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Heme oxygenase-1 protects regulatory T cells from hypoxia-induced cellular stress in an experimental mouse brain tumor model

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Abstract

Two characteristic features of malignant gliomas (MG) are the presence of hypoxia and accumulation of regulatory T cells (Treg). Heme-oxygenase-1 (HO1) is a cytoprotective enzyme expressed in high level by Tregs in glioma. In this study, we show that higher HO1 expression in Treg is associated with increased survival under hypoxic conditions and that HO1 inhibitor, tin protoporphyrin (SnPP), abrogate the survival benefits. Moreover, SnPP preferentially eliminates Tregs and treatment of tumor bearing mice with SnPP significantly increases survival (23 to 31 days ($p < 0.05$)). Thus HO1 inhibition provides another alternative way of therapeutically targeting Tregs in MG.

Keywords

Glioma; Regulatory T cells; Heme Oxygenase 1; Tin protoporphyrin; Immunization; Hypoxia

1. Introduction

CD4⁺CD25⁺ T cells play a critical role in suppression of autoimmunity and are known as regulatory T cells (Treg) (Sakaguchi et al., 1995). Our group, as well as several others, has independently shown that this subset of highly suppressive T cells plays a pathological role in brain tumor progression (El Andaloussi et al., 2006; Fecci et al., 2006; Wainwright et al., 2013). Malignant gliomas (MG), consisting of anaplastic astrocytoma and glioblastoma multiforme, are associated with a potentially immunosuppressive tumor microenvironment and efficiently evade the host anti-tumor response. One of the major players in the induction and/or maintenance of immunosuppression in MG are the infiltration and accumulation of Treg both peripherally and within the tumor itself (Crane et al., 2012; El Andaloussi et al.,

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2006; El Andaloussi and Lesniak, 2007; James, 2011). Previously, we demonstrated that brain tumor-infiltrating Tregs are predominantly thymus-derived natural Tregs (nTreg) that constitutively express high levels of the receptor, glucocorticoid-induced tumor necrosis factor receptor-related protein (GITR) (Shimizu et al., 2002; Wainwright et al., 2011). Treg suppress the function(s) of antigen presenting cells (APC) via the expression of immunosuppressive cytokines, such as interleukin-10 (IL-10) and transforming growth factor-beta (TGF- β) and inhibit the function of effector T cells by cytokine- and contact dependent-mechanisms (Corthay, 2009; Larmonier et al., 2007). Although chemokines have been suggested to be critical in recruiting Treg to brain tumors, several other factors involving tumor-associated cellular hypoxia and tumor-expressed indoleamine 2,3 dioxygenase have also been shown to regulate Treg recruitment and/or expansion (Facciabene et al., 2011; Fang et al., 2003; Wainwright et al., 2012). However, the factors that promote Treg survival in the tumor microenvironment has yet to be defined, *in vivo*.

Heme oxygenase-1 (HO1) is a stress-responsive cytoprotective enzyme that degrades free heme to carbon monoxide (CO) and iron, which is further converted to ferritin (an iron-sequestering protein) and biliverdin, which is converted to bilirubin (Lee and Chau, 2002). Expression of HO1 is regulated at the transcriptional level, with some notable exceptions including mRNA stabilization by hypoxia or acidosis (Gozzelino et al., 2010). The protective role of HO1 during periods of cellular stress induced by hypoxia, reactive oxygen species and/or acidosis has been previously established and characterized (Gozzelino et al., 2010). Coincidentally, our group previously showed that Tregs have high expression of HO1 and that the frequency of HO1 and Tregs simultaneously increase with the grade of astrocytoma (El Andaloussi and Lesniak, 2007). Although there are studies demonstrating that, Treg development, maintenance and function are independent of HO1 activity under physiological conditions in mice (Zelenay et al., 2007), the role of HO1 in Treg has yet to be investigated in the context of a brain tumor.

Metalloporphyrins, a class of compounds whereby the central iron molecule of the heme complex is replaced by other metals such as cobalt, zinc, manganese, chromium, or tin, function as competitive inhibitor of the HO1 reaction due to inefficient binding to molecular oxygen, thereby preventing HO from degrading natural metalloporphyrin, heme (Drummond, 1987; Kappas and Drummond, 1986). Zinc protoporphyrin (ZnPP) has been tested in the context of several solid tumors and was shown to effectively decrease the size of tumor (Fang et al., 2003; Metz et al., 2010). Of the metalloporphyrins currently characterized, tin protoporphyrin (SnPP) is the most potent inhibitor of HO1 both *in vitro* and *in vivo* and the only metalloporphyrins with the ability to cross the blood brain barrier efficiently (Drummond, 1987; Huang et al., 2005; Khelifi et al., 2003).

The significance of hypoxia in malignant glioma is well established (Jensen, 2009). Moreover, cellular hypoxia increases with the grade of brain tumor and plays a critical role in glioma-induced immunosuppression (Evans et al., 2004; Facciabene et al., 2011; Wei et al., 2011). Evidence from the literature demonstrates that cellular hypoxia, frequency of Treg and expression of HO1 in Treg increase with the grade of glioma. In this study, we established the role of HO1 in Treg biology in the context of hypoxic areas within brain tumors. We show that Treg preferentially express HO1 and survive the hypoxic environment of the brain tumor. Inhibiting HO1 with SnPP led to an increased level of Treg cell death, which was associated with increased survival in mice bearing intracranial (ic) tumor. Finally, we demonstrate that peripheral immunization following intracranial ic tumor implantation in combination with HO1 inhibition, is a promising strategy of inducing effective anti-brain tumor immunity.

