade II	WHO grade III	WHO grade IV
N		3	6	3	17
Median age (range)		33 (20–76)	50 (22–71)	44 (26–48)	54 (33–78)
Males		2 (66.7%)	2 (33.3%)	3 (100%)	10(58.8%)
Max. tumor diameter	≤3 cm	3 (100%)	4 (66.7%)	2 (66.7%)	7 (41.1%)
Resection					
GTR		1 (33.3%)	2 (33.3%)	1 (33.3%)	6 (35.3%)
STR		1 (33.3%)	3 (50.0%)	1 (33.3%)	8 (47.0%)
Biopsy		1 (33.3%)	1 (16.7%)	1 (33.3%)	3 (17.6%)
Preop. KPS	90–100%	3 (100%)	6 (100%)	2 (66.7%)	12 (70.5%)
Preop. seizures	Yes	3 (66.7%)	2 (33.3%)	2 (33.3%)	6 (35.3%)
Eloquence	Yes	1 (33.3%)	3 (100%)	2 (66.7%)	10 (58.8%)
Radiotherapy	Yes	0 (0.0%)	1 (16.7%)	3 (100%)	17 (100%)
Chemotherapy	Yes	0 (0.0%)	0 (0.0%)	3 (100%)	17 (100%)

2.2. Flow cytometry

Absolute numbers and relative proportions of T-lymphocyte (CD4+, T regs, ICOS + T regs) and dendritic cell subpopulations were determined by flow cytometry using six different fluorochromes: fluorescein isothiocyanate (FITC), phycoerythrin (PE), peridininchlorophyllprotein (PerCP), allophycocyanin (APC), phycoerythrin-Cy7 (PE-Cy7) and allophycocyanin-Cy7 (APC Cy7). The following surface and intracellular anti-human monoclonal antibodies were used: CD45-APC Cy7 (clone 2D1), CD4-PE (clone RPA-T4), CD3-PerCP (clone SK7), CD25-APC-Cy7 (clone M-A251), HLA-DR-PerCP (clone L243), CD11c-APC clone (S-HCL-3), lineage-FITC (lin-1 cocktail), CD34-FITC (clone 8G12), CD16 PE (clone B73.1, Becton Dickinson Biosciences, San Jose, CA), CD1c-PE (clone AD-58E7), CD141-PE (clone AD5-14H12, Miltenyi, Bergisch Gladbach, Germany), ICOS-PE-Cy7 (clone ISA-3), FoxP3-APC (clone PCH101), CD123-PE-Cy7 (clone 6H6, ebioscience, San Diego, CA) as well as isotype controls.

Cells were surface stained according to the manufacturers' protocols. Briefly, aliquots of whole blood (100 μl) were incubated with the appropriate monoclonal antibodies. After addition of ammonium chloride lysing solution the cells were washed twice with PBS before further analysis. The results were expressed as a percentage of the antibody-positive cells in the overall cell population which was defined as the number of cells with specific fluorescence higher than the isotype control and autofluorescent samples.

Consistent with previously published data (Savary et al., 1998; Autissier et al., 2010) DCs were isolated as mononuclear cells expressing major histocompatibility complex (MCH) II molecules (HLA-DR) while lacking common lineage markers (Lin−) such as CD3 (T cells), CD4 (T-helpers), CD25 (T regulatory cells), and CD34 (hematopoietic stem cells). DCs were gated as HLA-DR positive, lineage-negative, CD34 negative, and CD45 positive (HLA DR+/lin−/CD34−/CD45+) (Fig. 1). DCs were further subclassified as myeloid or plasmacytoid based on their reciprocal expression of CD11c (a-integrin) and CD123 (IL-3 receptor a), respectively. Three nonoverlapping populations of mDCs expressing CD16+, CD1c+ (BDCA-1), CD141+ (BDCA-3) were also isolated (Fig. 2). We classified as T regs those lymphocytes expressing common non-specific lineage markers such as CD3, CD4 and CD25 as well as the T reg specific intracellular marker forkhead box P3 (Foxp3) (Humphries et al., 2010; Sonabend et al., 2008). ICOS + T regs cells were isolated as previously reported (i.e. CD3+/CD4+/CD25 + bright/FOXP3 + bright/ICOS + cells; bright = >Mean Fluorescence Intensity, MFI) (Ito et al., 2008).