Published in final edited form as:

Photochem Photobiol. 2008 ; 84(2): 366–370. doi:10.1111/j.1751-1097.2007.00270.x.

The immunosuppressive effects of phthalocyanine photodynamic therapy in mice are mediated by CD4+ and CD8+ T cells and can be adoptively transferred to naïve recipients

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Abstract

Photodynamic therapy (PDT) is a promising treatment modality for malignant tumors but it is also immunosuppressive which may reduce its therapeutic efficacy. The purpose of our study was to elucidate the role of CD4+ and CD8+ T-cells in PDT immunosuppression. Using silicon phthalocyanine 4 (Pc4) as photosensitizer, non-tumor bearing CD4 knockout (CD4^{-/-}) mice and their wild type (WT) counterparts were subjected to Pc4-PDT in a manner identical to that used for tumor regression (1 cm spot size, 0.5 mg/kg Pc4, 110 J/cm² light) to assess the effect of Pc4-PDT on cell-mediated immunity. There was a decrease in immunosuppression in CD4^{-/-} mice as compared to WT mice. We next examined the role of CD8+ T-cells in Pc4-PDT induced immunosuppression using CD8^{-/-} mice following the same treatment regimen used for CD4^{-/-} mice. Similar to CD4^{-/-} mice, CD8^{-/-} mice exhibited less immunosuppression than WT mice. Pc4-PDT induced immunosuppression could be adoptively transferred with spleen cells from Pc4-PDT treated donor mice to syngenic naïve recipients (p<0.05) and was mediated primarily by T cells, although macrophages were also found to play a role. Procedures that limit PDT induced immunosuppression but do not affect PDT induced regression of tumors may prove superior to PDT alone in promoting long term anti-tumor responses.

Keywords

Pc4 (Phthalocyanine 4); Photodynamic therapy (PDT); T-cells; Immunosuppression; DNFB (1-Fluoro-2,4-dinitrobenzene)

INTRODUCTION

Photodynamic therapy (PDT) is a promising treatment modality for the management of various tumors and non-malignant diseases. It employs non-toxic photosensitizers which are then activated by visible light in combination with oxygen to produce cytotoxic reactive oxygen species that kill malignant cells directly by apoptosis and/or necrosis, and indirectly by damage to the tumor microvasculature (1). The photosensitizers function as catalysts when they absorb visible light and then convert molecular oxygen to a range of highly reactive oxygen species (ROS).

There are several reports that PDT can induce various forms of immunosuppression (7). In particular, PDT can cause suppression of contact hypersensitivity (CHS) in mice (8). In contrast to UVB irradiation that suppresses both CHS and delayed type hypersensitivity (DTH) responses, PDT does not suppress DTH (9). Contact hypersensitivity is a T-cell mediated cutaneous immune/inflammatory reaction to haptens. There is substantial evidence that the generation and maintenance of CHS is dependent on effective memory CD8⁺ T-cells although there are conflicting reports (10–13).

Studies from our group have focused on the photosensitizer silicon phthalocyanine 4 (Pc4) (14). This agent has a large macrocycle ring with silicon chelated in the center. The compound is activated by 675 nm light that penetrates more deeply into tissues than the wavelengths of light used to activate porphyrin photosensitizers. Mice treated with Pc4-PDT 3 days before dinitrofluorobenzene (DNFB) sensitization exhibit significant suppression of their cell mediated immune response when compared to mice that are not exposed to PDT. The response is dose dependant, requires both Pc4 and light, and occurs at a site remote from that exposed to the laser. The immunosuppression is not reversed by *in vivo* pretreatment of mice with antibodies to TNF or IL-10 (15).

The purpose of this study was to examine the nature of Pc4-PDT induced immunosuppression and to determine the specific cell types involved in the generation of the immunosuppressive state. *In vivo* experiments were designed using C3H/HeN mice to: (1) determine whether the immunosuppression induced by PDT treatment was adoptively transferable; (2) determine the characteristics of cells responsible for the transfer of suppression; and (3) evaluate the T-cell type involved in immunosuppression.

