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CpG oligodeoxynucleotide as immune adjuvant enhances photodynamic therapy response in murine metastatic breast cancer

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Abstract

Breast cancer is the most common cause of cancer death in women. The side effects and complications following current breast cancer therapy can be devastating. Moreover, the prognosis in late stages of the diseases is usually poor. Photodynamic therapy (PDT) is a promising cancer treatment modality that is capable of both local tumor destruction and immune stimulation. However, treatment with PDT alone is often non-curative due to tumor-induced immune cell dysfunction and immune suppression. This phenomenon has motivated a new approach by combining immunostimulants with PDT to enhance anti-tumor immunity. In the present study, we investigated PDT mediated by verteporfin and 690 nm light delivered 15 min later, in combination with an immunomodulation approach using CpG oligodeoxynucleotide for the treatment of 4T1 metastatic breast cancer in a BALB/c immunocompetent mouse model. *In vitro*, CpG primed immature dendritic cells (DC) via toll like receptor 9 to phagocytose PDT killed tumor cells leading to DC maturation and activation. Peritumoral injection of CpG after PDT in mice gave improved local tumor control and a survival advantage compared to either treatment alone ($p < 0.05$). CpG may be a valuable dendritic cell targeted immunoadjuvant to combine with PDT.

Keywords

breast cancer; CpG oligodeoxynucleotide; dendritic cells; photodynamic therapy

1. Introduction

Despite significant progress in treatment, cancer continues to be the leading cause of death in developed countries [1] and among all the cancers, breast cancer is the commonest cause of cancer death in women [2]. The side effects and complications following current breast cancer therapy can be devastating. Moreover, the prognosis in late stages of the diseases is usually poor [3]. Therefore, there is an urgent need for better treatment alternatives.

Photodynamic therapy (PDT) is a promising cancer treatment modality in which a photosensitizer (PS) localized at the tumor site is activated through absorption of photons of an appropriate wavelength leading to the transfer of light energy to molecular oxygen and generation of reactive oxygen species (ROS) [4]. The ROS-mediated damage induces acute local inflammation and causes cell death, vascular shutdown and tumor ablation [5, 6]. There is also an induction of a host immune response [7] via creation of damage associated molecular patterns (DAMPs) [8] and stimulation of pro-inflammatory cytokines [9, 10]. However, the precise mechanisms involved in the PDT-mediated induction of anti-tumor immunity are not yet clearly understood [11]. PDT has been shown to induce both a systemic neutrophilia and a strong and prolonged tumor infiltration by neutrophils [12, 13]. In addition tumor infiltration by dendritic cells (DC), macrophages and mast cells has been observed [14]. Complement activation has also been observed both in the tumor and serum after PDT [15]. DC are the antigen presenting cells that are responsible for initiating adaptive immune response and tolerance [16]. Toll-like receptors (TLR) are responsible for recognizing antigens and subsequently provoke DC maturation. Numerous TLR such as TLR-2, TLR-3, TLR-4, TLR-9 and TLR-13 are expressed on DC [17]. The PDT killed tumor cells may be phagocytosed by DC that then migrate to draining lymph nodes and prime naïve T-cells that recognize tumor associated antigen (TAA) epitopes. Furthermore, PDT can increase DC maturation and differentiation through increased expression of HSP70 which is internalized by TLR-4 and in turn leading to generation of tumor specific CD8+ T cells that can destroy distant deposits of untreated tumor [7]. This process is however, often sub-optimal, in part due to tumor-induced DC dysfunction and the presence of immune suppression caused by regulatory T cells (Tregs) [18–20]. This phenomenon has initiated a new alternative approach by combining immunostimulants with PDT to potentiate immune recognition of tumor antigens that are released and in turn enhance anti-tumor immunity [21]. A few examples of these combination immunostimulant approaches are; use of cytokine growth factors, various antimicrobial preparations that activate TLR and other receptors for pathogen-associated molecular patterns and approaches targeting Tregs [7, 11]. Recently PDT-mediated DC immunotherapy has been shown to be effective against solid tumors [22] and use of CpG oligodeoxynucleotide (ODN) in conjunction with PDT has been demonstrated to be successful [23]. This enhanced anti-tumor effect was most probably through activation of TLR-9 through CpG in addition to activation of TLR-4 through HSP70 induced by PDT. CpG ODN, synthetic oligonucleotides that contain unmethylated CpG dinucleotides and a phosphorothioate or chimeric backbone that renders them nuclease resistant, are the potent stimulators of innate immune system [24, 25]. CpG DNA stimulates immune cells regardless of whether the DNA is in the form of genomic bDNA or in the form of synthetic ODN [26]. The level of immune stimulatory effects of an ODN depends to a

