Stimulation of dendritic cells enhances immune response after photodynamic therapy

Citation: Mroz, Pawel, Ana P. Castano, and Michael R. Hamblin. "Stimulation of dendritic cells enhances immune response after photodynamic therapy." Biophotonics and Immune Responses IV. Ed. Wei R. Chen. San Jose, CA, USA: SPIE, 2009. 717803-10. © 2009 Society of Photo-optical Instrumentation Engineers.

As Published: http://dx.doi.org/10.1117/12.809630

Publisher: Society of Photo-optical Instrumentation Engineers

Persistent URL: http://hdl.handle.net/1721.1/54779

Version: Final published version: final published article, as it appeared in a journal, conference proceedings, or other formally published context

Stimulation of dendritic cells enhances immune response after photodynamic therapy

Pawel Mroz[1,2], Ana P Castano[1,2], Michael R Hamblin[1,2,3*]

[1] Wellman Center for Photomedicine, Massachusetts General Hospital, Boston MA
[2] Department of Dermatology, Harvard Medical School, Boston, MA
[3] Harvard-MIT Division of Health Sciences and Technology, Cambridge, MA
[*] corresponding author: BAR414, 40 Blossom Street, Boston, MA. 02114; hamblin@helix.mgh.harvard.edu

ABSTRACT

Photodynamic therapy (PDT) involves the administration of photosensitizers followed by illumination of the primary tumor with red light producing reactive oxygen species that cause vascular shutdown and tumor cell necrosis and apoptosis. Anti-tumor immunity is stimulated after PDT due to the acute inflammatory response, priming of the immune system to recognize tumor-associated antigens (TAA). The induction of specific CD8+ T-lymphocyte cells that recognize major histocompatibility complex class I (MHC-I) restricted epitopes of TAAs is a highly desirable goal in cancer therapy. The PDT killed tumor cells may be phagocytosed by dendritic cells (DC) that then migrate to draining lymph nodes and prime naïve T-cells that recognize TAA epitopes. This process is however, often sub-optimal, in part due to tumor-induced DC dysfunction. Instead of DC that can become mature and activated and have a potent antigen-presenting and immune stimulating phenotype, immature dendritic cells (iDC) are often found in tumors and are part of an immunosuppressive milieu including regulatory T-cells and immunosuppressive cytokines such as TGF-beta and IL10. We here report on the use of a potent DC activating agent, an oligonucleotide (ODN) that contains a non-methylated CpG motif and acts as an agonist of toll like receptor (TLR) 9. TLR activation is a danger signal to notify the immune system of the presence of invading pathogens. CpG-ODN (but not scrambled non-CpG ODN) 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 may be a valuable immunoadjuvant to PDT especially for tumors that produce DC dysfunction.

Keywords: CpG oligodeoxynucleotide, dendritic cells, toll like receptors, anti-tumor immunity, photodynamic therapy

1. INTRODUCTION

1.1 PDT, cancer and the immune system.

Despite major efforts in cancer research, cancer is still the leading cause of death in Western countries. Deaths are largely due to tumors that have metastasized despite local control. Photodynamic therapy (PDT) is a promising cancer treatment in which a photosensitizer (PS) accumulates in tumors and is subsequently activated by visible light of an appropriate wavelength [1-3]. The energy of the light is transferred to molecular oxygen to produce reactive oxygen species that produce cell death and tumor ablation. Mechanisms include direct cytotoxicity to tumor cells, shutting down of the tumor vasculature, and the induction of a host immune response. The precise mechanisms involved in the PDT-mediated induction of anti-tumor immunity are not yet understood. Among the potential contributing factors are alterations in the tumor microenvironment via stimulation of proinflammatory cytokines and direct effects of PDT on the tumor that increase immunogenicity.

PDT is an anti-cancer modality that can efficiently destroy local tumors in the context of acute local inflammation. PDT can increase DC maturation and differentiation and leads to generation of tumor specific CD8 T cells that can destroy distant deposits of untreated tumor [4]. Successful PDT of tumors growing in immunocompetent syngeneic mice can in some cases cause a long-term memory anti-tumor immunity as demonstrated by a resistance to a

