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Combination approaches to potentiate immune response after photodynamic therapy for cancer†

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Abstract

Photodynamic therapy (PDT) has been used as a cancer therapy for forty years but has not advanced to a mainstream cancer treatment. Although it has been shown to be an efficient way to destroy local tumors by a combination of non-toxic dyes and harmless visible light, it is its additional effects in mediating the stimulation of the host immune system that gives PDT great potential to become more widely used. Although the stimulation of tumor-specific cytotoxic T-cells that can destroy distant tumor deposits after PDT has been reported in some animal models, it remains the exception rather than the rule. This realization has prompted several investigators to test various combination approaches that could potentiate the immune recognition of tumor antigens that have been released after PDT. This review will cover these combination approaches using immunostimulants including various microbial preparations that activate Toll-like receptors and other receptors for pathogen-associated molecular patterns, cytokines growth factors, and approaches that target regulatory T-cells. We believe that by understanding the methods employed by tumors to evade immune response and neutralizing them, more precise ways of potentiating PDT-induced immunity can be devised.

1. Introduction

In the past thirty years, research and understanding of the treatment, diagnosis, and biology of cancer has grown exponentially. In spite of this explosion in knowledge, cancer rates are still increasing, and novel approaches for the management of malignancies in the clinical setting are urgently needed. Currently, the major therapeutic approaches against cancer are surgical resection, systemic chemotherapy, and radiation therapy. But these modalities only go so far, and are generally insufficient in the treatment of advanced stage malignancies.

Photodynamic therapy (PDT) is a novel and revolutionary therapeutic technique¹ that has shown pre-clinical and clinical effectiveness in the treatment of macular degeneration,² localized infections,³ and cancerous tumors.^{4,5} PDT involves light excitation of an

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exogenous, light-sensitive dye, known as a photosensitizer (PS); usually these PS are compounds with macrocyclic structures, including porphyrins, chlorins and other tetrapyrroles. For a reason not entirely understood, hyperproliferating cells (responsible for macular degeneration and cancerous lesions) and rapidly multiplying cells (*e.g.* bacteria) have a preferential uptake of PS. After PS localization occurs, light excitation of the PS yields an electronically excited PS that can give a long-lived triplet state. The PS triplet then can react subsequently with molecular oxygen (O_2) *via* energy transfer to produce singlet oxygen (1O_2), a highly reactive and transient molecular species.⁶ Additional reactions of the PS with oxygen caused by electron transfer form reactive oxygen species (ROS) including hydroxyl radicals, superoxide, and hydrogen peroxide, where hydrogen peroxide may participate in the self-perpetuating Fenton reaction, yielding more hydroxyl radical. These ROS have been shown to destroy tumors by three distinct mechanisms (Fig. 1): (1) direct cytotoxicity towards tumor cells producing apoptosis and necrosis; (2) destruction of tumor capillaries and microvasculature mediated by PDT damage towards endothelial cells; (3) an acute inflammatory response that can activate host-defense mechanisms involving neutrophils and dendritic cells.⁷

Despite the well-understood mechanisms responsible for PDT-mediated cell death and tumor destruction, there are still major problems in the development of effective PS. As of 2008, no PS has all of the properties of an ideal PS, as outlined by Castano *et al.*⁸ As a result of the deficiencies of many PS, and in an effort to enhance the effectiveness of anticancer PDT, researchers have begun to explore the use of combination therapies in conjunction with PDT. So far, radiotherapy, chemotherapy, and hypothermia in conjunction with PDT have been analyzed.⁹ One of the most promising dual therapies under development consists of selective immunostimulation in concert with PDT.

The mammalian immune system consists of two interlocking arms: the innate immune system and the adaptive immune system (Fig. 2). Innate immunity happens very soon after infection. It includes humoral and soluble elements, like the complement system and antimicrobial peptides, and cellular elements like innate leukocytes. The innate leukocytes include the phagocytes (macrophages, neutrophils, and dendritic cells), mast cells, eosinophils, basophils, and natural killer cells (NK cells). Adaptive immunity is triggered in vertebrates when a pathogen produces a threshold level of a specific antigen recognized as foreign. Subsequent activity includes antigen presentation by dendritic cells leading to antigen-specific effector T-lymphocyte cells, (CD4+ and CD8+), and antigen presentation by B-lymphocytes for eventual antibody production.

