

CD4⁺CD25⁺FoxP3⁺ T-cell infiltration and heme oxygenase-1 expression correlate with tumor grade in human gliomas

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Abstract Regulatory T-cells play an important role in the regulation of the immune response and the mediation of dominant immunologic tolerance. We have previously shown that these cells are elevated in tumors and blood of patients with glioblastoma multiforme. Heme oxygenase-1, a rate-limiting enzyme in heme catabolism, has also been shown to accumulate during glioma progression and to play a critical role in FoxP3 mediated immune suppression. In this study, we investigated the correlation between FoxP3 and HO-1 expression in patients with various grades of astrocytoma (WHO grade II–IV). Using qualitative and quantitative reverse transcriptase–polymerase chain reaction and quantitative flow cytometry analyses, we analyzed FoxP3 and HO-1 expression in 19 patients with different grades of astrocytoma. We observed the highest level of FoxP3 expression in patients with grade IV tumors ($11.54 \pm 1.95\%$) vs. grade III ($6.74 \pm 0.19\%$) or grade II ($2.53 \pm 0.11\%$) ($P < 0.05$). Moreover, in grade IV tumors, the frequency of HO-1 mRNA expression in CD4⁺CD25⁺ cells was $11.8 \pm 2.45\%$ vs. $7.42 \pm 0.31\%$ in grade III and $2.33 \pm 0.12\%$ in grade II. Tumor infiltrating Treg stained positively with anti-HO-1 antibody. The expression of HO-1 correlated with CD4⁺CD25⁺FoxP3⁺ infiltration ($r = 0.966$). Our results confirm that HO-1 expressing Treg accumulate during glioma progression. This study also suggests that HO-1

mRNA expression is linked to the induction of Foxp3 in CD4⁺CD25⁺ glioma infiltrating Treg. These findings support the suppressive role played by regulatory T-cells in the growth of malignant brain tumors.

Keywords Regulatory T-cells (CD4⁺CD25⁺ T-cells) · Heme oxygenase-1 (HO-1) · Forkhead box P3 (FoxP3) · Glioblastoma multiforme (GBM)

Introduction

Regulatory T-cells (also known as CD4⁺CD25⁺FoxP3⁺, Treg) are a specialized subpopulation of T-cells that act to suppress activation of the immune system and thereby maintain immune system homeostasis and tolerance to self. There is increasing evidence for the existence of elevated numbers of regulatory T-cells in solid tumors and hematological malignancies [1–10]. The presence of infiltrating Treg may be detrimental to the host defense against the tumor, while the presence of effector T lymphocytes, including CD8⁺ and non-regulatory CD4⁺ helper T-cells may be beneficial. We have previously shown that there is an increase in the number of Treg both in tumor infiltrating lymphocytes as well as peripheral blood of patients with glioblastoma multiforme (GBM) [11]. Moreover, we have also shown that depletion of Treg prolongs the survival of mice with experimental brain tumors [12].

Heme oxygenase (HO) is the rate-limiting enzyme in the catabolism of heme to biliverdin, carbon monoxide (CO), and free iron. To date, three isoforms of mammalian HO have been identified: HO-1, HO-2, and HO-3 [13]. HO-1 is inducible by a variety of stimuli, in particular, oxidative stress [14, 15]. HO-2

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and HO-3 are constitutive isozymes, found ubiquitously throughout the body, with high expression in brain and testes [16, 17]. Induction of HO-1 expression has been associated with neuroprotection during hyperthermia in glial cells [18] and during hypoxia [19]. HO-1 expression has also been observed in a wide range of experimental diseases of the rodent brain such as traumatic brain injury [20], ischemia [21], and human Alzheimer's disease [22]. In brain tumors, elevated HO-1 expression has been observed [23, 24], and in at least one report, it would appear that HO-1 accumulates during oligodendroglioma progression [25].