Biophotonics and Immune Responses IV, edited by Wei R. Chen,
Proc. of SPIE Vol. 7178, 717803 · © 2009 SPIE
CCC code: 1605-7422/09/$18 · doi: 10.1117/12.809630

rechallenge with the tumor from which they were cured, but not a different syngeneic tumor [5-7]. This effect is not observed when the same tumors are grown in immunosuppressed nude or SCID mice [8]. Splenocytes adoptively transferred from immunocompetent mice cured of tumors by PDT can restore the curative effect of PDT in immunosuppressed animals, and demonstrate specific lysis of tumor cells growing in vitro in a classical CTL assay. Tumor cells killed with PDT in vitro are more effective as tumor vaccines than the same cells killed by other methods [9]. PDT has effects on cancer cells that make immune activation more likely in an in vivo tumor treated with PDT. PDT can induce strong expression of heat shock proteins (especially HSP70) [10-12] that has been shown to potentiate immune recognition of tumors. PDT can cause activation of the transcription factors nuclear factor kappa B (NFκB) [13] and activator protein (AP)-1 [14] leading to production of a large variety of inflammatory mediators including eicosanoids and interleukins (IL) 1, 6, 8 and 10. Neutrophils are an important cell type for the PDT response [15] and if mice are depleted of neutrophils before PDT, the curative effect is lost [16]. PDT has been shown to induce both a systemic neutrophilia and a strong and prolonged tumor infiltration by neutrophils. In addition tumor infiltration by dendritic cell (DC), macrophages and mast cells has been observed [17]. Complement activation is also observed both in the tumor and serum after PDT [18]. Nevertheless, it is clear that although PDT has the potential to stimulate a systemic anti-tumor immune response in animal models of cancer, this favorable result is not always observed. The explanation for this observation may be due to variations in the immunogenicity of different syngeneic mouse tumors, the presence of immune suppression caused by Tregs or the existence of DC dysfunction caused by the tumor.

1.2 Dendritic cells in cancer.

Several lines of evidence have pointed to DC as critical players in the balance between T cell tolerance vs. T cell priming. The two well-established maturation states for DC include the "immature" and "mature" states [19]. Immature DC (iDC) constantly sample their environment, capture antigens and migrate in small numbers to draining lymph nodes. They display a phenotype reflecting their specialized function as antigen-capturing cells. They are highly endocytic, able to acquire fluid-phase antigens by macropinocytosis, take up protein or antigen-antibody immune complexes by receptor-mediated endocytosis, and ingest entire cells by phagocytosis. They express relatively low levels of surface MHC-I and MHC-II gene products and costimulatory molecules such as CD80 and CD86. In the absence of inflammation, the DC remain in an immature state, and antigens are presented to T cells in the lymph node without co-stimulation, leading to either the deletion of T cells or the generation of inducible regulatory T cells.

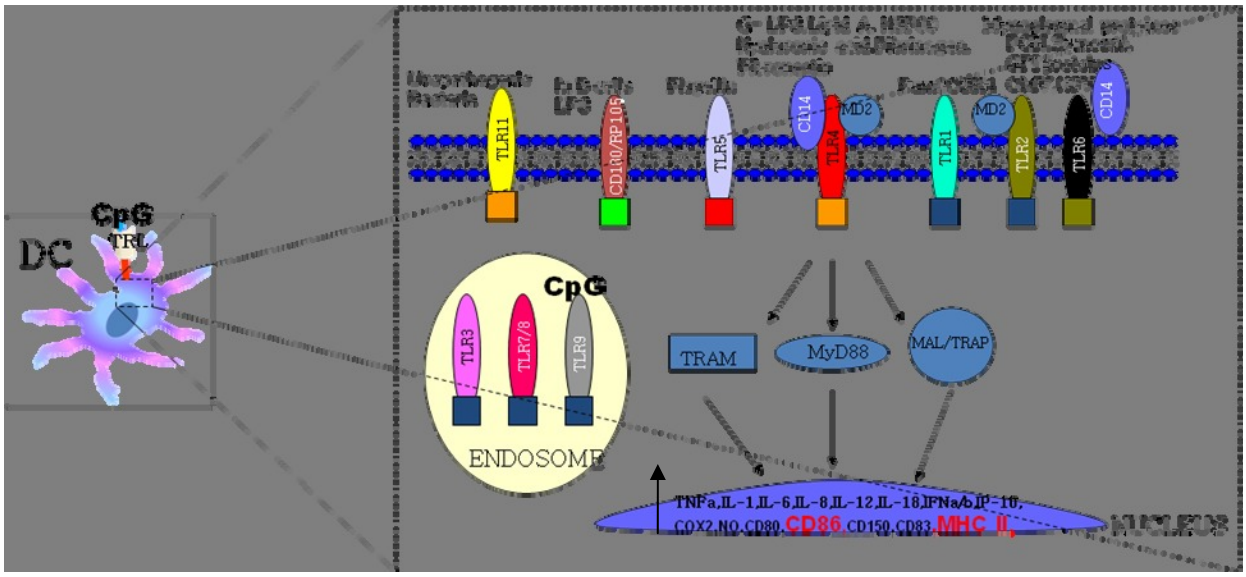

Figure 1. Activation of TRL signaling by various ligands in DC.