The immunostimulation combination therapy approach attempts to enhance cancer cell death and tumor regression by utilizing components of the immune system to attack a malignancy. It is known that PDT of tumors alone signals the immune system by the production of diverse inflammatory mediators and a class of molecules known as damage associated molecular patterns (DAMPs) – similar to pathogen-associated molecular patterns (PAMPs). This class of molecules includes molecular chaperones such as heat shock proteins¹⁰ and a diverse array of molecules that are normally considered to be intracellular but when they are released to the extracellular milieu represent a signal that tissue damage has occurred that might lead to invasion by pathogens.¹¹ Thus, the use of immunomodulatory techniques in combination with PDT – so as to enhance an already robust immune response – has been proposed and investigated by many researchers.¹²

Here, we summarize previous investigations into combination PDT and immunostimulant therapies, with the hope of suggesting approaches that may help forward PDT into the clinical setting. Fig. 3 gives an overview of how the combinations of immunostimulants and PDT have been delivered. The majority of immunostimulants have been delivered by

intratumoral injection. This is because powerful activation of the innate immune system has the possibility to be toxic and even fatal, if the cytokine storm – grossly enhanced positive feedback between cytokines and immune cells – released is too severe.

2. Exogenous and microbial immunostimulation strategies

2.1. Glycated chitosan and PDT

Chitosan is a polymer formed by glycosidic linkages of 2-amino-2-deoxy- β -D-glucose and is derived by alkaline hydrolysis (deacetylation) of chitin (poly-*N*-acetyl-2-amino-2-deoxy- β -D-glucose), a ubiquitous polymer in nature that comprises the cell wall of fungi and the exoskeleton of crustacean species.¹³ Chitosan is known for its ability to stimulate the immune system. This stimulation is believed to occur through the production of various mediators of the immune system including cytokines^{14,15} like tumor necrosis factor alpha and interleukin-6. Chitosan is also capable of boosting the immune responses through T-cell upregulation, as demonstrated in animal models. Recently, a water-soluble compound formed from chitosan and galactose, named glycated chitosan has been explored for combination therapy of PDT. Chen and colleagues¹⁶ looked at the effect of glycated chitosan as an enhancer of the immune system combined with PDT for the treatment of EMT6 mammary sarcoma and Line 1 lung adenocarcinoma in syngeneic BALB/c mice.

Photofrin (porfimer sodium) was used as a PS for the PDT treatment of EMT6 mammary sarcomas in mice. Immediately after conventional PDT, glycated chitosan solution was injected peritumorally. The Photofrin-PDT treatment resulted in a 37.5% survival rate, however, but when combined with the peritumoral injection of glycated chitosan solution, survival rates increased to 62.5% at 0.5% GC (w/v) and 75% at 1.5% GC (w/v), respectively.

When *m*-THPC (*m*-tetrahydroxyphenylchlorin) was used for the PDT treatment of Line 1 lung adenocarcinoma, following standard PDT treatment, all of the tumors recurred within 3 weeks. However, a localized treatment of the tumors with 1.67% concentration of glycated chitosan after PDT gave a 37.5% cure rate at a single dose. Line 1 lung adenocarcinoma has weak immunogenicity, thus the host is usually incapable of launching a sufficient immune response for the elimination of these cells.¹⁷ The effect observed with glycated chitosan PDT dual treatment thus emphasizes the immunostimulatory properties of glycated chitosan. The PDT treatment resulted in the destruction of the tumor cells releasing tumor antigens and initiating an inflammatory cascade. The co-presence of the immunoadjuvant (glycated chitosan) and the liberated tumor antigens together enhanced the immune responses against the tumor and gave a significantly better outcome. This forms a strong basis for future studies aimed at improving the understanding of different pathways and substantiates the practical application of chitosan in concert with PDT for clinical practice.

2.2. Complement activators and PDT

The complement cascade is an extremely important non-cellular mediator of immune responses following PDT.¹⁸ This suggests that enhancement of this pathway in combination with PDT might improve the effectiveness of PDT treatment. The complement cascade has three main pathways for its activation: the classical, alternative and lectin pathways. It is thought that the alternative complement pathway is the one that is relevant to PDT (Fig. 4). PDT itself has been shown to activate complement *via* ROS-induced breakdown of the unstable thioester bond in C3 to fragments C3a and C3b. Upon binding with a cellular membrane C3b is bound by factor B to form C3bB. This complex in presence of factor D will be cleaved into Ba and Bb. Bb will remain covalently bonded to C3b to form C3bBb, which is the alternative pathway C3-convertase. After hydrolysis of C3, C3b complexes to become C3bBb3b, which cleaves C5 into C5a and C5b. C5b with C6, C7, C8, and C9

(C5b6789) complex to form the membrane attack complex, also known as MAC, which is inserted into the cell membrane, “punches a hole”, and initiates cellular lysis.

