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Stimulation of anti-tumor immunity by photodynamic therapy

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Abstract

Photodynamic therapy (PDT) is a rapidly developing cancer treatment that utilizes the combination of nontoxic dyes and harmless visible light to destroy tumors by generating reactive oxygen species. PDT produces tumor-cell destruction in the context of acute inflammation that acts as a 'danger signal' to the innate immune system. Activation of the innate immune system increases the priming of tumor-specific T lymphocytes that have the ability to recognize and destroy distant tumor cells and, in addition, lead to the development of an immune memory that can combat recurrence of the cancer at a later point in time. PDT may be also successfully combined with immunomodulating strategies that are capable of overcoming or bypassing the escape mechanisms employed by the progressing tumor to evade immune attack. This article will cover the role of the immune response in PDT anti-tumor effectiveness. It will highlight the milestones in the development of PDT-mediated anti-tumor immunity and emphasize the combination strategies that may improve this therapy.

Keywords

anti-tumor immunity; cancer vaccines; cytotoxic T-lymphocytes; damage-associated molecular patterns; dendritic cells; photodynamic therapy; Toll-like receptor agonists; tumor-associated antigens

Since Richard Nixon's declaration to make the 'conquest of cancer a national crusade', our understanding of the development and propagation of cancer has considerably improved. As a result of major investments in cancer research and cancer prevention, treatment and survival has significantly improved over the last 40 years [1]. Consequently, the increasing knowledge created by basic scientific research becomes gradually translated into more (and sometimes more effective) treatment options [2]. Despite the increasing emergence of drugs produced by biotechnological techniques, in 2008 half a million individuals diagnosed with cancer died from their disease in the USA [1].

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Some of these drugs directed against tumor-associated factors such as ligands, receptors and transduction signaling factors are expensive, intrinsically cannot be used in a broad population of cancer patients and often fail to demonstrate their superiority over conventional chemotherapeutic drugs [3–6]. Furthermore, treating tumors with such 'one-target' drugs poses other problems to physicians. Some tumors remain persistently resistant to treatment and others are only detectable in advanced stages [7–9]. In addition, some tumors seem to adapt to these specialized medicines. Each time a portion of their biological pathways is blocked, they circumvent these obstacles by developing alternative routes for survival. Despite their known drawbacks, conventional intervention including surgery, radiation therapy and chemotherapy remain the first option in the oncologist's toolbox for the treatment of patients.

Photodynamic therapy (PDT) has been proven to be an interesting alternative to the three described treatment modalities in several indications [10,11]. This technique is based on the administration of a photosensitizing agent to a patient via topical or parenteral routes. Depending on its pharmacokinetic and pharmacodynamic properties, the intrinsically nontoxic photosensitizer (PS) selectively accumulates in the tumor cells and in the associated (morphologically modified) vasculature (Figure 1). Activation with light of an appropriate wavelength results in the activation of the PS from its electronic ground state (S_0) into one of its excited states (S_n). In biological media, the excited PS then returns to its lowest excited state (S_1) through internal conversion. From there, it can return to its ground state through heat dissipation, emission of fluorescence or convertion through spin-forbidden intersystem crossing (ISC) into its lowest triplet state. This long-lived triplet state can lose its energy through the emission of phosphorescence or by exchange of energy with its direct environment via collisional energy transfer. In the case of the presence of molecular oxygen, $^3O_2$ is converted into highly reactive $^1O_2$, which in turn gives rise to reactive oxygen species, ultimately destroying vital cellular targets in the close proximity [12]. Importantly, only the concomitant presence of all three constituents in PDT will result in a notable photodynamic action. Therefore, even in the case of a less selective accumulation of the PS in the target tissue, the selectivity of PDT can be managed by the presence/absence of one of the other two components.

Photodynamic therapy has several advantages over conventional treatment modalities. Along with its aforementioned selectivity, it can be repeated several times, side effects are rare, it is relatively inexpensive compared with biotechnological products, and resistance to this treatment has only been reported in rare cases [13,14]. To date, several PSs are commercially available and some of them are approved for the treatment for oncologic indications. Most of these marketed PSs belong to chemical class of porphyrins, chlorins or precursors thereof (Figure 2) [15]. Since its early beginnings, PDT has made substantial progress. Prolonged skin photosensitization, a problem that essentially hampered the broad implementation of PDT, has been nearly eliminated by the development of second-generation PSs [16]. Furthermore, these compounds absorb in regions of the visible spectrum, optimal for deep-tissue penetration, and problems encountered with respect to the formulation of mostly lipophilic PSs have been resolved by pharmaceutical sciences [17]. However, PDT is not yet a front-line therapy for most indications, presumably owing to the lack of large randomized clinical trials. Furthermore, severe side effects can be observed with suboptimal PDT parameters, such as PDT schedules, PS and light doses, especially in hollow organs.

In vivo, the curative or palliative effect from a photodynamic insult can be attributed to several, sometimes interconnected, biological and physiological effects (Figure 1). Depending on the localization of the PS within the organism and target cell, its concentration and also the light dose administered, PDT can exert its effect either through

direct cell killing, occlusion of the tumor-associated vasculature or modulation of the immune system. At a cellular level, both necrosis and apoptosis have been observed as a primary reaction to the photodynamic insult [11,18–21]. Recently, it has also been reported that autophagy seems to play a role in PDT [22,23].

Previously, it was assumed that PDT-induced damage is confined to the irradiated area. However, in 1941, Blum hypothesized the presence of so-called dilator substances leading to the increased permeability of the minute vasculature due to the release of a 'histamine-like' or H-substance [24]. Indeed, it is now accepted that the direct damage of tumor cells initiates the initiation of several cell-signaling cascades. Furthermore, damage to, and damage of, endothelial cells (ECs) will ultimately lead to the formation of thromboses and eventually to vascular occlusion. In both cases, the release of cell fragments, cytokines and inflammatory mediators is triggered. This reaction, in turn, activates a multifaceted spectrum of the host's response elements, including inflammation and innate or adaptive immunity. Both events are of essential importance for a pronounced systemic effect and the therapeutic outcome after PDT. In this article we will summarize how PDT stimulates the immune system. Early and late effects of PDT on the key players of the immune system and the consequences for the tumor on a local and systemic level will also be described.

PDT & anti-tumor immune response

The ideal cancer treatment modality should cause local tumor regression and eradication, as well as inducing a systemic anti-tumor immunity that could effectively eradicate distant metastases without toxicity to normal tissues. PDT may meet these expectations, since it produces acute inflammation and attracts immune cells to treat distant tumors. PDT-treated dying cells produce danger signals, which both increase the antigen presentation by dendric cells (DCs) and the recruitment of antigen-specific cytotoxic T lymphocytes (CTLs). This favorable response to the treatment can be impaired, however, by the mechanisms of immune escape employed by the tumor, in particular by intratumoral accumulation T regulatory cells (Tregs) [25]. It is therefore of the utmost importance that we understand the mechanisms that govern the PDT-induced anti-tumor immunity to be able to successfully exploit this phenomenon for the benefit of cancer patients.

The last 20 years of dedicated research have cast some light on PDT-mediated anti-tumor immune responses and our understanding of these processes has never been greater. In the following section, we review the significant body of work in this area.

