ARTICLE
Journal of Cellular Biochemistry 112:3035–3043 (2011)

Journal of Cellular Biochemistry

Photodynamic Therapy Boosts Anti-Glioma Immunity in Mice: A Dependence on the Activities of T Cells and Complement C3

Fei Li,[1] Yingxin Cheng,[1] Jiayou Lu,[1] Rong Hu,[1] Qi Wan,[2] and Hua Feng[1*]

[1]*Department of Neurosurgery, Southwest Hospital, The Third Military Medical University, Chongqing 400038, China*

[2]*Department of Physiology & Cell Biology, School of Medicine, University of Nevada, Reno, Nevada 89557-0352*

ABSTRACT

Photodynamic therapy (PDT) involves the systemic administration of a tumor-specific photosensitizer and local irradiation of visible light, can generate highly cytotoxic molecular species in the tumor and kill malignant cells directly or by shutting down the tumor microvasculature. Collectively data show that anti-tumor immunity is an important mechanism that mediates the PDT-induced tumor-destroying effects in many types of cancers. However, it is unknown whether PDT also promotes anti-tumor immunity in gliomas in the central nervous system (CNS). Here we show that the PDT generates regional and systemic anti-tumor immunity in mice with G422 gliomas in the brain. The infiltration of immune cells and the release of inflammatory factors, such as TNF-α and IFN-γ, are increased in animals with G422 gliomas following PDT when compared with those without receiving PDT. The lymphocytes that are isolated from PDT-treated mice are able to induce anti-tumor immunity in nude mice. The anti-glioma immunity fostered by PDT is inhibited in complement C3 knockout mice and the nude mice indicate the requirement of the activities of complement C3 and T cells. Thus, T cells that produce cytokines, along with complement C3, may play crucial roles in mediating PDT-induced anti-glioma responses. J. Cell. Biochem. 112: 3035–3043, 2011.

KEY WORDS: PHOTODYNAMIC THERAPY; ANTI-TUMOR IMMUNITY; T CELLS; COMPLEMENT C3

Gliomas are the most common primary brain tumor. The hallmarks of glioma cells include their ability to deeply penetrate the surrounding tissue and to inhibit anti-tumor immune responses. Although progress has been made in glioma treatment in the past few decades, the resistance of gliomas to radiotherapy and chemotherapy leads to devastating tumor growth and a dismal prognosis [Tait et al., 2007; Simon and Schramm, 2009].

Photodynamic therapy (PDT), an adjuvant treatment following surgical resection, involves the systemic administration of a tumor selective-localizing photosensitizer that is only excited when exposed to a specific wavelength of light [Dolmans et al., 2003]. PDT has been shown to be an effective treatment for malignant gliomas [Muller and Wilson, 2006; Xiao et al., 2009]. It has been reported that 73% of patients with astrocytomas multiforme and 75% of patients with glioblastoma multiforme treated with PDT have prolonged survival [Stylli et al., 2005]. Recent evidence suggests that PDT-induced tumor-destroying effects may be mediated through direct cytotoxicity, microvascular disruption, and inflammation [Nowis et al., 2005]. The development of anti-tumor immunity is thought to contribute to PDT-induced inflammatory responses [Castano et al., 2006; Mroz et al., 2010]. However, the mechanism through which PDT induces anti-tumor immunity in the brain has not been explored.

The present study was designed to investigate the effects of PDT on anti-tumor immunity in mice with G422 gliomas. This study provides evidence that PDT elicits anti-tumor immunity in the mouse brain by inducing an inflammatory infiltration in immunocompetent mice. However, PDT provides poor tumor control in immunodeficient mice that lack T cells or complement C3. The lymphocytes isolated from PDT-treated mice are able to generate anti-tumor immunity in vitro and in nude mice. Taken together, these results suggest that anti-tumor immunity may play a crucial role in mediating PDT-induced tumor-destroying effects.

Fei Li and Yingxin Cheng contributed equally to this work.

Grant sponsor: National Natural Science Foundation of China; Grant numbers: 30670506, 30973494; Grant sponsor: Post-Doctor Science Foundation of China; Grant number: 20070420768.

*Correspondence to: Dr. Hua Feng, Department of Neurosurgery, Southwest Hospital, Third Military Medical University, Chongqing 400038, China. E-mail: fenghua8888@yahoo.com.cn

Received 15 April 2011; Accepted 6 June 2011 • DOI 10.1002/jcb.23228 • © 2011 Wiley-Liss, Inc.

Published online 15 June 2011 in Wiley Online Library (wileyonlinelibrary.com).

MATERIALS AND METHODS

MOUSE GLIOMA MODELS

All animal experiments were approved by the Subcommittee on Research Animal Care of the Third Military Medical University of China (ID: 2007181). G422 and GL261 mice glioblastoma cells were purchased from the Cell Bank of the Chinese Academy of Sciences and cultured in RPMI-1640 media containing 10% fetal calf serum and penicillin/streptomycin. Male BALB/c mice (immunocompetent mice, n = 60), nude mice (T cells-related immunodeficient mice, n = 30), and complement C3 knockout mice (complement C3-related immunodeficient mice, C3-KO mice, n = 24), weighing 18–22 g, were provided by the laboratory animal center of the Third Military Medical University. The G422 tumor cells (1×10^5, 5 μl) were implanted using a microinjector that was placed 2 mm anterior and 2 mm lateral to the junction of the sagittal and lambdoid sutures [Sun et al., 2010]. All procedures were performed aseptically. Two mouse models were used in this study: an orthotopic tumor model that was implanted in the brain and a subcutaneous model. To distinguish between these models, the mice with the gliomas that were implanted in the brain were referred to as brain glioma-bearing mice, whereas the mice with the gliomas that were implanted in the subcutaneous region were referred to as subcutaneous glioma-bearing mice. The subcutaneous model was only used to study the in vivo immunoactivity of splenocytes that were isolated using the brain model.

PDT AND TUMOR RESPONSE ASSESSMENT

Animals were given PDT after implantation when the tumors reached a size of 3 mm in diameter. For the PDT, each mouse was given an intravenous injection of hematoporphyrin derivatives (HPDs, 10 mg/kg, Huading Ltd., Chongqing, China) [Li et al., 2006]. After 24 h, gliomas were subjected to irradiance. The skin was cut open to expose a skull area (3×3 mm^2) where the tumor was located for irradiance. The light was produced by a Lumacar-051 light source (Citec UK Ltd., London, UK) with a 150-W QTH lamp and a 630-nm interference filter. The light was delivered through an 8-mm core diameter fiber optic catheter with an irradiance of 80–90 mW/cm^2 and a radiant exposure of 150 J/cm^2. Sham mice that were given a microinjection of vehicle instead of the HPD injection were also subjected to the light therapy as a control.