Many substances that have the ability to activate the alternative complement pathway have also been shown to possess anti-tumor activity.^{19,20} These may consist of macromolecules derived from pathogens (*e.g.* zymosan from yeast), enzymes (urokinase) or proteins (gamma-globulin). The most widely explored has been zymosan, a glucan cell wall polymer of β -1,3-glycosidic linkages found in various fungal species.^{18,21} Studies carried out by Korbelik and colleagues explored the use of different complement activators, including zymosan, in combination with PDT.^{22,23} This study elucidated the effects of complement activators in Lewis lung carcinoma (LLC) and squamous cell carcinoma (SCCVII) mouse models syngeneic with C57BL/6 and C3H/HeN mice respectively.

The PS used were Photofrin and mTHPC. Complement activators used were zymosan, heat-aggregated gamma globulin, streptokinase (secreted by several Gram-positive *Streptococcus* species) and urokinase. Although LLC responded well to the standard PDT treatment, all tumors regrew within two weeks after treatment. When intra-tumoral injection of zymosan was used in combination with PDT, it resulted in a cure rate of 60%. When heat-aggregated gamma globulin, was used as a localized immunoadjuvant with PDT, there were no statistically significant differences between the experimental groups. Additionally, when zymosan was combined with PDT in immunodeficient mice, there were no cures. This result strongly suggested that the host immune response needs to be involved in effective destruction of tumors treated with combination therapy. When mTHPC was used as a PS and combined with intravenous injection of streptokinase, which activates the complement cascade systemically *via* binding to plasminogen,^{24,25} the cure rates raised to 80%. This curative effect was however not observed in immunodeficient mice. Urokinase, another systemic complement activator, also improved the cure rates of SCC in mice when combined with Photofrin-PDT. All aforementioned results imply that the activation of complement cascade plays a major role in the anti-tumor immune responses and therefore enhances the therapeutic effects of PDT.

An experimental trial for a combination therapy comprised of gamma-inulin and PDT was tested using different PSs on mouse models of multiple myelomas and fibrosarcomas. The results showed evidence of increased complement activation in tumors treated with combination therapy compared to those treated with single therapies. Single line therapy with gamma-inulin showed no significant reduction in tumor growth, while PDT and combination therapy resulted in complete tumor ablation. However, the time to tumor recurrence was significantly longer in mice treated with combination therapy. In addition, combination therapy resulted in significantly higher tumor C3 accumulation as compared to those recorded with single line therapy and there was increased infiltration of CTLs engaged in tumoricidal activity *via* the granzyme-perforin mechanism at the tumor site. These findings warrant further investigation for possible clinical application of complement activators in combination with PDT.

2.3. Schizophyllan in combination with PDT

Schizophyllan is a beta-D-glucan polysaccharide derived from fungal cells and has been shown to induce both humoral and cell mediated immunity mainly by two different mechanisms.^{19,21,26,27} Firstly, schizophyllan works by activating macrophage dectin-1 receptor, and secondly by enhancing the activity of Toll-like receptors (TLRs). It has been previously shown^{22,27} that schizophyllan potentiates PDT effects in SCCVII-bearing mice. When Photofrin was used in conjunction with red light, with prior schizophyllan intramuscular injection, there was an increase in the number of mac-1 positive host cells in the tumor. Moreover, schizophyllan-potentiated PDT treatment resulting in a threefold

increase in tumor cure rates as compared to the mice that received PDT alone. Treatment with schizophyllan after PDT however, produced no cures, suggesting that schizophyllan may have strong immunostimulatory properties that can potentiate PDT anti-tumor effects thus encouraging further exploration of this approach.