great degree on the precise bases flanking the CpG dinucleotide [27]. The resultant antigen-specific immunity is characterized by the production of high-affinity antibodies and the generation of cytotoxic T cells that provide long-lasting protection. The primary receptor for CpG ODN is pathogen associated molecular pattern recognition receptor called TLR9 [28]. TLR9 interacts with MyD88, which recruits IL-1 receptor associate kinase 4 [27]. CpG ODN induces T-helper-1 biased complex immune response by both direct activation of TLR9 and indirect effects resulting in the activation of DC, natural killer cells, T cells, B cells and macrophages which in turn leads to secretion of cytokines [29–31]. Multiple studies have demonstrated the potential use of CpG ODN in wide variety of diseases including allergic asthma [32] and cancer [33, 34]. However, as of today, in vivo effects of CpG in combination with PDT have not been investigated. Among all the cells activated by CpG ODN, DC are the most potent antigen-presenting cells (APC) for naive T cells, due to their high expression of MHC class 2 (MHC II) and co-stimulatory molecules such as CD80 and CD86 [35]. The two well-established maturation states for DC are known as the “immature” and “mature” states [36]. In the absence of inflammation, the DC remain in an immature state expressing low levels of MHC II, CD80 and CD86. At this stage, DC are known as “tolerogenic” and antigens are presented to T cells in the draining lymph node without co-stimulation, leading to either the deletion of the T cells or to the generation of inducible regulatory T cells. Mature DC on the other hand, known as “immunogenic”, allow the priming of CD4⁺ T helper cells and CD8⁺ cytotoxic T lymphocytes, the activation of B cells and the initiation of an adaptive immune response. The decision “to mature” is heavily influenced by the signals coming from the environment. The tumor microenvironment not only fails to provide pro-inflammatory signals needed for efficient DC activation, but also provides additional immunosuppressive mechanisms mediated by cytokines such as interleukin 10 (IL-10), transforming growth factor beta (TGF- β) and vascular endothelial growth factor (VEGF). These factors inhibit the differentiation of DC leading to decreased levels of functionally competent, mature APC, and accumulation of immature DC (iDC) that are unable to up-regulate MHC II or co-stimulatory molecules or produce appropriate cytokines [37]. The application of DC based vaccines, based on the concept that the host immune system can effectively attack and lyse tumor cells, is an emerging potent form of immunotherapy for cancer treatment.

In the present study, we used orthotopic metastatic 4T1 breast tumors growing in the mammary fat pad in BALB/c mice that represents a model of stage III breast cancer [38]. We used liposomal benzoporphyrin derivative mono-acid ring A (verteporfin, BPD) as a PS and delivered light after 15 minutes for a vascular PDT regimen and tested PDT alone or in combination with immunomodulation approach that consisted of CpG-ODN injected peritumorally three times (once every 2 days) before or after PDT.