Damage-associated molecular patterns

Researchers have recently discovered a second array of molecular motifs that may play a similar role to that of pathogen-associated molecular patterns (PAMPs). Instead of being associated with pathogenic microbes, these molecules are associated with host tissue damage. By analogy with the terminology used for PAMPs, this second class of early warning signals to the immune system was named damage-associated molecular patterns (DAMPs) [26–28]. DAMPs are intracellular molecules normally 'hidden' within live cells, which acquire various different properties such as immunostimulation upon exposure or secretion by sudden and uncontrolled induction of damaged and/or dying cells. DAMPs are thought to mediate the possible immunogenicity of dying cells. It has been proposed that hydrophobicity is probably the most 'ancient' danger signal capable of activating the innate immune system and hence most known DAMPs tend to contain many hydrophobic regions such as the chaperones belonging to the heat-shock protein (HSP) family [29]. A list of reported DAMPs is presented in Table 1.

The discovery of DAMPs may explain some contradictory reports concerning whether or not tumor cells that have been killed by apoptotic or by necrotic cell death pathways are immunogenic [30]. While it is generally accepted that necrotic tumor cells are proinflammatory and therefore likely to be immunogenic, the importance of necrotic tumor cell death for generating an immune response has not been specifically demonstrated in the case of PDT of cancer. It was considered that apoptotic cells in general and tumor cells that have been killed by apoptosis in particular were silently disposed of by macrophages and other phagocytic cells in a non-inflammatory fashion and were therefore unlikely to stimulate anti-tumor immunity [31,32]. However, other reports suggested that under certain circumstances, apoptotic tumor cells could be effective in generating an immune response [33,34]. Recently, researchers have begun to refer to immunogenic and non-immunogenic apoptosis [32,35,36]. It is likely that the original concept of programmed cell death that is known to be a major aspect of embryogenesis is non-inflammatory and non-immunogenic, but some modalities of cancer therapy that cause tumor damage (by certain chemotherapy agents or by PDT regimens) produce an inflammatory and therefore an immunogenic form of apoptosis, largely characterized by the release of DAMPS.

Although there have not as yet been many studies that have looked extensively at the release of DAMPs after PDT, the subject has begun to be studied [27,28]. The most frequently reported example of DAMP expression after PDT is the upregulation and translocation of HSPs to the cell membrane [37,38].

Involvement of innate immune response in anti-tumor PDT

The innate immune system consists of all the immune defenses that lack immunologic memory. Thus, a characteristic of innate responses is that they remain unchanged regardless of how often the antigen is encountered. The innate immune system consists of several immune cells that all participate is the first line of defense [39] and PDT has been shown to effectively engage them in the host's inflammatory responses to cancer [40,41]. PDT alters the tumor microenvironment by stimulating the release or expression of various proinflammatory and acute-phase response mediators from the PDT-treated site (Figure 3). They include complement proteins, HSPs, arachidonic acid derivatives, chemokines and cytokines such as TNF-α, IL-6 and IL-1 [42]. It is thought that PDT causes this inflammatory response in treated solid tumors by causing a significant rise in oxidative stress, thereby severely damaging cellular membranes and cytoplasmic structures. The body recognizes the presence of local trauma threatening the integrity of the affected site, and releases proinflammatory mediators to maintain homeostasis [43]. PDT thereby prompts a powerful acute inflammatory response, causing activation of complement and the accumulation of neutrophils and other inflammatory cells in large numbers at the treated site and to attack tumor cells [44,45]. In particular, the complement system has emerged as a powerful mediator of the effects of PDT on tumor cells and *in vitro* studies have indicated that PDT induces fixation of complement C3 protein to tumor cells [46]. Complement fixation, in turn, marks cells as targets for destruction by the innate immune system [47–49]. Complement not only acts as a direct mediator of inflammation but also stimulates cells to release secondary inflammatory mediators, including the cytokines IL-1β, TNF-α, IL-6, IL-10, granulocyte colony-stimulating factor, thromboxane, prostaglandins, leukotrienes, histamine and coagulation factors [50].

In addition to stimulating local inflammation, PDT acts systemically to induce a potent acute phase response [45]. Using animal tumor models subjected to PDT, researchers observed a dramatic rise in serum levels of established acute-phase reactants, including serum amyloid P component and mannose-binding lectin A. Upregulation of genes encoding C-reactive protein was also noted [51]. Furthermore, the acute-phase response causes marked neutrophilia by accelerating maturation of neutrophils in the bone marrow as well as

increasing neutrophil recruitment from storage pools [50]. In the following section, we discuss in detail the involvement of several classes of immune cells in the PDT anti-tumor response.

PDT & macrophages—Macrophages are phagocytic cells derived from blood-borne monocytes that are known to express a wide range of membrane cellular receptors that can recognize numerous endogenous and exogenous ligands [52]. In addition, macrophages have receptors for antibodies and complement, so that the coating of microorganisms with antibodies, complement or both enhances phagocytosis. The subsequent response is central to their functions in homeostasis as well as to host defense and they can be directly cytotoxic to tumor cells as well as engage in the activation of adaptive immunity through presentation of tumor antigens (TAs).

There are reports based on *in vitro* data that PDT can have an effect on monocyte/macrophage cell lineages. Macrophages can be activated by low sublethal doses of PDT [53] and secrete TNF-α [54] by a PDT-related increase in macrophage -activating factor [55,56]. Evidence also indicates that macrophages can show preferential cytotoxicity towards tumor cells treated with a sub-lethal dose of PDT [57] and that this effect may be due to potential interaction between macrophages and natural killer cells (NK) [58]. Macrophage functions can also be enhanced by several cytokines and when Krosl *et al*. repeatedly injected lethally irradiated squamous cell carcinoma (SCC) VII cells genetically engineered to produce granulocyte/macrophage colony stimulating factor (GM-CSF), they observed higher cytotoxic activity of tumor-associated macrophages against PDT-treated tumors [59].

PDT & neutrophils—The granulocytes are another group of cells that form the innate immune system. This group of cells is formed by neutrophils, basophils and eosinophils [60]. Contrary to macrophages, they are only weakly phagocytic and their main function is to secrete leukotrienes, prostaglandins and other cytokines to facilitate the development of the inflammatory response. The neutrophils and their responses are probably the most studied subject in the 'PDT and immunology' field.

