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Review

Immunological aspects of photodynamic therapy

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ABSTRACT

Photodynamic therapy (PDT) involves the administration of a photosensitizer, followed by local irradiation of tumor tissues using a light of the appropriate wavelength to activate the photosensitizer. Since multiple cellular signaling cascades are concomitantly activated in cancer cells exposed to the photodynamic effect, understanding the responses of cancer cells to PDT will aid in the development of new interventions. The main effect of PDT is induction of localized tumor cell death and it could create a local depot of tumor associated antigens, which would be available for uptake and presentation to the immune system. In this review, we describe our latest findings regarding the induction of anti-tumor immunity following PDT against cancer cells. A more detailed understanding of the molecular mechanisms related to PDT immune-stimulation will potentially help to improve long-term survival of PDT treated patients.

KEYWORDS: photodynamic therapy, cancer, tumor immunity, dendritic cells, T cells

1. INTRODUCTION

Photodynamic therapy (PDT) is a photochemical modality approved for the treatment of various cancers and diseases in which neovascularization occurs [1, 2]. PDT is being considered not only as a palliative therapy, but also as a treatment option for early skin, lung, cervical, and esophageal cancers as well as basal cell carcinomas. Currently, PDT has been approved for localized diseases and precancerous lesions such as bladder cancers, pituitary tumors, and glioblastomas [3, 4]. Furthermore, numerous ongoing clinical studies have been designed to optimize the conditions for PDT; subsequently, PDT has been approved in several countries.

The PDT process consists of injecting a photosensitizer, which selectively accumulates at the lesion site, followed by local irradiation of the tumor with a light of the appropriate wavelength to activate the specific drug [5]. Absorption of a photon causes the absorbing molecule to be electronically excited (S_n: singlet states, T_n: triplet states). S_1 may react with neighboring molecules, transition to a different excited state (T_1), or relax to S_0. T_1 is longer-lived than S_1; consequently, the triplet state often mediates the biologically important reactions. T_1 can initiate photochemical reactions directly, yielding free radicals, or collisionally transfer energy to oxygen molecules, potentially resulting in $^1O_2$-mediated photo-oxidative reactions. The $^1O_2$-mediated photodynamic mechanism is generally accepted as a mode of PDT-induced cytotoxicity [1].

The direct destruction of cancer cells (necrosis) by PDT is caused by irreversible damage to the plasma membrane and intracellular organelles, such as the mitochondria, lysosomes, Golgi apparatus, and endoplasmic reticulum (ER). Necrosis may occur only after high photosensitizer and/or light doses, but these are generally to be avoided in order to suppress side effects. The mechanisms of PDT-induced apoptosis have been described by

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many researchers. Apoptosis, or programmed cell death, is one mechanism that mediates toxicity in the target tissue following PDT [6]. Apoptosis involves a cascade of molecular events leading to orderly cellular death without an inflammatory response [7, 8, 9]. The initiation of apoptosis involves a complex network of signaling pathways, both intrinsic and extrinsic to the individual cell, which are regulated in part by pro- and anti-apoptotic factors [7]. PDT has a direct effect on cancer cells, producing cell death by necrosis and/or apoptosis, in contrast to most conventional cytotoxic agents, which usually only trigger apoptotic cell death. The initial damage can involve different molecules, ultimately leading to activation of specific death pathways. Mitochondria-localized photosensitizers can cause immediate and light-dependent photodamage to mitochondrial components such as the anti-apoptotic Bcl-2 and Bcl-xL proteins, prompting the release of caspase-activating molecules [10]. Photosensitizers that accumulate in the lysosomes or ER also induce Bax-mediated caspase activation.