Tissue inflammation induces the maturation of DCs and the migration of large numbers of mature DCs to draining lymph nodes. The mature DCs express peptide–MHC complexes at the cell surface, as well as appropriate co-stimulatory molecules. This allows the priming of $CD4^+$ T helper cells and $CD8^+$ cytotoxic T lymphocytes (CTLs), the activation of B cells and the initiation of an adaptive immune response. The decision "to mature" is hugely 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 like IL10 and VEGF. These factors inhibit the differentiation of DC leading to decreased levels of functionally competent, mature antigen-presenting cells, and accumulation of iDC that are unable to up-regulate MHC class II and co-stimulatory molecules or produce appropriate cytokines [20].

The innate immune response is a first-line defense system in which individual Toll-like receptors (TLRs) recognize distinct pathogen-associated molecular pattern (PAMP) molecules that are expressed by a diverse group of infectious microorganisms [21] and thereafter stimulate immune responses against a variety of pathogens .The extracts of the attenuated mycobacterium bacillus Calmette Guerin (BCG) have been used as a therapy for human bladder cancer [22]. It was discovered that the active component of BCG was DNA with a potential to activate natural killer (NK) cells and induce tumor regression in mice [23]. Yamamoto et al sequenced mycobacterial genes and synthesized constituent oligodeoxynucleotides (ODN) [24]; they concluded that certain self-complementary palindromes in these ODN were responsible for the immune stimulatory effects. The active palindromes contained at least one CpG dinucleotide and were more common in the genomes of bacteria compared to humans. Immunostimulatory sequences in bacterial (bDNA) that are structurally defined by their content of unmethylated CpG motifs (5'-purine-purine/T-CpG-pyrimidine-pyrimidine-3') are underrepresented in mammalian DNA [25]. CpG DNA stimulates B cells, natural killer (NK) cells, dendritic cells (DC), and macrophages, 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. TLR 9 recognizes d(CpG) dinucleotides present in specific sequence contexts (CpG motifs) in bacterial DNA, plasmid DNA, and synthetic oligodeoxynucleotides . TLR9 interacts with MyD88, which recruits IL-1 receptor associate kinase 4 [27]. CpG DNA has strong stimulatory effects on murine and human lymphocytes in vitro and murine lymphocytes in vivo, such as: triggering B cell proliferation, release of IL-6 and IL-12; natural killer cell secretion of IFN-γ and increased lytic activity; and macrophage secretion of IFN-α/β, IL-6, IL-12, granulocyte-monocyte colony-stimulating factor, chemokines, and TNF-α.

We used 4T1 tumors growing in the mammary fat pad (orthotopic) in BALB/c mice that represents a model of stage III breast cancer. We used liposomal benzoporphyrin derivative mono-acid ring A, (BPD) as a PS and delivered light after 15 minutes for the PDT regimen and we tested PDT alone or in combination with immunomodulation approache that consisted of CpG-ODN injected intratumorally three times before or after PDT. Our hypothesis was to allow the immune system to be more effective against the tumor by increasing the effectiveness of the immune system by a multifunctional immunostimulant such as CpG-ODN.

2. MATERIALS AND METHODS

2.1 Chemicals and reagents

BPD (liposomal benzoporphyrin derivative mono-acid ring A, Verteporfin for Injection) was a kind gift from, QLT Inc, Vancouver, BC, Canada. The lyophilized powder was reconstituted with 5% dextrose before injection. CpG ODN and control non CpG ODN were obtained from Coley Pharmaceutical Group (Wellesley, MA); these are ODN consisting of 20 bases and have two CpG motifs. The ODN used was: 1826 Class B. Sequence. A non- CpG Control ODN was, 2138 Class B (control). 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 rat anti-mouse CD86 antibody and anti-mouse -MHC class II were obtained from BD Pharmingen .

2.2 Isolation of bone marrow derived dendritic cells.

On day 0 of the experiment 6 weeks old BALB/c mice 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 was washed from bone marrow cavities with the 29 gauge needle and RPMI. The harvested cells were centrifuged at 300g for 10 minutes and the resulting pellet was resuspended in ice cold AKC buffer to lyse erythrocytes. After another round of washing the cells were counted, resuspended and transferred to 10 cm Petri dishes at the final concentration of $1x10^7$/ml. To each Petri dish GM-CSF at 20ng/ml/10^6 cells was added together with the 10ng/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 Preparation of PDT treated 4T1 cells.

The BALB/c mammary adenocarcinoma 410.4 sub-line 4T1 cells were grown in RPMI 1640 media containing HEPES, glutamine, 10% fetal calf serum (FCS), 100 U/mL penicillin and 100 μg/mL streptomycin. They were collected for injection by washing with PBS without Ca^{2+} and Mg^{2+}, and adding trypsin-EDTA to the plate for 10 minutes at 37°C.

