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T-cell mediated anti-tumor immunity after photodynamic therapy: Why does it not always work and how can we improve it?

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Abstract

Photodynamic therapy (PDT) uses the combination of non-toxic photosensitizers and harmless light to generate reactive oxygen species that destroy tumors by a combination of direct tumor cell killing, vascular shutdown, and activation of the immune system. It has been shown in some animal models that mice that have been cured of cancer by PDT, may exhibit resistance to rechallenge. The cured mice can also possess tumor specific T-cells that recognize defined tumor antigens, destroy tumor cells in vitro, and can be adoptively transferred to protect naïve mice from cancer. However, these beneficial outcomes are the exception rather than the rule. The reasons for this lack of consistency lie in the ability of many tumors to suppress the host immune system and to actively evade immune attack. The presence of an appropriate tumor rejection antigen in the particular tumor cell line is a requisite for T-cell mediated immunity. Regulatory T-cells (CD25+, Foxp3+) are potent inhibitors of anti-tumor immunity, and their removal by low dose cyclophosphamide can potentiate the PDT-induced immune response. Treatments that stimulate dendritic cells (DC) such as CpG oligonucleotide can overcome tumor-induced DC dysfunction and improve PDT outcome. Epigenetic reversal agents can increase tumor expression of MHC class I and also simultaneously increase expression of tumor antigens. A few clinical reports have shown that anti-tumor immunity can be generated by PDT in patients, and it is hoped that these combination approaches may increase tumor cures in patients.

Graphical Abstract

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Anti-tumor PDT liberates antigens that are taken up by dendritic cells that migrate to lymph nodes, prime naïve T-cells that proliferate and return to destroy remaining tumor cells.

1 Introduction

Photodynamic therapy (PDT) is an effective, clinical procedure against several solid tumors ¹. Although the use of photosensitizers (PS) dates back thousands of years ², the concept of PDT was first described about 100 years ago. Around 40 years ago, PDT using the combination of porphyrin derivatives and red light was first introduced by Thomas Dougherty and colleagues at Roswell Park Cancer Center in Buffalo NY ².

PDT involves intravenous, oral or topical administration of PS, followed by delivery of light of a specific wavelength, in the presence of molecular oxygen ³⁻⁵. In some circumstances near-infrared light can be used taking advantage of upconverting nanoparticles containing rare-earth salts ⁶, or two-photon excitation of the PS using femtosecond pulsed lasers ⁷. A PS (in its non-excited state) has its HOMO (highest occupied molecular orbital) in a low-energy singlet state. Once light is absorbed the electron moves into a high energy singlet state in the LUMO (lowest unoccupied molecular orbital) ¹. This excited singlet state can undergo a transition to a long-lived excited triplet state by the process known as intersystem crossing. In a type I reaction the excited state PS converts molecular oxygen to superoxide and hydroxyl radicals by electron transfer, while in a type II reaction, singlet oxygen is formed by energy transfer from the triplet PS to ground state triplet oxygen (**Figure 1**) ^{8,9}. Both types of reactive oxygen species (ROS) can cause cell damage ^{10,11}. A combination of direct tumor cytotoxicity, the destruction of vasculature and subsequent deprivation of nutrients, added to a possible antigen specific immune response, results in tumor death and, in some cases, in long-term cures ¹¹⁻¹³. PDT-mediated tumor destruction *in vivo* involves cellular mechanisms with photodamage of mitochondria, lysosomes, nuclei, and cell membranes. This photodamage activates apoptotic, necrotic and autophagic signals, leading to cell death ^{3,14,15}. PDT is approved in the US for treatment of various cancers including endobronchial and endoesophageal tumors ^{16,17}, bladder, stomach, oral cavity, breast and skin cancer ⁹.

2 Effects of the immune system on PDT for cancer

While nowadays surgical treatment of a localized tumor is often successful, the treatment of metastatic tumors remains a challenge. Tumor therapies such as ionizing radiation, as well as chemotherapy, can sometimes have a stimulatory effect on the immune system at low doses, but at the doses needed to destroy tumors they are in general immunosuppressive¹⁸⁻²⁰. Moreover, surgery has also been reported to have immunosuppressive effects²¹. The ideal tumor therapy, therefore, would enhance the body's natural defense against tumor cells at the same time as it destroyed the actual tumor. A large number of spontaneous regressions in patients with metastasized tumors have been reported, mostly after infection, leading to attempts to create a tumor therapy that activates the immune system such as tumor vaccines²², and adoptive T-cell immunotherapy²³. Recently there has been substantial progress in the use of checkpoint inhibitors or inhibitors of co-inhibitory pathways such as CTLA4 and PD-1 that can unleash anti-tumor immunity²⁴. Although the effects of PDT on the immune system can be either stimulatory or immunosuppressive^{8, 11, 25}, when PDT is able to locally destroy tumor cells as well as to create a systemic response then the best hope of cures is achieved²⁶.