2. Materials and Methods

2.1 Mice

C57BL/6 (WT; Cat # 000664) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and maintained in the University of Chicago Carlson Barrier Facility. Mice were intracranially (ic) injected with syngeneic GL261 cells between the ages of 6 and 8 weeks. All animal work was reviewed and approved by the University of Chicago Institutional Animal Care and Use Committee. All mice were provided autoclaved food pellets and water ad libitum. All surgical procedures were completed in accordance with NIH guidelines on the care and use of laboratory animals for research purposes. Mice were euthanized by cervical dislocation. For intracranial implantation, mice were first anesthetized with an intraperitoneal injection of 0.1 mL of stock solution containing ketamine HCl (25 mg/ml), xylazine (2.5 mg/ml), and 14.25 % ethyl alcohol (diluted 1:3 in 0.9 % NaCl). The surgical site was shaved and prepared with 70 % ethyl alcohol. A midline incision was made and a 1 mm diameter right parietal burr hole, centered 2 mm posterior to the coronal suture and 2 mm lateral to the sagittal suture, was drilled. Mice were placed in a stereotactic frame and 4×10^5 GL261 cells in 2.5 μ L saline were intracranially injected with a 26-gauge needle at a depth of 3 mm. The needle was removed and the skin was sutured with 4-0 nylon threads. For peripheral immunization, mice were injected in the flank with 4×10^5 GL261 cells in 100 μ L saline.

2.2 Tumor Cells

GL261 cells were obtained from the NCI Frederick National Tumor Repository Lab and cultured in Dulbecco's Modified Eagle's Media supplemented with 10% fetal calf serum, as well as streptomycin (100 mg/mL) and penicillin (100 U/mL) at 37°C in a humidified atmosphere of 95% air/5% CO₂. All cell culture products were purchased from Gibco Invitrogen.

2.3 Antibodies and flow cytometry

Single cell suspensions were made from brain, cervical draining lymph node (dLN) or spleen by mashing cells through a sterile 70 μ M nylon mesh cell strainer (Fisher Scientific) using the rubber end of a 3 mL syringe into ice-cold PBS (Gibco, Grand Island, NY). Red blood cells were removed by treatment with ACK Lysis Buffer (Lonza, Walkersville, MD) for four minutes on ice. For brain isolates, cells were mixed in a PBS/30% Percoll solution and gently pipetted onto a 70% Percoll cushion. The samples were centrifuged at $1200 \times g$ for 30 min., with no brake activated at the end of the centrifugation. The top layer (composed mostly of tumor cells, neurons and non-myeloid-derived glia) was aspirated, followed by isolation of the leukocyte layer. The cells were washed in cold PBS, twice, to remove any Percoll from the solution. Cells from the brain, dLN and/or spleen were then quantified in the TC10 Automated Cell Counter (Biorad, Hercules, CA). For intracellular cytokine analysis, cells were incubated with cell stimulation cocktail with protein transport inhibitors (PMA/ionomycin with brefeldin A and monensin) (eBioscience) for 5 hrs at 37°C in a humidified atmosphere of 95% air/5% CO₂ before cellular staining. Cells were incubated in PBS + 2 % bovine serum albumin (Sigma-Aldrich, St. Louis, MO) and stained with: anti-CD4-PB (RM4-5; eBioscience, San Diego, CA), anti-CD3-PE-Cy7 (145-2C11; eBioscience), anti-CD8-APC (53-6.7; eBioscience) and GITR-PE (DTA-1; eBioscience), PD1-PE (J43; eBioscience), IL-10-FITC (JES5-16E3; BD-biosciences) and HO1-PE (bs-2075R; Bioss) for 30 min on ice. Cells were then permeabilized and fixed overnight at 4°C using the Mouse Regulatory T Cell Staining Kit (eBioscience) according to manufacturer's instructions and stained with anti-FoxP3-FITC/APC (FJK-16s; eBioscience) for 30 min on ice. For *in-vitro* analysis, single cell suspensions were made from dLN and spleens and then were sorted into CD4+Foxp3+ and CD4+ Foxp3- cells, which were further

analyzed for HO1 expression and apoptosis. For apoptosis analysis, cells were resuspended in Annexin V binding buffer (eBioscience) and co-incubated with a combination of Annexin V antibody (eBioscience) and propidium iodide (BD Biosciences) to discriminate viable from nonviable cells. After staining, cells were analyzed within 4 hours. Cellular frequency was determined with the LSR II flow cytometer (BD) and Flowjo analysis software (TreeStar, Cupertino, CA).

2.4 SnPP

HO1 inhibitor, tin protoporphyrin (SnPP), was prepared as described earlier by Huang et al (Huang et al., 2005). Briefly, SnPP was obtained from Frontier Scientific (Newark, DE) and dissolved in 0.9% NaCl containing 0.1M NaOH and brought to the desired volume with additional saline. The pH was then adjusted to 7.4 with HCl. Mice received daily intraperitoneal injection of 50 μ mol/kg SnPP.