Tumor cells were plated at the concentration of $0.5x10^6$ in Petri dishes and allowed 24h for attachment. On the next day the cells were incubated with 400-nm BPD for 1h, washed and illuminated with 690nm light to a total fluence of 5J/cm^2. Cells were left in the dishes for 48 h at 37°C. Next the samples were harvested and centrifuged and PDT lysates were used for further studies.

2.4 Incubation of bone marrow derived DC with supernatants from PDT treated tumor cells.

The PDT lysates were harvested 48h after PDT of 4T1 tumors were added to previously prepared DC. DC were incubated with supernatants for 24h at 37 degrees of C. Additionally LPS alone (1ug/ml) or CpG alone (1 ug/ml or 10 ug/ml) or CpG together with 4T1 were added to DC cultures.

2.5 Analysis of DC maturation phenotype.

After 24h incubation with supernatants from 4T1 PDT treated tumor cells the DC were harvested, washed, fixed and incubated with fluorescently labeled anti-CD11c and anti-CD86 or anti-MHC class II monoclonal antibodies overnight in 4 degrees. Flow cytometry analysis was performed on FACSAria 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 either CD86 or MHC II in each tested group was determined.

2.6 Animal model of metastatic breast cancer.

All animal experiments were carried out with the approval of the Subcommittee on Research Animal Care of Massachusetts General Hospital and were in accordance with the NIH Guide for the Care and Use of Laboratory Female BALB/c mice, weighing 20-25 g were depilated on one mammary fat pad. One million 4T1 cells were injected in one mammary fat pad suspended in 100 μL PBS. Tumors grew as expected and reached a size of 5-6-mm diameter in 10-12 days after injection at which time they were used for PDT.

2.7 PS and light source for PDT.

Mice were anesthetized with an i.p. injection of ketamine/xylazine cocktail (90mg/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 at 15 minutes after injection for BPD. Illumination was carried out using a 1W solid state diode laser (High Power Devices, Newark, DE) emitting light at 690-nm (+/- 2-nm) for activation of BPD. The laser was coupled into a 400-μm fiber via a SMA connector and light from the distal end of the fiber was focused into a uniform spot with an objective lens (No 774317, Olympus, Tokyo, Japan). The spot had a diameter of 1.2 cm and was positioned so that the entire tumor and a surrounding 2-3 mm of normal tissue were exposed to light. Mice 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.

2.8 Immunostimulants

ODN concentrations in the final solution were 10 µg/100µl and 20 µg per mouse was injected peritumorally. After the tumor reached 5 mm diameter two possible treatments were done: CpG-ODN administered in 3 doses (Day 1, 4, 7) and PDT carried out on day 7. The second one was PDT day 1 and CpG-ODN on days, 1, 4, and 7. The total dose of CpG ODN per mouse was 60 µg.

2.9 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 would be considered cured when the tumor did not return after 90 days. Mice were sacrificed according to protocol when their primary tumors reached a volume of 200 mm^3 or when they reached a moribund state (loss of >15% body weight) due to uncontrolled progression of metastatic disease.

2.10 Data analysis and statistics.

All values are expressed as ± standard error of the mean. Comparison between two means was carried out using the Mann-Whitney U-test. 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 in the computer program GraphPad Prism (GraphPad Software Inc., San Diego, CA). The tumor growth curves were analyzed by transforming the data to a logarithmic scale and comparing the slopes. P values of < 0.05 were considered significant.

3. RESULTS

3.1 Model of metastatic breast cancer in BALB/c mice.

The 4T1 mammary carcinoma tumor is a poorly immunogenic and highly malignant tumor that rapidly and spontaneously metastasizes to lymph nodes, lung, liver, brain, and bone and is disseminated via the blood stream following growth of the primary tumor in the female mouse mammary gland [28, 29] (Figure 2). This disease progression closely parallels human breast cancer and makes the 4T1 tumor an excellent model for human disease [30] and a rigorous animal model of advanced spontaneous metastatic disease.

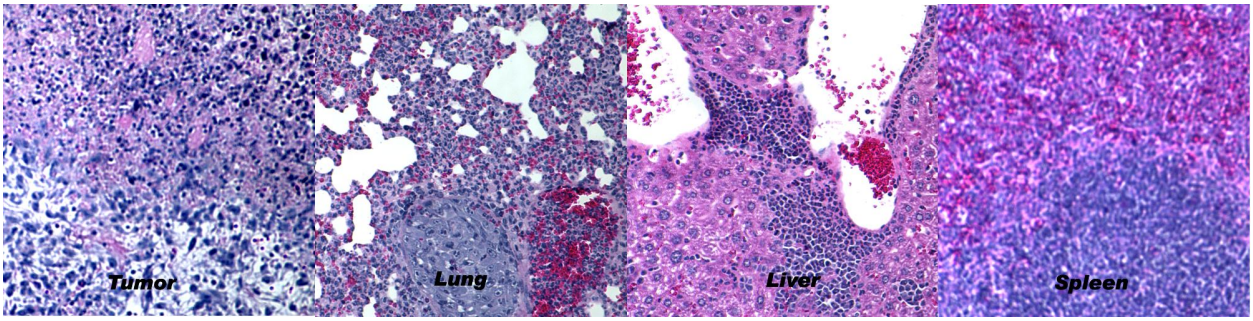

Figure 2. H&E stained paraffin fixed sections of tissues from mice with 4T1 tumors. There are metastases after twenty days, in the liver, spleen, and lung.