PDT causes an acute inflammatory response, involving many cells and elements of the innate and adaptive immune systems^{4, 14, 27-30, 31, 32}. Following PDT, neutrophils and other inflammatory cells are induced to migrate to the targeted area^{8, 33}. A rapid and strong migration of neutrophils is required for an effective anti-tumor response and enhanced immunity^{33, 34}. In fact, a correlation between the numbers of circulating neutrophils and effectiveness of PDT has been shown when rats were administered granulocyte colony-stimulating factor (G-CSF)³⁵. Vice versa, it was also shown that anti-G-CSF antibodies profoundly diminished neutrophils as well as reducing PDT efficacy³⁵. For the first 2 hours post-PDT, a rise in myeloid cells, including monocytes and mast cells, is observed³⁶. Furthermore TNF- α is produced after PDT, and acts as a promoter of neutrophilia in the early phase post therapy³⁷. In mice with squamous cell carcinoma VII (SCCVII) a 200-fold increase in neutrophils was found as early as 5 minutes after PDT, followed by a rise in macrophages³⁶. Cecic et al 2001 confirmed the former findings of increasing neutrophilia after Photofrin or mTHPC-mediated PDT³⁴. Neutrophil mobilization towards the tumor or tumor draining lymph nodes plays a key role in the activation of CD8+ T-cells after PDT³⁸.

Macrophages have been shown to be activated by PDT at low doses^{39, 40} although they can be killed at high PDT doses^{41, 42}. Macrophages, as well as lymphocytes, secrete lysophosphatidylcholine after PDT. Lysophosphatidylcholine acts through β -galactosidase and NEU1 sialidase from B- and T-lymphocytes, and these enzymes act in concert to modify vitamin D3 binding protein, leading to production of macrophage-activating factor (MAF)^{43, 44}. MAF acts in a feedback loop to activate more macrophages, that are then primed to attack and destroy tumor cells⁴⁵. Several studies have also shown an increase in natural killer (NK) cell activity after PDT that has been proposed to contribute towards the anti-tumor immune response⁴⁶⁻⁴⁸.

Another mechanism by which PDT can cause acute inflammation is by activation of transcription factors. Nuclear factor κ B (NF- κ B) and activator protein 1 (AP1) are two

transcription factors that are activated by oxidative stress. The oxidative stress produced by PDT activates these transcription factors and produces acute inflammation⁴⁹⁻⁵¹. The expression of cyclooxygenase-2 (COX-2) is regulated by NF- κ B, which produces inflammatory mediators such as prostaglandin E2 (PGE-2) and leukotrienes¹¹. PGE-2 plays a role in inflammation and fever. Ferrario et al. 2002 showed that PDT enhances the upregulation of COX-2 and PGE-2. However, COX-1 was not affected by PDT⁵². This study suggests that PDT could be made even more effective with additional administration of selective COX-2 inhibitors.

PDT is also involved in the modulation of the complement system, which functions as an important part of anti-tumor immunity⁵³⁻⁵⁶. The complement system causes the release of pro-inflammatory cytokines such as IL-1 β , IL-6, IL-8, IL-10, TNF- α , thromboxane, histamine, prostaglandins, leukotrienes, G-CSF and coagulation factors^{8, 37, 57}. The release of thromboxane affects endothelial cells, causing them to shut down the microvasculature, leading to post-irradiation tumor ischemia^{58, 59}. Increased expression of complement factors C3 and C5a may be responsible for the neutrophilia that occurs after PDT, because inhibition of these complement fragments has been shown to lead to neutropenia⁶⁰. Korbely et al. 2005 showed that tumor-C3 was elevated 1 and 24 hours after Photofrin-PDT, but serum-C3 was only increased 24 hours after PDT. When C3a or C5a were blocked, efficacy of PDT was decreased in Lewis lung carcinoma (LLC)⁵⁴. Intercellular adhesion molecule (ICAM) 1, also referred to as CD54, seems to be upregulated after PDT and may contribute to tumor destruction. However, when ICAM 1 is blocked with monoclonal antibodies, cures are reduced³⁷.

IL-6 is a cytokine that influences the production of megakaryocytes and hematopoietic progenitor cells⁶¹. IL-6 can trigger inflammation and, in some cases, can promote tumor growth⁶¹⁻⁶⁵. Although it is known that IL-6 is induced in large quantities after PDT, there are conflicting opinions about whether its overall influence is in favor of better PDT response, or whether it can act to promote tumor resistance to PDT. IL-6 plays a major role in T-cell proliferation and function⁶⁶. Since PDT of tumors can induce the production of IL-6⁵⁹, it has been proposed that this IL-6 enhance T-cell mediated anti-tumor immunity^{11, 27, 38}. In Colo26 colon carcinoma and 4T1 mammary carcinoma models, IL-6 inhibited tumor growth control by PDT, mostly due to the inhibition of the proapoptotic protein Bax²⁵, IL-6 increases the killing ability of NK cells⁶⁷⁻⁷⁰ and aids the differentiation of cytotoxic killer cells⁶¹. Generally, IL-6 is considered to promote anti-tumor immune response⁷¹. IL-6 and TNF- α can act together to synergize the anti-tumor effect of PDT⁶¹.

Kick et al found in HeLa cells that expression of IL-6 after PDT was mediated by activated AP-1, but not by NF- κ B⁶¹. IL-6 leads to neutrophil mobilization that may be mediated by activation of several intracellular signaling pathways such as STAT-3, mitogen-activated protein kinases (MAPK), and phosphoinositol 3 kinase (PI3K)²⁵. IL-6/STAT-3 signaling is assumed to promote the expression of prosurvival and antiapoptotic proteins^{25, 72}. It was shown that IL-6 over-expression in human basal cell carcinoma cell (BCC) lines increased resistance to PDT by decreasing apoptosis via elevation of the anti-apoptotic protein, Mcl-1^{25, 73}. Contrary to this, elevated levels of IL-6 in lung carcinoma cells produced

increased sensitivity to PDT. A possible explanation for this was the increased expression of proapoptotic Bax protein ⁷⁴.