FOLLOW-UP OF MICE

The orthogonal diameters of the tumors were measured twice a week using 7.0 MRI (BioSpec In-vivo MR Spectroscopy/Imaging System. Bruker BioSpin International AG, Switzerland) after tumor implantation (Fig. 1D–F) and were verified using hematoxylin and eosin (H&E) stain at specific time points. The maximal diameters of the tumors were measured in three trans-planes: coronal (d1), sagittal (d2), and transverse (d3). In addition, the tumor volume was calculated with the following formula: $d1 \times d2 \times d3 \times \pi/6$ [Gentilini et al., 2010]. The orthogonal growth was measured by the using of vernier caliper in the mice with subcutaneous tumor. The specimens were subjected to flow cytometry analysis, cytokine determination and in vitro cytotoxicity measurements at 24, 48, 72, 96, and 120 h after light exposure. For the survival duration observations, each mouse was sacrificed when either the tumor reached a diameter of 0.6 cm or when the mouse lost 15% or more of body weight.

FLOW CYOMETRY

The flow cytometry analysis was carried out to determine the percentage of apoptotic cells of tumors out of the total number of cells after PDT [Korbelik and Sun, 2006]. Tumors were excised, weighed, enzymatically disaggregated, and obtained as single cell. The cells were stained with fluorescein isothiocyanate (FITC)-conjugated annexin V followed by phycoerythrin (PE)-conjugated rabbit monoclonal antibody against active caspase-3. The apoptotic cells were identified when they were positively stained with both antibodies [Vermes et al., 2000].

Fresh isolated tumor was dissociated, centrifuged, washed, and the Percoll-gradient isolation technique was used to isolate inflammatory cells from tumor tissues [Hussain et al., 2006]. Peripheral blood was collected by orbital sinus venipuncture after PDT and erythrocytes were depleted with the lysis buffer. Cells were stained with monoclonal antibodies (mAb) against specific cell-surface antigens (CD4, CD8, and CD68) as previously described [Kabingu et al., 2007]. The CD4, CD8, and CD68 mAbs were conjugated with FITC, PE, PerCPCy5.5, and allophycocyanin (PharMingen, SanDiego, CA), respectively. The flow cytometry analysis was performed using a two-laser FACStar Plus flow cytometer (Becton-Dickinson, San Jose, CA) at 488 nm by employing 420/20, 530/30, and 575/30 band pass filters and a 640 long pass filter. A minimum of 20,000 cells were used for the data analysis.

DETERMINATION OF CYTOKINE CONCENTRATIONS

The concentrations of tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) in the splenic lymphocytes culture supernatants tumor tissue homogenates of glioma pre- and post-treatment and were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Wiesbaden-Nordenstadt, Germany) according to the manufacturer's instructions [Mabrouk et al., 2007].

SPLENOCYTE ISOLATION FOR CO-CULTURE AND TRANSPLANTATION

Single cell suspensions were obtained by passing spleens through a 70 mm mesh nylon cell strainer (BD Falcon) to make single cell. The cells were harvested using centrifugation, were depleted of erythrocytes with the lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH 7.3) and were washed with PBS. About 5×10^6 G422 cells per well were seeded to 12-well plates, followed by the addition of 1×10^5 splenocytes to each well. After 3 days, the number of cells was counted. For the adoptive transfer studies, T lymphocytes (1×10^7 cells/mouse) that were separated from splenocytes were immediately injected into the recipient mice through the tail vein. Flow cytometry verified that the purity of the T cell population was >90% by fluorescence-labeled anti-CD3 mAb [Korbelik et al., 1996].

STATISTICAL ANALYSIS

All measured values were presented as mean ± SD. The survival analysis was performed using the Kaplan–Meier method. The