2.4. Topical imiquimod and PDT combination

Imiquimod is known to stimulate the innate immune response through TLRs, thus causing a localized inflammatory reaction at the site of application. It acts by activating the antigen presenting cells, thereby increasing their capacity to present the tumor antigens to T-lymphocytes. Imiquimod acts on the TLR 7 and TLR 8 which are key components of the innate immunity of the human body and help to protect against bacterial, viral, fungal and parasitic infections.^{28,29}

The immunostimulating effects of Imiquimod have been recently explored in combination with PDT for vulvar intraepithelial neoplasia (VIN). Treatment of VIN using PDT has varied, but increase in the levels of the interlesional infiltrating lymphocytes under the lesion has been observed, thus suggesting PDT can stimulate local immunity in these type of lesions.^{28,30} Topical Imiquimod and PDT dual therapy involved the application of topical Imiquimod to the vulval skin, followed by treatment of the affected area using PDT. In the course of the study 20 female patients with VIN were treated initially with topical Imiquimod for 8 weeks followed by PDT in order to increase T cell infiltration.^{24,31} Two PDT treatments were carried out at weeks 12 and 16, after application of methyl-aminolevulinate cream (a precursor to protoporphyrin IX, PPIX) to the affected areas for 3 h. The area was then exposed to red light delivering a dose of 50 J cm⁻². After 52 weeks of follow up there was a response rate of 55% by interpretation and 64% per protocol and the non-responders showed a higher level of T regulatory cells. 13 patients improved while 2 developed peripheral lesions. Also, the VIN lesions were biopsied and checked for HPV DNA and inflammatory cells and the size compared to that before treatment. Overall the increase in infiltration by CD4+, CD68+ and CD1-a+ cells was not found to be significant, however CD8+ T cells were significantly increased. The study was unable to show whether the observed effects were due to Imiquimod alone or due to the combination therapy as no control group was included, but responders showed a greater response after PDT administration and there is still need to further explore the treatment of VIN with a combination therapy using Imiquimod and PDT.

2.5. CpG-Oligodeoxynucleotide in combination with PDT

Oligonucleotides with nonmethylated CpG motifs are considered pathogen-associated molecular patterns (PAMPs) due to their abundance in microbial genomes and their rarity in vertebrate genomes. The CpG motif is recognized by the pattern recognition receptor, Toll-like receptor 9 (TLR9), which is only constitutively expressed in B cells and plasmacytoid dendritic cells. Synthetic CpG ODN differ from microbial DNA in that they have a partially or completely phosphorothioated backbone instead of the typical phosphodiester backbone to protect the ODN from being degraded by nucleases. Acting through TLR 9, CpG-ODN triggers B cells, natural killer cells, monocytes and macrophages to proliferate and release various chemicals that enhance the inflammatory responses of the host immune system (Fig. 5).

In a recent study³² on BALB/c mice inoculated with a highly metastatic 4T1 mammary tumor cell line in the mammary fat pad, an adjuvant administration of CpG-ODN was tested in combination with PDT. It was proposed that such treatment would lead to enhanced stimulation of the host immune system and the production of the CD8+ T lymphocytes, which play a vital role in tumor destruction. The treatment of intratumoral CpG injection

plus benzoporphyrin derivative mediated PDT with a 15 min drug–light interval, showed significant higher effect on the tumor compared to PDT alone or CpG alone, and the rate of growth decreased leading to a longer survival rate.