The exact functional role of HO-1 expression is not fully understood in T-cells. However, growing evidence has shown that human Treg constitutively express HO-1 and that HO-1 inhibits T-cell proliferation [26]. Over-expression of HO-1 further renders T-cells resistant to Fas-mediated apoptosis [27]. Most recently, FoxP3, which encodes a forkhead/winged-helix transcription repressor specifically expressed in Treg, has been shown to induce HO-1 expression [28]. Taken together, these studies suggest that HO-1 may be an important effector of FoxP3-mediated immune suppression and an important target for further clinical development.

In the present study, we investigated the expression of HO-1 and Treg infiltration in various grades of human glioma. Both qualitative and quantitative reverse transcription polymerase chain reaction was used to demonstrate HO-1 and FoxP3 gene expression. Flow cytometry was used to quantify Treg infiltration. We report here for the first time that HO-1 and FoxP3 expression directly correlates with tumor grade in patients with malignant glioma.

Materials and methods

Patient samples

Resected specimens from 19 patients who underwent operations in the Division of Neurosurgery at the University of Chicago from 2004 to 2006 were evaluated in this study. According to the WHO classification, tumors included three grade II astrocytomas, four grade III astrocytomas, and 12 grade IV astrocytomas (Table 1). Healthy brain controls ($n = 4$) consisted of surgical specimens obtained during temporal lobe resections for seizure focus. All patients received perioperative steroids. Histological confirmation of the diagnosis, for both tumors and normal cortex, was obtained in all cases. Of note, only normal and healthy

Table 1 Clinical characteristics of patients with glioma

WHO grade	N=
<i>Grade II</i>	
Male	1
Female	2
Average age	3
Newly diagnosed	3
Recurrent	–
History of prior radiotherapy and/or chemotherapy	–
<i>Grade III</i>	
Male	3
Female	1
Average age	48
Newly diagnosed	4
Recurrent	–
History of prior radiotherapy and/or chemotherapy	–
<i>Grade IV</i>	
Male	7
Female	5
Average age	55
Newly diagnosed	4
Recurrent	8
History of prior radiotherapy and/or chemotherapy	8

brain rather than diseased tissue, was used for controls. All surgical specimens were analyzed immediately after removal. This study was approved by the Institutional Review Board of the University of Chicago.

Cell sorting

The separation of CD4⁺CD25⁻ cells from CD4⁺CD25⁺ cells from brain tumor samples was done by positive selection using Treg Isolation Kit (Miltenyi Biotec, Auburn, CA). Briefly, 1×10^7 cells were resuspended in 80 μ l buffer and the cells were magnetically labeled by adding 20 μ l of anti-CD25 microbeads. The cells were incubated for 15 min and then washed by centrifugation and resuspended in 1 ml of buffer per 1.25×10^8 cells. CD4⁺CD25⁺ regulatory T-cells were isolated by positive selection with MS Columns. The effluent was collected; the purity of cells was analyzed by flow cytometry and was more than 90%.

Multi-color flow cytometry analysis

One million tumor infiltrating lymphocytes (TIL) were resuspended in staining buffer (3% FBS in PBS). Tumor cell suspensions were prepared by homogenization of the tumor with a cell strainer and washing of the cells with staining buffer. Erythrocytes were lysed and cells were then stained with various antibodies. Monoclonal antibodies to CD3, CD4, CD25, and Foxp3 were obtained from BD Biosciences (San Jose, CA). The analysis of CD4⁺CD25⁺ T-cells was

performed as a percentage of total CD3⁺ cells in TILs. Monoclonal antibody to HO-1 was from Stressgen Biotechnologies (Victoria, BC). These monoclonal antibodies were directly coupled to fluorescein isothiocyanate, phycoerythrin, or phycoerythrin-indotri-carbocyanin. Antibodies were used at 5 µg/ml and staining was done in fluorescence-activated cell sorting (FACS) buffer on ice for 45 min. Surface marker expression on TIL cells was visualized with FAC-SCalibur™ (BD Biosciences) and was analyzed with FlowJo software (BD Biosciences). Tumor cells sorting without tumor infiltrating lymphocyte was done with a MoFlo cell sorter (Cytomation, Fort Collins, CO).