MATERIALS AND METHODS

Animals and reagents

C3H/HeN mice were purchased from Charles River Laboratories (Wilmington, MA). Female mice 6–8 weeks of age were used in the experiments. CD4 knockout (CD4^{-/-}) and CD8 knockout (CD8^{-/-}) mice on a C57BL/6 background were purchased from Jackson Laboratories (Bar Harbor, ME) and were backcrossed onto a C3H/HeN background. The CD4 deletion was backcrossed for 12 generations via traditional methods. These mice had greater than 99% C3H/HeN background genes. The CD8 deletion was placed on the C3H/HeN background via rapid backcross methodology (16, 17). Briefly, mice at each backcross generation were genotyped with two sets of polymorphic microsatellite repeat markers (Research Genetics, Huntsville, AL), making it possible to select progeny which retained the desired C3H genome and had lost a larger portion of the undesired C57BL/6 DNA. By selective breeding of these mice, it was possible to eliminate the C57BL/6 genome and replace it with C3H DNA after 4 generations of backcrossing. These mice had greater than 99% C3H/HeN background genes before they were used. All animal procedures were performed according to National Institute of Health guidelines under protocols approved by the Institutional Animal Care and Use Committee of the University of Alabama at Birmingham. Each panel of mice consisted of 4–5 mice.

The silicon phthalocyanine (Pc), Pc4, employed for these studies was obtained from National Cancer Institute. Silicon phthalocyanine 4 was dissolved in vehicle, for administration to animals (14). 2,4-Dinitro-1-fluorobenzene (DNFB) was purchased from Sigma Chemical Co. (St. Louis, MO) and was dissolved in an acetone:olive oil solution (4:1). The antibodies Thy-1.2 and CD11b were purchased from Pharmingen (San Diego, CA), control Rat IgG from Rockland (Gilbertsville, PA) and sheep anti-rat IgG dynabeads M-450 were purchased from Invitrogen (Carlsbad, CA).

Photodynamic therapy with Pc4

Mice were given intravenous injections of 0.5mg/kg Pc4. The volume of Pc4 was adjusted to 100µl for every 10g of body weight. Twenty four hours later, the dorsal skin was exposed to 135 J/cm² 675 nm light from a diode laser (Applied Optronics Corp, Newport, RI) coupled to a fiberoptic cable terminating in a microlens, which distributes light uniformly throughout the treatment field. Hair was removed from the irradiation site by depilation at least 48 h prior to injection with the photosensitizing agent. Mice were anesthetized with an intraperitoneal injection of 0.2 ml of 1:1 ketamine:xylazine prior to treatment with the laser. Then 675 nm light was delivered using a 1 cm-diameter spot size aimed at the depilated aspect of the back of each mouse. A 1 cm-diameter spot size was chosen because that is the size that has been employed to treat murine tumors in our other studies (14). Ears were not within the field of light administration. After treatment with light, the mice were kept in a dark environment and were shielded from further light exposure.

Assessment of cell mediated immunity

Cell mediated immune function following Pc4-PDT was assessed with DNFB contact hypersensitivity. Induction and elicitation of DNFB contact hypersensitivity was performed, modifying the method described previously (15). Unless otherwise stated, mice were sensitized to DNFB on days 3 and 4 after exposure to light from the laser by applying 25µl of 0.5% DNFB in an olive oil/acetone solution to the shaved abdomen. Four days after the second treatment with DNFB, one ear was challenged with topical administration of 20µl of 0.2% DNFB to both sides of the ear. Measurements of ear thickness were taken prior to challenge and 24 and 48 h after challenge using a dial thickness gauge micrometer. Positive control panels were sensitized and challenged with DNFB but were not given Pc4 or light prior to sensitization. Negative control panels did not receive Pc4-PDT or DNFB sensitization but were challenged with DNFB on the ear. The difference in ear thickness when pre- and post-DNFB challenge measurements were compared was used to quantify the development of cell mediated immunity. Suppression of the immune response was calculated using the following formula.