2.6. Microbial vaccine in combination with PDT

2.6.1. OK-432—OK-432 is a penicillin and heat-killed Su strain of *Streptococcus pyogenes* A3 developed by Okamoto. OK-432 is known to be a biological response modifier^{33,34} with immunostimulatory properties.^{34–36} Many previous studies in which OK-432 was used along with radiation and hyperthermia³³ have proposed that OK-432 has the ability to enhance anti-tumor immune responses. Consequently, a study conducted by Uehata *et al.*³⁷ has shown the results of OK-432 used in combination with PDT. In this study NR-S1 mouse squamous cell carcinoma was transplanted into male C3H/HeNCrj mice and hematoporphyrin oligomers were used as a PS along with red light. The PS was administered through intra-peritoneal injections 48 h before light therapy. The comprised 4 groups of mice. Group 1 received OK-432 by local injection into the tumor 3 h before irradiation, whereas, group 2 received OK-432 treatment following PDT. Group 3 was the PDT only group and group 4 contained mice receiving OK-432 only. The anti-tumor effects produced by each treatment were measured and analyzed 48 h after each laser irradiation. The group receiving OK-432 treatment 3 h before laser irradiation showed the highest amount of hemorrhaging vessels, necrosis and infiltrating inflammatory cells. Group 1 had significant differences in the number of infiltrating immune cells and the area of tumor necrosis after 48 h as compared to the other groups. The volume of tumor after 3 days was also significantly smaller in Group 1 as compared to group 4 which received OK-432 treatment only. The average tumor volumes measured at 7 and 10 days post-PDT were also significantly smaller in Group 1 as compared to the other groups. This study also looked at the number of infiltrating immune cells in the tumor site up to 48 h after laser irradiation. After 48 h, the number of infiltrating immune cells in the Group that received standard PDT treatment alone was not significantly different from the control group. OK-432 treatment led to activation of macrophages and neutrophils within 3 h of treatment that may have contributed to increases in IFN- γ and TNF- α .³⁵ PDT has also been shown to enhance the activity of macrophages.³⁸ This study showed that the OK-432 administration prior to PDT leads to significantly better results; administration of OK-432 after PDT produced no significant differences.

2.6.2. Intralesional *Corynebacterium parvum*—Studies have shown the effectiveness of *Corynebacterium parvum* (CP), a killed bacterial vaccine, in a C3H/He mouse model implanted with a poorly differentiated transitional cell carcinoma of the bladder (MBT-2).³⁹ CP inhibited the growth of subcutaneously transplanted tumors, the most significant effects of which were seen when CP was injected directly into the tumor.

A study by Myers *et al.*⁴⁰ combined this immunotherapy with PDT. Mice receiving a dose of 25 μ g CP in concert with PDT showed the smallest mean tumor volume when compared to those receiving CP at 250 μ g. Interestingly, CP administered alone showed a greater effect at 250 μ g as compared to 25 μ g. A previous study had shown that CP 250 μ g was superior to 25 μ g dose in controlling the MBT-2 tumor *in vivo*,⁴¹ but surprisingly in the study by Myers,⁴⁰ the high dose of CP (250 μ g) had less effect than that of the low dose. CP has tumoricidal or tumorigenic effects, which are mediated through indirect stimulation of immune cells, including phagocytes and natural killer (NK) cells.³⁹ Previous studies have shown that 250 μ g of CP causes an acute inflammatory response involving the recruitment of mainly phagocytes and NK cells. On the other hand, lower doses of CP (25 μ g) involves a slower inflammatory response mediated mainly *via* T-lymphocytes. Thus, when high doses of CP are given it leads to the lysis of tumor cells, such that subsequent PDT may also

results the phototoxic killing of immune cells attracted into the tumor by CP 42. As a result of the delayed response of the immune system to low dose CP-25 μg , a therapeutic window exists for PDT damage to tumor cells that will not affect slower CP-attracted leukocytes. Results of the study also demonstrated the importance of the sequence of CP, as shown by previous studies on hyperthermia and ionizing radiation as an adjuvant to PDT.

2.6.3. Bacillus Calmette-Guérin—*Bacillus Calmette-Guérin* (BCG) is a live, but weakened, mycobacterial preparation (derived from *Mycobacterium bovis*) used as a vaccine against tuberculosis but also may elicit inflammatory reactions leading to the development of anti-tumor immunity.⁴² Currently, BCG vaccine is used in the treatment of bladder cancer after intra-vesicular instillation and works *via* immunostimulation. BCG activation of various cellular elements of the immune system is known to play a significant role in the immune responses that come into action against cancerous lesions.¹⁵ BCG is now widely used for the treatment of superficial bladder cancer and investigations have looked at the outcome of combining PDT with BCG treatment to increase therapeutic effectiveness.⁴³ The study by Korbely *et al.*⁴² employed PDT using six different PSs (Photofrin, benzoporphyrin derivative, mTHPC, ce6, ZnPC and lutetium texaphyrin). Murine subcutaneous EMT6 tumors were used as a model. The cure rates were significantly better in mice treated with BCG and PDT regardless of which PS was used or the time of BCG administration. This study also examined the number of immune cells in tumors and draining lymph nodes to compare any differences found between the experimental groups. Although the number of activated myeloid cells was not statistically different between BCG alone and combination treatments, the number of memory T-cells in tumor-draining lymph nodes increased twofold when PDT was combined with BCG. It was also suggested that adjuvant BCG therapy with PDT would be more capable of targeting small slow growing tumors as compared to rapidly dividing tumors, because tumor recurrence rates were decreased with BCG-PDT treatment, while the time to recurrence was unchanged.