Gollnick *et al*. demonstrated that 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-α (HPPH)-mediated-PDT caused neutrophil migration into the treated tumor area due to a transient and local increase in the expression of the chemokine macrophage inflammatory protein-2 (the murine equivalent of IL-8), together with increased expression of the adhesion molecule E-selectin [42]. Interestingly, they also found that the local and systemic increase in expression of IL-6 was not necessary for neutrophil recruitment. A subsequent report compared a low and a high fluence (total light energy) each delivered at a low and high fluence rate against Colo 26 murine tumor treated with HPPH [61]. The oxygen-conserving low fluence rate PDT at a high fluence yielded 70–80% tumor cures, whereas the same fluence at the oxygen-depleting high fluence rate yielded 10–15% tumor cures. The highest levels of inflammatory cytokines and neutrophilic infiltrates were measured with low fluence delivered at low fluence rate (10–20% cures) while the optimally curative PDT regimen (high fluence at low fluence rate) produced minimal inflammation. Interestingly, the depletion of neutrophils did not significantly change the high cure rates of that regimen but abolished curability in the maximally inflammatory regimen. This group went on to subsequently demonstrate that mice defective in neutrophil homing to peripheral tissues ($CXCR2^{(-/-)}$ mice) or mice depleted of neutrophils were unable to mount strong anti-tumor $CD8^+$ T-cell responses following PDT [62]. The lack of neutrophils seemed to be disturbing T-cell proliferation and/or survival and these results further support the notion that tumor-infiltrating neutrophils play an essential role in the establishment of anti-tumor immunity following PDT. Sluiter *et al*. first observed that neutrophils adhere to the microvascular wall after PDT *in vivo* [63] and that EC retracted after PDT allowing the adherence of neutrophils

by their β2-integrin adhesion receptors to the subendothelial matrix [64]. In agreement with this finding was a report describing that expression levels of the adhesion molecules ICAM-1 and VCAM-1 were downregulated on ECs after PDT [65]. The administration of anti-neutrophil serum together with PDT in rhabdomyosarcoma-bearing rats completely abrogated the expected anti-tumor PDT effects, providing additional information that neutrophil infiltration of the PDT-treated area is essential for an effective anti-tumor response [66]. Blocking ICAM-1 with monoclonal antibodies also reduced the number of tumor cures and a noticeable upregulation of ICAM1 ligands CD11b/c expressed by neutrophils was also associated with PDT-treated tumors [67]. An increase in the number of peripheral blood neutrophils was also found 4 h after PDT treatment and lasted for 24 h. It was preceded by an increase in serum levels of IL-1β. Anti-G-CSF antibodies decreased neutrophil numbers and decreased the efficacy of PDT. Krosl and colleagues investigated cellular infiltrate in the murine SCCVII model treated with Photofrin® PDT (Axcan Pharma, AL, USA) [44]. They reported a 200-fold rise in neutrophils. Cecic *et al*. established prominent and rapid neutrophilia after PDT (Photofrin or m-tetrahydroxyphenylchlorin) of mice with SCCVII or EMT6 mammary carcinomas and found that complement inhibition completely prevented this [68]. de Bruijn *et al*. published a study in which a treatment of rat rhabdomyosarcoma tumors with 5-aminolevulinic acid-PDT lead to a significant increase in blood neutrophils during the first few days after illumination, with the highest observed levels at 16 and 24 h post-treatment [69].

PDT & NK-cell recruitment—In a recent report, Kabingu *et al*. used NK cells depletion in severe combined immunodeficient (SCID) mice to assess the involvement of this cell lineage in PDT-induced immunity [70]. Mice were injected subcutaneously and intravenously at the same time with EMT/6 tumor cells to establish both lung and subcutaneous tumors; the subcutaneous tumors alone received PDT treatment. They observed that the number of lung tumors per mouse 10 days after PDT was significantly higher in NK-depleted animals, suggesting that NK cells participate in PDT-induced immune control of tumors. Moreover, in the absence of NK cells, SCID mice replenished with $CD8^+$ T cells exhibited a significant increase in lung tumor number. The authors concluded that that NK cells may play an important role in post-PDT activity of $CD8^+$ T cells and control of distant nontreated metastases.

PDT & DCs—Dendritic cells are the most potent APCs and a key element in the development of an anti-tumor immune response. There are two well-established maturation states for DCs that include the ‘immature’ and ‘mature’ states [71]. The decision ‘to mature’ is hugely influenced by the signals coming from the environment. In many instances, the tumor microenvironment not only fails to provide the proinflammatory signals needed for efficient DC activation, but also provides additional immunosuppressive mechanisms that actively inhibit it. These factors affect the differentiation of DCs, leading to decreased levels of functionally competent, mature APCs, and accumulation of immature dendritic cells [72]. The immature DCs are those that 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 MHCI and MHCII gene products and costimulatory molecules such as CD80 and CD86.

In the absence of inflammation, the DCs remain in an immature state, and antigens are presented to T cells in the lymph node without costimulation, leading to either the deletion of T cells or the generation of inducible Tregs. 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 costimulatory molecules. This allows the priming of $CD4^+$ T helper cells and $CD8^+$ CTLs, the activation of B cells and the initiation of an adaptive immune response.

It has been rapidly realized that DC may play an important role in PDT-mediated anti-tumor immunity. One of the major cellular factors induced by PDT and released from tumor cells is extracellular HSP70. HSP70 expression is prompted by cellular stress and, when HSP remains intracellular, it chaperones unfolded proteins and inhibits cell death by preventing the aggregation of cellular proteins [73]. This forms stable complexes with cytoplasmic TAs that can then either be displayed at the surface of cellular membrane or escape intact from dying necrotic cells to interact with APCs such as DCs and stimulate an anti-tumor immune response [37]. DCs as well as other APCs have high-affinity receptors on their surface and binding of HSP–antigen complexes leads to the effective activation and maturation of DCs and the subsequent presentation of the peptide antigen to $CD8^+$ cytotoxic T cells [74]. PDT mediated by three different PSs has been shown to increase HSP70 mRNA, but only mono-L-aspartyl chlorin-e6 and tin etiopurpurin, and not Photofrin, increased HSP70 protein levels in mouse tumor cells *in vitro* and in tumors *in vivo* [38]. The release of HSP-bound TAs that can easily be taken up by DCs from PDT-induced necrotic tumor cells may therefore explain the particular efficiency of PDT in stimulating an immune response against tumors. It has also been observed that PDT-generated lysates were able to induce phenotypic DC maturation and IL-12 expression. Korbelik and Sun produced a vaccine by treating SCCVII cells with benzoporphyrin derivative (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 cure [75]. Importantly, vaccine cells retrieved from the treatment site at 1 h postinjection were intermixed with DCs, exhibited HSP70 on their surface, and were opsonized by complement C3.

PDT & adaptive immunity

Adaptive immunity is provided by antigen-specific B and T cells. B cells produce immunoglobulins, the antigen-specific antibodies responsible for eliminating extracellular microorganisms. T cells are divided into several populations and they can be involved in helping B cells to make antibody or eradication of intracellular pathogens, activation of macrophages, killing of virally infected or cancer cells and finally immunosuppression. The involvement of the adaptive immune system in PDT is depicted in Figure 4.

PDT activation of $CD8^+$ T cells

The first evidence on the involvement of cytotoxic T cells in the PDT anti-tumor effects comes from Canti and colleagues [76] who examined the effects of PDT in both immunosuppressed and normal mice bearing MS-2 fibrosarcomas. All mice were cured and survived indefinitely, but resistance to MS-2 rechallenge was evident only in normal surviving animals cured by PDT, while immunosuppressed surviving animals and animals cured by surgery died after tumor rechallenge. Different syngeneic murine leukemias were not rejected. The subsequent studies with the adoptive transfer of splenic T lymphocytes from naive BALB/c mice into SCID mice performed before PDT provided additional evidence. It postponed the recurrence of treated tumors, while adoptive transfer performed immediately or 7 days after PDT had no benefit [77]. Adoptive transfer of non adherent splenocytes (a mixture of $CD4^+$ and $CD8^+$ T cells together with some B cells, NK cells and monocytes) from normal mice cured of EMT6 by PDT 5 weeks previously fully restored the curative effect of PDT on EMT6 tumors growing in SCID mice. Splenocytes obtained from donors cured by x-rays were much less effective. Additional studies used depletion to prove the role of cytotoxic T cells. The depletion of specific T-cell populations from donor

splenocytes indicated that $CD8^+$ CTLs had the most effect, while $CD4^+$ helper T cells played a supportive role [78]. Analogous studies were performed by a different group using PDT with the PS 2-iodo-5-ethylamino-9-diethylaminobenzo-phenothiazinium chloride [79].