2.5 Hypoxyprobe, immunofluorescence and histology

Anti-mouse hypoxypore kit was purchased from Hypoxypore (Burlington, MA). Mice were intracranially injected with 4×10^5 GL261 cells, followed by a tail vein injection of hypoxypore per manufacturers published protocol at 3 weeks post-ic injection. Mice were euthanized 30 min. after tail vein injection, the brain was extracted and flash frozen [in 62.5% n-Butyl Bromide (Fisher Scientific, Pittsburg, PA) + 37.5% 2-methylbutane (Fisher Scientific) surrounded by crushed dry ice]. Frozen brains were sectioned on the Microm cryostat with vacutome system (Thermo Scientific) with a temperature of -24°C and immersed into Tissue Teck O.C.T. Compound (Sakura Finetek USA, Inc.) at 8 mm intervals and thaw-mounted onto pre-cleaned SuperFrost slides (Fisher Scientific). Sections were post-fixed with 4% paraformaldehyde and blocked for nonspecific staining with 10% bovine serum albumin (BSA; A4503; Sigma-Aldrich) in PBS for 1 hour. Sections were incubated overnight with hypoxypore antibody per manufacturers instructions, mouse anti-CD4 alexa fluor 488 (GK1.5; eBioscience), mouse anti-Foxp3 FITC (FJK-16s; eBioscience) and mouse anti HIF-1 α (HA111a; Abcam, Cambridge, MA) and poly-clonal goat anti-mouse secondary Cy2 (Abcam) antibodies in PBS with 1% BSA at 4°C . Sections were washed extensively (PBS), 1 drop of glycerol was added and the slides were cover slipped. Images of antibody-stained sections were captured using the Sp5 Tandem Scanner 2-photon confocal microscope (Leica Microsystems) running LAS AF software (Leica Microsystems) using the argon and red NeHe laser lines. Fluorescent images were captured using the 20 $\times$ objective.

Hypoxia Induction—Pre-sorted CD4⁺Foxp3⁺ (1×10^5) and CD4⁺Foxp3⁻ (1×10^6) cells were separately pipetted into 96-well plates in medium consisting of Rosewell Park Memorial Institute medium supplemented with 1% fetal calf serum as well as streptomycin (100 mg/mL) and penicillin (100 U/mL), with or without N-acetyl cysteine amide (NACA) and/or SnPP, or Treg medium consisting of regular medium plus 10 ng TGF- β and 10 ng IL-2, with or without 10 mM N-acetyl cysteine amide (NACA) and/or SnPP, and incubated under normoxic (37°C in a humidified atmosphere of 95% air/5% CO₂) or hypoxic conditions at 1% O₂ in hypoxic chamber for another 18 h (Sanyo Manufacturing)

2.6 Statistics

Survival curves were calculated according to the method of Kaplan–Meier. Overall, survival is defined as the time from injection of GL261 cells to day 80 of the time course. The P value was obtained by log-rank statistical analysis and considered significant when $P \leq 0.05$. All other analysis are presented as $\pm$ SEM and were analyzed by 2-way ANOVA, 1-way ANOVA or the 2-tailed Student's t test with a P value ≤ 0.05 considered to be significant.

Post-hoc analyses were conducted using the Newman–Keuls method for multi-comparison procedures. All analyses were conducted using GraphPad Prism version 4.00 (GraphPad Software, Inc.).

3. Results

3.1 Treg has higher expression of HO1 when compared to conventional CD4⁺ T cells (Tconv)

Previously, we showed that the frequency of CD4⁺Foxp3⁺ T cells and the expression of HO1 simultaneously increase with the grade of the glioma; correlating to poor patient outcome (El Andaloussi and Lesniak, 2007). It has also been shown that cellular hypoxia within the malignancy increases with tumor grade (Evans et al., 2004). Since successful tumor clearance requires activation of the cytotoxic arm of the immune system, while simultaneously inhibiting the immunosuppressive arm, we sought out to investigate the relationship between CD4⁺Foxp3⁺ (Treg), CD4⁺Foxp3⁻ (Tconv) cells, HO1 and hypoxia. To determine the effect of hypoxia on these 2 T cell subsets, we isolated CD4⁺ T cells from the spleen and cervical lymph nodes (cLN) of C57BL/6 mice, sorted them into CD4⁺Foxp3⁺ and CD4⁺Foxp3⁻ cells and analyzed HO1 expression in these 2 separate compartments under normoxic and hypoxic conditions. We found that under normoxic conditions, the frequency of HO1⁺ Treg is significantly higher (2.17–3.35%) when compared to the frequency of HO1⁺ Tconv cells (0.02–0.12%) ($p < 0.01$). Also, the MFI for HO1 in HO1⁺ Treg is significantly higher than that in HO1⁺ Tconv cells ($p < 0.01$) (Fig. 1A). Under hypoxic conditions, this difference is even more pronounced (frequency of HO1⁺ Treg (5.86–5.98%) when compared to the frequency of HO1⁺ Tconv cells (0.16–0.24 %) ($p < 0.001$)) (Fig. 1B). Culturing Treg and Tconv cells in media treated with 10 mM NACA (N-acetyl cystine) or SnPP does not significantly alter the expression of HO1 in either cell compartment (Fig 1C). Thus, we show that Treg possess significantly higher expression of HO1 when compared to Tconv under normoxic conditions. This effect is amplified by hypoxia. Treatment with the HO1 competitive inhibitor, SnPP, or the antioxidant, NACA, does not alter the expression of HO1 in either cell compartment.