2. Materials and methods

2.1 Materials

BPD was a kind gift from, QLT Inc, Vancouver, BC, Canada. The lyophilized powder was reconstituted with 5% dextrose before injection. CpG ODN was obtained from Coley Pharmaceutical Group (Wellesley, MA). Recombinant lyophilized murine GM-CSF was

ordered from R & D Systems (Minneapolis, MN USA) and reconstituted in PBS with 0.1% serum albumin and stored at -20°C . The recombinant lyophilized murine IL-4 was ordered from Peprotech Inc. (Rocky Hill, NJ) and reconstituted in water and stored in aliquots in -20°C . The PE-conjugated anti-CD11c antibody, FITC conjugated anti-mouse CD86, anti-mouse CD80 and anti-mouse-MHC class II antibodies were obtained from BD Pharmingen. Anti-CD3 antibody (Abcam; ab5690); anti-rabbit secondary antibody conjugated to cyanine 3 (Cy3) and normal rabbit IgG (Jackson ImmunoResearch, Westgrove, PA); VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA).

2.2 Isolation of dendritic cells from bone marrow

The research protocol of this study was approved by the Institutional Animal Care and Use Committee of the Massachusetts General Hospital and was in accordance with the guidelines of the National Institutes of Health. The BALB/*c* mice (7 weeks old, male) were used for isolation of bone marrow. Mice were euthanized and the long bones of front and hind limbs were harvested and the bone marrow cavities were washed with the 29 gauge needle and RPMI 1640 medium. The harvested cells were centrifuged at 300 g for 10 minutes and the resulting pellet was resuspended in ice cold ACK buffer (0.15 M NH_4Cl , 10 mM KHCO_3 and 0.1 mM EDTA, pH 7.35) to lyse erythrocytes. After another round of washing, the cells were counted, resuspended and cultured (final concentration of $1 \times 10^7/\text{ml}$) in 5 cm Petri dishes containing RPMI 1640 medium for 2 hours. Then the medium was replaced gently to remove the non-adherent lymphocytes. The GM-CSF at 10 ng/ml/ 10^6 cells was added together with the 50 ng/ml/ 10^6 cells of IL-4. Cells were incubated overnight at 37°C . On day 1 the non-adherent cells were removed and the cells transferred to fresh Petri dishes and incubated overnight. On day 3 half of the medium in the Petri dish was replaced with the fresh medium containing GM-CSF and IL-4. On day 5 of the experiment the non-adherent cells were again discarded and the adherent cells were counted and used for further experiments.

2.3 Incubation of DC with supernatants from PDT treated 4T1 cells and CpG-ODN

The BALB/*c* mammary adenocarcinoma 410.4 sub-line of 4T1 cells (ATCC, Manassas, VA) were grown in RPMI 1640 media containing 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 $\mu\text{g}/\text{mL}$ streptomycin. The 4T1 cells were washed with PBS without Ca^{2+} and Mg^{2+} , trypsinized, and collected. Next, tumor cells were plated at a concentration of 0.5×10^6 in Petri dishes and allowed 24 h for attachment. On the next day the cells were incubated with 400 nm BPD for 1 h, washed and illuminated with 690 nm light to a total fluence of 5 J/cm². Cells were left in the dishes for 12 h at 37°C . Next the samples were harvested and centrifuged and PDT supernatant was collected for further studies. The DC cultures were incubated with PDT supernatant for 24 h at 37°C . Additionally, lipopolysaccharide (LPS), a TLR 4 agonist generally employed as a positive factor to stimulate and activate DC, alone (0.1 $\mu\text{g}/\text{ml}$) or CpG alone (10 $\mu\text{g}/\text{ml}$) or CpG together with 0.1 ml of PDT lysates were added to 1.0 ml of DC cultures.

2.4 Flow cytometric analysis of DC maturation phenotypes

After 24 hour incubation with supernatant from 4T1 PDT treated tumor cells, the DC were harvested, washed and incubated with fluorescently labeled anti-CD11c, CD80, CD86 or anti-MHC class II monoclonal antibodies for 0.5 h at 37 °C. Flow cytometry analysis was performed on FACS Aria flow cytometer. The cells were first gated for side scatter and forward scatter and then for the expression of CD11c. Next among the CD11c population the percentage of cells positive for CD80, CD86 or MHC II in each tested group was determined.