PDT & $CD4^+$ T cells

Gollnick *et al*. demonstrated that $CD4^+$ T cell depletion had no effect on the ability of PDT to control tumors present outside the treated area [70]. Splenocytes transferred to SCID mice after depletion of $CD4^+$ T cells successfully controlled the growth of tumors and these observations were also confirmed in mice lacking CD40, a $CD4^+$ T cell costimulatory molecule necessary for interaction with DCs. The tumor growth control after PDT was not significantly different compared with wild-type mice. To further elucidate the necessity of $CD4^+$ T cells for PDT-induced immunity, they reconstituted SCID mice with only $CD8^+$ cells and next inoculated them with EMT/6 tumors. The following PDT treatment resulted in 40 days of tumor-free survival and the development of memory immunity demonstrated by rejection of rechallenge with intravenous administration of the same tumor.

PDT & TAs

The mechanism by which the immune system can tackle tumors is by recognition of TAs presented by MHC class I molecules on the tumor cell surface and the subsequent destruction of these tumor cells by CTLs. The molecular identity of a number of these TAs has been well defined, both in mouse and human tumors [80]. The TAs defined to date broadly belong to three major groups:

- Antigens encoded by cancer-testis genes of the melanoma antigen (MAGE)-type expressed in various tumors but not in normal tissues such as the mouse gene *P1A* and human genes of the MAGE, B melanoma antigen (BAGE) and G antigen (GAGE) families [81–86]. These antigens are tumor specific and absent on most normal adult cells. If they are present in normal tissues they are usually expressed in the immune-privileged sites like the testes and therefore are not exposed to the host immune system. They are also shared because they are present on many tumors of several histological types;
- Differentiation antigens of the melanocytic lineage, which are present on most melanomas but also on normal melanocytes [86–88];
- Antigens that result from tumor-specific mutations in genes, which are expressed in all tissues [89–93]. In most cases, the mutation is unique to a single tumor and these antigens are therefore individually specific.

Efforts have been made to introduce defined TAs into mouse tumor cell lines in order to have reproducible models to study. These are usually ‘foreign’ proteins such as β-galactosidase from bacteria [94], ovalbumin from chicken eggs [95] and viral influenza hemagglutinin [96], among others. For many of these TAs, the peptide sequence displayed in MHC-I molecules is known, and specific CTL lines that kill the tumor cells expressing these TAs have been produced. However successful this approach may be in the laboratory, the artifcial TAs are not clinically applicable and it would be preferable to study naturally occurring TAs. Therefore, we propose to study the antigen-specific PDT-induced anti-tumor immune response in a more clinically relevant setting by employing two naturally occurring cancer antigens: the mouse MAGE-type P1A antigen, the best described unmutated mouse cancer/testis tumor antigen that has been identified [81,97,98], and the E7 antigen associated with human papilloma virus-induced cervical cancer [99].

Our laboratory was the first to recognize the role of TAs in the anti-tumor immune response after PDT. We showed that a vascular PDT regimen was able to produce 100% long-term

cures and rejection of rechallenge when tumors were formed in C3H mice by RIF1 cells that had been transduced to stably express enhanced green fluorescent protein by a retroviral vector [100]. The recently completed study goes even further and showed that PDT can induce a highly potent antigen-specific systemic immune response capable of causing regression in distant established tumors that received no light [101].

Additional information on the role of TAs in the PDT immune response comes from other laboratories. The report by Abdel-Hady *et al*. observed a short-term response in a third of patients with high-grade vulval intraepithelial neoplasia (VIN 2–3) treated with 5-aminolevulinic acid PDT [102]. In this study, Abdel-Hady investigated the level of human papilloma virus infection, the levels of HLA expression as well as the infiltration of the lesions by the cells of the immune system in biopsies from responders and nonresponders. Interestingly the nonresponders were more likely to show HLA class I loss while the responders showed increased infiltration by $CD4^+$ and $CD8^+$ CTLs. These data indirectly point to the role of tumor antigen presentation in the anti-tumor PDT immunity.

Recently, the role of tumor antigen in PDT anti-tumor immunity has been studied in the clinical setting. In the study by Kabingu *et al*., basal cell carcinoma lesions were either treated with PDT or surgically removed and the results showed that the immune recognition of hedgehog-interacting protein 1, a TA that is increased in some patients with basal cell carcinoma lesions, was significantly better in patients treated with PDT [103]. These findings showed that local tumor PDT can enhance systemic immune responses to tumor antigen in patients, and helped to validate previous preclinical findings.

PDT & Tregs

A special population among $CD4^+$ T cells is Tregs. Tregs can be defined as a T-cell population that functionally suppresses an immune response. Tregs were initially described by Gershon *et al*. in the early 1970s and were called suppressor T cells [104]. There has recently been an explosion of interest in the role of Tregs in both mice and humans that led to many studies describing their involvement in both autoimmune disease [105] and cancer [106]. Tregs were initially characterized by coexpression of CD4 and high levels of CD25 (the high-affinity component of the IL-2R complex [IL-2R α-chain]) [107]. It was subsequently determined that the most specific marker for Tregs is the transcription factor *Foxp3* [108], as other Treg markers (CD25, CTLA-4 and glucocorticoid-induced tumor necrosis factor receptor [GITR]) can be found on other T-cell subsets, especially on activated $CD4^+$ T cells [109,110]. The transcription factor *Foxp3* is specifically expressed in Tregs and is required for their development [111]. $CD25^+$ Tregs comprise 5–10% of $CD4^+$ T cells and have been divided into two main classes: naturally occurring Tregs found in the thymus and inducible Tregs found in the periphery [112]. Naturally occurring Tregs are thought to have T-cell receptors (TCRs) that recognize self-antigens and to play a major role in the prevention of autoimmune disease. Inducible Tregs can be induced and differentiate in the periphery, such as in the tumor microenvironment. Thymus-derived Tregs in the tumor microenvironment might clonally expand following stimulation by tumor-associated DCs that frequently have an immature phenotype. Tregs can also be present in a tumor as a result of conversion from $CD4^+CD25^-$ T cells [113] under the influence of TGF-β, which is present at high levels in the tumor microenvironment [114]. Therefore the tumor (or tumor draining lymph nodes) might contain thymus-derived natural Tregs, expanded and converted natural Tregs, and locally differentiated and expanded T inducible regulatory type 1 cells.