Semiquantitative RT-PCR and real-time PCR

Total cellular RNA was isolated using RNeasy kit (Ambion Inc. Austin, TX) according to the manufacturer's protocol. Equivalent amounts of RNA were reverse-transcribed with the superscript II-RT kit (Invitrogen). The cDNA amplification was analyzed quantitatively by incorporation of SYBR Green (Applied Biosystems) into double stranded DNA. PCR was done in a total volume of 25 µl containing 1 µl cDNA template, 0.5 µM of each primer, and SYBR Green Master Mix (Applied Biosystems, Foster City, CA) under the following conditions: 15 min hot start at 95°C, 15 s denaturation at 95°C, 20 s annealing of primers at 54°C, and 15 s elongation at 72°C, for 32–35 cycles. Gene expression was analyzed with the ABI PRISM 7300 Sequence Detection Software version 1.9.1 (Applied Biosystems). Sample results were normalized by dividing the value of the unknown gene by the value of the endogenous reference gene encoding glyceraldehyde phosphate dehydrogenase (GAPDH). Quadruplicate reactions were done with all cDNA samples. We used the following primers (Invitrogen, Carlsbad, CA): HO-1 forward (5'-ACT TTC AGA AGG GTC AGG TGT CC-3'); HO-1 reverse (5'-TTG AGC AGG AA G GCG GTC TTA G-3'); Foxp3 forward (5'-ATG CCT CCT CTT CTT CCT TGA-3'); Foxp3 reverse (5'-ATT GTG CCC TGC CCT TCT CA-3'); GAPDH forward (5'-GGC ATG GAC TGT GGT CAT GA-3'), and GAPDH reverse (5'-TGC ACC ACC AAC TGC TTA GC-3'). HO-1 and Foxp3 mRNA levels were normalized to total CD3⁺ T-cells.

Statistical analysis

Statistical analysis was performed with StatView software (SAS). Differences in continuous variables between groups were tested by ANOVA with the Scheffé *F*-test used as a post hoc test. Correlation was

analyzed by multiple linear regression method. The observed differences were considered statistically significant if *P* was less than 0.05. All data are expressed as mean ± SEM.

Results

CD4⁺CD25⁺ T-cell infiltration increases with tumor grade

The CD4⁺CD25⁺ T-cells were gated via the forward and side scatter properties of TIL (Fig. 1A). Analysis of TIL from glioma patients shows an increase in CD4⁺CD25⁺ frequency with increasing tumor grade (Fig. 1B). The percentage of Treg was 11.33 ± 1.52% (range, 10.46–13.5%) in grade II, 15.75 ± 1.5% (range, 14.88–17.5%) in grade III, and 24.85% ± 2.67% (range, 20–28.4%) in grade IV. The observed increase in Treg between grades II and III glioma as well as grades III and IV was statistically significant (*P* < 0.02).