2.5 Animal model of breast cancer

Female BALB/*c* mice, weighing 20–25 g were depilated on one mammary fat pad. 1×10^6 4T1 cells suspended in 100 μ L PBS were injected in one mammary fat pad. Tumors grew as expected and reached a size of 5–6 mm diameter in 12 days after injection at which time they were used for PDT. Some of animals were euthanized at day 20 to examine the metastases in the distant organs.

2.6 PDT protocol for breast cancer treatment

Mice were anesthetized with an intraperitoneal injection of ketamine/xylazine cocktail (90 mg/kg ketamine, 10 mg/kg xylazine) and BPD at 2 mg/kg was injected in 0.1 mL of PBS in the lateral tail vein. Tails were warmed in water (50 °C) to facilitate the procedure. PDT was carried out after 15 minutes of BPD injection. Illumination was carried out using a 1 W solid state diode laser (High Power Devices, Newark, DE) emitting light at 690 nm for activation of BPD. The light spot had a diameter of 0.8 cm and was positioned so that the entire tumors were exposed to light. Animals were anesthetized as described above and the tumor bearing breast positioned under the spot. A total fluence of 150 J/cm² was delivered at a fluence rate of 100 mW/cm². At the completion of the illumination mice were allowed to recover in an animal warmer until they resumed their normal activity.

2.7 CpG and PDT combination

Peri-tumoral injection of 20 μ g CpG-ODN/mouse (solution concentration 10 μ g/100 μ L, $4 \times 50 \mu$ L aliquots around the tumor) administered. At day 12 after initial injection of cells two possible treatments were done: CpG-ODN administered in 3 doses (Day 12, 15, 18) and PDT carried out on day 18. The second one was PDT on day 12 and CpG-ODN on days 12, 15, and 18. The total dose of CpG-ODN per mouse was 60 μ g.

2.8 Animal follow-up

Mice were examined and weighed three times a week. Two orthogonal tumor dimensions (*a* and *b*) were measured with Vernier calipers and the volume calculated according to the formula $= 4/3\pi [(a + b)/2]^3$. Mice were sacrificed according to protocol when their primary tumors reached a volume of 2000 mm³ or when they reached a moribund state (loss of >15% body weight) due to uncontrolled progression of metastatic disease. In some cases mice were sacrificed 5 days after the last treatment (PDT or last injection of CpG respectively) and tumors were removed and fixed in formalin for immunofluorescence staining.

2.9 Histology and immunofluorescence

The formalin fixed tumors were embedded in paraffin. Thin sections (5 μ m) were obtained using microtome. Sections were stained with hematoxylin and eosin (H & E) for histological studies. Immunohistological studies were performed as per following protocol. After deparaffinization and rehydration, antigen retrieval was performed prior to immunostaining. Sections were incubated for 2 h in block/permeabilizing solutions containing PBS, 0.25% Triton X-100, 0.1 bovine serum albumin (BSA), and 5% (v/v) goat serum at room temperature. The slides were subsequently incubated with rabbit anti-mouse CD3 antibody (1 : 100 antibodies, PBS, 0.1% Triton X-100, 10 mg/ml BSA and 1% serum) at 4 °C overnight. After washing with PBS containing 0.1% BSA three times for 5 min each, an anti-rabbit Cy3 secondary antibody (1 : 200) was applied to the sections for 1 hour in the dark. Negative controls were run in parallel with normal rabbit IgG. Sections were washed with PBS three times for 5 min. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI). Images were taken within 1 hour of mounting using a confocal microscope (Fluoview FV1000, Olympus America Inc., PA, USA).

2.10 Statistical analysis

Values of all measurements were expressed as mean $\pm$ SD. In order to determine the statistical significance, data was analyzed using GraphPad Prism 5.0 biochemical statistical package (GraphPad Software, Inc., San Diego, CA). Statistical analysis was performed using one way ANOVA test to compare between the groups. Survival analysis was performed using the Kaplan-Meier method. Survival curves were compared, and differences in survival were tested for significance using a log rank test. The tumor growth curves were analyzed by transforming the data to a logarithmic scale and comparing the slopes. Differences at $p < 0.05$ were considered significant.