2.6.4. Mycobacterial cell wall extracts—A preparation made from the glycocalyx from the cell walls of the species *Mycobacterium phlei* emulsified in oil known as mycobacterial cell wall extract (MCWE) is a bacterial vaccine. Several compounds such as trehalose dimycolate, muramyl dipeptide and lipoarabinomannan contained in MCWE are known to have immunostimulatory properties.⁴⁴ A study by Korbely and Cecic⁴⁵ combined administration of MCWE peritumorally immediately after PDT using several different PSs (Photofrin, BPD, mTHPC and ZnPC). Murine EMT6 tumors were selected as a model. The results showed that in groups receiving MCWE immediately after laser irradiation, there was an improved cure rate with only 25% of tumors recurring as compared to groups which received PDT treatment alone and had 100% tumor recurrences within two weeks. MCWE injection given 24 h before PDT did not give any significant potentiation. The improvement in cure rates was consistent for all the mice receiving MCWE-PDT treatment regardless of which PS was used. The tumors were also examined for any changes in the cell density of infiltrating leukocytes. Although the increase in number of neutrophils was not statistically significant, the numbers of malignant cells were significantly reduced by MCWE-PDT combination as compared to groups that received no treatment or have received PDT alone. Since MCWE has been shown to stimulate both humoral and cell-mediated immunity as well as to lead to secretion of various cytokines from macrophages,⁴⁶ it holds a lot of potential as a combination strategy used with PDT to enhance anti-tumor effects produced by host immune responses.

3. Growth factors, hormones, and other immunostimulants

3.1. Serum vitamin D3-binding protein derived macrophage-activating factor

This immunotherapy involves the exogenous administration of vitamin D3 binding protein (DBP), which is a precursor of macrophage activating factor (MAF). This can be enzymatically treated to yield DBPMAF, if of mouse origin, or GcMAF, if obtained from humans.⁴⁷ Under natural conditions, MAF formation through this pathway is triggered by the release of lipid metabolites in response to inflammation.⁴⁸ It has been demonstrated that MAFs can stimulate the phagocytic potential of such cells as granulocytes, monocytes and osteoclasts.^{47,49} In a study that employed squamous cell carcinoma SCCVII in mice,⁵⁰ DBPMAF administration was combined with PDT. DBPMAF alone did not show any effects on the growth of the tumors however, in combination with PDT the multiple DBPMAF administrations led to a 100% cure rate. The number of tumor-free mice in combination group was also significantly increased as compared to PDT alone indicating DBPMAF may synergize with the immune stimulatory effects of PDT.

3.2. Low-dose cyclophosphamide

In the 1970s it was realized^{51,52} that treatment of mice with low dose cyclophosphamide (CY) had an additional and different effect on tumor growth that involved the host immune system, rather than a direct cytotoxic effect on the tumor cells. Low dose CY in combination with adoptive transfer of splenocytes from normal mice that had been previously vaccinated against the MethA tumor, caused complete and permanent regressions of established tumors.⁵³ This effect was abrogated by additional adoptive transfer of splenocytes from tumor-bearing mice and the conclusion was that a particular population of suppressor T-cells were sensitive to low dose CY. More recent studies have shown that low dose CY administration depletes Tregs⁵⁴ and also increases the fraction of both CD4+ and CD8+ T-cells with a memory phenotype and induces type I interferon production.⁵⁵ It has been proposed that the reason for the selective action of low dose CY is its preferential induction of apoptosis in Treg and reduction in their suppressor function⁵⁶ or to the ability of CY to inhibit or reduce the activity of inducible nitric oxide synthase (iNOS).⁵⁵ There have been some published studies that have compared the anti-tumor effects of low dose CY with high dose CY in mouse models. Motoyoshi *et al.*⁵⁷ found an equal effect of 20 mg kg⁻¹ and 200 mg kg⁻¹ CY on slowing the growth of a murine hepatoma MH129 in immunocompetent mice but only the high dose CY had any anti-tumor effect in nude mice that lack T-cells. Low-dose CY depleted T-regs, while high-dose CY depleted all classes of T-cells. Brode and others reported⁵⁸ that CY can induce autoimmune diabetes in NOD mice and that this can be prevented by adoptive transfer of islet antigen-specific CD4+CD25+FoxP3 T-regs to CY-treated recipients. Taieb and coworkers reported⁵⁹ that CY administration to tumor-bearing mice reduced T-regs and also enhanced secondary CTL response induced by a tumor-vaccine.