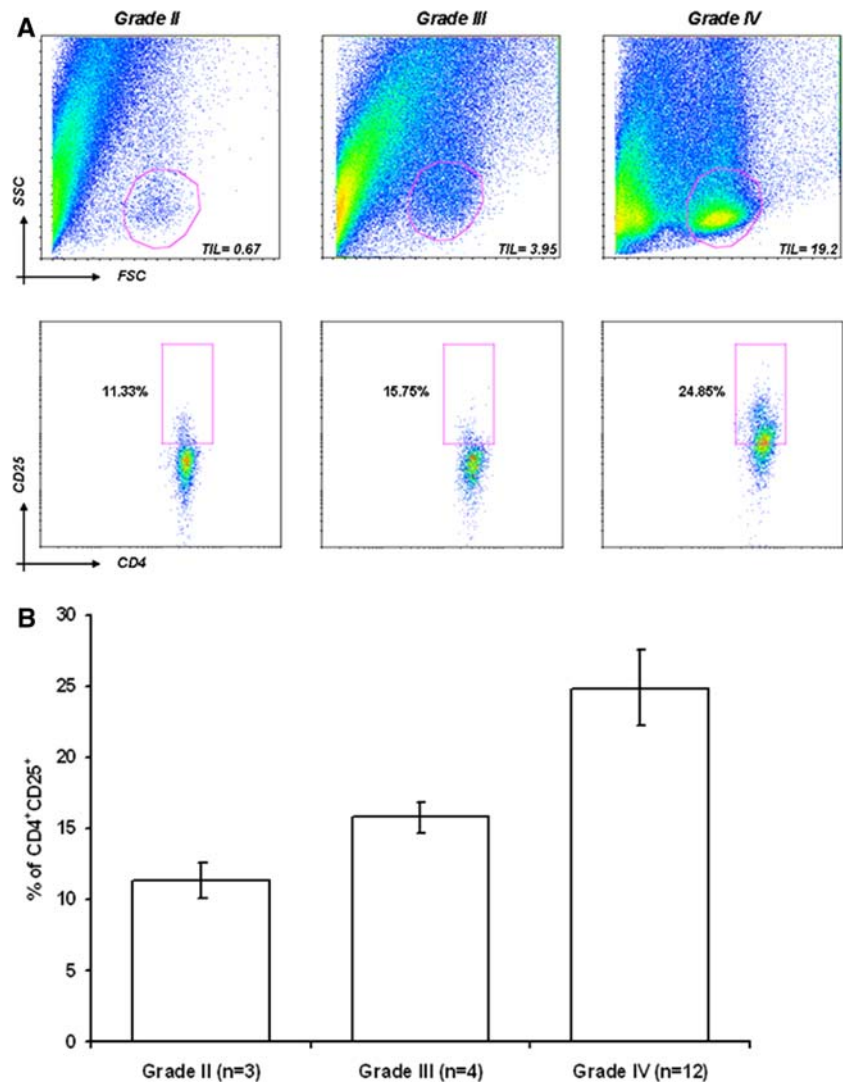
Tumor infiltrating CD4⁺CD25⁺ T-cells express FoxP3

Given that FoxP3 is a marker for Treg cells, we examined FoxP3 mRNA expression in sorted CD4⁺CD25⁺ and CD4⁺CD25⁻ in different tumor grades. First, FoxP3 mRNA expression was analyzed by semiquantitative RT-PCR and then quantified by real-time PCR. As shown in Fig. 2, the expression of FoxP3 mRNA increased with tumor grade. In fact, we observed the highest level of FoxP3 expression in patients with grade IV tumors (11.54 ± 1.95; range [8.12–13.74%]) vs. grade III (6.74 ± 0.19%; range [6.61–6.88%]) or grade II (2.53 ± 0.11%; range [2.54–2.61%]) (*P* < 0.05), confirming that CD4⁺CD25⁺FoxP3⁺ T-cells preferentially accumulate in high-grade tumors.

Expression of HO-1 depends on the presence of tumor infiltrating CD4⁺CD25⁺FoxP3⁺ T-cells

To determine whether the reported expression of HO-1 in brain tumors is a function of tumors cells or the presence of regulatory T-cells, we examined HO-1 expression in both tumor cells and glioma infiltrating lymphocytes. The expression of HO-1 was first analyzed by semi-quantitative RT-PCR. As shown in Fig. 3A, HO-1 mRNA was detected in all glioma samples. However, the detection of HO-1 depended on the presence of tumor infiltrating lymphocytes as no HO-1 was detected in sorted tumor cells alone.