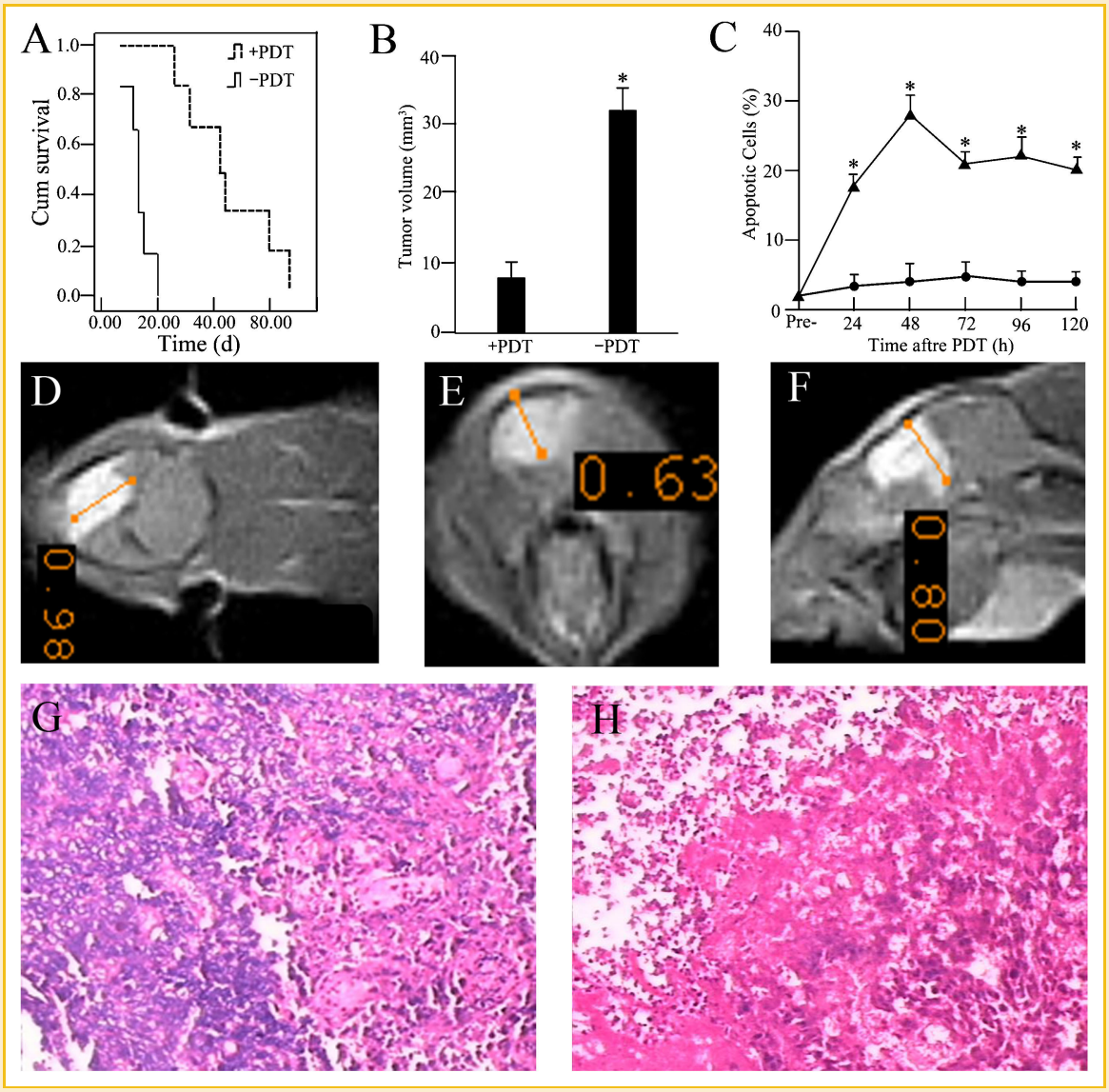

Fig. 1. Effects of PDT on G422 gliomas in mice brain. G422 glioma cells were injected into the brain of BALB/c mice and PDTs were performed after the tumor growth to 3 mm in diameter. The survival time, tumor growth, and apoptosis of tumor cells were measured in the mice treated with PDT (+PDT group) or without PDT (−PDT). A: Kaplan–Meier analysis shows that PDT significantly improves the overall survival duration of glioma-bearing BALB/c mice (Log Rank, n = 6 for each group, Chi-square = 12.03, $P = 0.01$). B: A statistical analysis suggests that PDT significantly reduces the G422 tumor volume in tumor-bearing mice (Student's t test, n = 6 for each group, $^{*}P < 0.05$). C: PDT induces apoptosis of G422 tumor cells (ANOVA, n = 6 for each group, $^{*}P < 0.05$, vs. 0 h). D–F: Tumors were detected by MRI. G: H&E staining shows that the tumor invasion into the brain. H: Necrosis and hemorrhage could be found in the tumor 1 day after PDT (H&E ×200). [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

survival curves were compared for statistical differences using the log rank test and SPSS13.0 software. The differences between groups were tested for significance using a one-way analysis of variance (ANOVA). P values <0.05 were considered to be statistically significant.

RESULTS

PDT INHIBITS GLIOMA GROWTH AND INCREASES APOPTOTIC CELL DEATH IN GLIOMA

We employed a glioma model in which G422 glioma cells were injected into the brain of immunocompetent BALB/c mice. We first measured survival time in the brain glioma-bearing mice treated with PDT and without PDT. The median post-PDT survival time was 42.5 ± 6.1 days with PDT, significantly prolonged than those without PDT (9.0 ± 1.5 days) (Fig. 1A, Chi-square = 12.03, $P = 0.01$). This prolonged survival duration was accompanied by a reduced tumor volume in animals at the 72-h post-PDT. As shown in Figure 1B, the average tumor volume was reduced by 78.32% after PDT. In addition, the percentage of apoptotic tumor cells was significantly increased, with a peak value at 48 h after PDT (Fig. 1C). The tumor cell growth before PDT, and the necrosis and hemorrhage of tumor tissue 24 h after PDT could also be observed (Fig. 1G,H).

PDT POTENTIATES ANTI-GLIOMA IMMUNITY

Because the increase in $CD68^+$ cells indicates an activated inflammatory infiltration in the CNS including the macrophages, myeloid/mononuclear lineage cells, gamma/delta T cells, and activated $CD4^+$ and $CD8^+$ T cells [Asai et al., 1999; Strik et al., 2004], the proportions of $CD68^+$ cells in the isolated inflammatory infiltration cells from glioma tissue at 0, 24, 48, and 72 h after PDT (+PDT) were measured. Take the brain glioma-bearing BALB/c mice that did not receive an HPD injection were used as a sham control (−PDT). As shown in Figure 2A, the proportion of $CD68^+$ cells is increased after PDT in the tumor tissues at 24 h and peaked at 48 h.

Inflammatory factors, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ), secreted from immunocytes mediate inflammatory reactions [Herman et al., 1996]. The levels of TNF-α and IFN-γ in glioma tissues pre- and post-treatment were measured. The results showed that PDT significantly increased the levels of TNF-α and IFN-γ in the tumor tissue after PDT, with peak levels of these factors occurring at 72 h post-treatment (Fig. 2B,C).

PDT ENHANCES SYSTEMIC ANTI-GLIOMA IMMUNITY

The ratio of $CD4^+/CD8^+$ lymphocytes in the blood reflects the activation status of systemic immunity [Kabingu et al., 2007]. We compared the proportion of blood $CD4^+/CD8^+$ lymphocytes in BALB/c mice without tumor implantation and without PDT (control group), in brain glioma-bearing mice without PDT (−PDT) and in brain glioma-bearing mice with PDT for 72 h (+PDT) (Fig. 3A). The data showed that the increase of the $CD4^+$ cells and the decrease of the $CD8^+$ cells, the ratio of blood $CD4^+/CD8^+$ lymphocytes in glioma-bearing mice without PDT was remarkably lower than that in the control mice (Fig. 3A). However, PDT elevated the ratio of $CD4^+/CD8^+$ lymphocytes to a normal level at 72 h after treatment (Fig. 3A). The levels of TNF-α and IFN-γ secreted by splenic lymphocytes that were collected from the culture supernatants in these three groups of mice were also measured. PDT significantly increased the levels of TNF-α and IFN-γ in splenic lymphocytes of brain glioma-bearing mice (Fig. 3B,C).

To provide evidence for the involvement of splenic lymphocytes in anti-glioma immunity, G422 glioma cells and GL261 glioma cells (also derived from mouse) were respectively co-cultured in vitro with the splenic lymphocytes that were collected from mice without tumor implantation (control), glioma-bearing BALB/c mice without PDT (−PDT), and with PDT for 72 h (+PDT). The death rate of both G422 and GL261 cells was significantly increased after co-culture with splenic lymphocytes collected from mice treated with PDT (Fig. 4A).

PDT SUPPRESSES GLIOMA GROWTH IN VIVO THROUGH LYMPHOCYTE-MEDIATED ANTI-TUMOR IMMUNITY

The positive in vitro findings led us to investigate whether PDT exerted its inhibitory effect on glioma growth by enhancing systemic immunity in vivo. Splenic lymphocytes, harvested from the brain glioma-bearing mice treated with or without PDT (+Lym+PDT/+Lym−PDT) were injected into nude mice with intracerebral and subcutaneous G422 gliomas. The tumor volumes in these nude mice were measured at 14 days after the lymphocyte injection. As shown in Figure 4B, the splenic lymphocytes collected from the brain glioma-bearing mice treated with or without PDT were effective in inhibiting not only the intracerebral but also subcutaneous tumor growth of the nude mice. However, PDT remarkably enhanced splenic lymphocyte-mediated suppression of intracerebral and subcutaneous tumor growth (Fig. 4B).