Our laboratory was the first to demonstrate that low-dose CY treatment when combined with PDT treatment could lead to cures and long-lasting immunity.⁶⁰ We used J774 tumors and CY (50 or 150 mg kg⁻¹ IP) was injected 48 h before light delivery. PDT in combination with low dose CY led to 70% permanent cures. Cured mice had tumor specific T-cells in spleens and low dose CY was shown to reduce CD4+FoxP3+ T-regulatory cells.

Our recent preliminary data (not shown) revealed that Treg depletion by CY could also unravel PDT mediated immune response to mouse colon adenocarcinoma CT26WT. Moreover, this combination treatment led to the development of long lasting immune memory that could be only uncovered by CY administration prior to rechallenge.

3.3. Granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony stimulating factors (GM-CSF) and PDT

Neutrophils are considered extremely important players in the cellular cascade that leads to the anti-tumor immune response.⁶¹ Enhancement of neutrophil activity is one of the mechanisms of stimulating the innate immune system, increasing the tumoricidal activity. G-CSF is a hematopoietic cytokine synthesized endogenously by the human body, but is also available as an exogenous recombinant preparation. It is imperative for normal neutrophil function, both to maintain basal neutrophil counts in the body and to create a state of neutrophilia in response to infection.⁶² It causes the potentiation of neutrophil production from precursor cells in bone marrow, accelerated entry of matured cells from marrow to blood, increased chemotaxis towards immunogenic sites, increased phagocytic activity and a decrease in activation of apoptotic pathways.⁶³

Neutrophils have also been implicated as the major players in the antitumor response induced by PDT,⁶¹ with increased tumor infiltration followed by direct cytotoxicity or indirect effects through pathways involving lymphoid cells.⁶⁴ They may also play a role in the regulation of the increased stimulation of tumor-specific T-cell mediated immune responses.⁶⁵ Neutrophils have been also reported as the main immune cells to infiltrate tumors in the early hours after PDT. When a study examined the effectiveness of a combined therapy of localized G-CSF and PDT in murine models of colon-26 and Lewis lung carcinomas,⁶⁶ it was found that the survival time of the mice treated with combination therapy was significantly greater than in groups treated with individual therapies. One third of the mice were completely cured and rejected tumor rechallenges, demonstrating the development of memory immunity. Moreover, PDT seemed to potentiate the granulopoietic effects of G-CSF on the spleen and bone marrow, increasing the efficacy of the immune system by increased production of immune cells. Thus, the results indicate the prognosis post-combination therapy to be superior to that seen with single line therapy.

GM-CSF is an immune-stimulatory cytokine, stimulating the differentiation and function of macrophages, dendritic cells and other antigen presenting cells, *i.e.* antigen presentation, adhesion, chemotaxis, phagocytosis and cytokine production.^{67,68} Considering the effects of GM-CSF administration in the context of anti-tumor immunity, it enhances an anti-tumor responses mediated by CD4+ and CD8+ T-cells^{69,70} and also enhances tumoricidal activity of the granulocytic and monocytic cells at the tumor sites. Combination therapy of GM-CSF with PDT using a hematoporphyrin PS was carried out against murine squamous cell carcinoma (SCCVII).²² Single line therapy with GM-CSF potentiated the tumoricidal activity of macrophages but did not bring about any significant alteration in the growth of the tumors. PDT treatment, both alone and in combination with GM-CSF, brought about complete tumor ablation. Disappointingly, it did not bring about a long-term cure and tumor recurrence occurred in all these groups. The tumor free interval was longer in the mice treated with combination therapy as compared to those with PDT alone. This indicates that GM-CSF indeed potentiates the stimulation of the immune system brought about by PDT. However, the degree of potentiation is not great enough to prevent recurrence of the tumor or stimulate immunological memory.