3. Results

3.1 Effect of PDT treated 4T1 cells together with CpG-ODN on DC activation and maturation

We incubated bone marrow-derived iDC isolated from BALB/c mice with either CpG alone, the supernatant from PDT treated 4T1 cells alone or with the supernatant from PDT treated 4T1 cells combined with CpG. LPS was used as a positive control. Flow cytometric analysis was performed to assess the mature phenotype of DC by measuring the levels of expression of three maturation markers MHC class II, CD80 and CD86. As shown in Figure 1, iDC alone had about 10–20 percent of cells that expressed maturation markers. LPS treatment approximately doubled this percentage. CpG alone had only a slight effect in increasing the percentage of cells that expressed activation markers. However, when PDT treated 4T1 tumor cells supernatant was incubated with iDC there was a marked increase in the percentage of cells that express CD86 and to a lesser extent an increase in the percentage of cells that expressed MHC class II and CD80. However the combination of CpG and PDT killed 4T1 cells supernatant synergistically increased all three makers of DC maturation and activation (MHC class II, CD80 and CD86) more than treatment alone ($p < 0.001$) and up to or above the level of activation achieved with LPS.

3.2 Development of metastatic breast cancer in BALB/c mice

To induce tumors in the wild type BALB/c mice, 1×10^6 4T1 cells were injected in a mammary fat pad. Mice were examined and weighed three times a week and the growth of the primary tumor in the mammary fat pad was measured by caliper. Primary breast tumors developed as expected and reached a size of 5–6 mm diameter in 10–12 days. At day 20 animals were euthanized. As assessed by histology, significant metastasis was observed in lung, liver and spleen (Figure 2).

3.3 Effect of PDT and peri-tumoral CpG-ODN treatment on tumor progression and survival

At day 7, mice with 4T1 tumors were divided into five groups, (i) control (no treatment), (ii) a group treated with BPD-PDT alone, (iii) a group treated with CpG alone, injected in three doses of 20 μ g (200 μ l) distributed in four different intradermal points around the site where the tumor was implanted, CpG was injected on days 12, 15 and 18. The last two groups were treated with a combination of BPD-PDT and CpG, (iv) a group treated on the day 12 with BPD-PDT and subsequently CpG was injected on days 12, 15, and 18; (v) a group treated with CpG on days 12, 15, and 18 and BPD-PDT was carried out on day 18. The results in terms of tumor growth curves (Figure 3A) and tumor size (Figure 3B) showed significant improvement with PDT treatment which is further improved with combination treatment. Tumor size at day 32 was significantly reduced in treatment groups; control (1338.7 ± 286.5) compared to PDT (1058.2 ± 113.2 mm³) $p < 0.05$, CpG + PDT (861.7 ± 92.5) $p < 0.001$, PDT + CpG (693.4 ± 112.3) $p < 0.001$. However, treatment with CpG alone group did not have any difference in tumor size compared to control group.

The median survival times for groups of mice were as follows (Figure 4): no treatment control: 40 ± 2.20 days; CpG alone: 36 ± 1.89 days; PDT alone: 41 ± 2.16 days; PDT + CpG: 46 ± 1.99 days; CpG + PDT: 43 ± 1.50 days (P for both orders of combination vs. PDT alone < 0.01 ; and P for both orders of combination vs. CpG alone < 0.001).

The results in this series of experiments show that combination of BPD-PDT and CpG immunostimulation regardless of the order of administration is significantly better than either treatment alone both in suppressing the local tumor growth and extending the survival. CpG alone actually appeared to be worse than no treatment in terms of local tumor growth (Figure 3) but significantly better ($P < 0.005$) than no treatment in prolonging survival (Figure 4) presumably due to a weak anti-metastatic effect attributed to immune stimulation.