3.2 PDT treated 4T1 cells together with CpG mature and activate DC.

We incubated bone marrow derived immature DC isolated from BALB/c mice with CpG (or with LPS as a positive control) and with either PDT killed 4T1 cells alone or with PDT treated 4T1 cells combined with CpG. We used flow cytometry analysis to assess the mature phenotype of DC by measuring the levels of expression of two maturation markers MHC class II molecules and CD86. The results shown in Figure 3 indicate that iDC alone had

about 10% of cells that expressed maturation markers. LPS (a toll like receptor 4 agonist generally employed as a positive factor to stimulate and activate DC) approximately doubled this percentage. CpG alone had only a slight effect in increasing the percentage of cells that expressed activation markers. When PDT killed 4T1 tumor cells were 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. However the combination of CpG and PDT killed 4T1 cells synergistically increased both makers of DC maturation and activation (MHC class II and CD86) more than either treatment alone and up to or above the level of activation achieved with LPS. 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.

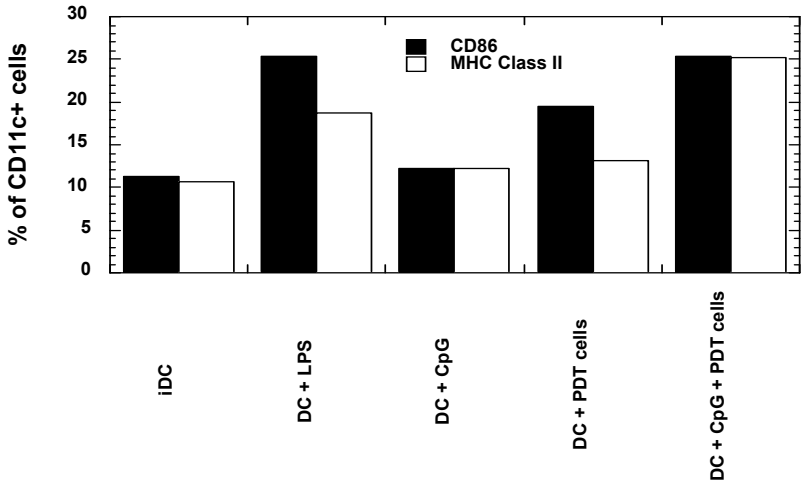

Figure 3. Percentages of iDC (gated for CD11c) that express maturation markers (CD86 and MHC class II) after indicated treatments.

3.3 Combination of PDT and intratumoral CpG oligonucleotides.

Mice with 4T1 tumors at day seven were distributed in 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 μg (200 μl) distributed in four different intradermal points around the site where the tumor was implanted, counting first day as the day of treatment, CpG was injected on days 1, 4 and 7. The last two groups were treated with a combination of BPD-PDT and CpG, (iv) a group of mice was treated the first day with BPD-PDT and subsequently CpG was injected at day 1, 4, and 7; (v) a group treated with CpG on day 1, 4, and 7 and BPD-PDT carried out on day 7. The results in terms of tumor growth curves are shown in Fig 4A and the survival curves are given in Fig 4B. The median survival times for groups involving CpG were CpG alone: 32 days; PDT + CpG: 53 days; CpG + PDT: 55 days (P for both orders of combination vs PDT alone < 0.0001; and P for both orders of combination vs CpG alone < 0.0001).The results in this series of experiments show that combination of BPD-PDT and CpG immunostimulation regardless of 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 (Fig 4A) but significantly better ($P < 0.005$) than no treatment in prolonging survival (Fig 4B) presumably due to a weak antimetastatic effect.