Fig. 1 Frequency of tumor infiltrating lymphocytes (TIL) in patient with astrocytoma (grade II–IV). **(A)** Treg infiltration in human astrocytoma was examined by flow cytometry analysis of CD4 and CD25 expression of electronically gated TIL cells. **(B)** Figure **B** shows the frequency of Treg with grade of disease. The mean frequency of regulatory T-cells was $11.33 \pm 1.52\%$ (range: 10.46–13.5%) in grade II, $15.75 \pm 1.5\%$ (range: 14.88–17.5%) in grade III, and $24.85 \pm 2.67\%$ (range: 20–28.4%) in grade IV glioma tumors ($P < 0.02$) in total $CD3^+$ T-cells



Furthermore, real-time PCR demonstrated a significant increase in HO-1 mRNA expression ($P < 0.05$) depending on the tumor grade. In grade IV tumors, the frequency of HO-1 mRNA expression in $CD4^+CD25^+$ cells was $11.8 \pm 2.45\%$ (range: 8.14–15.33%) vs. $7.42 \pm 0.31\%$ (range: 7.2–7.65%) in grade III, and $2.33 \pm 0.12\%$ (range: 2.25–2.42%) in grade II (Fig. 3B).

Induction of HO-1 expression in $CD4^+CD25^+FoxP3^+$ T-cells is dependent on tumor grade

To further confirm that HO-1 was expressed only by glioma infiltrating Treg, we performed a FACS analysis of total $CD4^+$ T-cells and $CD4^+CD25^+FoxP3^+$ after gating in total $CD3^+$ T-cells, using anti-HO-1 mAb. The frequency of HO-1 expression was analyzed in

various tumor grades. The results, shown in Fig. 4A, illustrate progressive HO-1 expression in glioma infiltrating total $CD4^+$ T-cells. The percentage of HO-1 expressing Treg was $31.1 \pm 2.2\%$ in grade IV tumors as compared to $12 \pm 1.7\%$ grade III tumors, and $7.78 \pm 1.1\%$ grade II tumors ($P < 0.02$). Furthermore, the induction of HO-1 expression was highest in $CD4^+CD25^+FoxP3^+$ subset purified from patients with grade IV glioma ($92.3 \pm 1.75\%$) as compared to grade III ($70.3 \pm 2.3\%$) or grade II tumors ($56.4 \pm 2.86\%$) ($P < 0.02$) (Fig. 4B).

The level of HO-1 directly correlates with FoxP3 expression

The expression of HO-1 was correlated with $CD4^+CD25^+FoxP3^+$ infiltration. The coefficient of correlation between HO-1 mRNA and Foxp3 mRNA