3.3.1 Infiltration of T-cells into treated tumors—In order to assess if the advantage of combining CpG with PDT over either treatment alone, could be attributed to stimulation of the adaptive immune response, we stained the tumor sections removed 5 days after the last treatment with an antibody that recognizes CD3 on T-cells. We reasoned that it would take several days for the matured DC to migrate to the draining lymph nodes, present their TAA to naïve T-cells to produce antigen specific CD8+ T-effector cells and for the latter to migrate back to the tumor site. Indeed, there was significantly higher number of CD3 expressing cells in the tumor sections from the animals that received combination of BPD-

PDT and CpG immunostimulation treatment, regardless the order of administration, compared to the animals from the control and treatment alone groups as shown in Figure 5.

4. Discussion

To our knowledge this is the first report demonstrating that the combination of PDT with immunoadjuvant CpG treatment significantly prolongs survival and reduces tumor size in a mouse model of metastatic breast carcinoma. Moreover, PDT treated 4T1 cell supernatant along with CpG synergistically increased DC maturation and activation (MHC class II CD80 and CD86) markers.

Human tumors express a number of protein antigens that can be recognized by T cells, thus providing potential targets for cancer immunotherapy [39, 40]. DC are rare leukocytes that are uniquely potent in their ability to present antigens to T cells, and this property has prompted their recent application in therapeutic cancer vaccines [41]. Even though DC vaccines have been shown to activate antitumor immunity, their efficacy is however, still limited [20]. PDT can increase the expression of antigen in the dying tumor through enhanced expression of tumor antigens which can be detected by APC resulting in immune response towards local and metastatic tumor [7]. Studies have shown that CpG-ODN preferentially activates CD8 T cells through induction of DC [42, 43]. Our in vitro results showing that incubation of DC with CpG and PDT treated 4T1 cell supernatants resulted in phenotypic activation and maturation of DC cells led us to be optimistic about in vivo testing. By adding CpG as an immunoadjuvant to PDT in vivo, we expected to obtain increased activation and phenotypic maturation of DC at the tumor site leading to higher CD8 T cell levels and antitumor immunity. In order to investigate this hypothesis, a 4T1 mouse orthotopic mammary breast cancer model with a capacity to metastasize efficiently to sites affected by human breast cancer such as lung, liver and spleen was used [44]. Since this model is syngeneic in BALB/c mice, it provides the ability to study the role of the immune system in tumor growth and metastasis [44].

4T1 is a highly virulent and metastatic BALB/c breast cancer that is considered to be poorly immunogenic. It is also noted for being highly immunosuppressive, so much so that BALB/c mice with 4T1 tumors will allow unrestricted growth of allotrans-plant B16 melanoma tumors that are syngeneic to C57BL/6 mice [45]. Previous studies have extensively investigated the immunology of this tumor focusing its ability to escape from immune control [38, 46]. Myeloid derived suppressor cells (MDSC) are immature myeloid cells that suppress the activation of CD4+ and CD8+ T lymphocytes and thereby inhibit immune surveillance [47, 48]. The accumulation of MDSC in the spleen and blood of tumor-bearing hosts is associated with increased tumor burden. A study by Sinha et al. showed that mice that were never exposed to tumor, have less than 8% Gr1+ CD11b+ cells in their spleens; in contrast, BALB/c with primary 4T1 mammary carcinomas, have 30–60% Gr1+ CD11b+ cells [49]. Macrophages polarized towards an M1 phenotype produce nitric oxide (NO) and are cytotoxic for tumor cells, whereas alternatively activated M2 macrophages produce arginase which facilitates tumor growth and progression [50]. Ostrand-Rosenberg has stated [46, 51] that regulatory T-cells (T-regs) are not involved in the immunosuppressive mechanisms of 4T1 tumors.