3.2 Hypoxia preferentially induces Tconv cell death

HO1 is a cytoprotective enzyme and has been shown to have anti-apoptotic effects in various cell types, including hepatocellular carcinoma cells (Lee et al., 2012), as well as neurons (Chen et al., 2000). However, the role of HO1 in Treg biology has not been studied. Mougiakakos et al reported that naturally occurring Treg possess a decreased level of sensitivity toward oxidative stress-induced cell death when cultured with H₂O₂ for 18hrs (Mougiakakos et al., 2009). To determine if HO1 provides a protective, anti-apoptotic effect to Treg when compared to Tconv cells, in the context of hypoxic cellular stress, we cultured pre-sorted Treg and Tconv cells under 1% hypoxia for 18 hours and then measured apoptosis in each compartment based on positive signal for annexin V. We found that after being cultured in 1% hypoxia for 18 hours, there was a significantly higher frequency of apoptotic cells in the Tconv group (20.6–22%) when compared to the Treg group (4.88–4.98%) ($p < 0.001$) (Fig 2A). Similar results were observed when Treg and Tconv cells were cultured under the same hypoxic conditions, but in the presence of NACA. In the presence of the HO1 inhibitor, SnPP, there was a significantly higher frequency of apoptotic cells in the Treg group (54–57%) when compared to the Tconv group (33–35%) ($p < 0.001$) and combining NACA with SnPP did not produce a synergistic effect (Fig 2B). Thus, we demonstrate that HO1 provides an anti-apoptotic protective effect that preferentially benefits Treg under hypoxic conditions and that inhibiting HO1 with SnPP removes the benefit leading to an increase in the frequency of Treg apoptosis when compared to Tconv cell.

3.3 Treg localize in close proximity to the hypoxic areas within brain tumors

Although several studies have shown an accumulation of various subsets of immune cells in brain tumors, a specific niche for Treg have not yet been described. Since our *in vitro* work suggests a proactive role of HO1 in Treg survival under hypoxic condition, to determine if a correlation between the hypoxic area and localization of Treg and Tconv cells in brain tumors exist, we performed *in vivo* immunofluorescence with hypoxyprobe, as well as antibodies against HIF-1 α , FoxP3 and CD4. We verified the ability of the hypoxyprobe to bind to hypoxic areas within the brain tumor by co-immunostaining with HIF-1 α (a marker for hypoxia) and hypoxyprobe antibody (Fig 3A). Moreover, we found that CD4⁺Foxp3⁺ T cells preferentially localize in the close proximity to the hypoxic area of the tumor when compared to CD4⁺Foxp3⁻ T cells (Fig 3B and C). When compared with non-hypoxic area, hypoxic area of the brain tumor contains higher number of Tregs (Fig 3D). It has been previously shown that tumor hypoxia promotes recruitment of Tregs into the tumor microenvironment by inducing the expression of chemokine ligand 28 (CCL28) (Facciabene et al., 2011). Along with this previous finding, our data suggests that hypoxia provides a favorable environment for the recruitment and maintenance of highly immunosuppressive Tregs in the brain tumor.

3.4 An HO1 inhibitor increases survival and decreases Treg in brain tumors

Based on our *in vitro* results, we postulated that treatment of mice bearing ic tumor with HO1 inhibitor would decrease the frequency of Treg in the tumor microenvironment and impart a survival advantage. The HO1 inhibitor, ZnPP, has been shown to decrease tumor volume in several solid tumor models including hepatoma and colon cancer (Doi et al., 1999; Fang et al., 2003). The primary limitation of ZnPP is its inability to cross the blood brain barrier (BBB). To overcome this potential limitation, we chose to use SnPP, the only metalloporphyrin that has been demonstrated to cross the BBB, at previously described dose (Huang et al., 2005). Initially, we treated mice bearing ic tumor with subcutaneously (s.q) administered SnPP or PBS, starting at the day of ic tumor implantation and for 5 consecutive days, followed by 2 days off the treatment regimen, with this cycle being repeated throughout the duration of the mouse life span. However, there was no survival difference between the treated and control group (Fig 4A). We then modified our strategy so that mice bearing ic tumor received SnPP or PBS, only between days 3 to day 7, post-tumor implantation. This modified treatment schedule resulted in an increase in median survival from 23 to 31 days ($p < 0.05$) (Fig 4B). This effect was associated with a decrease in brain tumor-infiltrating Treg both at 1 week (22–50%: mean 41% control vs. 14–46%: mean 33.5% treated; $p < 0.05$)- and 3 week (20–50%: mean 39.1% control vs. 13–39%: mean 23.95% treated; $p < 0.01$)- post-tumor implantation. There was also a significant increase in CD4⁺Foxp3⁻ (45 to 78%: mean 59% control vs. 58–86%: mean 74% treated; $p < 0.001$) and CD8⁺ T cells (11 to 25%: mean 16.4% control vs. 22 to 41%: mean 28.6%; $p < 0.001$) in the treated group at 3 weeks post-tumor implantation (Fig 5A). The pronounced decrease in Treg frequency and increase in CD8⁺ T cell frequency was neither replicated in the dLN (Fig 5B) nor in the spleen (data not shown).