T CELLS AND COMPLEMENT C3 ARE REQUIRED FOR THE INHIBITION OF G422 GLIOMA GROWTH

The T lymphocyte mediated immune response is proved to be mainly response for the PDT-induced anti-tumor immunity, and the complement C3 was identified as a major chemoattractant in the advanced phase of PDT-induced inflammatory infiltration [Cecic and Korbelik, 2002; Stott and Korbelik, 2006; Mroz et al., 2010]. To test the possible roles of T cells and complement C3 in PDT-induced anti-glioma effects, we used nude mice with T cell-related immunodeficiency, the complement C3 knockout mice (C3-KO mice), and BALB/c mice. The G422 cells were implanted into the

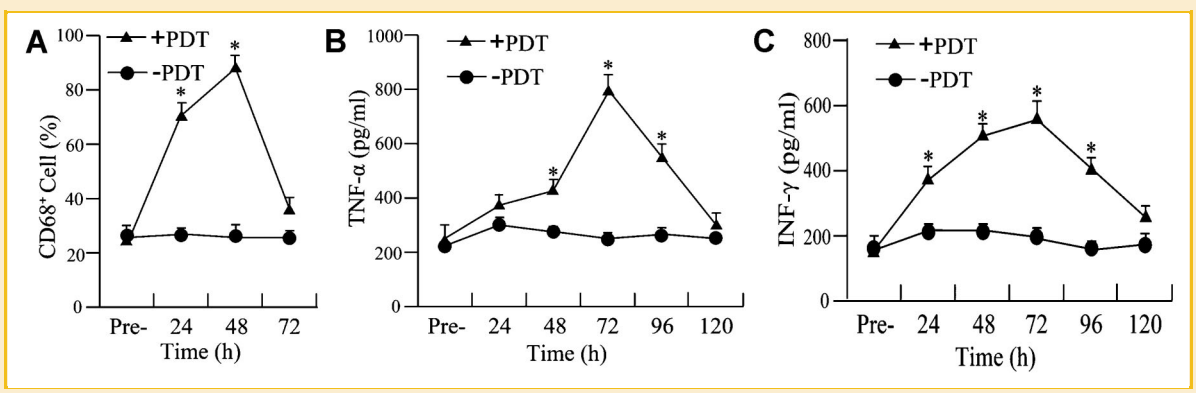

Fig. 2. PDT enhances regional anti-glioma immunity. The inflammatory cells were isolated from tumors with or without PDT. The proportion of $CD68^+$ cells were quantified by cytometry and the level of TNF-α and IFN-γ of the tumor homogenates were measured by ELISA. A: A statistical analysis shows that PDT increases the number of $CD68^+$ cells in the glioma tissue of BALB/c mice (ANOVA, n = 6, $^*P < 0.05$, vs. −PDT); B: PDT increases TNF-α levels in the glioma tissue (ANOVA, n = 6, $^*P < 0.05$, vs. −PDT); C: PDT increases levels of IFN-γ in the glioma tissue (ANOVA, n = 6, $^*P < 0.05$, vs. −PDT).

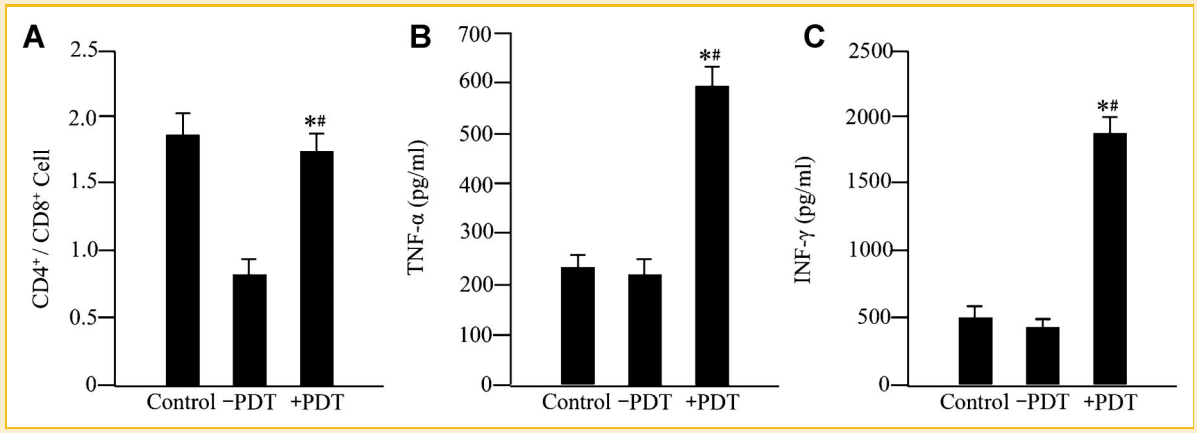

Fig. 3. PDT enhances systemic anti-tumor immunity in mice with G422 gliomas. The proportion of blood $CD4^+/CD8^+$ lymphocytes and the levels of TNF-α and IFN-γ secreted by splenic lymphocytes in BALB/c mice without PDT (control group), brain glioma-bearing mice without PDT (−PDT) and brain glioma -bearing mice with PDT for 72 h (+PDT) were analyzed. A: A statistical analysis shows that the fractions of blood $CD4^+/CD8^+$ lymphocytes are increased at 72 h after PDT in glioma-bearing BALB/c mice (ANOVA, n = 6 for each group; $^*P < 0.05$ vs. −PDT; $^\#P > 0.05$ vs. control). B,C: Statistical analyses show that the levels of TNF-α and IFN-γ secreted by splenic lymphocytes of tumor-bearing mice with PDT (+PDT) are significantly elevated (ANOVA, n = 6 for each group; $^*P < 0.05$ vs. −PDT; $^\#P < 0.05$ vs. control).

brain of these three strains of mice (Fig. 5A–C), and the volumes of the gliomas were measured at various time points after tumor cell injection (Fig. 5D). The growth rate of the gliomas was the fastest in the C3-KO mice, followed by that in the nude mice and the BALB/c mice. Thus, the BALB/c mice had the longest median survival duration (44.3 ± 6.0 days), followed by 24.8 ± 5.2 days for the nude mice and 18.6 ± 5.8 days for the C3-KO mice. However, when the host mice died, the average size of the tumors (37.43 ± 1.67 mm^3 in BALB/c mice, 37.46 ± 2.31 mm^3 in nude mice and 39.10 ± 2.89 mm^3 in C3-KO mice) was similar for the three strains of mice (Fig. 5A–D).