Considering the poor immunogenicity of 4T1 we decided to combine PDT with an immune stimulation approach. Our results suggest that CpG-ODN significantly increased bone-marrow DC activation after exposure to PDT-killed tumor cells, and significantly increased tumor response to PDT and mouse survival after peri-tumoral administration. CpG administration, both prior and after PDT, resulted in significantly better treatment outcomes when compared to PDT alone. However when CpG was administered prior to PDT, the outcome was better in terms of control of local tumor growth which was most likely due generation of inflammatory response by attracting the DC to the local tumor site before PDT and in turn enhancing effects of PDT in the area. Another possible mechanism could be changed BPD accumulation profile in the tumor after CpG pretreatment. On the other hand, administering PDT before CpG resulted in more prolonged survival and more pronounced T-cell infiltration into the tumor. This may be explained by CpG-induced accumulation of DC at the tumor treatment site, and these DC would be expected to be subsequently killed by PDT. When the CpG is injected after the PDT the DC are attracted at an appropriate time to phagocytose PDT induced tumor debris and moreover the DC are safe from destruction. The photosensitizer BPD that we have used has been shown to localize in the vasculature when injected 15 minutes prior to PDT [19]. It has been shown that the vascular type of PS work by shutting down the vascular supply to the tumor area, which could also result in restricted access of immune cells to the local tumor site. For this reason, CpG was injected peri-tumorally to attract DC to the tissue in the tumor periphery.

In summary, these data suggest that CpG can prime iDC to recognize and phagocytose PDT killed tumor cells, and that this phagocytosis can lead to DC maturation and activation. The improved tumor specific T-cell resulting can control both local and systemic tumor spread. Therefore, CpG may be a valuable immunoadjuvant to PDT both in terms of local tumor growth control as well as survival.

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Photomicrographs showing immunohistochemical expression of CD3 in thin sections.

Figure 2.

Detection of metastasis with 4T1 tumor. Female BALB/*c* mice, weighing 20–25 g were depilated on one mammary fat pad. 1×10^6 4T1 cells suspended in 100 μ L PBS were injected in one mammary fat pad. Animals were euthanized at day 20 to examine the metastases in the distant organs. Tissues were harvested and processed for histological examination with HE staining in (A) liver (B) lung (C) spleen (D) abdominal muscle (E) skin. Scale bar 100 μ M. ($N = 3$).

Figure 3.

(A) Tumor growth curves and (B) Tumor volume of mice with 4T1 tumors after receiving either; no treatment, BPD-PDT alone, CpG alone in 3 injections, or CpG combined with BPD-PDT either before or after: Female BALB/c mice, weighing 20–25 g were depilated on one mammary fat pad. 1×10^6 4T1 cells suspended in 100 μ L PBS were injected in one mammary fat pad. Two orthogonal tumor dimensions (a and b) were measured with Vernier calipers and the volume calculated according to the formula $= 4/3\pi [(a + b)/2]^3$. P value * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ ($N = 5$).

Figure 4.

Kaplan-Meier curves of mouse survival: Female BALB/*c* mice, weighing 20–25 g were depilated on one mammary fat pad. 1×10^6 4T1 cells suspended in 100 μ L PBS were injected in one mammary fat pad and animals were followed for Survival time. Control 40 ± 2.20 days; CpG alone: 36 ± 1.89 days; PDT alone: 41 ± 2.16 days; PDT + CpG: 46 ± 1.99 days; CpG + PDT: 43 ± 1.50 days (P for both orders of combination vs. PDT alone <0.01 ; and P for both orders of combination vs. CpG alone <0.001) ($N = 5$).

Figure 5.

Photomicrographs showing immunohistochemical expression of CD3 in thin sections from (A) normal mouse (B) untreated tumor (C) CpG alone treatment (D) PDT alone treatment (E) PDT + CpG treatment (F) CpG + PDT treatment. Female BALB/*c* mice, weighing 20–25 g were depilated on one mammary fat pad. 1×10^6 4T1 cells psuspended in 100 μ L PBS were injected in one mammary fat pad. Paraffin section from tumor site were stained using anti-CD3 antibody. DAPI was used to stain the nuclei. Significantly higher numbers of CD3 expressing cells were seen in treatment groups which received combination (both CpG and PDT) therapy. Scale bar 100 μ M.