It is thought that Tregs mediate their immunosuppressive effects by multiple pathways [115]. Tregs express CTLA-4, which binds to B7-1 and B7-2 costimulatory molecules on APCs but with affinities much higher than CD28, and produces negative signaling, rather

than the positive signaling produced by the equivalent molecule, CD28 expressed on normal T cells [116]. T-cell activation is a dynamic process that is determined by the strength of the TCR signal; the strength of costimulation provided by CD28; and the magnitude of inhibitory signals generated by CTLA-4. Tregs also express GITR, however, this appears to reduce suppressor function on binding its ligand [117]. Treg can express TGF-β, which is an immunosuppressive cytokine [118] that can induce further proliferation of Tregs [119]. Recent evidence also suggests that Tregs control T-cell activation by suppression of DC activation [120]. In addition, imaging studies have suggested that Tregs diminish the ability of DCs to form stable contacts with self-reactive T cells and thereby diminish their activation [121].

Tumor-induced expansion of Tregs is a major obstacle to successful cancer immunotherapy. It has been shown that Tregs inhibit the generation of immune responses against tumors [112]. Many studies report that increased tumor Tregs predict reduced survival or treatment response. Tregs mediate their immunosuppressive effects by multiple pathways [115,116,118–120], and the targeting of these cells using antibodies[112] or cyclophosphamide (CY) [122] promotes rejection of several transplantable murine tumors [123,124], leading to complete and permanent regressions of established tumors [125] or an increase in the fraction of both $CD4^+$ and $CD8^+$ T cells with a memory phenotype [126].

Our laboratory was the first to realize that Tregs may play an important and negative role in PDT anti-tumor immunity. We observed that Tregs can be efficiently depleted by a low dose of CY and that low-dose CY combined with BPD-PDT led to a significant number of long-term J774 reticulum cell sarcoma cures and resistance to tumor rechallenge, while either treatment alone led to 100% death from progressive tumors or metastasis [127]. Cured mice had tumor-specific T cells in spleens and low-dose CY was shown to significantly reduce the numbers of Tregs. Our recent preliminary data revealed that Treg depletion by CY can unravel PDT-mediated immune response to mouse autoantigen gp70 in the colon adenocarcinoma CT26WT model [Mroz P *et al.*, Unpublished Data]. Moreover, this combination treatment leads to the development of long-lasting immune memory that could only be uncovered by CY administration prior to rechallenge.

PDT & immunostimulants

Because of the infrequency and generally unsatisfactory nature of the observable immune response after PDT, a considerable amount of work has gone into testing strategies designed to administer some sort of immunostimulant or adjuvant in combination with PDT to increase the frequency or strength of the anti-tumor immune response. Many of these combination adjuvants fall into the classification of Toll-like receptor (TLR) agonists. TLRs were discovered in 1994 and named after the Toll gene in the fruit fly *Drosophila melanogaster* responsible for immunity to fungal infection, which it achieved by activating the synthesis of antimicrobial peptides [128]. It is now known that most mammalian species have between ten and 15 types of TLR and their role is to act as early warning systems for microbial invasion [129]. Various molecular motifs (PAMPs) derived from Gram-positive bacteria (TLR-1, −2 and −9), from Gram-negative bacteria (TLR-4, −5 and −9) and from viruses (TLR-3, −7 and −8) bind to TLR and thereby activate a cell-signaling pathway that results in secretion of cytokines, activation of the inflammatory cascade and the recruitment of innate immune cells that will destroy the microbial invaders [130]. Because of this ability to activate the immune system, several groups have studied whether or not it is beneficial to combine these substances with PDT. Owing to the nature of most TLR agonists, they cannot be administered systemically because they would cause unacceptable toxicity due to indiscriminate activation of innate immune cells all over the body and uncontrolled secretion of cytokines such as TNF-α. For these reasons, the combination of TLR agonists and other

related immunostimulatory preparations is usually carried out by local administration by means of an intratumoral or peritumoral injection of small amounts of the substance. This is schematically illustrated in Figure 5. Table 2 gives a listing of the studies in which microbial preparations have been successfully combined with PDT.

In addition to TLR agonists, other microbial substances (especially those derived from fungi) activate other aspects of the innate immune system such as complement and C-type lectins [131,132]. The adjuvants known as glycated chitosan [133] and schizophyllan [134] that have been combined with PDT are both probably recognized by C-type lectins. On the other hand, zymosan (also a fungal constituent) was proposed to act as a classical activator of complement when combined with PDT [135].

PDT & combination approaches

There have been studies that have used combination therapies to increase response of tumors to PDT by affecting various elements of the immune system (see Table 3 for a summary). These combination approaches include cytokines such as TNF-α, which was shown by Bellnier to potentiate Photofrin-mediated PDT of murine SMT-F adenocarcinoma after a single dose of intravenously administered recombinant human material [136]. Photofrin-mediated PDT was tested against SCCVII tumors in combination with serum vitamin D3-binding protein-derived macrophage-activating factor [137]. This markedly improved the outcome of PDT, but as a single agent had no significant effect on the growth of SCCVII tumors. Localized tumor treatment with G-CSF in combination with Photofrin-mediated PDT resulted in a significant reduction of tumor growth and an increase in the length of survival of BALB/c mice bearing two types of tumor: colo 26 tumors and Lewis lung carcinomas [138]. Moreover, 33% of colo 26 tumor-bearing mice were completely cured after combined therapy and developed a specific and long-lasting immunity. Korbelik and Cooper used intralesional γ-inulin (a potent classical complement activator) in delaying the recurrence of B16BL6 melanomas after PDT [139]. This effect of γ-inulin was further enhanced by IFN-γ pretreatment. Tumor C3 protein levels, already elevated after individual PDT or γ-inulin treatments, increased much more after their combination. With fibrosarcomas MCA205 and FsaR, adjuvant γ-inulin proved to be highly effective in reducing recurrence rates following PDT using four different PSs (BPD, ce6, Photofrin and m-tetrahydroxyphenylchlorin). At 3 days after PDT plus γ-inulin treatment, over 50% of cells found at the tumor site were CTLs engaged in killing specific targets via the perforin–granzyme pathway.

PDT-induced Immunity & cancer vaccines

If the antimicrobial vaccines can be considered as one of the seminal accomplishments of the 21st Century, the cancer vaccines that may reduce or eliminate morbidity and mortality from debilitating and fatal malignancies show considerable promise to be the next generation's greatest exploit [140]. Recent research indicates that PDT may be used to produce such cancer vaccines. PDT-derived anticancer vaccines have clinical potential to become beneficial adjuvant or primary therapy in the treatment of various cancers.