PDT-INDUCED ANTI-GLIOMA EFFECTS DEPEND ON THE ACTIVITY OF T CELLS AND COMPLEMENT C3

Our data thus far suggest that T cells and complement C3 play critical roles in the suppression of G422 glioma growth. We therefore examined the effects of PDT on the survival time, tumor volume, apoptotic rate of tumor cells, proportion of $CD68^+$ cells, and levels of inflammatory factors in BALB/c mice (BALB/c + PDT), nude mice (Nude + PDT), and C3-KO mice (C3-KO + PDT) with brain G422 tumors. The BALB/c mice with brain G422 tumors that were treated with light but not HPD (BALB/c + light) were used as a

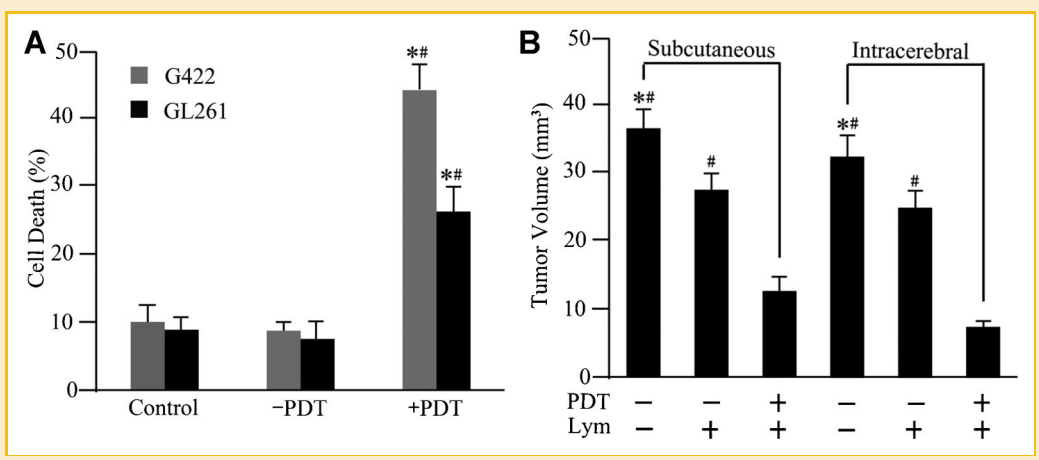

Fig. 4. Splenic lymphocytes of tumor-bearing mice with PDT suppress glioma growth in vitro and in vivo. Splenic lymphocytes, harvested from the brain glioma-bearing mice treated with or without PDT (+Lym+PDT/+Lym−PDT) were co-cultured of G422 and GL261 cells or injected into nude mice with intracrebral and subcutaneous G422 gliomas. The tumor volumes were observed at 14 days after the lymphocyte injection. A: A statistical analysis indicates that the co-cultures of G422 and GL261 cells, respectively, with splenic lymphocytes from tumor-bearing mice with PDT (+PDT) increase apoptotic cell death of both G422 and GL261 cells (ANOVA, n = 6 for each group; $^*P < 0.05$ vs. −PDT; $^\#P < 0.05$ vs. control). B: A statistical analysis shows that the tumor volume in the nude mice after the injection of splenic lymphocytes collected from the G422 tumor-bearing mice treated with PDT (+Lym+PDT) is significantly reduced (ANOVA, n = 5 for each group, $^*P < 0.05$ vs. +Lym−PDT, $^\#P < 0.05$ vs. +Lym+PDT).

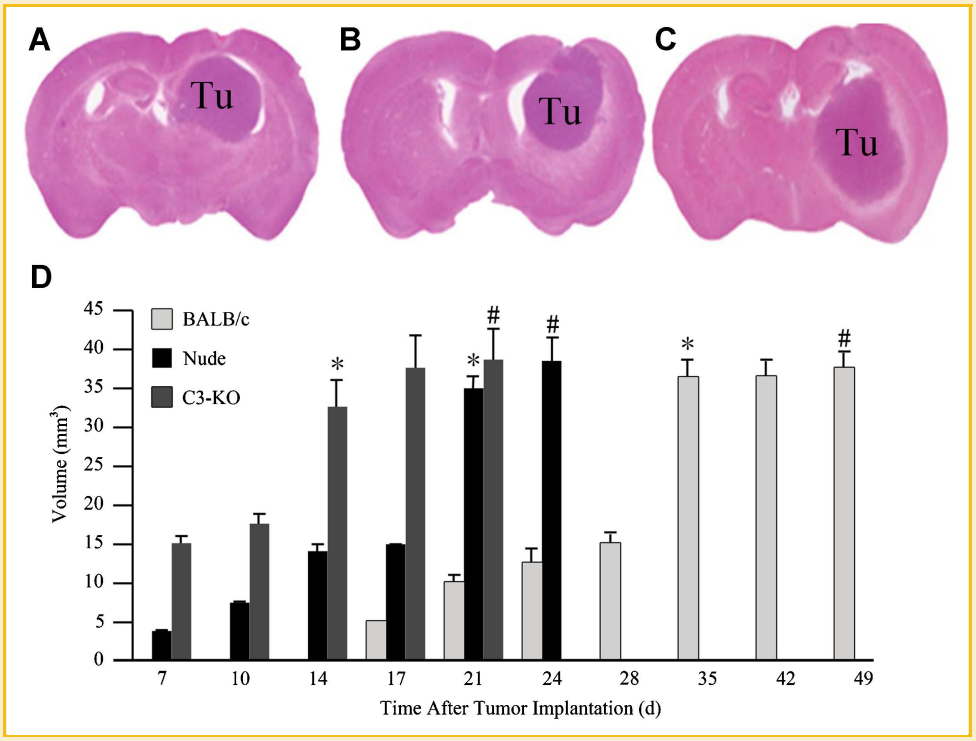

Fig. 5. Effects of T cells and complement C3 on glioma growth. The G422 cells were implanted into the brains of nude mice with T cell-related immunodeficiency, the complement C3 knockout mice (C3-KO mice) and BALB/c mice. The tumor volumes of the gliomas were measured. A–C: Sample histological sections of G422 tumor with H&E staining. The sections were prepared from gliomas in the BALB/c mice at 49 days (A), the nude mice at 24 days (B), and the complement C3 knockout mice at 17 days (C) after tumor implantation. D: Summarized data shows the volumes of the G422 tumors in the brains of the three mouse strains at various time points after tumor implantation, indicating that the G422 tumors grows the fastest in the complement C3 knockout mice, followed by the nude mice and the BALB/c mice. The average sizes of the tumors were similar at the time point when the host mice died (ANOVA, n = 6 for each group, $^{\#}P > 0.05$). The average survival duration is 35 days for the BALB/c mice, 21 days for the nude mice, and 14 days for the C3-KO mice (ANOVA, n = 6 for each group, $^{*}P > 0.05$). Tu, tumor.

control. We showed that the median survival durations were 43 days for the BALB/c mice, 18 days for the nude mice and 9 days for the C3-KO mice after PDT. The PDT resulted in a 78.32% reduction of tumor volume in BALB/c mice, 23.54% in nude mice and 11.76% in C3-KO mice at 72 h after treatment. At 72 h after PDT, the proportions of the apoptotic tumor cells were dramatically increased in the brain glioma-bearing BALB/c mice with a peak value at 48 h. This proportion was significantly higher than nude mice (Fig. 6A). However, PDT had no significant effects on the proportions of apoptotic tumor cells in the C3-KO mice.