3.5 SnPP decreases the expression of GITR and production of IL-10 by Treg

Our *in vitro* data showed that SnPP had no effect on the expression of HO1 in neither Treg nor Tconv. We further verified this finding, *in vivo*, at 1 and 3 weeks after treatment with SnPP and confirmed that HO1 expression remained unchanged between the treated and control groups at both the 1 and 3 week post-tumor implantation (Fig 6A). Our previous work showed that Treg infiltrating brain tumors have constitutively high expression of GITR when compared to Treg in the spleen (Wainwright et al., 2012; Wainwright et al., 2011). To determine if treatment with SnPP had any effect on GITR expression by brain tumor-

infiltrating Treg, we analyzed the GITR MFI at 1 and 3 weeks post-tumor implantation. We found that treatment with SnPP significantly decreases the expression of GITR on Treg isolated from brain tumors (8741–11476: Mean 10512 control vs. 1902–3245: Mean 2671 treated; $p < 0.001$) and dLN (6449–7548: Mean 7111 control vs. 5776–6123: Mean 5979 treated; $p < 0.01$) but not the spleen (2347–6481: Mean 4339 control vs. 2648–4607: Mean 3902 treated; p not significant) at 1 week post-tumor implantation (Fig 6B). Previously it has been shown that high expression of GITR by T cells protects them from apoptosis (Ronchetti et al., 2002; Ronchetti et al., 2004). Since HO1 inhibitor significantly decreases GITR expression on Treg and also causes Treg apoptosis, this provides a possible mechanism by which HO1 plays a role in the survival of Treg.

Apart from providing a survival advantage to Treg, HO1 also affects the functional properties of Treg. One of the major immunosuppressive cytokine produced by Treg is IL-10. In our *in vivo* cytokine analysis with phorbol 12-myristate 13-acetate (PMA) and ionomycin stimulation, we found that treatment with SnPP decreases IL-10 production by brain tumor infiltrating Tregs both at 1 wk (83.3–100: Mean 91.66 control vs. 30.8–41.7: Mean 36.9 treated; $p < 0.001$) and 3wk (93.5–95.5: Mean 94.76 control vs. 33.3–66.7: Mean 53.33 treated; $p < 0.05$) (Fig 7). This effect was only seen in the brain but not in cLN or spleen. More interestingly, we found that by 3wk of tumor progression Tregs isolated from brain produce IL-10 even without PMA/ionomycin stimulation and IL-10 production even in this setting is significantly less in the animal group treated with SnPP when compared to the control group (76–90.2: Mean 83.16 control vs. 16.7–56.2: Mean 34.3 treated; $p < 0.05$). Analysis of the Tconv subset showed very insignificant amount of IL-10 production by this sub-set at any point in tumor progression and it did not change with the cell stimulation protocol. To the best of our knowledge, this is the first time HO1 has been shown to play a role in IL-10 production by Treg and inhibition of HO1 by SnPP significantly decreases IL-10 production by the Treg in the context of brain tumor.

3.6 SnPP treatment enhance peripheral immunization

Although we noticed some survival advantage by treating brain tumor-bearing mice with the HO1 inhibitor, given the significant decrease in Treg frequency noted in the treated group, we decided to further investigate the effect of combining the HO1 inhibitor with an immune stimulatory strategy. There are some earlier studies that alluded towards a potentially beneficial role of peripheral immunization in inducing an effective anti-brain tumor systemic immune response that extends beyond the BBB. However, whether or not a particular glioma lines can induce this type of immunity and respond to it, seemed to depend on their individual immunobiological characteristics (Naujocks et al., 1995). One of our preliminary pilot studies showed that peripherally immunizing mice with live GL261 cells, 1 month prior to ic implantation of GL261 cells, significantly prolonged the survival of mice (5 out of 6 animals live longer than 100 days post ic injection). This effect persisted with or without treatment with the HO1 inhibitor (data not shown). Since immunization prior to ic tumor implantation doesn't recapitulate a clinically-relevant situation, we investigated the role of peripheral immunization after ic tumor implantation, with or without the HO1 inhibitor. We hypothesized that peripheral immunization following ic tumor implantation would result in an effective anti-tumor response, in the context of reduced frequency of Treg with SnPP treatment. After implantation with the GL261 cells, intracranially, the mice were peripherally immunized with GL261 cells on post implantation day 3 and treated with SnPP from post implantation day 3 to day 7 and followed for survival. We noticed that peripheral immunization in combination with SnPP significantly increases the survival (median survival 39 days, with 2 out of 6 mice surviving longer than 150 days vs. 26 days for control mice with tumor and 31 days for mice with ic tumor and peripheral immunization without SnPP, $p < 0.05$) of mice bearing ic tumor (Fig 8A).

4. Discussion

One of the primary obstacles in designing effective immune-mediated anti-glioma therapy is the profoundly immunosuppressive tumor microenvironment (Lesniak, 2011). Regulatory T cells are a subset of T helper cells that play a critical role in maintaining the immunosuppressive milieu in brain tumors and depletion of these cells increases the survival of animals bearing ic tumor (El Andaloussi et al., 2006). Another factor that has been found to play a critical role in glioma-induced immunosuppression is cellular hypoxia (Facciabene et al., 2011; Wei et al., 2011). Hypoxia not only increases with the grade of glioma, it has also been shown to be linked to poor clinical outcome in patients (Evans et al., 2004; Jensen, 2009). Hypoxia ubiquitously induces the expression of HO1, which is in accordance with our earlier observation that HO1 expression by Treg increases with the grade of glioma (El Andaloussi and Lesniak, 2007). There is also evidence emerging from patient data that in the context of GBM there exists an inverse relationship between the number of Treg and survival (Jacobs et al., 2010). Although these cells have been therapeutically targeted both in pre-clinical and clinical settings using Treg depleting mAb, the functional outcome has been less than satisfactory (Wainwright et al., 2013). Thus understanding the biology of these cells and targeting them with a different strategy is fundamental to developing effective anti-glioma therapy.