The concept behind conventional vaccination is the introduction of attenuated or killed forms of the microbe (or its toxins), which the body recognizes as foreign and produces protective antibodies against. Researchers have produced cancer vaccines with a similar mechanism in mind, exposing tumor cells to lethal doses of radiation and then introducing these killed tumor cells to animal subjects with the hope that the host's immune system will recognize the killed tumor cells and develop immunity. However, many tumor cells are poorly immunogenic and therefore are not the best target for vaccine-based therapies. It has been postulated, however, that PDT may be used to augment the immunogenicity of the

tumor cells and increase the effectiveness of anticancer vaccines. Korbelik and Sun incubated SCC cells with the photosensitizing agent BPD and exposed them to light (690 nm, 1 J/cm^2) before killing them via radiation exposure [75]. These PDT-treated tumor cells, which constitute a cancer vaccine, were injected peritumorally in mice bearing subcutaneous SCC. The PDT vaccine resulted in a significant therapeutic effect, including tumor growth retardation, regression and cures. Interestingly, increasing the inoculum of cancer cells in the PDT vaccine further improved the outcome, reaching the maximum benefit with 2×10^7 cells/vaccination. A further increase in cells per inoculum proved counterproductive and decreased vaccine efficacy, presumably due to a self-regulatory downregulation of host immune response due to excessive antigen load [141]. Remarkably, no significant difference was found between two concentrations of the PS agent BPD and both doses were equally effective. In an attempt to dissect the mechanism responsible for the observed phenomenon, Korbelik *et al*. excised lymph nodes from the mice 4 days post-vaccination, and noted a dramatic rise in lymph node cells in PDT-vaccinated mice as compared with controls [75]. A five-to-sixfold increase in T cells, an over 17-fold increase in B cells and a greater than tenfold increase in DCs has been noted. Furthermore, a greater percentage of T cells bore the $CD44^+CD45RB^-$ memory phenotype. Retrieved PDT vaccine tumor cells were found to be coated by C3 surface proteins, as were lymph node cells. The relevance of complement involvement was demonstrated by the fact that the PDT vaccine was deemed ineffective in C3 complement-deficient mice. Retrieved tumor cells were also found to express HSP70 on their cell surface. Gollnick *et al*. also explore the effectiveness of PDT vaccines in cancer therapy [142]. They compared the PDT vaccine potential with vaccines generated by ultraviolet radiation or ionizing irradiation. PDT-generated vaccines were tumor specific, induced a cytotoxic T-cell response and, unlike the other methods of producing vaccines, did not require coadministration of an adjuvant to be effective. Moreover, PDT-generated lysates were able to induce phenotypic DC maturation and IL-12 expression.

Expert commentary

It is now widely accepted that there is a pronounced activation of the immune system after PDT for cancer in both animal models and also in patients. However, there is no agreement on the molecular and cellular determinants of the effect and more importantly on how to improve it. T-cell responses (CD4 and CD8) have been observed and these have been shown to be tumor specific and to lead to memory immunity. Other reports have demonstrated the activation of neutrophils, NK cells and macrophages after PDT. It has been argued that PDT regimens that have a high degree of acute inflammation are better at immune activation than those in which the acute inflammation is lower. However, it is conceivable that PDT-induced inflammation may instead actually increase the rate of recurrence or regrowth of remaining tumor cells. It is known that an increase in inflammatory mediators can promote tumor cell growth in many situations [143]. Acute-phase response and complement activation have been proposed to be important pathways in immune responses after PDT. The realization that tumors have evolved particular pathways and mechanisms for evading the host immune response has only recently been appreciated by the PDT community. Large and diverse arrays of therapies that combine PDT and an immunostimulant have been tested but there is no agreement on which is best and which could be clinically applied.

Five-year view

We believe that the next 5 years will see great advances in the field of anti-tumor immune response after PDT. These advances will be largely driven by three considerations. First, there will be increased understanding from the tumor immunology community of how tumors interact with the immune system of their hosts. The different pathways that tumors use to evade the host immune response and suppress the immune system will be better

understood and more effective ways of overcoming these pathways will be discovered. Second, there will be advances from the PDT community. These will include better PSs and improved light sources and light delivery technology that will make obtaining a good local response in PDT-treated tumors more predictable and achievable. The PDT community will also increasingly study immunological aspects, especially in small animal tumor models. Combination studies will continue to increase. It is important that more collaboration and discussion between PDT specialists and tumor immunologists take place. Third, there will be more involvement by clinicians who treat cancer patients with PDT. We are only aware of one clinical paper that demonstrated effective induction of adaptive anti-tumor immunity after PDT [144]. Clinical PDT trials will include one or more measures of immune function and immune response in patients, and these metrics will be correlated with outcome. We must realize that in all probability it will take considerably longer than 5 years for the use of PDT to induce a sufficiently powerful anti-tumor immune response that results in improved survival in advanced cancer patients to become clinically accepted practice.

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Norbert Lange would like to dedicate his participation to this review to his beloved father.

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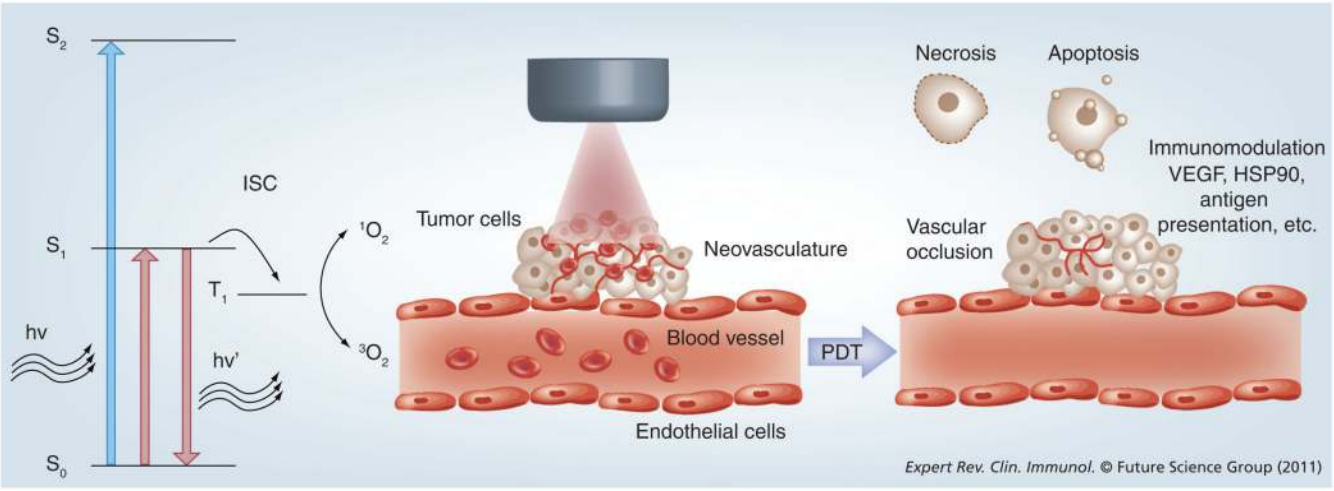

Figure 1. Anti-tumor mechanisms of photodynamic therapy

Jablonski diagram illustrates the absorption of light by photosensitizer ground state to form a short-lived excited singlet state that can lose energy by fluorescence, internal conversion to heat, or can undergo intersystem crossing to long-lived PS triplet state that can carry out photochemistry. Subsequently this photochemistry leads to the local production of reactive oxygen species that are cytotoxic to tumor and endothelial cells.

HSP: Heat-shock protein; hv: Light; ISC: Intersystem crossing; PDT: Photodynamic therapy; S_0: Ground state; S_1: First excited singlet state; S_2: Second excited singlet state; T_1: First excited triplet state.

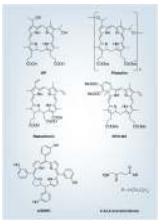

Figure 2. Clinically approved photosensitizers for photodynamic therapy

5-ALA: 5-aminolevulinic acid; BPD-MA: Benzoporphyrin derivative monoacid ring A; HP: Hematoporphyrin; mTHPC: m-tetrahydroxyphenylchlorin.