MAPK also play a role in PDT. Hendrickx et al. 2003 showed that p38 MAPK blocked the up-regulation of COX-2 in human cancer cells and hence increased the effect of PDT ⁷⁵. IL-8 has been shown to help tumor growth and functions as an important modulator of the innate immune system that is chiefly secreted by macrophages ⁷⁶. IL-8 is pro-angiogenic, increases survival and proliferation of endothelial cells and tumor cells, and promotes tumor infiltration by neutrophils ⁷⁷. Koon et al. 2010 showed that IL-8 production was down-regulated after Zn-BC-AM PDT in Epstein-Barr virus-infected nasopharyngeal carcinoma cells. This suggests that Zn-BCAM PDT might indirectly reduce tumor growth by lessening IL-8 secretion ⁵⁷.

Coutier et al. correlated the fluence rate during the PDT irradiation with the amount of tumor destruction in nude mice using meta-tetra(hydroxyphenyl)chlorin (mTHPC) as PS. They showed that using low fluence rates and thus reducing the extent of oxygen depletion yielded the best results in tumor destruction ⁷⁸. Oxygen depletion during PDT should be avoided since it has been shown that oxygen as well as the PS itself is needed in the tumor. Overall the efficacy of PDT is increased by low fluence rates ^{78, 79}. Moreover, Sluiter et al. in 1996 showed that PDT at low fluence rates was successful in 70–80% of mice, while PDT at high fluence rates was only effective in 10–15% ⁸⁰. Immunological activation may have a role in this difference in outcomes (*vide infra*).

3 T-cell mediated anti-tumor immunity

When antigen presenting cells (APCs), such as mature dendritic cells (DCs), interact with T-cells through binding between antigen containing MHC- complexes I and II, and T-cell receptors, the adaptive immune response is initiated. B cells are induced to secrete antigen-specific immunoglobulins (antibodies), while antigen specific T-cells are also activated and induced to proliferate ⁸¹. MHC class II molecules on the APC surface display peptides derived from foreign or exogenous pathogens, while MHC class I molecules display peptides derived from endogenous proteins that function as self-antigens. While MHC class I antigen complexes generally activate CD8+ T-cells, MHC class II antigen complexes activate CD4+ T-cells ³⁰. CD8+ T cells play an important role in the facilitation of PDT-mediated anti-tumor immunity, contributing to the long-term control of tumors ^{1011, 19, 82}. On the other hand CD4+ helper T cells have been shown to play only a supportive role ⁸³. The removal or depletion of CD8+ T cells by injection of anti-CD8 antibodies led to faster tumor growth and worse PDT treatment outcome. By contrast, a similar depletion of CD4+ T cells had only a minor effect on treatment outcome ¹⁰. However, CD4+ T-cells can act as “T-helper” cells to improve activation and proliferation of CD8+ cytotoxic T lymphocytes, which can be mediated by IL-17 and other cytokines ⁸⁴.

Contact hypersensitivity (CHS) is a T-cell mediated immune response towards antigens in the skin formed from conjugates between skin proteins and reactive molecules called haptens. CHS is a type IV hypersensitivity and requires a first encounter, also known as the sensitization phase, with a hapten such as 2,4-dinitrofluorobenzene⁸⁵. The specific protein

and the hapten bind covalently and this complex is taken up by APC, that then present the hapten-protein conjugate to T-cells. In the elicitation phase, this time using a low concentration of hapten, the process described above repeats itself. However, this time memory T-cells are recruited and destroy skin cells that are in contact with the hapten ⁸⁶. PDT can suppress CHS, depending on the PS used. For instance, HpD and meso-tetra(4-sulfonatophenole)porphine have shown to suppress CHS, while no immunosuppression with Photofrin II or mesotetra(4-carboxyphenyl)porphine-mediated PDT was found ⁸⁷. When benzoporphyrin-derivative monoacid ring (BPD-MA) was compared with BPD monoacid ring B (BPD-MB) and BPD diacid ring B (BPD-DB), the monoacid forms proved to be most efficient in suppressing CHS ⁸⁸. Also silicon phthalocyanine 4 (Pc4), a second generation PS, was tested and showed dose-dependent immunosuppression ⁸⁹. Additionally, the anatomical site of PDT seems to play a role in T-cell mediated immunity in CHS ⁹⁰.

Several studies have shown that T-cell mediated anti-tumor immunity is antigen specific. In a report by Mroz et al. in 2010 who used BALB/c mice bearing two different colon adenocarcinomas, the results of PDT were found to be dependent on specific antigen-expression ¹³. They used the CT26 wild-type (CT26WT) or the CT26.CL25 analogue expressing the bacterial protein beta-galactosidase (that functions as a model tumor antigen) and found cures and resistance to rechallenge only in the mice with antigen expressing tumors (CT26.CL25). The immune response against the CT26.CL25 tumor after PDT was sufficiently strong to destroy a distant established untreated tumor in the contralateral leg. Canti et al were the first to show that PDT could induce immune effects when they used aluminium disulphonated phthalocyanine mediated PDT to cure MS-2 fibrosarcomas in both normal mice and mice that had been immunosuppressed with cyclophosphamide (CY). When these groups were rechallenged, only the group with the unaltered immune system survived. A second rechallenge was performed with L1210 and P388 murine leukemias. None of the mice survived showing that anti-tumor immunity caused by PDT is tumor-type specific ⁹¹.