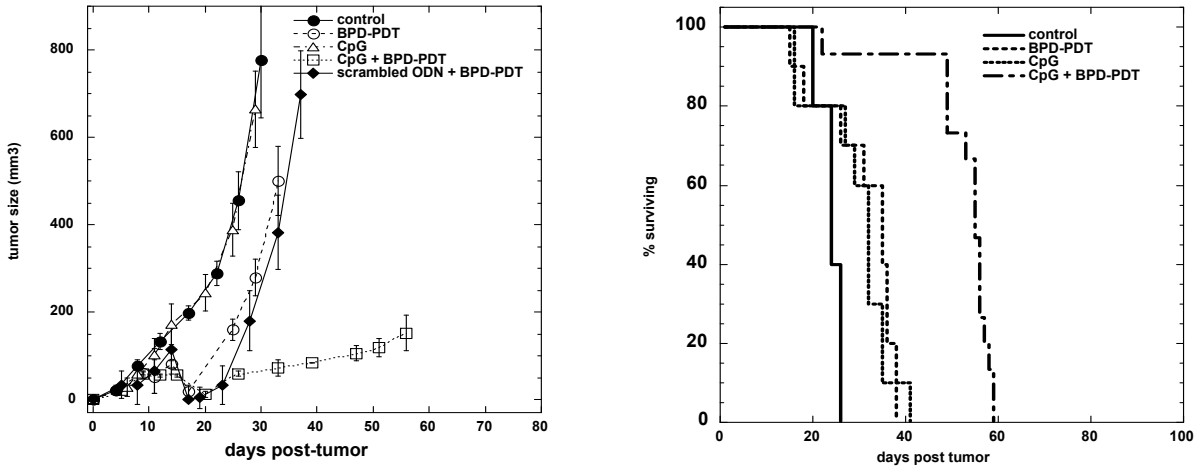

Figure 4. A. Tumor growth curves of mice with 4T1 tumors receiving either; no treatment, BPD-PDT alone, CpG alone in 3 injections, or CpG combined with BPD-PDT either before or after. B. Kaplan-Meier curves of mouse survival of the treatment groups described in Fig 2A. Survival times were: CpG alone: median 32 days; PDT + CpG: 53 days; CpG + PDT: 55 days (P for both orders of administration vs PDT alone < 0.0001; and P for both orders of administration vs CpG alone < 0.0001).

4. DISCUSSION

Antigen presenting cells (APC) possess the unique ability to induce primary immune responses. They capture and transfer the information from the outside world to the cells of the adaptive immune system. One of their most characteristic features is the expression of the MHC class II molecules and the ability to present exogenous antigens to the T helper lymphocytes. Among professional APCs there are DC, macrophages and B lymphocytes. Dendritic cells are the key players among all APC in the process of the induction of immune response. They can effectively acquire the antigens, process them and present in the context of MHC class II molecules. They express Toll-like receptors as well as costimulatory molecules necessary for successful DC – T cells interactions. They can travel to the lymph nodes and interact with T cells to initiate the primary immune response. Therefore the attempts have been made to understand the role and utilize the abilities of DC in the PDT mediated immunity.

To study the unique interaction between PDT mediated killing of tumor cells and DC activation we have prepared supernatants from 4T1 treated cells and incubated fresh, bone marrow derived DC to assess the maturation phenotype. In some cases we also added simultaneously CpG to further boost the activation status of DC. We have observed that the DC incubated with both CpG and PDT killed 4T1 cell supernatants obtained activation and maturation status comparable or even surpassing levels obtained after incubation with LPS. It is interesting that, despite many publications describing the synergistic activation of DC by PDT and toll like receptor ligands, there are not that many reports investigating the observed effects in vitro. However some groups reported the increased expression of heat shock proteins on the surface of PDT treated cancer cells. Extracellular HSP70 binds to high-affinity receptors on the surface of the APCs, leading to the activation and maturation of DCs, a process that enables the cross-presentation of the peptide antigen cargo of HSP70 by the APC to CD8 cytotoxic T-cells [31]. PDT-mediated by three different PS increased HSP70 mRNA, but only mono-L-aspartyl chlorin-e6 and tin etio-purpurin but not Photofrin increased HSP70 protein levels in mouse tumor cells *in vitro* and in tumors *in vivo* [10]. The release of HSP-bound tumor antigens that can easily be taken up by APC, from PDT-induced necrotic tumor cells may therefore explain the particular efficiency of PDT in stimulating an immune response against tumors.

A related approach has been implemented by Gollnick et al [9]. This group compared the cancer vaccine potential of PDT-generated cell lysates (EMT6 and P815 tumor cells) compared to lysates generated by UV or ionizing irradiation. PDT-generated lysates were able to induce phenotypic DC maturation and IL-12 expression. Korbelik and Sun [32] produced a vaccine by treating SCCVII cells with BPD-PDT and later with a lethal X-ray dose, and showed that these cells, when injected peritumorally in mice with established SCCVII tumors, produced a significant therapeutic effect, including growth retardation, tumor regression and cures. Importantly, vaccine cells retrieved from the treatment site at 1-h postinjection were intermixed with dendritic cells (DCs), exhibited HSP70 on their surface, and were opsonized by complement C3. This observation verifies some of the earlier findings in mouse models and *in vitro*.