HO1 has been shown to play a central protective role in physiology and several disease processes (Soares and Bach, 2009). Numerous studies indicate that the protective action of HO1 in diseases is mediated by CD4⁺CD25⁺ Treg (Xia et al., 2008), thus implicating a major role of this enzyme in Treg function. In this investigation, we demonstrate that HO1 promotes a cytoprotective effect on Treg, thereby protecting them from cellular damage. In the context of hypoxia, there is differential expression of HO1 between CD4⁺Foxp3⁺ and CD4⁺Foxp3⁻ T cell compartments. Under 1% hypoxia, the frequency of HO1⁺ Treg is significantly higher than HO1⁺ Tconv cells. The competitive inhibitor of HO1, SnPP, does not affect the expression of HO1 in either T cell compartment. When we compared hypoxia-induced apoptosis between the two T cell subsets, we found a significantly higher number of Treg surviving under hypoxic conditions when compared to Tconv cells. Under the same hypoxic conditions, treatment with SnPP significantly increases the frequency of cell death in the Treg subset when compared to Tconv cell subset. For the first time, we show the cytoprotective effect of HO1 in Treg survival, where inhibition of HO1 in the presence of cellular stress leads to Treg cell death. Using a hypoxia indicator, we also demonstrate the difference in anatomical localization when comparing Treg and Tconv cells in brain tumors. Although both Tregs and Tconv cells infiltrate brain tumors, Treg tend to localize near hypoxic regions of the tumor.

SnPP preferentially caused Treg apoptosis *in vitro*. We therefore chose to pursue this effect therapeutically in the GL261 cell-based orthotopic mouse model of glioma. Our initial strategy of treating tumor-bearing mice with SnPP during the duration of the animals' life span after implantation of the tumor did not result in any survival advantage. We attributed this to the potential toxic effect of high SnPP levels. We have previously established that brain tumor-infiltrating Treg are predominantly thymus-derived nTregs (Wainwright et al., 2011) and not converted from Tconv cells, and progressively increase with glioma progression (Grauer et al., 2007). We therefore hypothesized that depleting nTreg early during gliomagenesis would result in a survival advantage. We thus modified our treatment schedule whereby tumor-bearing mice were treated with SnPP for 5 days, starting at day 3 after tumor implantation. This strategy provided a significant survival advantage coincident with a decrease in the frequency of Treg while simultaneously increasing the frequency of CD8⁺ T cells. This demonstrates that the effects of depleting Treg early in glioma

progression extend beyond the 1 week treatment period, as the frequency of Treg remained low up to the 3 week time point.

Several studies from our lab have shown that brain tumor-infiltrating nTreg constitutively express high levels of GITR (Wainwright et al., 2012; Wainwright et al., 2011). Previous studies from other groups have shown that although GITR is clearly a negative regulator of Treg, it does not appear to be a critical regulator of contact-dependent suppressor activity (Ronchetti et al., 2004). Interestingly, there have been several studies showing that high GITR expression by T cells conveys protection from apoptosis (Nocentini and Riccardi, 2005; Ronchetti et al., 2002; Ronchetti et al., 2004). Accordingly, we show that Treg isolated from the brain of control tumor bearing mice has a high level of GITR expression, whereas treatment with SnPP significantly decreased the expression of GITR of tumor-infiltrating Treg. This provides a potential mechanism by which high HO1 and GITR expression by Treg promote a survival advantage to these cells, while the inhibition of HO1 eliminates this effect. Our finding that, SnPP doesn't change the expression of HO1, either *in vitro* or *in vivo*, and only affects its function, whereas it decreases the expression of GITR, suggests that cellular stress induces HO1 expression and activity, which, regulates GITR expression. GITR-GITR ligand interaction in turn sends survival signals via TRAF2/NF- κ B pathway (Nocentini and Riccardi, 2005). The exact mechanism of HO1 governing GITR expression needs to be further investigated. Collectively, these data suggest that inhibition of HO1 activity removes the survival signal, thereby promoting Treg apoptosis. Apart from its cytoprotective property, HO1 also alters the functional characteristics of Tregs by regulating IL-10 production by Tregs. One of the major mechanisms of immunosuppression by Tregs is via production of immunosuppressive cytokine IL-10 and TGF- β (Larmonier et al., 2007). Our *in vivo* cytokine analysis shows that SnPP decreases IL-10 production by Treg but does not affect TGF- β production. Although it is known that HO1 regulates IL-10 production by dendritic cells (DC) (Schumacher et al., 2012), we for the first time show that in the context of brain tumor HO1 regulates IL-10 production by the Tregs. Even though SnPP was administered systemically the decreased IL-10 producing Tregs were only seen in the brain, not systemically. This suggests that even though HO1 is expressed by Tregs systemically, factors associated with brain tumor microenvironment like hypoxia ultimately regulate the activity and resulting cytoprotective and functional effect of this enzyme.