$$\% \text{ Suppression} = \frac{[\text{ear swelling (positive control)} - \text{ear swelling (experimental)}]}{[\text{ear swelling (positive control)} - \text{ear swelling (negative control)}]} \times 100$$

Adoptive transfer studies

Groups of mice were injected with Pc4 and exposed to 675 nm light from the diode laser as described above. Three days later they were sensitized on the shaved abdominal skin with either 25µl of 0.5% DNFB for two consecutive days (in 4:1 acetone:olive oil). Five days after the first sensitization mice were sacrificed and spleen cells were prepared by passing them through a wire screen. Red blood cells were removed by osmotic lysis and the remaining cells were washed two times in Hanks balanced salt solution (HBSS). The cells were then counted and their viability was assessed using trypan blue. Fifty million viable cells in a volume of 0.2 ml were injected intravenously into naive recipients. Following injection, the mice were immediately sensitized with 25 µl of 0.5% DNFB to the shaved abdomen. Four days later the ear thickness was measured and the mice were challenged with 20 µl of 0.2% DNFB to each side of one ear. Measurements of ear thickness were taken prior to challenge and 24 and 48 h after challenge using a dial thickness gauge micrometer as described earlier.

Antibody mediated cell depletion

In order to assess the cell type responsible for transfer of PDT induced immunosuppression, the mice were treated as they were treated for adoptive transfer studies. Prior to adoptive transfer of spleen cells from the Pc4 PDT treated mice, different cell populations were removed from suspension by using antibody mediated depletion using sheep anti-rat IgG dynabeads according to the manufacturer's instructions. Briefly, after antibody treatment, prewashed dynabeads were added to the prepared sample and incubated with gentle tilting for 30 min at 4°C. The tube was placed on a magnet for 2 min and the supernatant containing the unbound cells was transferred to a fresh tube for further experiments. Fifty microliter dynabeads were added to 1×10^7 cells/ml sample. Different cellular populations were removed individually in this manner using their respective antibodies. Anti-Thy-1.2 was used to remove T-cells and anti-CD11b was employed for macrophages. Fifty million cells were then adoptively transferred into untreated recipients and their CHS response was evaluated as described above.

Statistical analysis

The significance of the differences between the means of groups was analyzed using the Student's *t* - test.

RESULTS

Phthalocyanine 4 PDT (Pc4-PDT) is immunosuppressive

Prior studies by our group have demonstrated that suppression of cell mediated immunity is a consequence of Pc4-PDT in C3H/HeN mice (15). In order to assess the time dependence for light administration after injecting Pc4 on immunosuppression, mice were exposed to light from the diode laser 24 h or 48 h after having given Pc4 intravenously. There was slightly less immunosuppression (44%) when mice were exposed to light 48 h after Pc4 injection compared to 24h (53%) (Figure 1).

Immunosuppression by Pc4-PDT can be adoptively transferred

The immunosuppressive effect of PDT using hematoporphyrin derivative (16) could be adoptively transferred with spleen cells. Studies by Lynch et al showed that macrophages were responsible for the immunosuppression in their system (18). We therefore did experiments to determine if (1) Pc4 PDT induced immunosuppression was could be adoptively transferred and, if so, (2) which cell type was responsible for transfer of immunosuppression. Mice that received cells from Pc4 PDT-treated mice exhibited a cell mediated immune response that was 64% suppressed compared to untreated control mice (Figure 2). Mice that received spleen cells from mice receiving Pc4 only or light only did not exhibit significant immunosuppression.

In order to determine whether the adoptively transferable suppression was mediated by T-cells or macrophages, the splenocytes from Pc4 PDT treated mice were depleted either of T-cells using anti-Thy-1.2 antibody or of macrophages using anti-CD11b antibody by immunomagnetic separation. The remaining cells were adoptively transferred in recipient naive mice. In addition, a separate panel of mice received spleens cells that had been treated with a control antibody. Transfer of spleen cells showed different degrees of suppression as compared to the isotype control (69%). Spleen cells lacking the T cells showed 28% and macrophages population 53%, indicating that T cells are the primary mediators of Pc4 PDT mediated immunosuppression, although macrophages participate to some extent as well (Figure 3).