BPD-PDT only slightly delayed the progression of 4T1 tumors. It is known that BPD is a vascular photosensitizer, and can destroy the tumor microcirculation therefore leading to apoptosis and necrosis of the tumor cells. PDT can increase the expression of antigens in the dying tumor, with the possibility that antigen-presenting cells could recognize these tumor antigens and consequently increase the immune response towards the local tumor and/or distant metastases. However the relative lack of major tumor destruction by PDT alone in this model of orthotopic mammary fat-pad 4T1 tumors makes this mechanism less likely. This model of breast cancer is stage III, at the time of the treatment the tumor has already metastasized and therefore an approach is needed that has both a local effect and a distant or systemic effect. Therefore we decided to combine PDT with immune stimulation regimens.

The use of CpG-ODN in combination with BPD-PDT had a bigger impact in the treatment of this model of breast cancer. There was a significant delay in the local tumor progression and a corresponding increasing in the survival of the mice. One important question to answer in designing any combinatory therapy is the order of administration of the respective component treatments. We had supposed that a chief role of CpG ODN is to attract host immune cells such as dendritic cells, natural killer cells and macrophages, into the tumor and surrounding tissue [33]. Therefore CpG ODN should perform better if administered before PDT rather than afterwards, because PDT can shut down the blood vessels thus preventing leukocyte access into the tumor after PDT. However if the chief role of CpG ODN is to potentiate the phagocytosis of necrotic or apoptotic tumor cells by already present dendritic cells and to induce dendritic cell maturation and migration to draining lymph nodes [34], then the reverse order (i.e. CpG ODN after PDT) may be superior because the PDT induced damage will be there for the DCs to take up when stimulated with CpG. The results showed that both orders of administration were significantly better than either treatment alone. CpG first and PDT afterwards gave a somewhat better control of local tumor growth but somewhat less of a survival advantage, compared to PDT first and CpG afterwards where the control of the local tumor was not so good but the survival was slightly better. This may suggest that the local inflammation caused by intratumoral injection of CpG could potentiate the effect of PDT on the primary tumor (perhaps by increasing the amount of BPD accumulating in the tumor due to increased microvascular permeability). PDT first and CpG later could produce a better systemic immune response due to the creation of potential tumor antigenic fragments before the immune stimulation led to their take up by antigen presenting cells.

ACKNOWLEDGEMENTS

Ana P Castano was supported by Department of Defense CDMRP Breast Cancer Research Grant (W81XWH-04-1-0676). Michael R Hamblin was supported by the U.S. National Institutes of Health (Grants R01-CA/AI838801 and R01-AI050875). We are grateful to QLT Inc, Vancouver, BC, Canada for the kind gift of BPD.

REFERENCES

[1] Castano, A. P., Demidova, T. N., and Hamblin, M. R., "Mechanisms in photodynamic therapy: part one - photosensitizers, photochemistry and cellular localization," Photodiagn Photodyn Ther. 1, 279-293 (2004)

[2] Castano, A. P., Demidova, T. N., and Hamblin, M. R., "Mechanisms in photodynamic therapy: part two - cellular signalling, cell metabolism and modes of cell death," Photodiagn Photodyn Ther. 2, 1-23 (2005)

[3] Castano, A. P., Demidova, T. N., and Hamblin, M. R., "Mechanisms in photodynamic therapy: part three - photosensitizer pharmacokinetics, biodistribution, tumor localization and modes of tumor destruction," Photodiagn Photodyn Ther. 2, 91-106 (2005)