Korbelik et al treated three strains of mice, two of which were immunosuppressed (severe combined immunodeficient (SCID) and nude mice) with immunocompetent BALB/c mice, all bearing EMT6 mammary sarcomas receiving Photofrin-based PDT. Only the immunocompetent BALB/c mice were cured. Adoptive transfer of splenic T-lymphocytes from naive BALB/c mice into SCID mice showed positive effects, prolonging-disease free time before recurrence, when performed either before, immediately or 7 days after. The best result was when SCID mice were reconstituted with BALB/c bone marrow, as opposed to BALB/c mice, which were reconstituted with SCID bone marrow. Thus, the assumption can be made that the activity of host lymphoid populations was the key to successful PDT results ⁹². Saji et al. treated BALB/c mice with intratumoral dendritic cells and PDT. A chlorin-type PS ATXS10 Na(II) was chosen. The combination therapy was more successful than either treatment alone. Moreover distant metastases were reduced in size. This result was attributed to the presence of tumor-specific lymphocytes, whose presence was proven by chromium-release cytotoxic T-lymphocyte (CTL) assay and by IFN γ production ⁹³.

Although PDT has been shown to be an effective treatment for local tumor destruction, the generation of systemic responses through tumor-specific cytotoxic T-cells that can destroy

distant tumors remains the exception rather than the rule²⁶. The question is what factors determine whether anti-tumor immunity is generated or not^{11, 94, 95}. To enhance the occurrence of T-cell anti-tumor immunity, treatment with a single dose of BCG in combination with PDT has been shown to increase the number of memory-T cells in tumor draining lymph nodes⁹⁶. In another study CTLs and NK cells were stimulated by injection of immature dendritic cells into tumors and subsequent Photofrin-mediated PDT leading to a superior tumor response⁹⁷.

T- cells also play a role in the immunostimulatory effects of PDT produced cancer vaccines. Vaccines were produced by making lysates of PDT-killed tumor cells in vitro, that when injected into mice, induced a cytotoxic T-cell response and phenotypic DC maturation and slowed tumor growth⁹⁸. By contrast, lysates from UV-radiation or ionizing radiation killed tumor cells did not affect tumor growth. Another study showed that these PDT-induced vaccines were presented to T-cells after being taken up by antigen-presenting cells⁹⁹.

4 Importance of tumor antigens

Cancer immunotherapy uses various strategies to discover and take advantage of tumor antigens in order to improve the long-term survival after surgical removal or local cancer ablation^{100, 101}. Antigens that have been found to elicit an immune response against tumors can be divided into four different groups: (a) unique tumor-specific antigens that are caused by somatic mutations in genes¹⁰²; (b) antigens which are found in both normal and tumor tissues, but are more highly expressed in tumors¹⁰³; (c) tumor-antigens, which are found in several different tumors and are not specific to a single tumor type^{103, 104}; and (d) antigens of viral etiology¹⁰⁵. However, most tumors only exhibit weak immunogenicity and therefore escape immunosurveillance¹⁰⁶. Some recognized classes of tumor antigen are: (1) “Cancer-Testis” (antigens with normal expression restricted to male cells inside of the testis, but no expression on the somatic cells)¹⁰⁷⁻¹¹⁰; (2) Melanocyte lineage-related antigens (differentiation antigens that are expressed on most melanomas but they are not expressed on normal melanocytes)¹¹¹⁻¹¹³; and (3) some antigens expressed after mutations on the host cells that are caused by viruses^{102, 114-116}. Tumor antigens can play two different roles in anticancer therapy: (1) they can act as a target for drug or radioisotope-carrying vehicles that recognize the antigen such as monoclonal antibodies; and (2) they can act as specific antigens recognized by the host immune system.

The type and degree of antigen expression plays an important role in PDT. It has been demonstrated by Mroz and co-workers that PDT can induce T-cell recognition of antigens expressed on the surface of tumor cells. This process can lead to a potent systemic immune response that is capable of not only increasing the primary tumor destruction, but also can lead to destruction of a distant established untreated tumor¹³. As mentioned earlier, CTLs recognize antigens presented by tumor cells (via MHC class I) derived from proteins that have been translocated to the cell surface, secreted into the cytoplasm or released extracellularly. The epitopes (a small peptide that was part of a protein antigen and can be recognized by T-cells and antibodies) are presented by MHC class I on the tumor cell surface and, most importantly, by MHC class II on the DC surface, and this MHC-epitope complex is recognized by the T-cell receptor (TCR). This binding forms the core of the so-

called immunological synapse and triggers the T-cell activation, clonal expansion and differentiation into effector cells^{117, 118} (**Figure 2**). Two PDT-induced processes are likely to be helpful for increasing the expression and liberation of antigens from tumor cells: endoplasmic reticulum (ER) stress and ROS production. The expression of co-stimulatory molecules is also necessary. These considerations explain why some degree of T-cell immune response can occur¹¹⁹, however, the tumor can escape from immune surveillance by decreasing or losing the expression of both MHC molecules and also tumor antigens^{120, 121}.

The involvement of T-cells was also shown in two studies by Håkerud et al^{122, 123} who studied vaccination of mice with the model antigen, chicken ovalbumin (OVA). The group used photochemical internalization mediated by the PS meso-tetraphenylchlorin disulfonate to disrupt the endosomes that had taken up the exogenously administered protein. This endosomal disruption ensured that the antigen could be processed by the cytosolic proteasome-based processing machinery and directed to MHC class I for priming of CD8+ T-cells. The vaccinated mice were significantly protected from B16 melanoma cells expressing OVA antigen.