4. Cell-based therapies and PDT

Golab's group⁷¹ showed that administration of immature dendritic cells into tumors treated with Photofrin-PDT resulted in effective homing to regional and peripheral lymph nodes and stimulation of cytotoxic T lymphocytes and natural killer cells. The combination treatment produced the best tumor-response and some resistance to a tumor rechallenge. In another study of combination therapy of intratumoral dendritic cells and PDT⁷² a chlorin-type PS, ATX-S10 Na(II) was used against CT26 tumors in BALB/c mice, and the resulting tumor

resorption with combination therapy was greater than that of individual therapies observed alone. Furthermore when mice bearing two tumors had only one treated with the combination of PDT and dendritic cells, the contralateral untreated tumor underwent regression. Tumor-specific lymphocytes were demonstrated by chromium release CTL assay and by IFN- γ production. Korbelik and Sun⁷³ used adoptive transfer of a human NK cell line genetically altered to produce IL-2 combined with mTHPC-PDT of subcutaneous human squamous cell carcinoma growing in SCID mice. Peritumoral or intravenous injection of cells immediately after PDT produced an improvement in the outcome of PDT, not seen with a cell line that does not produce IL-2.

5. Conclusion

We believe that in the coming years there will be great advances in the area of understanding the induction of an anti-tumor immune response after PDT and particularly in the area of combination therapies that can be used with PDT to potentiate the immune response. As more complete understanding of the pathways by which tumors manage to evade the host immune response is achieved by tumor immunologists, we expect better, more efficient and more selective ways of overcoming this tumor immune evasion will be discovered. These innovative strategies to overcome tumor immunosuppression will be ideal to test in combination with PDT. Most studies in the foreseeable future will likely continue to be performed in mouse models, as the body of knowledge concerning mouse tumor immunology is considerable and it is hugely challenging for physicians to explore combinations of two experimental therapies in the clinical setting. We must realize that in all probability it will take considerably longer for the use of combination-PDT to induce a sufficiently powerful anti-tumor immune response that results in improve survival in advanced cancer patients to become clinically accepted practice.

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Fig. 1.

The mechanisms of PDT. Type I and Type II photochemical processes deriving from the PS triplet state are classically presented by a Jablonski energy diagram. PDT induces necrosis and apoptosis of tumor cells, destruction of tumor blood vessels, and an acute inflammatory response resulting increased infiltration by macrophages, activation of neutrophils (PMN) and dendritic cells (DC), and increased antigen presentation.

Fig. 2.

Two arms of the mammalian immune system. The innate arm reacts to pathogenic invaders using phagocytes (neutrophils, macrophages and dendritic cells), the complement cascade and natural killer (NK) cells. The adaptive arm involves professional antigen presenting cells (APC) that can prime naïve T-cells to become cytotoxic T-cells that can proliferate and destroy tumor cells *via* perforin/granzyme. Regulatory T-cells (T-reg) can inhibit this process. B-cells also recognize antigens, proliferate and become plasma cells secreting antibodies.

Fig. 3.

Delivery routes for PDT and immunostimulants. The combination immunostimulants delivered by intratumoral injection include various microbial preparations that activate Toll-like receptors and other receptors for pathogen associated molecular patterns, cytokines, growth factors, and some complement activators. Imiquimod is applied topically. Those delivered systemically include cyclophosphamide, some complement activators and macrophage activating factor (GC-MAF). CpG-ODN = CpG containing oligodeoxynucleotide; OK-432 = killed *Streptococcus pyogenes*; G-CSF = granulocyte colony stimulating factor; GM-CSF = granulocyte macrophage colony stimulating factor.

Fig. 4.

The alternative complement pathway is activated by ROS production after PDT. All pathways converge at the level of C3 and lead to the cleavage products, C3a and C5a, as well as the terminal membrane attack complex, C5b-9, which forms pores in the membranes of bacteria and cells, ultimately causing their lysis.

Fig. 5.

CpG ODNs facilitate innate and adaptive immune responses. CpG oligodeoxynucleotides (ODNs) can directly activate human B cells and plasmacytoid dendritic cells, stimulating generation of T helper 1 (TH1)-type cytokines. Moreover, *Response*, CpG ODNs facilitate the generation of humoral and cellular antigen-specific immunity. CD40, CD86 = markers of maturation and activation of dendritic cells; CTL = cytotoxic T lymphocyte; IFN- γ = interferon- γ ; IgM = immunoglobulin M; IL = interleukin; MHC = major histocompatibility complex; TNF = tumor-necrosis factor.