Although PDT significantly increased the number of $CD68^{+}$ cells in the glioma tissue in all three strains of mice (Fig. 6B), the treatment only significantly enhanced the release of TNF-α and IFN-γ in the BALB/c mice (Fig. 6C,D), as well as IFN-γ in the nude mice (Fig. 6D).

DISCUSSION

Accumulating evidence suggests that the failure of host immune systems to eradicate glioblastomas is due to the inability of glioblastomas to stimulate an effective anti-tumor immune response. Thus, active immunization of patients with malignant gliomas is regarded as a therapeutic strategy to promote anti-tumor response [Grauer et al., 2009; Albesiano et al., 2010; Waziri, 2010; Yang et al., 2010]. Indeed, recent findings indicate that anti-cancer treatment, such as chemotherapy, radiotherapy, hyperthermia, and high intensity focused ultrasound, can induce necrosis in tumors and boost anti-tumor immunity [Hirschberg et al., 2004; Wu et al., 2004; Heisel et al., 2008; Zitvogel et al., 2008]. Using the well established brain glioma models by implanting G422 or GL261 mouse glioma cells in mice brain, which can mimic the growth of human glioma such as the forming of spheres and invasion to the normal brain [Zhang et al., 2004], our study provides evidence to further support the anti-tumor immune strategy in the CNS. At the same time, PDT, an effective adjuvant treatment for gliomas, showed no obvious harm to the normal brain and no severe side effects were observed as we had reported [Li et al., 2006].

A PDT-induced inflammatory reaction is thought to be enhanced by the recruitment of neutrophils, mast cells, and monocytes [Vonarx et al., 1997; Dolmans et al., 2003; Nowis et al., 2005; Castano et al., 2006; Kousis et al., 2007], and the inflammatory mediators that are released from these cells enable the massive recruitment of immune cells to the tumor site [Krosl et al., 1996; Cecic and Korbelik, 2002]. In the present study, the inflammatory cells isolated from the tumor tissue were stained with monoclonal

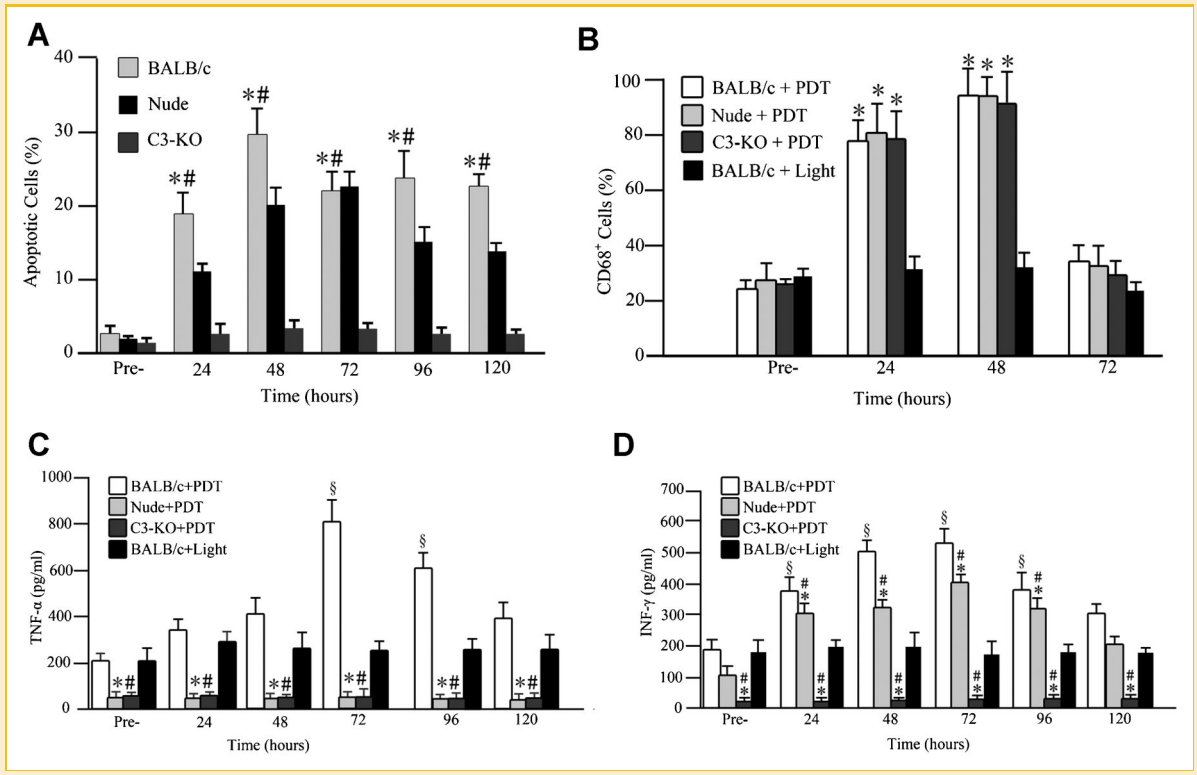

Fig. 6. Effects of T cells and complement C3 on PDT-induced anti-glioma effects. The apoptotic rate of G422 tumor cells, proportion of $CD68^+$ cells and levels of inflammatory factors in BALB/c mice (BALB/c + PDT), nude mice (Nude + PDT) and C3-KO mice (C3-KO + PDT) after PDT were measured. A: A statistical analysis shows the proportions of apoptotic tumor cells out of the total number of cells before and after PDT in the three types of mice (ANOVA, n = 6 for each group, $^*P < 0.05$ vs. C3-KO mice; $^\#P < 0.05$ vs. nude mice). B: A statistical analysis shows the effects of PDT on the proportions of $CD68^+$ cells out of the total number of cells in the glioma tissue in all three strains of mice (ANOVA, n = 6 for each group, $^*P < 0.05$ vs. BALB/c + light). C: A statistical analysis shows the effects of PDT on TNF-α release in the tumor tissue of all three strains of mice (ANOVA, n = 6 for each group, $^*P < 0.05$ vs. BALB/c + PDT, $^\#P < 0.05$ vs. BALB/c + light, $^\S P < 0.05$ vs. BALB/c + light). D: A statistical analysis shows the effects of PDT on IFN-γ release in the tumor tissue of all three strains of mice (ANOVA, n = 6 for each group, $^*P < 0.05$ vs. BALB/c + PDT, $^\#P < 0.05$ vs. BALB/c + light, $^\S P < 0.05$ vs. BALB/c + light).

antibody against specific CD68. The proportion of $CD68^+$ cells is dramatically increased in the glioma tissue despite the immuno-background of the mice after PDT. The treatment also markedly enhances the release of TNF-α and IFN-γ in the BALB/c mice. Although the subtypes of lymphocyte were not identified, but the $CD68^+$ cells indicates an activated inflammatory infiltration in the CNS [Asai et al., 1999; Strik et al., 2004], the increasing of proportions of $CD68^+$ cells could indicate the higher activation of infiltrated inflammatory in the tumor tissue in the brain. Thus, PDT may enhance anti-glioma immunity by promoting regional infiltration of inflammatory cells and increasing the release of cytokines.