It is difficult to correlate the outcomes from laboratory animal models to naturally occurring cancers in patients, therefore efforts must be made towards a better comprehension of the mechanisms that lead the cancer cells to express tumor-related antigens, and how they can be killed in an immunogenic manner via PDT. Some relevant data have been obtained in the last decade, for example the paper by Garg and co-workers¹¹⁹, who treated T24, CT26, HeLa and MEF cells with the ER-localizing PS, hypericin, and found that PDT caused immunogenic apoptosis with phosphatidylserine externalization and ATP secretion through an overlapping PERK- and phosphoinositide 3-kinase (PI3K)-mediated mechanism (not associated with caspase signaling).

Mroz and co-workers were able to identify a correlation between a population of CD8+ T-cells that recognized an epitope of the murine P1A antigen and PDT efficacy. P815 tumors that expressed P1A were cured by PDT, while isotype P1.204 tumors that did not express P1A were not cured. They also showed that PDT was not efficient in nude mice (mice lacking adaptive immune system) bearing P815 tumors⁴. It is clear that approaches capable of inducing increased antigen expression by tumor cells are a good strategy to improve long-term tumor response and could increase the survival rates of patients, therefore it is important to unravel the mechanisms related to PDT-induced antigen expression and the consequent CD8+ cell proliferation/differentiation in order to successfully apply those strategies in the clinic.

5 Stimulation of dendritic cells

Dendritic cells (DC) are professional antigen-presenting cells (APCs) and a critical part of the innate and adaptive immune system. DC are derived from hematopoietic bone marrow progenitor cells¹²⁴, or from monocytes in the presence of GM-CSF and IL-4¹²⁵. Immature DCs can phagocytose both pathogens and damaged tumor cells, and subsequently undergo a transformation to DCs with a mature phenotype¹²⁶. In vitro the maturation of DCs is

typically measured by increased up-regulation of MHC II (for example HLA-DR in humans and I-A/I-E in mice), and by increased CD86¹¹⁹. Mature DC home to lymph nodes, where the tumor specific-antigen is presented to naive lymphocytes. This mechanism leads to proliferation of antigen specific CD4+ and CD8+ lymphocytes and thus activation of the adaptive immune system (**Figure 3**)¹²⁷. An immune response can be created and also distant metastases can be successfully targeted if effective activated tumor-specific CD8+ T-cells can be created^{8, 13, 128}.

For different kinds of anti-tumor immunotherapy, DCs play an important (not to say an essential) role. The death of tumor cells after PDT that can be recognized by the immune system is referred as immunogenic cell death (ICD)¹²⁹. In ICD the PS is often found to accumulate in the ER. The combination of ER stress and ROS production occurring after light delivery sets off danger signals, in the form of damage-associated molecular patterns (DAMPs) or cell death associated molecular patterns (CDAMPs)¹³⁰. These molecules are released, exposed and secreted by the dying cells¹³¹. Examples of DAMPs include heat shock protein (HSP)70 and HSP90, surface calreticulin, secreted ATP¹³², and high-mobility group protein B1 (HMGB1)¹³³. At times between 1 to 4 hours after PDT, calreticulin becomes expressed on the surface of damaged cells^{119, 134, 135}, then ATP¹³⁶⁻¹³⁸ is secreted from dying cells, followed by the expression of certain proteins such as, HSPs^{119, 139} and HMGB1^{140, 141}. These DAMPs play a role as activators of APCs and DCs¹³⁹. DAMPs can interact with antigen-presenting surface receptors on DC such as low density lipoprotein receptor-related protein 1 (LRP1)¹⁴² and CD40 ligands^{143, 144} leading to maturation and activation of DC and increased production of IL-1 β ^{8, 145, 146}. In similar manner to DAMPs, PDT also causes an increased expression of IL-1 β and IL-6, as well as a reduction in TNF- α from DCs. These changes increase tumor-antigen uptake by DC and increased anti-tumor immunity¹⁴⁷. The expression of pattern-recognition receptors (PRRs) such as toll-like receptors (TLRs)-2 and 4 on APCs is linked to the recognition of DAMPs¹⁴⁸. When DCs have homed to lymph nodes, interactions with T-lymphocytes through MHC molecules occurs, which leads to proliferation of specific- CD4+ or CD8+ T cells as well as production of IFN γ by T-cells and induction of anti-tumor immunity^{8, 127, 145, 146, 149, 150}.

PDT leads to oxidative damage of cellular macromolecules, including proteins that undergo multiple modifications such as fragmentation, cross-linking, and carbonylation, that result in protein unfolding and aggregation. Because the major mechanism for elimination of carbonylated proteins is their degradation by proteasomes, a combination of PDT with proteasome inhibitors might lead to accumulation of carbonylated proteins in ER, aggravated ER stress, and thus to potentiated cytotoxicity toward tumor cells. When Bortezomib (or another proteasome inhibitor, PSI) was combined with PDT in a mouse model of cancer (EMT6 and C-26) a significant anti-tumor effect was seen with 60% to 100% complete responses¹⁵¹. In patients with human myeloma, Bortezomib has been shown to increase anti-tumor immunity by promoting the expression of HSP90¹³².

Unfortunately, a variety of different resistance mechanisms operate to prevent PDT from universally eliciting a systemic anti-tumor response. Systemic steroids can function as an immunosuppressant, impairing systemic inflammation by disruption of DC differentiation and activation⁸. A possible correlation of ROS-damaged tumor cells and suppression of DC

stimulation is being discussed¹⁵². Although one phenotype of DC can be immunogenic, a different phenotype can also have pro-tolerogenic effects⁸. Tolerogenic DC activation may trigger counteraction that leads to the suppression of PDT-mediated immunity. Thus the DC may not only deliver the antigen to lymph nodes in a stimulatory way but also in a tolerogenic manner¹⁵³.