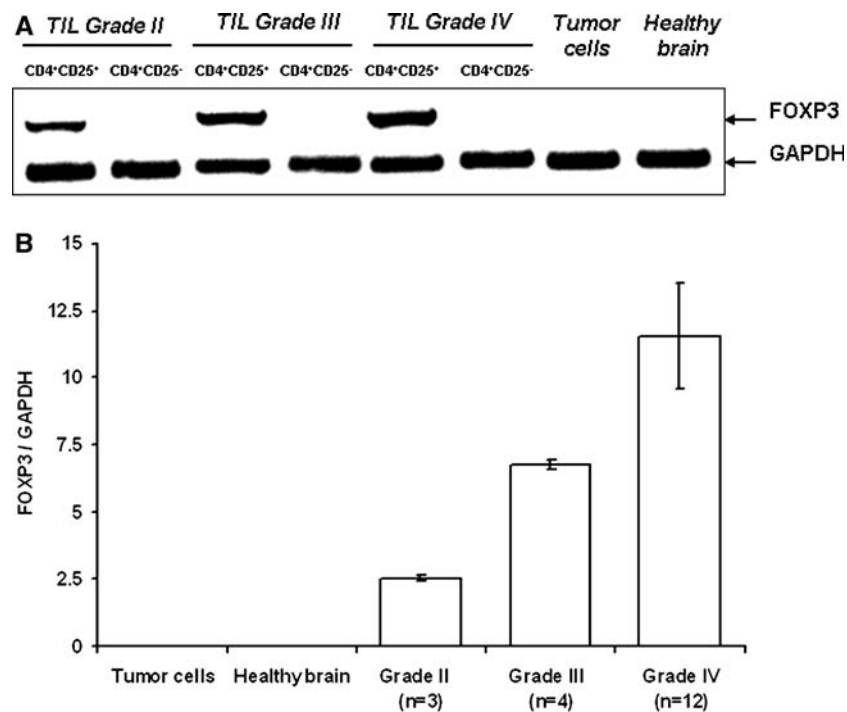


Fig. 2 Foxp3 expression in Treg from glioma patients compared to glioma tumor sorted without TIL or healthy brain controls. **(A)** RT-PCR of Foxp3 mRNA purified from isolated CD4⁺CD25⁺, CD4⁺CD25⁻ T-cells, sorted tumor cells without TIL, or healthy brain. The reaction was positive in CD4⁺CD25⁺ isolated from patients at different stage of disease, but negative in CD4⁺CD25⁻ T-cells, sorted tumor cells without TIL, and healthy brain. **(B)** Foxp3 quantification in purified CD4⁺CD25⁺ in different tumor grades. The level of Foxp3 expressed is grade

IV tumors was higher ($11.54 \pm 1.95\%$; range: 8.12–13.74%) compared to grade III ($6.74 \pm 0.19\%$; range: 6.61–6.88%) or grade II ($2.53 \pm 0.11\%$; range: 2.54–2.61%) ($P < 0.05$). Of note, there was a total absence of Foxp3 expression in healthy brain and sorted tumor cells. GAPDH expression was used to normalize gene expression. Relative expression was calculated for Foxp3 gene by using ΔC_T method. The data shown is the mean \pm SD

was 0.966 (Fig. 5). This result confirms that HO-1 mRNA expression correlates with the induction of Foxp3 in CD4⁺CD25⁺ glioma infiltrating Treg.

Discussion

Heme oxygenase-1, a key enzyme in heme catabolism, has recently been reported to be expressed by human brain tumors [23], to represent a useful marker for angiogenesis in human gliomas [24], and to accumulate during oligodendroglioma progression [25]. To date, only macrophages/microglia present in the stromal compartment of tumors have been shown to express HO-1 [24, 25]. In this study, we provide the first report to show that the expression of HO-1 and Foxp3 directly correlate with tumor grade. Moreover, we show that the expression of HO-1 is not unique to gliomas but is rather a function of Treg infiltration, and the increased infiltration of high-grade tumors with Treg is an important determinant of the immunosuppressive

environment exhibited by malignant brain tumors. These are significant findings, which elucidate the mechanism of HO-1 induction and expression in human gliomas.

HO-1 has an important role in rapid tumor growth because of its antioxidative and antiapoptotic effects [29, 30]. Indeed, there is convincing evidence that accumulation of HO-1 expressing macrophages in necrotic areas of malignant brain tumors contributes to neoplastic growth and tissue damage [24]. The expression of HO-1 is activated in response to various environmental insults, including hypoxia, heat shock, heavy metal toxicity, and UV light irradiation [31, 32]. Although the accumulation of HO-1 expressing Treg may be induced by irradiation and chemotherapy administered to the patients (Table 1), our published findings of Treg infiltration even in newly diagnosed gliomas [11] would argue against this hypothesis. Indeed, even in the present study we did not detect any significant difference in Treg infiltration between newly diagnosed versus recurrent