T cell-mediated immune responses represent the main type of cellular anti-tumor immunity in cancer patients. The $CD4^+/CD8^+$ ratio is usually used as an indicator of a patient's anti-tumor immunity and also as prognostic markers for cancer patients receiving immunomodulative therapy [Korbelik and Dougherty, 1999; Kabingu et al., 2007]. In the brain glioma-bearing mice, the blood $CD4^+/CD8^+$ ratio is significantly lower, but PDT increases $CD4^+/CD8^+$ ratio, implying that PDT reverses the immune ability of the host and exerts anti-tumor immunity through systemic $CD4^+$ T cells.

Although in the early stage of the PDT, the cell death mainly causes by direct cytotoxicity and microvascular disruption, increasing evidence indicates that PDT promotes systemic immunity and generate specific vaccines to antagonize tumors, and the localized inflammatory effect of PDT also initiate the formation of anti-tumor immunity [Krosl et al., 1995; Krosl et al., 1996; Hendrzak-Henion et al., 1999; Jiang et al., 2002; Skivka et al., 2004; Korbelik and Sun, 2006; Kabingu et al., 2009]. PDT induces systemic anti-tumor immunity in mice with G422 gliomas by increasing the ratio of blood $CD4^+/CD8^+$ lymphocytes, which enhances the ability of splenic lymphocytes to kill glioma cells and promotes the release of TNF-α and IFN-γ. Interestingly, the splenic lymphocytes from mice after PDT also have inhibitory effect on the growth of intracerebral and subcutaneous implanted G422 tumors in the nude mice, indicating that PDT-mediated systemic immunity can suppress tumor growth outside the treatment region.

It is interesting to note that PDT increases the infiltration of inflammatory cells, regardless of the immunity status of the host, in all three strains of mice. However, the treatment had the greatest anti-glioma effects in the normal immunocompetent BALB/c mice, suggesting that PDT-induced anti-glioma effects depend on the activity of T cells and complement C3. The T lymphocyte mediated

immune response is proved to be mainly response for the PDT-induced anti-tumor immunity, and the complement C3 was identified as a major chemoattractant in the advanced phase of PDT-induced inflammatory infiltration [Cecic and Korbelik, 2002; Stott and Korbelik, 2006; Mroz et al., 2010]. Thus, the activation of T cells and complement C3 may play critical roles in mediating PDT-induced anti-glioma immunity.

PDT-mediated tumor destruction may provide tumor-specific antigens to T cells and activates the immune cascade that stimulates the release of a wide variety of potent mediators, such as vasoactive substances, components of the complement cascades, cytokines (IL-6, IL-1β, IL-2, tumor necrosis factor-α, and granulocyte colony stimulating factor), growth factors, and other immunoregulators [Castano et al., 2006; Korbelik and Sun, 2006; Kabingu et al., 2009; Mroz et al., 2010]. As one of the key effect molecules engaged in immune reactions, complement C3 is activated after PDT, and this activation has been identified as a major PDT-induced host response [Stott and Korbelik, 2006]. PDT did not induce a sufficient immune response in nude mice and C3-KO mice in our study, indicating that the activation of T cells and their complements are crucial in PDT-induced anti-glioma immunity.

Collectively, the present data showed that the PDT generates regional and systemic anti-tumor immunity in mice with gliomas in the brain. The infiltration of immune cells and the release of inflammatory factors, such as TNF-α and IFN-γ, were response for the regional effects of PDT-induced anti-glioma immunity. Furthermore, we showed that the lymphocytes isolated from the PDT-treated mice were able to induce anti-tumor immunity in nude mice suggested the activation of systemic immune by PDT. We also provided evidence that the anti-glioma immunity fostered by PDT was inhibited in complement C3 knockout mice and the nude mice, indicated the requirement of the activities of complement C3 and T cells. Although more details, such as the identification of the subtypes of lymphocytes, especially the role of microglia infiltration and activation in the CNS should be studied deeply, the present study provided clear evidence that the T cells along with complement C3, might play crucial roles in mediating PDT-induced anti-glioma responses.

ACKNOWLEDGMENTS

This work was supported by National Natural Science Foundation of China 30670506 (Hua Feng) and 30973494 (Fei Li) and Post-Doctor Science Foundation of China 20070420768 (Yingxin Cheng).

REFERENCES

Albesiano E, Han JE, Lim M. 2010. Mechanisms of local immunoresistance in glioma. Neurosurg Clin N Am 21:17–29.

Asai J, Suzuki R, Fujimoto T, Suzuki T, Nakagawa N, Nagashima G, Miyo T, Hokaku H, Takei A. 1999. Fluorescence automatic cell sorter and immunohistochemical investigation of CD68-positive cells in meningioma. Clin Neurol Neurosurg 101:229–234.

Castano AP, Mroz P, Hamblin MR. 2006. Photodynamic therapy and anti-tumour immunity. Nat Rev Cancer 6:535–545.

Cecic I, Korbelik M. 2002. Mediators of peripheral blood neutrophilia induced by photodynamic therapy of solid tumors. Cancer Lett 183:43–51.

Dolmans DE, Fukumura D, Jain RK. 2003. Photodynamic therapy for cancer. Nat Rev Cancer 3:380–387.

Gentilini D, Besana A, Vigano P, Dalino P, Vignali M, Melandri M, Busacca M, Di Blasio AM. 2010. Endocannabinoid system regulates migration of endometrial stromal cells via cannabinoid receptor 1 through the activation of PI3K and ERK1/2 pathways. Fertil Steril 93:2588–2593.

Grauer OM, Wesseling P, Adema GJ. 2009. Immunotherapy of diffuse gliomas: Biological background, current status and future developments. Brain Pathol 19:674–693.

Heisel SM, Ketter R, Keller A, Klein V, Pallasch CP, Lenhof HP, Meese E. 2008. Increased seroreactivity to glioma-expressed antigen 2 in brain tumor patients under radiation. PLoS ONE 3:e2164.

Hendrzak-Henion JA, Knisely TL, Cincotta L, Cincotta E, Cincotta AH. 1999. Role of the immune system in mediating the antitumor effect of benzophenothiazine photodynamic therapy. Photochem Photobiol 69:575–581.