There are several mechanisms that can inhibit DC maturation, one of which is elevated levels of vascular endothelial growth factor (VEGF)¹⁵⁴. PDT can increase VEGF levels and thus could inhibit DC maturation¹⁵⁵ and increase the immature phenotype¹⁵⁶. Studies have shown that the PDT treatment benefit is diminished when increased VEGF is present^{154, 155}. This accumulation of immature DC can induce regulatory T-cells (Treg) causing immunosuppression and thus increase PDT resistance¹⁵⁷. Not surprisingly, low levels of VEGF, or the blocking of VEGF has led to physiologic DC maturation and function^{154, 156, 158}. When VEGF blocking via anti-VEGF antibody was carried out, specific anti-tumor CTL were enhanced⁸.

Resistance may also occur through upregulation of immunosuppressive cytokines and growth factors such as IL-10 (or TGF- β), which are secreted by apoptotic cells or macrophages⁸. Broady et al investigated the pro-tolerogenic effects of PDT with 4,5-dibromorhodamine methyl ester (TH9402) in dendritic and peripheral blood mononuclear cells (PBMCs). When PBMCs were examined 24 h after TH9402-PDT, the DCs remained immature with low phagocytosis; they had a reduced allostimulatory capacity and expressed increased IL-10 levels¹⁵³.

As it is not uncommon for PDT to fail to show a systemic anti-tumor response, immune cell dysfunction and immune suppression have been proposed as possible causes. The resistance of tumors to immune activation after PDT has led to combining immunostimulants with PDT, hoping to find an increased or synergistic tumor response. Xia et al. used PDT-mediated by verteporfin combined with injection of CpG oligodeoxynucleotide (a TLR-9 agonist) in BALB/c immunocompetent mice bearing 4T1 metastatic breast cancer. CpG was injected peritumorally before or after performing PDT. Immature DC were primed via TLR-9 activation to enhance phagocytosis. DC maturation and subsequent antigen-presentation to T-cells were followed. CpG administration resulted in improved local tumor control, and a survival advantage, showing that CpG can be an effective DC-targeted immunoadjuvant¹⁵⁹. Marrache et al. were able to enhance the immuno-stimulatory effect of PDT by combining a zinc phthalocyanine (ZnPc) based PS encapsulated within polymeric nanoparticles made from poly(d,l-lactic-co-glycolic acid)-b-poly(ethylene glycol) (PLGA-b-PEG) with addition of CpG oligodeoxynucleotide. Using a mouse model of 4T1 metastatic breast cancer, a synergistic effect of the ZnPc and CpG on the immune response was found¹⁶⁰.

6 Depletion of regulatory T-cells (Tregs)

Another possible mechanism of PDT resistance to T-cell mediated immune-response, involves Tregs, also referred to as suppressor T cells. Tregs have an important role within the immune system. Out of all the classes of regulatory cells, CD25+ CD4+ FoxP3+ T cells

are the most common ¹⁶¹⁻¹⁶³. Their purpose is to maintain tolerance to self-antigens, which they do by deactivating the immune system ^{106, 164, 165}. Their inhibitory effect on T-cells has been shown both in vitro and in vivo studies ^{163, 166}. Tregs in normal homeostasis function to prevent the developing of autoimmune diseases such as colitis and diabetes ¹⁶⁷⁻¹⁶⁹. IL-10, and TGF- β enhance proliferation of CD4+CD25+ Tregs ¹²⁰.

Since tumors are known to progress, despite the fact that they contain antigens that can be recognized by T-cells, it can be presumed that the body's immune defense must be suppressed either systemically or locally. An alternative explanation is that the tumors change in order to escape the immune attack, or else never activated the immune system at all ^{102, 170}. Moreover when depletion of Tregs is carried out, induction of immunity to weak and self-like antigens can be revealed¹⁶³. In order to demonstrate the correlation between Tregs and unrestricted tumor growth, Tregs were depleted using a monoclonal antibody, anti-CD25 mAb (PC61). The tumor size increased for 10 days and then declined. However, when the time of depletion of Tregs was more than 2 days after tumor inoculation, the tumors grew continuously and no regressions were observed ¹⁷¹. Nevertheless the combination of PC61 with PDT in a mouse model has not yet been investigated ²⁹.

It has been suggested that suppression of immune response by Tregs occurs predominantly at the tumor site, and that local reversal of suppression (even at a later stage of tumor development) can be an effective treatment ¹⁷². Depletion of Tregs and the subsequently decline in tumor growth has been shown to occur in several studies including models of myeloma, leukemia, melanoma and fibrosarcoma ^{171, 173, 174}. However, it is important to note that immune responses to malignant tumors often are weak, and therefore the depletion of Tregs might not of itself be sufficient to cause tumor regression; therefore combining Treg depletion with other immunologic interventions, such as transfer of activated T cells ¹⁷⁵, DC-based vaccines ¹⁷⁶ or tumor destructive approaches such as PDT have been suggested and are under investigation.

Treg levels seem to be comparable before, 7 day, and 14 days after PDT, but there is a transient increase in Treg numbers in the spleen and lymph nodes in the first few days after treatment ^{121, 177}. On the other hand, according to the study by Reginato et al. on patients with esophageal squamous cell carcinoma, despite the fact that measured Treg levels were comparable 7 and 14 days after PDT, the suppressive function of peripheral Tregs was abrogated after PDT ¹⁷⁷. This phenomenon may be attributed to the DAMPs generated by PDT, and to the increased expression of IL-6 (a cytokine involved in inhibition of Treg immunosuppressive capacity) following PDT ^{71, 177}.