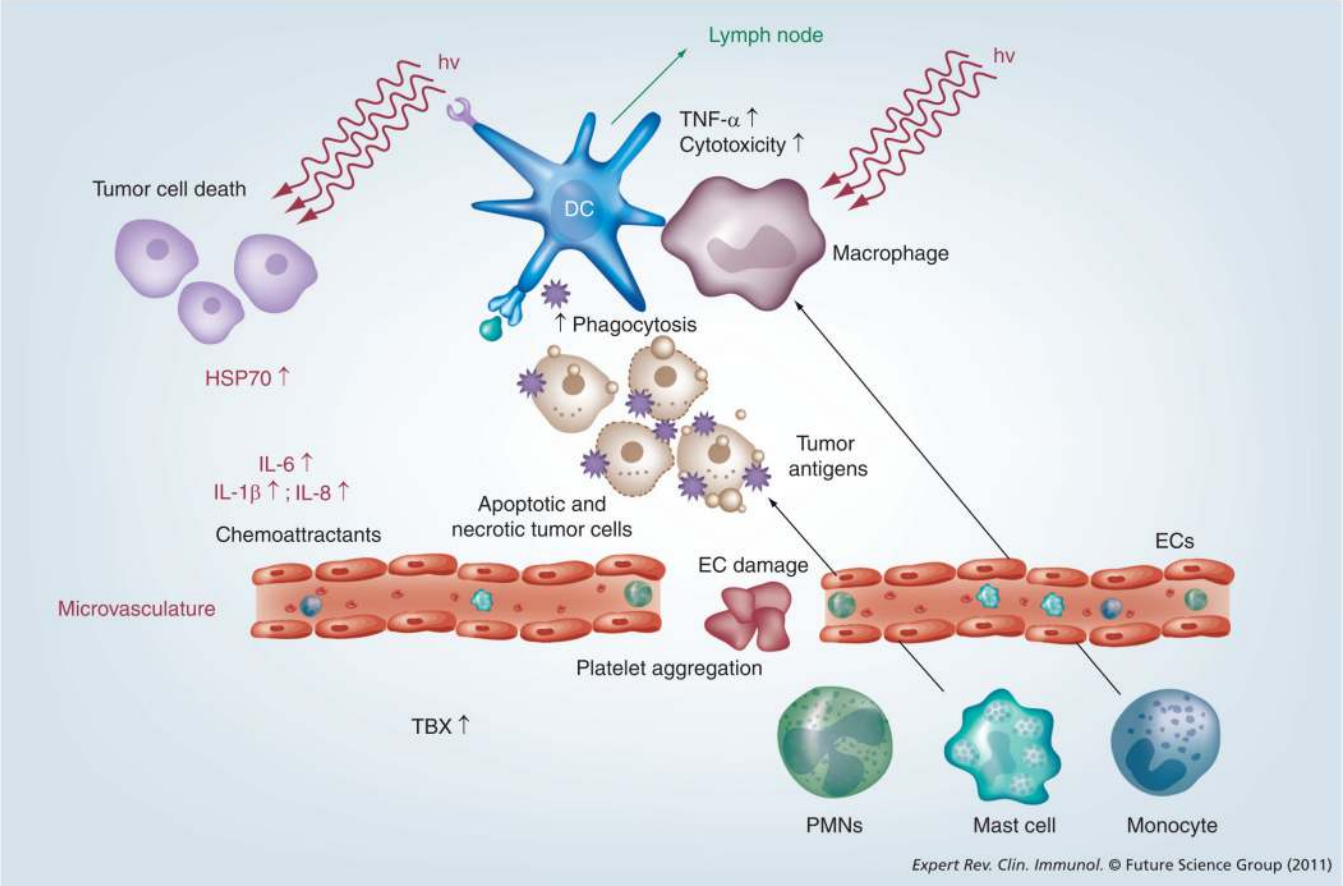

Figure 3. Photodynamic therapy of tumors leads to the development of local inflammation mediated by the localized release of danger signals, cytokines and derivatives of arachidonic acid

The infiltration of the treated area by various cells of the immune system follows.

EC: Endothelial cell; HSP: Heat-shock protein; hv: Light; PMN: Polymorphonuclear neutrophil; TBX: Thromboxane.

Adapted with permission from [25].

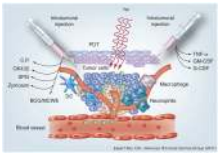

Figure 4. Photodynamic therapy-induced local inflammation leads to the development of systemic immunity

Antigens released from PDT-treated tumor cells are phagocytosed by DCs and presented to naive T cells in regional lymph nodes. Activated T cells return to the circulation and then track down and destroy tumors. BCG: Bacillus Calmette–Guérin; C.P.: *Corynebacterium parvum*; DC: Dendritic cell; G-CSF: Granulocyte colony-stimulating factor; GM-CSF: Granulocyte-macrophage colony-stimulating factor; hv: Light; MCWE: Mycobacterium cell wall extract; PDT: Photodynamic therapy; SPG: Schizophylan.

Adapted with permission from [25].