Role of CD4⁺ T-cells in immunosuppression by Pc4-PDT

To elucidate the role of CD4⁺ T-cells in Pc4-PDT mediated immunosuppression, panels of CD4^{-/-} mice and wild type C3H/HeN mice were injected intravenously with 0.5 mg/kg of Pc4. Twenty four hours later, a 1 cm spot of depilated skin on the back was exposed to 135 J/cm² 675 nm light. After three days, attempts were made to immunize mice to DNFB by applying DNFB to the abdomen. The ears were challenged four days later with DNFB, and the increment in ear thickness was used to quantify the magnitude of immune response. Positive controls in both groups were not subjected to Pc4-PDT but were sensitized and ear challenged with DNFB; negative control mice in both groups were ear challenged with DNFB only. The Pc4-PDT treated CD4^{-/-} mice showed 49% immunosuppression as compared to their wild type C3H/HeN mice which showed 81% immunosuppression 24 h after challenge (Figure 4), indicating that CD4⁺ T-cells are at least in part responsible for Pc4 PDT-induced immunosuppression.

Role of CD8⁺ T-cells in immunosuppression by Pc4-PDT

We next examined the role of CD8⁺ T-cells in Pc4-PDT mediated immunosuppression. Panels of CD8^{-/-} mice and wild type C3H/HeN mice were subjected to Pc4 PDT in a manner identical to that of the CD4^{-/-} mice. CD8^{-/-} mice showed 57% immunosuppression compared to 80% suppression observed in wild type C3H/HeN mice (Figure 5). These results indicate that CD8⁺ T-cells also contribute to Pc4 PDT-induced immunosuppression.

DISCUSSION

Previous studies from our laboratory have shown that inhibition of cell mediated immunity is a consequence of Pc4-PDT and that the mechanism by which immunosuppression is achieved is different from that of UV-induced immunosuppression (15). In this regard, Pc4-PDT is similar to other modalities, such as chemotherapy, used to treat solid tumors, which are immunosuppressive as well, but which are effective as a cancer therapy.

In other studies, it has been shown that the immunosuppression produced by PDT can be adoptively transferred with splenocytes (16). Lynch et al. confirmed these results, and also found in their system, that it was mediated by macrophages (18). Musser and Oseroff (9) showed that immunosuppression mediated by dorsal PDT using Photofrin® as a sensitizer was found to be long lived, requiring light exposure to a much smaller surface area than anticipated for its development. In contrast to Lynch et al's results, the immunosuppression resembled that caused by UVB in that could be adoptively transferred; was antigen specific; and was CD4⁺ T-cell mediated. It differed principally from UVB-induced immunosuppression at the cellular level in that the DTH response was not suppressed.

The relationship between PDT and cell-mediated immune function is complex. While our studies have shown that PDT is immunosuppressive, it is also clear that the immunosuppression is only partial and that the antigen specific T-cell mediated response to the tumor contributes significantly to tumor ablation and to tumor cures following PDT. It is well-known that tumor specific T-cells can eliminate small foci of malignant cells, and this is likely to be the way in which tumor cells are removed that have escaped the direct cytotoxicity and vascular effects of PDT. Strong evidence that PDT, in fact, does lead to the induction of tumor specific immunity comes from the studies of Korbelik et al. (19, 20). When the EMT-6 mammary carcinoma cell line was transplanted to BALB/c mice, PDT caused marked tumor regression and resulted in long-term tumor cures. In contrast, when the same tumors were transplanted to immunodeficient nude or scid mice and then subjected to Photofrin® PDT, tumor regression could be achieved, but there were no long-term cures. When cells from the spleen or bone marrow of immunocompetent BALB/c mice were

transferred to SCID mice, recurrence was delayed, providing further evidence that the immune system contributes in an important way to the efficacy of PDT.