[4] Castano, A. P., Mroz, P., and Hamblin, M. R., "Photodynamic therapy and anti-tumour immunity," Nat Rev Cancer. 6, 535-545 (2006)
[5] Canti, G., et al., "Antitumor immunity induced by photodynamic therapy with aluminum disulfonated phthalocyanines and laser light," Anti-Cancer Drugs. 5, 443-447 (1994)
[6] Hendrzak-Henion, J. A., et al., "Role of the immune system in mediating the antitumor effect of benzophenothiazine photodynamic therapy," Photochem. Photobiol. 69, 575-581 (1999)
[7] Korbelik, M., and Dougherty, G. J., "Photodynamic therapy-mediated immune response against subcutaneous mouse tumors," Cancer Res. 59, 1941-1946 (1999)
[8] Korbelik, M., et al., "The role of host lymphoid populations in the response of mouse EMT6 tumor to photodynamic therapy," Cancer Res. 56, 5647-5652 (1996)
[9] Gollnick, S. O., Vaughan, L., and Henderson, B. W., "Generation of effective antitumor vaccines using photodynamic therapy," Cancer Res. 62, 1604-1608. (2002)
[10] Gomer, C. J., et al., "Photodynamic therapy-mediated oxidative stress can induce expression of heat shock proteins," Cancer Res. 56, 2355-2360 (1996)
[11] Mitra, S., et al., "Activation of heat shock protein 70 promoter with meso-tetrahydroxyphenyl chlorin photodynamic therapy reported by green fluorescent protein in vitro and in vivo," Photochem. Photobiol. 78, 615-622 (2003)
[12] Korbelik, M., Sun, J., and Cecic, I., "Photodynamic therapy-induced cell surface expression and release of heat shock proteins: relevance for tumor response," Cancer Res. 65, 1018-1026 (2005)
[13] Granville, D. J., et al., "Nuclear factor-kappaB activation by the photochemotherapeutic agent verteporfin," Blood. 95, 256-262 (2000)
[14] Kick, G., et al., "Strong and prolonged induction of c-jun and c-fos proto-oncogenes by photodynamic therapy," Br J Cancer. 74, 30-36 (1996)
[15] Sun, J., et al., "Neutrophils as inflammatory and immune effectors in photodynamic therapy-treated mouse SCCVII tumours," Photochem. Photobiol. Sci. 1, 690-695 (2002)
[16] de Vree, W. J., et al., "Evidence for an important role of neutrophils in the efficacy of photodynamic therapy in vivo," Cancer Res. 56, 2908-2911 (1996)
[17] Krosl, G., Korbelik, M., and Dougherty, G. J., "Induction of immune cell infiltration into murine SCCVII tumour by photofrin-based photodynamic therapy," Br. J. Cancer. 71, 549-555 (1995)
[18] Cecic, I., et al., "Characteristics of complement activation in mice bearing Lewis lung carcinomas treated by photodynamic therapy," Cancer Lett. 225, 215-223 (2005)
[19] Adams, S., O'Neill, D. W., and Bhardwaj, N., "Recent advances in dendritic cell biology," J Clin Immunol. 25, 177-188 (2005)
[20] Gabrilovich, D. I., et al., "Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells," Nat Med. 2, 1096-1103 (1996)
[21] Werling, D., and Jungi, T. W., "TOLL-like receptors linking innate and adaptive immune response," Vet Immunol Immunopathol. 91, 1-12 (2003)
[22] Kassouf, W., and Kamat, A. M., "Current state of immunotherapy for bladder cancer," Expert Rev Anticancer Ther. 4, 1037-1046 (2004)
[23] Tokunaga, T., Yamamoto, T., and Yamamoto, S., "How BCG led to the discovery of immunostimulatory DNA," Jpn. J. Infect. Dis. 52, 1-11 (1999)
[24] Yamamoto, S., et al., "Discovery of immunostimulatory CpG-DNA and its application to tuberculosis vaccine development," Japanese journal of infectious diseases. 55, 37-44 (2002)
[25] Yamamoto, S., et al., "Unique palindromic sequences in synthetic oligonucleotides are required to induce IFN and augment IFN-mediated natural killer activity," J Immunol. 148, 4072-4076 (1992)
[26] Kuramoto, E., et al., "Oligonucleotide sequences required for natural killer cell activation," Jpn J Cancer Res. 83, 1128-1131 (1992)
[27] Han, J., et al., "Selection of antisense oligonucleotides on the basis of genomic frequency of the target sequence," Antisense Res Dev. 4, 53-65 (1994)
[28] Miller, F. R., Miller, B. E., and Heppner, G. H., "Characterization of metastatic heterogeneity among subpopulations of a single mouse mammary tumor: heterogeneity in phenotypic stability," Invasion Metastasis. 3, 22-31 (1983)

[29] Pulaski, B. A., and Ostrand-Rosenberg, S., "Reduction of established spontaneous mammary carcinoma metastases following immunotherapy with major histocompatibility complex class II and B7.1 cell-based tumor vaccines," Cancer Res. 58, 1486-1493 (1998)
[30] Heppner, G. H., Miller, F. R., and Shekhar, P. M., "Nontransgenic models of breast cancer," Breast Cancer Res. 2, 331-334 (2000)
[31] Todryk, S., et al., "Heat shock protein 70 induced during tumor cell killing induces Th1 cytokines and targets immature dendritic cell precursors to enhance antigen uptake," J. Immunol. 163, 1398-1408 (1999)
[32] Korbelik, M., and Sun, J., "Photodynamic therapy-generated vaccine for cancer therapy," Cancer Immunol. Immunother. Epub ahead of print, 1-10 (2005)
[33] Hacker, G., Redecke, V., and Hacker, H., "Activation of the immune system by bacterial CpG-DNA," Immunology. 105, 245-251 (2002)
[34] Pearce, E. J., Kane, C. M., and Sun, J., "Regulation of dendritic cell function by pathogen-derived molecules plays a key role in dictating the outcome of the adaptive immune response," Chem Immunol Allergy. 90, 82-90 (2006)