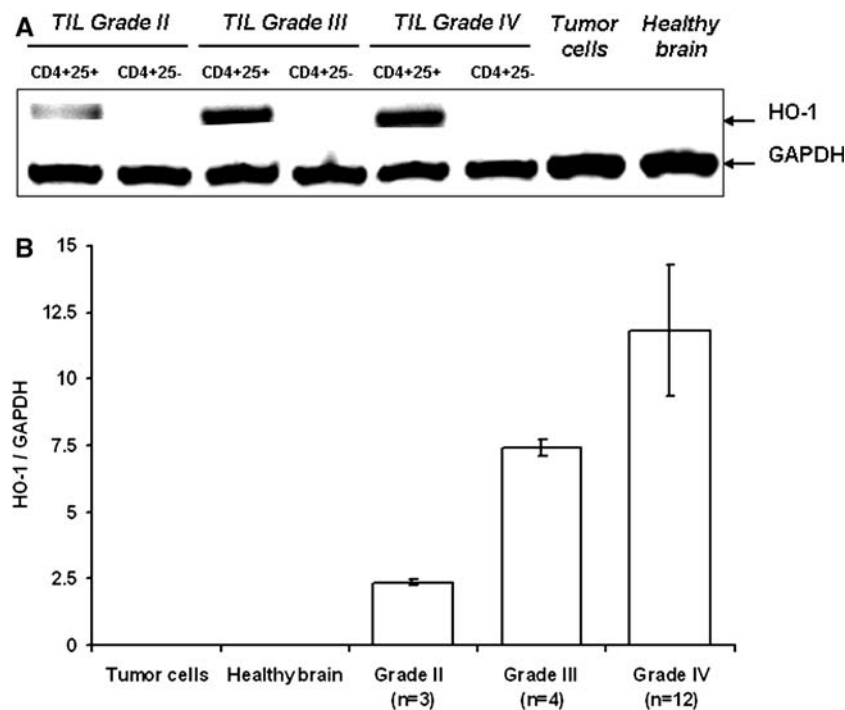


Fig. 3 HO-1 expression in Treg from glioma patients compared to glioma tumor sorted without TIL or healthy brain controls. **(A)** RT-PCR of HO-1 mRNA prepared from purified CD4⁺CD25⁺, CD4⁺CD25⁻ T-cells, sorted tumor cells without TIL, and healthy brain. The reaction was positive in CD4⁺CD25⁺ isolated from patients at different stage of disease, but negative in CD4⁺CD25⁻ T-cells, sorted tumor cells without TIL, or healthy brain. **(B)** HO-1 quantification in isolated CD4⁺CD25⁺

in different tumor grades. The level of HO-1 expressed in grade IV tumors was higher ($11.8 \pm 2.45\%$; range: 8.14–15.33%) compared to grade III ($7.42 \pm 0.31\%$; range: 7.2–7.65%) or grade II ($2.33 \pm 0.12\%$; range: 2.25–2.42%) ($P < 0.05$). Total absence of HO-1 expression was noted in healthy brain and sorted tumor cells. GAPDH expression was used to normalize gene expression. Relative expression was calculated for HO-1 gene by using ΔC_T method. The data shown is the mean \pm SD

grade IV astrocytomas. As such, we propose that Treg progressively infiltrate gliomas at various stages of the disease. In turn, this leads to localized HO-1 expression. The expression of HO-1 by Treg along with local tissue hypoxia/re-oxygenation stimulates a number of cytokines, proteolytic enzymes, and inhibitors, which play key role in the angiogenesis cascade. Indeed, HO-1 mediated heme degradation is the primary mechanism for cellular carbon monoxide (CO) production, and reactive oxygen intermediates, including CO, induce angiogenesis in vitro and in vivo [33]. Most recent studies confirm that HO-1 is a useful marker for neovascularization of human gliomas [24].

The exact role of HO-1 expression is not fully understood in T-cells. However, growing evidence has shown that HO-1 and its byproduct, CO, suppress T-cell proliferation [34, 35]. The over-expression of HO-1 results in the inhibition of several immune effector functions and thus provides an explanation for stress-induced immunosuppression [36]. Moreover, human CD4⁺CD25⁺ T-cell constitutively express HO-1 [26]. It

would appear the FoxP3 induces HO-1 expression and the suppressive effect of Foxp3 is not observed in the presence of HO-1 siRNA [28]. These findings provide a compelling argument that human FoxP3 may be a crucial regulatory gene for the expression of HO-1 and that HO-1 is engaged in FoxP3 mediated immune suppression.

In conclusion, we have shown that (1) Treg progressively infiltrate gliomas with increasing tumor grade; and (2) the expression of HO-1 directly correlates with the expression of Foxp3, a marker of Treg. Although we are aware that the small number of patients with grade II and grade III tumor may limit these conclusions, the data provides a compelling argument for a more extensive investigation of HO-1 and FoxP3 expression in a larger number of patients. Our findings suggest that, at least in part, the expression of HO-1 by gliomas is dependent on the presence of CD4⁺CD25⁺ regulatory T-cells. Selective targeting of either HO-1 or FoxP3 may therefore have important therapeutic implications in the setting of malignant gliomas.