The finding that treatment with the adjuvant serum vitamin D₃-binding protein-derived macrophage-activating factor (DBPMAF) enhanced the curative effect of PDT against the SCCVII tumor provides additional support for a role of the immune system in PDT-induced tumor regression and cure (21).

The observation that PDT is immunosuppressive may open new opportunities for optimizing protocols for its use clinically in tumor ablation protocols. For example, it is conceivable that PDT induced tumor regression may occur at the expense of the generation of an effective anti-tumor response. Thus, procedures that limit PDT-induced immunosuppression but do not affect PDT induced regression of tumors may prove to be superior to PDT alone in promoting long-term tumor responses.

It may also be possible to exploit the immunosuppressive properties of Pc4 PDT for therapeutic benefit. In animal models, PDT has been shown to impair organ transplant rejection (22, 23) and to be effective at reducing the severity of experimental autoimmune encephalitis (24) and collagen-induced arthritis (25). There are also a number of clinical observations that support the hypothesis that PDT can be an effective therapy in diseases with overactive cell-mediated immunity (26, 27). For example, in small series of patients, PDT has been reported to be effective in psoriasis, alopecia areata and cutaneous sarcoidosis.

Acknowledgments

This work was supported by NIH Grants P30 AR050948, P30 CA13148 and Veterans Administration Merit Review Award 18-103-02 to C.A.E.

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Figure 1.

Time dependence of light after Pc4 administration for immunosuppression. Comparison was made between mice that were subjected to light, 24h and 48h after intravenous Pc4 injection. After exposure, mice were subsequently sensitized and challenged with DNFB. There was significant immunosuppression in the Pc4-PDT groups (* $p < 0.05$) treated for 24h and 48h as compared to the non-PDT group. Results are the mean of change in auricular thickness $\pm$ SEM with 3 mice per group and each experiment was repeated twice.

Figure 2.

Phthalocyanine 4 PDT mediated immunosuppression can be adoptively transferred. Fifty million spleen cells from the animals after various treatments and subsequent DNFB sensitization were injected intravenously into naïve recipients which were subsequently sensitized and challenged with DNFB. There was significant immunosuppression (* $p < 0.05$) in the group which received cells from Pc4-PDT treated mice as compared to the group which received cells from non-treated mice. Results are the mean $\pm$ SEM with 3 mice per group and each experiment was repeated twice.

Figure 3.

Phthalocyanine 4 PDT mediated immunosuppression can be adoptively transferred by T cells and also macrophages. Spleen cells from the animals after Pc4 PDT treatment and subsequent DNFB sensitization were depleted of T cells (Thy1.2 antibody) or macrophages (CD11b antibody). Isotype control antibody was used as a depletion control. The above groups of depleted cells (50×10^6) were injected intravenously into naïve recipients which were subsequently sensitized and challenged with DNFB. Results are the mean $\pm$ SEM with 3 mice per group and each experiment was repeated twice.

Figure 4. Phthalocyanine 4 PDT induced immunosuppression is mediated in part by CD4⁺ T cells. Animals were treated with Pc4 PDT on their normal dorsal skin. Their cell mediated immune response was then assessed by sensitizing the animals with the contact allergen DNFB as described in the Methods section. There was significant immunosuppression (* $p < 0.05$) in the wild type C3H/HeN mice as compared to CD4^{-/-} mice. Results are the mean $\pm$ SEM with 3 mice per group and each experiment was repeated twice.

Figure 5.

Phthalocyanine 4 PDT mediated immunosuppression is partially mediated by CD8⁺ T cells. Animals were treated with Pc4-PDT on their normal dorsal skin. Their cell mediated immune response was then assessed by sensitizing the animals with the contact allergen DNFB as described in the Methods section. There was significant immunosuppression (* $p < 0.05$) in the wild type C3H/HeN mice as compared to CD8^{-/-} mice. Results are the mean $\pm$ SEM with 3 mice per group and each experiment was repeated twice.