Besides Treg, other cells like macrophages/microglia (Liu et al., 2013), dendritic cells (Braudeau et al., 2004; Chora et al., 2007), tumor cells (Do et al., 2013) etc, are also known to express HO1. Therefore, systemic delivery of HO1 inhibitor is most likely to have some off target effects, which can ultimately impact survival. The interaction between HO1 expressing DCs and Treg resulting in indirect influence on Tregs immune suppressive property is well described in the literature (George et al., 2008; Moreau et al., 2009). Even though the survival advantage *in vivo* cannot be attributed just to the HO1 inhibition in Tregs, nonetheless, our study clearly shows that HO1 plays a critical role in Treg survival and function in the setting of brain tumor. To further delineate the role of HO1 expressing Treg in extending survival of glioma bearing mice, it will be important to study this in HO1 knockout mice.

Finally, we investigated the potential role of mounting effective anti-brain tumor immunity by peripheral immunization. Several studies have reported encouraging results, whereby tumor-bearing animals peripherally immunized with irradiated tumor cells or tumor cells modified to produce immune stimulatory cytokines, effectively induce an anti-brain tumor immune response (Iwadata et al., 2003; Visse et al., 1999). Whether or not glioma induces peripheral immunity and responds to it, depends on individual immunobiological characteristics (Naujocks et al., 1995). We found that, GL261 cells injected subcutaneously 1 month prior to intracranial tumor implantation is highly effective at inducing anti-tumor

immunity, such that almost all animals implanted with ic tumor after peripheral immunization survived longer than 100 days (at the time of experimental termination). We used non-irradiated non-modified normal GL261 cells. We used this strategy since one of the criticism of vaccination with irradiated cells is that a greater proportion of intracellular molecules are combined with a smaller proportion of cell surface-associated molecules, thereby reducing the concentration of cancer cell-specific antigens recognized for generating effective anti-tumor immunity (Lokhov and Balashova, 2010). Given this highly immunogenic profile of GL261 cells, we decided to investigate the role of peripheral immunization after ic tumor implantation with and without an HO1 inhibitor. We show that there is a significant survival advantage in the group of mice treated with a combination of peripheral immunization and Treg depletion by HO1 inhibition.

In summary, we show that HO1 plays a critical role in Treg survival under cellular stress such as hypoxia (Fig 7B). Additionally, the HO1 inhibitor significantly decreases the level of Treg in brain tumors, lowers IL-10 production by the existing Treg and decreases the cell-associated expression of GITR, translating into a survival advantage. This effect was further amplified when combining SnPP with peripheral immunization.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- We examined role of HO1 in Treg survival in hypoxic condition.
- We analyzed role of HO1 inhibition in brain tumor model.
- HO1 provides anti-apoptotic effect that preferentially benefits Treg compared to Tconv cells under hypoxic conditions.
- Tregs localize in close proximity to the hypoxic areas within brain tumors.
- HO1 inhibitor, SnPP, increases survival, decreases the number of Tregs in brain tumor and decreases IL-10 production by the existing Tregs.

Fig 1.

Tregs have higher expression of HO1 compared to Tconv cells. Cells from cervical lymph nodes (cLN) and spleen were isolated (as described in *materials and methods*) and sorted by initially gating on CD3 to identify T cell population then into CD4+Foxp3+ and CD4+Foxp3- groups. Cells were cultured in either normoxia or 1% hypoxia for 18h. HO1 expression was measured in both compartments. Under both normoxia (A) and hypoxic (B) condition CD4+Fox3+ cells have higher expression of HO1 compared to CD4+Foxp3- cells. Representative FACS dot plots are shown on the left, and the summary bar graphs of percentage changes and MFIs are shown on the right. Expression of HO1 by CD4+Fox3+ cells is higher in hypoxia compared to normoxia. (C) Under hypoxic conditions, treatment of CD4+Fox3+ and CD4+Fox3- cells with the anti-oxidant NACA or HO1 inhibitor SnPP or a combination of both doesn't significantly alter the expression of HO1. Error bars are derived from two to three separate experiments, each done in triplicate, representing the standard error between experiments throughout the manuscript. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Fig 2.

HO1 preferentially protects CD4+Foxp3+ cells from hypoxic cellular damage. Pre-sorted CD4+Foxp3+ and CD4+Foxp3- cells were cultured in hypoxic conditions for 18hrs. After that cellular apoptosis was analyzed using Annexin V and propidium iodide (PI) assay. Dead PI+ cells were gated out, early apoptotic cells were identified by gating on Annexin V. Representative FACS dot plots are shown on the left, and the summary bar graphs of percentage changes are shown on the right. (A) High percentage of CD4+Foxp3- cells undergoes apoptosis when exposed to hypoxia and larger percentage of CD4+Foxp3+ cells survives hypoxic condition. (B) Treatment of the cells with NACA makes no difference in the frequency of apoptosis between the two compartments. However treatment with HO1 inhibitor SnPP drastically increases apoptosis in the CD4+Foxp3+ cells. SnPP is more potent at inducing CD4+Foxp3+ cell apoptosis compared to combination of SnPP and NACA. ** $p < 0.01$, *** $p < 0.001$

Fig 3.