Herman S, Kalechman Y, Gafter U, Sredni B, Malik Z. 1996. Photofrin II induces cytokine secretion by mouse spleen cells and human peripheral mononuclear cells. Immunopharmacology 31:195–204.

Hirschberg H, Sun CH, Tromberg BJ, Yeh AT, Madsen SJ. 2004. Enhanced cytotoxic effects of 5-aminolevulinic acid-mediated photodynamic therapy by concurrent hyperthermia in glioma spheroids. J Neurooncol 70:289–299.

Hussain SF, Yang D, Suki D, Aldape K, Grimm E, Heimberger AB. 2006. The role of human glioma-infiltrating microglia/macrophages in mediating antitumor immune responses. Neuro-Oncology 8:261–279.

Jiang H, Granville DJ, North JR, Richter AM, Hunt DW. 2002. Selective action of the photosensitizer QLT0074 on activated human T lymphocytes. Photochem Photobiol 76:224–231.

Kabingu E, Oseroff AR, Wilding GE, Gollnick SO. 2009. Enhanced systemic immune reactivity to a basal cell carcinoma associated antigen following photodynamic therapy. Clin Cancer Res 15:4460–4466.

Kabingu E, Vaughan L, Owczarczak B, Ramsey KD, Gollnick SO. 2007. CD8+ T cell-mediated control of distant tumours following local photodynamic therapy is independent of CD4+ T cells and dependent on natural killer cells. Br J Cancer 96:1839–1848.

Korbelik M, Dougherty GJ. 1999. Photodynamic therapy-mediated immune response against subcutaneous mouse tumors. Cancer Res 59: 1941–1946.

Korbelik M, Krosl G, Krosl J, Dougherty GJ. 1996. The role of host lymphoid populations in the response of mouse EMT6 tumor to photodynamic therapy. Cancer Res 56:5647–5652.

Korbelik M, Sun J. 2006. Photodynamic therapy-generated vaccine for cancer therapy. Cancer Immunol Immunother 55:900–909.

Kousis PC, Henderson BW, Maier PG, Gollnick SO. 2007. Photodynamic therapy enhancement of antitumor immunity is regulated by neutrophils. Cancer Res 67:10501–10510.

Krosl G, Korbelik M, Dougherty GJ. 1995. Induction of immune cell infiltration into murine SCCVII tumour by photofrin-based photodynamic therapy. Br J Cancer 71:549–555.

Krosl G, Korbelik M, Krosl J, Dougherty GJ. 1996. Potentiation of photodynamic therapy-elicited antitumor response by localized treatment with granulocyte-macrophage colony-stimulating factor. Cancer Res 56:3281–3286.

Li F, Zhu G, Lin JK, Meng H, Wu N, Du Y, Feng H. 2006. Photodynamic therapy increases brain edema and intracranial pressure in a rabbit brain tumor model. Acta Neurochir Suppl 96:457–460.

Mabrouk GM, Ali EM, El-Rehany MA, El-Samoly HM. 2007. TGF-beta1, TNF-alpha and cytochrome c in human astrocytic tumors: A short-term follow up and correlation with survival. Clin Biochem 40:255–260.

Mroz P, Szokalska A, Wu MX, Hamblin MR. 2010. Photodynamic therapy of tumors can lead to development of systemic antigen-specific immune response. PLoS ONE 5:e15194.

Muller PJ, Wilson BC. 2006. Photodynamic therapy of brain tumors—A work in progress. Lasers Surg Med 38:384–389.

Nowis D, Makowski M, Stoklosa T, Legat M, Issat T, Golab J. 2005. Direct tumor damage mechanisms of photodynamic therapy. Acta Biochim Pol 52:339–352.

Simon M, Schramm J. 2009. Surgical management of intracranial gliomas. Recent Results Cancer Res 171:105–124.

Skivka LM, Gorobets OB, Kutsenok VV, Lozinsky MO, Borisevich AN, Fedorchuk AG, Kholin VV, Gamaleya NF. 2004. 5-Aminolevulinic acid mediated photodynamic therapy of Lewis lung carcinoma: A role of tumor infiltration with different cells of immune system. Exp Oncol 26:312–315.

Stott B, Korbelik M. 2006. Activation of complement C3, C5, and C9 genes in tumors treated by photodynamic therapy. Cancer Immunol Immunother 56:649–658.

Strik HM, Stoll M, Meyermann R. 2004. Immune cell infiltration of intrinsic and metastatic intracranial tumours. Anticancer Res 24:37–42.

Stylli SS, Kaye AH, MacGregor L, Howes M, Rajendra P. 2005. Photodynamic therapy of high grade glioma—Long term survival. J Clin Neurosci 12:389–398.

Sun CY, Hu Y, Huang J, Chu ZB, Zhang L, She XM, Chen L. 2010. Brain-derived neurotrophic factor induces proliferation, migration, and VEGF secretion in human multiple myeloma cells via activation of MEK-ERK and PI3K/AKT signaling. Tumour Biol 31:121–128.

Tait MJ, Petrik V, Loosemore A, Bell BA, Papadopoulos MC. 2007. Survival of patients with glioblastoma multiforme has not improved between 1993 and 2004: Analysis of 625 cases. Br J Neurosurg 21:496–500.

Vermes I, Haanen C, Reutelingsperger C. 2000. Flow cytometry of apoptotic cell death. J Immunol Methods 243:167–190.

Vonarx V, Foultier MT, Anasagasti L, Morlet L, Lajat Y, Patrice T. 1997. Photodynamic effect on the specific antitumor immune activity. Int J Immunopharmacol 19:101–110.

Waziri A. 2010. Glioblastoma-derived mechanisms of systemic immunosuppression. Neurosurg Clin N Am 21:31–42.

Wu F, Wang ZB, Lu P, Xu ZL, Chen WZ, Zhu H, Jin CB. 2004. Activated anti-tumor immunity in cancer patients after high intensity focused ultrasound ablation. Ultrasound Med Biol 30:1217–1222.

Xiao H, Liao Q, Cheng M, Li F, Xie B, Li M, Feng H. 2009. 5-Amino-4-oxopentanoic acid photodynamic diagnosis guided microsurgery and photodynamic therapy on VX2 brain tumour implanted in a rabbit model. Chin Med J (Engl) 122:1316–1321.

Yang I, Han SJ, Kaur G, Crane C, Parsa AT. 2010. The role of microglia in central nervous system immunity and glioma immunology. J Clin Neurosci 17:6–10.

Zhang Z, Tang LL, Zhan RY, Tong Y, Yao HP, Du LA. 2004. Immunotherapy of intracranial G422 glioblastoma with dendritic cells pulsed with tumor extract or RNA. J Zhejiang Univ Sci 5:1298–1303.

Zitvogel L, Apetoh L, Ghiringhelli F, Kroemer G. 2008. Immunological aspects of cancer chemotherapy. Nat Rev Immunol 8:59–73.