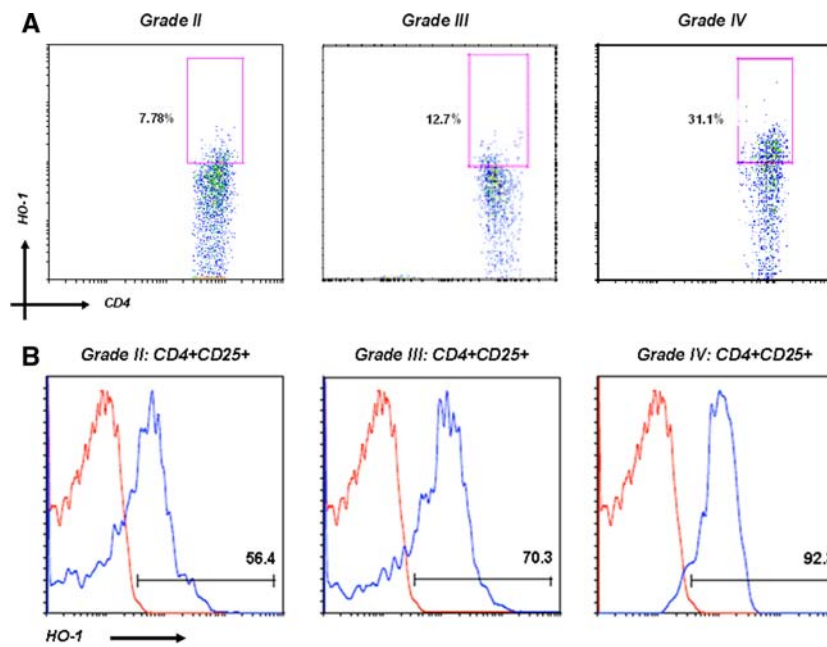


Fig. 4 FACS analysis of HO-1 expression in Treg. **(A)** Total CD4⁺ T-cells were gated electronically on the TIL and analyzed versus HO-1 in two-color. **(B)** Gated CD4⁺CD25⁺ or CD4⁺CD25⁻ were then analyzed versus HO-1 in tricolor. As shown in the figure, there was selective induction of HO-1 expression in CD4⁺CD25⁺ (red plot represents isotype control;

blue is specific staining). Specifically, the induction of HO-1 expression was highest in CD4⁺CD25⁺FoxP3⁺ subset purified from patients with grade IV glioma (92.3 ± 1.75%) as compared to grade III (70.3 ± 2.3%) or grade II tumors (56.4 ± 2.86%) (*P* < 0.02) (Fig. 4B). In contrast, there was no evidence of HO-1 expression in CD4⁺CD25⁻ (data not shown)

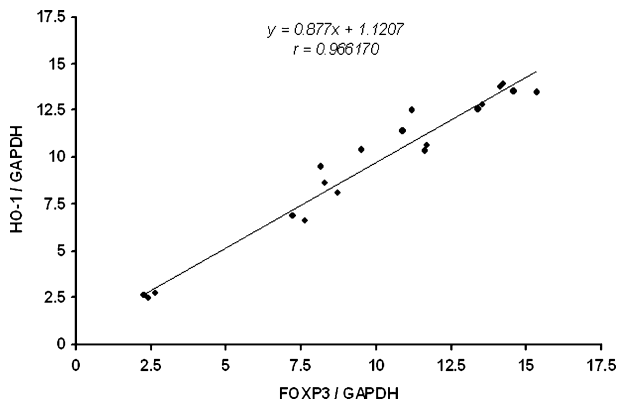


Fig. 5 Correlation between HO-1 and FoxP3 induction (*r* = 0.966)

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