CD4+Foxp3+ cells preferentially accumulate near the hypoxic area of the brain tumor *in vivo*. IC tumor bearing mice were injected with hypoxypromer, at 3 wk post tumor implantation, via tail vein injection. 30 min following the injection brains were removed and flash frozen and systematically sectioned throughout the tumor injection site. Fluorescent microscopy for CD4 (light blue), Foxp3 (green), HIF-1α (light blue) and hypoxypromer (red) as well as nuclear 4',6-diamidino-2-phenylindole (blue) staining, was conducted. Photomicrographs of the deep tumor parenchyma were imaged at X20 magnification. (A) Hypoxypromer stains the hypoxic area within the brain tumor. Hypoxypromer staining corresponds to the HIF-1α staining. (B) Non-hypoxic area within the brain tumor without significant Treg accumulation. (C) Most Tregs accumulate near the hypoxic area within the brain tumor (arrow indicates regulatory T cells). (D) Number of CD4+Foxp3+ cells per field of view is higher in the hypoxic area compared to nonhypoxic area.

Fig 4.

Early treatment with HO1 inhibitor increases survival. WT mice were intracranially injected with 4×10^5 GL261 cells. (A) Tumor bearing mice were treated with subcutaneously (S.q) 50 μ mol/Kg SnPP or PBS for 5 days starting day 0 post implantation then 2 days off, the cycle was repeated for the entire life span of the animal. The Kaplan–Meier curve represents mouse survival times over a time course of 30 days (n=8–9 mice/group). (B) Tumor bearing mice were treated with s.q. 50 μ mol/Kg SnPP or PBS for 5 days starting day 3 post implantation and then followed for survival. The Kaplan–Meier curve represents mouse survival times over a time course of 50 days (n = 8–9 mice/group) shows statistically significant increased median survival from 23 to 31 days (p<0.05).

Fig 5.

SnPP decreases Treg frequency and increases Tconv and CD8+ T cell frequency *in vivo*. WT mice were ic. injected with 4×10^5 normal GL261 cells. Tumor bearing mice were treated with s.q. 50 μ mol/Kg SnPP or PBS for 5 days starting day 3 post implantation. The frequency and absolute numbers of total CD4+ T cells, CD8+ T cells, and CD4+FoxP3+ regulatory T cells isolated from the (A) brain and (B) bilateral deep and superficial cervical dLN of tumor-bearing mice were analyzed at 1 and 3 wk post implantation. All T cell populations were initially identified by the expression of CD3. (C) Representative flow cytometric plots show the gating strategy used for the identification of total CD3+CD4+, CD3+CD8+, and CD3+CD4+FoxP3+ T cells. Bar graphs in A and B are shown as mean $\pm$ SEM and are representative of 2 independent experiments (n = 3–5 mice/group). * p<0.05, **p<0.01, ***p<0.001.

Fig 6.

SnPP decreases expression of GITR on Tregs. WT mice were ic. injected with 4×10^5 GL261 cells. Tumor bearing mice were treated with s.q. $50 \mu\text{mol/Kg}$ SnPP or PBS for 5 days starting day 3 post implantation. The mean fluorescence intensity of (A) HO1 and (B) GITR expressed by CD4⁺FoxP3⁺ regulatory T cells isolated from the brains, ipsilateral cervical dLN and spleen of tumor-bearing mice were analyzed at 1 and 3 wk post implantation. All T cell populations were initially identified by the expression of CD3. Bar graphs in A & B are shown as mean $\pm$ SEM and are representative of 2 independent experiments ($n = 3-5$ mice/group). SnPP does not alter HO1 expression *in vivo* however it significantly decreases the expression of GITR on Tregs in the brain and to some extent Tregs from dLN but not spleen. ** $p < 0.01$, *** $p < 0.001$

Fig 7.

SnPP decreases IL-10 production by Treg in the brain tumor. WT mice were ic. injected with 4×10^5 GL261 cells. Tumor bearing mice were treated with s.q. 50 $\mu\text{mol/Kg}$ SnPP or PBS for 5 days starting day 3 post implantation. T cells were analyzed for cytokine production by ex-vivo stimulation using cell stimulation cocktail containing PMA/ionomycin in combination with protein transport inhibitors. IL-10 expression by CD4+FoxP3+ regulatory T cells isolated from the brains, ipsilateral cervical dLN and spleen of tumor-bearing mice were analyzed at 1 and 3 wk post implantation. All T cell populations were initially identified by the expression of CD3, then CD4 and Foxp3 and IL-10 gate is based on un-stimulated Tconv cells. Bar graphs are shown as mean $\pm$ SEM ($n = 3$ mice/group). ** $p < 0.05$, *** $p < 0.001$

Fig 8.

Peripheral immunization along with SnPP increases survival. (A) Tumor bearing mice were treated with s.q. 50 μ mol/Kg SnPP or PBS for 5 days starting day 3 post implantation and were also peripherally immunized with 4×10^5 GL261 cells implanted subcutaneously on day 3 post ic tumor implantation then followed for survival. The Kaplan–Meier curve represents mouse survival times over a time course of 150 days (n = 6–12 mice/group) shows statistically significant increased survival ($p < 0.05$). (B) In the hypoxic tumor microenvironment inducing cellular stress, high expression of HO1 by highly suppressive Tregs provides them a survival advantage over the Tconv cells and potentiates glioma progression. In the presence of the HO1 inhibitor SnPP, Tregs lose their survival advantage and undergo apoptosis, which in turn helps CD8+ cell mediated tumor clearance.