Castano and colleagues reported that treatment of mice bearing J774 tumors (a highly metastatic BALB/c mice reticulum cell sarcoma) with low-dose (50mg/kg) cyclophosphamide (CY) administered 2 days before BPD-PDT, resulted in 70% permanent PDT cures, while no cures but only a small survival advantage was seen with either treatment alone (**Figure 4**) ²⁹. It was interesting to observe that high dose CY (150 mg/kg) combined with PDT had no benefit at all, and even may have made the PDT somewhat less effective. These data show that the beneficial effect of low dose CY were not related to its ability to act as an anti-cancer chemotherapy drug. In an attempt to demonstrate activation of

the host immune system and generation of long-term memory immunity, the group that received low-dose CY plus PDT and were cured were rechallenged with J774 tumor cells in the contralateral leg and 71% of the mice could reject the tumor rechallenge²⁹. In addition secretion of TGF- β , which functions as a stimulus for Tregs, was reduced^{178, 179}. An important role of Tregs in PDT-induced anti-tumor immunity was also demonstrated in a recent mouse study using CT26-wild type colon adenocarcinoma (a tumor expressing the self-antigen gp70)¹²¹. Upon selective depletion of Tregs with low-dose CY (50mg/kg) prior to PDT, a dramatically better survival was observed compared to PDT alone, or to low-dose CY treatment alone. Additionally, 65% percent of the mice treated with PDT+CY that were tumor free 90 days, rejected the rechallenge with the same tumor when a second dose of CY was administered before the rechallenge. This second rejection did not occur when the second low-dose CY was omitted, and tumors grew at the rechallenge site.

7 Epigenetic reversal

A great variety of molecular and cellular mechanisms contribute to tumor initiation, proliferation and metastatic development. Some of them are processes capable of interfering with gene expression and are, therefore, called epigenetic (from the greek epi-, meaning outside, plus the term genetics). Epigenetic events are often related to inactivation of tumor suppressive mechanisms and inactivation of DNA repair genes, both contributing to the progression of cancer¹⁸⁰⁻¹⁸². Epigenetic changes have been found as early as the very beginning of tumor development, and several lines of evidence suggest that epigenetic modifications play an important role in the predisposition of a tumor to metastasize¹⁸³⁻¹⁸⁶.

The downregulation of MHC class I expression by epigenetic modifications occurring in the tumor is one of the mechanisms that allow cancer cells to escape the surveillance of the immune system. Studies demonstrated that strategies directed towards the restoration of MHC class I expression can increase the efficacy of anti-cancer therapies¹⁸⁷⁻¹⁸⁹. Epigenetic processes include DNA methylation and demethylation, and modifications on histones, including methylation/demethylation, phosphorylation/dephosphorylation and acetylation/deacetylation. These processes regulate the RNA polymerase activity and control the access of transcription factors to gene promoters thus affecting gene expression¹⁹⁰. DNA methylation and histone modifications can be easily targeted and reversed by drugs, which has led to their investigation as possible clinical therapies¹⁹¹⁻¹⁹³.

Gong and co-workers demonstrated that breast cancer metastasis suppressor 1-like (BRMS1-L) was able to inhibit metastasis of breast cancer cells by epigenetically silencing FZD10, a seven-transmembrane receptor for the Wnt/ β -catenin pathway (related to the proliferation and loss of differentiation of cancer cells)¹⁹⁴. Metastases were also reversed in lung cancer after treatment with 5-aza-2'-deoxycytidine (5-aza-dC), which inhibits DNA methyltransferase at low doses, thus causing hypomethylation of DNA¹⁹⁵. Therefore, these could be strategies to control metastasis development in cancers with a metastatic profile, although methyltransferase inhibitors are not selective and can sometimes accelerate tumor progression^{10, 196}.

Oxidative stress generated by PDT could induce epigenetic changes *per se*, that may increase MHC class I expression on tumor cell surface, as demonstrated previously^{190, 197}. On the other hand, other authors have found that PDT (under certain conditions) could decrease MHC class I expression, as shown by King et al., who used a verteporfin-based PDT approach¹⁹⁸.

Epigenetic reversal agents such as demethylating agents can be used to remove the methylation of DNA and histones that govern gene expression (**Figure 5**). Therefore epigenetic reversal agents could be used in combination with PDT, to enhance the overall PDT response and especially increase the immune response. Both MHC class I molecules and tumor-associated antigens (TAAs) are often down-regulated in cancer cells, and when their levels are restored by epigenetic reversal agents, a better tumor antigen presentation occurs, facilitating tumor recognition by T-cells that will lead an antigen-specific effective antitumor response^{199, 200}. It has been shown that the pre-treatment of tumor cells with 5-aza-dC, can lead to restoration of P1A antigen expression in a range of P1A-negative mouse tumor cell lines as well as increasing MHC class I expression. When 5-aza-dC was combined with PDT, this combination induced much better long-term anti-tumor responses (including cures) in several different syngeneic mouse tumor models,¹⁰.

8 Clinical evidence of T-cell immunity and possible improvements

The first study to implicate immune response in the clinical outcome of PDT was by Abdel-Hady et al. High-grade lesions of vulval intraepithelial neoplasia (VIN 2–3) were resistant to PDT compared to low-grade lesions. It was suggested that high-grade lesions caused by high-risk HPV sub-types have lower MHC class I levels, and therefore a lack of cell-mediated immunity accounts for the observed poor PDT response of these genital lesions¹⁸⁸.