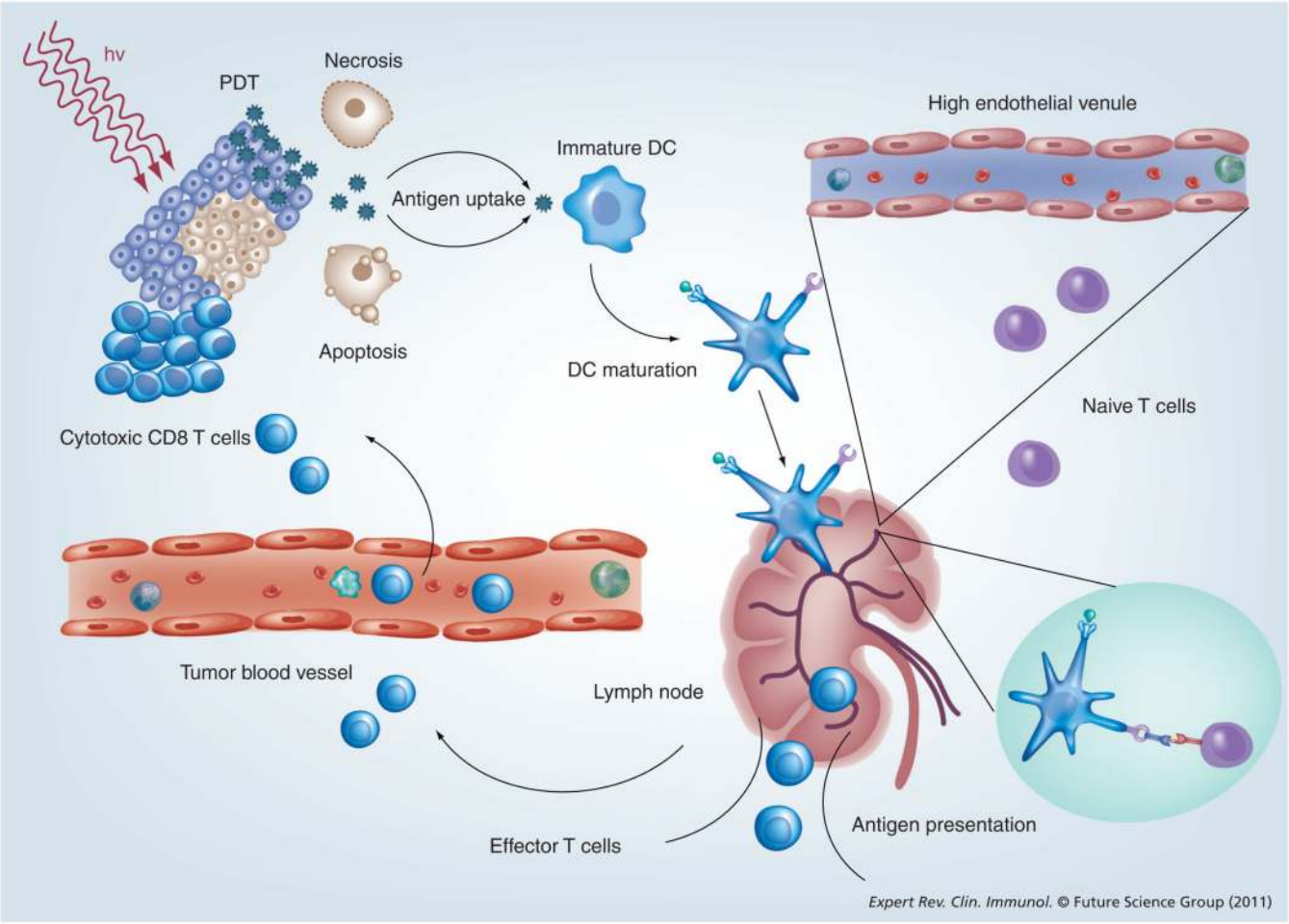

Figure 5. Local activation of the innate immune system can be strongly potentiated by strategies that facilitate better activation of dendritic cells, macrophages or neutrophils

Intratumoral injection greatly enhances the effectiveness of combination strategies.

DC: Dendritic cell; hv: Light; PDT: Photodynamic therapy.

Adapted with permission from [25].

Table 1

Damage-associated molecular pattern molecules that may be released after photodynamic therapy of tumors.

DAMP	Name	Function	Responder cells	Ref.
CRT	Calreticulin	ER-located calcium-binding protein		[145]
HSP70, HSP90, gp96	Heat-shock proteins	Inducible molecular chaperones on cell surface	Monocytes, neutrophils	[146]
HMGB1	High mobility group box-1	Nuclear chromatin-binding protein	Monocytes, neutrophils	[147]
ATP	Adenosine triphosphate	High-energy molecule, normally intracellular	Dendritic cells, microglia	[148]
S100S	Calgranulin family members	Calcium-binding proteins	Monocytes, neutrophils	[149]
SAP130	Spliceasome-associated protein 130	Histone deacetylase complex subunit	Macrophages	[150]
dRP S19	Covalent dimer of ribosomal protein S19	Constituent of small ribosomal subunit	Monocytes, neutrophils	[151]
Uric acid	Mono sodium urate	Derived from degradation of RNA and DNA	Dendritic cells, neutrophils, $CD4^+$ and $CD8^+$ T cells	[152]

DAMP: Damage-associated molecular patterns; ER: Endoplasmic reticulum; HSP: Heat-shock protein; PDT: Photodynamic therapy.

Table 2

Studies combining local application of microbial products with photodynamic therapy of tumors.

Adjuvant	Source	PDT agent	Tumor model	Ref.
OK432	Penicillin-killed streptococci	Hematoporphyrin derivative	Murine NR-S1 squamous cell carcinoma	[153]
CpG	Oligodeoxynucleotide	BPD (verteporfin)	Murine 4T1 mammary carcinoma	[154]
MCWE	Mycobacterium cell wall extract	Photofrin, BPD, mTHPC, ZnPc	Murine EMT6 sarcoma	[155]
BCG	Live mycobacterial vaccine	Photofrin, BPD, mTHPC, ZnPc	Murine EMT6 sarcoma	[156]
Cryptosporidium parvum	Killed bacterial vaccine	Hematoporphyrin derivative	Murine MBT2 transitional cell carcinoma	[157]
Glycated chitosan	Polysaccharide preparation	Photofrin, mTHPC	Murine EMT6 sarcoma, line 1 lung cancer	[133]
Schizophyllan	Fungal β-glucan	Photofrin	Murine SCCVII squamous carcinoma	[134]
Zymosan	Yeast cell wall extract	Photofrin, mTHPC	Murine SCCVII squamous and LLC lung carcinomas	[135]
Imiquimod topical	Small molecule TLR8 agonist	ALA–PPIX	Human HPV-associated vulval intraepithelial neoplasia	[158]

ALA–PPIX: 5-aminolevulinic acid–protoporphyrin IX; BCG: Bacillus Calmette–Guérin; BPD: Benzoporphyrin derivative; HPV: Human papilloma virus; LLC: Lewis lung carcinoma; MCWE: Mycobacterium cell wall extract; mTHPC: M-tetrahydroxyphenylchlorin; PDT: Photodynamic therapy; SCC: Squamous cell carcinoma; ZnPc: Zinc phtalocyanine.

Table 3

Studies of other immunostimulants that have been combined with photodynamic therapy of tumors.

Agent	Mechanism	PDT agent	Tumor model	Ref.
DBPMAF (ip. and peritumoral)	Serum vitamin D3-binding protein-derived macrophage-activating factor	Photofrin®	Murine SCCVII squamous carcinoma	[137]
TNF-α (single dose iv.)	Cytokine activation Antivascular effect	Photofrin	Murine SMT-F adenocarcinoma	[136]
DMXAA	TNF-α induction Antivascular effect	HPPH (Photochlor)	Murine CT-26 colon carcinoma	[159]
GM-CSF (intratumor)	Killed cells expressing GM-CSF Stimulates macrophages	BPD (Verteporfin)	Murine SCCVII squamous carcinoma	[160]
G-CSF	Stimulates neutrophils	Photofrin	Murine CT26 colon and LLC lung carcinomas	[138]
γ-inulin (intratumor)	Classical complement activator	BPD, ce6, Photofrin, mTHPC	Murine B16 melanoma MCA205 and FsaR fibrosarcomas	[139]
Low-dose CY (systemic ip.)	Regulatory T-cell depletion	BPD (15 min drug-light interval)	Murine J774 reticulum cell sarcoma	[161]

BPD: Benzoporphyrin derivative; CT: Colon tumor; CY: Cyclophosphamide; DBPMAF: Vitamin D-binding protein macrophage-activating factor; G-CSF: Granulocyte colony-stimulating factor; GM-CSF: Granulocyte-macrophage colony-stimulating factor; HPPH: 2- (1-hexyloxyethyl)-2-devinyl pyropheophorbide-a; ip.: Intraperitoneal; iv.: Intravenous; LLC: Lewis lung carcinoma; mTHPC: M-tetrahydroxyphenylchlorin; PDT: Photodynamic therapy; SCC: Squamous cell carcinoma.