In another clinical study Kabingu et al. demonstrated that local PDT of BCC enhanced systemic anti-tumor immune responses. Before, and 7-10 days after treatment of with PDT, blood was drawn. Peripheral blood leukocytes were isolated and the HLA-A2 status and the reactivity to Hip1, (a transmembrane protein which is over-expressed in BCC) were evaluated. The study showed that higher reactivity towards Hip1 was found in HLA-A2 positive patients (the class that could be evaluated) post-PDT compared to HLA-A2 patients that underwent surgery. Superficial lesions appeared to be especially susceptible to increased systemic anti-tumor immunity²⁰¹.

Tumor response to PDT also depends on the parameters used. Fluence-rates, drug as well as light doses, are thought to play an important role. Thong et al. showed, that high fluence-rate PDT using Fotolon (a chlorin-based PS) in an angiosarcoma patient, led to some success in local tumor control, but only for up to one year. Conversely when the tumor recurred, and it was treated again this time with low fluence-rate PDT, the treatment achieved tumor eradication, and some spontaneous remission of non-treated distant lesions occurred, showing that a systemic anti-tumor immune response had been activated. Immunohistochemical examination further confirmed the activation of a cell-mediated systemic immune response^{202, 203}.

To date there has only been a limited number of reports of anti-tumor immunity occurring in patients after PDT. Therefore it is fair to say that systemic effects remain the exception rather than the rule. A possible reason for the rarity of clinical anti-tumor immunity after PDT, is the weakness of the immune system in older people, as well as in patients with tumors in advanced stages that may often be treated with PDT “as a last resort“. Stage 4 cancer patients can often suffer from severe immunosuppression.

As mentioned in the pre-clinical studies referred to above, it is likely that some kind of combination therapy will need to be applied to increase the immune response after clinical PDT in patients. This combination could either be some of the various immunoadjuvants derived from microbial cells, or else an already approved treatment such as low dose CY, epigenetic reversal agents (5aza-dC or Vorinostat) or the proteasome inhibitor, Bortezomib could be used. In terms of T-cell mediated immunity there might also be another possible combination treatment. Ipilimumab (Yervoy), a monoclonal antibody targeting the CTLA-4 receptor, is approved for the treatment of melanoma. CTLA-4 receptor signaling suppresses T-cell mediated immunity, and Ipilimumab blocks this receptor leading to increased tumor killing by cytotoxic T-cells ²⁰⁴. Another new anti-cancer drug is pembrolizumab, which targets the programmed cell death 1 (PD-1) receptor. Pembrolizumab is also approved for the use against melanoma ²⁰⁵. PD-1 is expressed on the surface of T-cells and B-cells and negatively regulates the immune response ²⁰⁶⁻²⁰⁹. The inhibition of PD-1 prevents its cognate ligand (PD-L1, which is expressed on tumor cells) from binding to PD-1 and thereby prevents the tumor from killing the attacking T-cells. However, there have not been any trials conducted on combination therapies involving PDT with either ipilimumab or with pembrolizumab. Further studies are required.

In conclusion it is fair to state that stimulation of anti-tumor immunity after PDT is a real phenomenon. However robust immune responses after PDT remain the exception rather than the rule. The reasons for this variability are many and diverse. The PDT parameters such as choice of PS, doses of both PS and light, fluence rate and drug-light interval are all important in optimizing the immune response. The expression of right kind of antigens in the tumor is of critical importance. Identifying and overcoming the immunosuppressive mechanisms that allow the tumor to grow in the first place, provides a wealth of opportunities for devising combination treatments. These may include co-administration of various immunostimulatory adjuvants, strategies that involve DC, depletion of Tregs, and epigenetic reversal agents. Future research will be able to test and optimize many of these PDT-based combinations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Productions of reactive oxygen species (ROS)

When light ($h\nu$) is absorbed by the photosensitizer (PS) the electron moves from a non excited, low-energy singlet state into a high-energy singlet state.

By intersystem crossing a transition into a long-lived excited triplet state can occur. In the presence of molecular oxygen, superoxide and hydroxyl radicals are formed in type I reactions and singlet oxygen in a type II reactions.

Figure 2. Immunologic synapse

An example of antigen presentation from an antigen-presenting cell (i.e. dendritic cells) to a CD8+ T cell. The so-called immunologic synapse occurs when T-cell receptors (TCR) recognizes the complex MHC I + epitope, generating a signal cascade that leads to T-cell activation. Many clusters of differentiation (CDs), help this process by generating co-stimulatory signals.

Figure 3. Activation of T cells

During photodynamic therapy (PDT) light is absorbed by a photosensitizer (PS). Tumor cells are killed through several mechanisms and antigen-presenting cells (APC) like dendritic cells (DC) take up tumor-specific antigens. In the lymph node T cells are primed and can then attack tumor cells.

Figure 4. Synergistic effects and BPD-mediated PDT

Kaplan–Meier survival curves of mice treated with BPD-mediated PDT and CY in different dosis. The most effective treatment turned out to be photodynamic therapy combined with low dose CY, which inhibits T-regulatory cells.

Figure 5. Epigenetic reversal

The main epigenetic mechanisms are methylation of DNA and methylation of histones. The first can lead to upregulation or downregulation of genes, depending on the targeted DNA. Histone methylation can lead to enhanced or decreased gene expression due to the exposition of gene promoter sites in the presence of transcription factors or gene inhibitors.