Another important cellular factor induced by PDT and released from necrotic tumor cells is heat-shock protein 70 (Hsp70) [11]. Hsp70 is significantly induced after stress; when it remains within the cell, it chaperones unfolded proteins and prevents cell death by inhibiting the aggregation of cellular proteins. PDT induces rapid cytochrome c release, initiating an apoptotic cascade via an activation of different caspases. Hsp70 directly binds to the caspase-recruitment domain of apoptotic-protease activating factor 1 (Apaf-1), thereby preventing the recruitment of Apaf-1 oligomerization and association of Apaf-1 with procaspase 9. These properties not only enable intracellular Hsp70 to inhibit cancer cell death by apoptosis, but also promote the formation of stable complexes with cytoplasmic tumor antigens. These antigens can then either be expressed at the cell surface or escape intact from dying necrotic cells to interact with antigen-presenting cells and thereby stimulate an anti-tumor immune response. The mechanism of cell death following PDT has been thoroughly summarized in the literature [6, 12, 13, 14, 15]. A better understanding of the molecular differences between apoptosis and necrosis, and identification of the crosstalk between these programs, will certainly prove crucial to the development of new PDT modalities aimed at increasing the efficiency of cancer-cell killing.

On the other hand, one inherent consequence of PDT is local hypoxia, which can arise either directly, from oxygen consumption during treatment [16, 17, 18], or indirectly, from the destruction of tumor vasculature as a result of effective treatment [19, 20]. Hypoxia is a major stimulus for angiogenesis, via its stabilization of the hypoxia-inducible factor-1α (HIF-1α) transcription factor [21, 22]. HIF-1 is a heterodimeric complex of two helix-loop-helix proteins, HIF-1α and HIF-1β (ARNT). ARNT is constitutively expressed, whereas HIF-1α is rapidly degraded under normoxic conditions. Hypoxia induces the stabilization of the HIF-1α subunit, which in turn allows formation of the transcriptionally active protein complex. A number of HIF-1–responsive genes have been identified, including those encoding vascular endothelial growth factor (VEGF), erythropoietin, and glucose transporter-1 [23, 24]. Following PDT, increases in VEGF secretion and angiogenic responses stimulated via HIF-1 pathways have been documented *in vivo* [25, 26, 27, 28]. VEGF induction could contribute to tumor survival and regrowth, and therefore could represent one of the factors that prevent PDT from achieving its full tumoricidal potential. Recent evidence indicates that PDT can destroy cancer cells directly by the efficient induction of apoptotic as well as non-apoptotic cell-death pathways. The identification of the molecular effectors that regulate the crosstalk between apoptosis and other major cell-death programs is an area of intense research in cancer therapy. Detailed biological mechanisms for killing of cancer cells by PDT, including signaling cascades, have been proposed by several researchers [6, 12, 13, 14, 15], but a few recent reports have focused on the response of cancer cells following PDT [29, 30, 31].

PDT also has a significant effect on the immune system [32, 33, 34]. PDT increases the immunogenicity of dead tumor cells by exposing or creating antigens, and by inducing heat-shock proteins that increase the efficiency of antigen cross-presentation to form more effective tumor-specific cytotoxic T cells [35]. In addition, the pro-inflammatory effects of PDT increase

dendritic-cell migration, antigen uptake and maturation. PDT-induced inflammation is also accompanied by leucocyte infiltration into the treated tumor. A major fraction of the infiltrating cells are neutrophils, but also included are mast cells and monocytes/macrophages [33]. Despite increasing numbers of identified tumor-specific antigens, there are clear advantages of whole-cell or polypeptide vaccination over targeting specific epitopes. The polyvalent vaccines, such as autologous whole-cell vaccines represented by PDT vaccines, secure greater coverage of potential tumor antigens and include the necessary determinants for helper T cells [36, 37]. However, there are only a few reports of the immunostimulatory effects of PDT, but increasing recognition of the effect should lead to further work and possibly to improved patient outcome [35].

In this review article, we summarize the induction of tumor immunity as a result of PDT against cancer cells. We hope this review article will contribute to a greater understanding of PDT-related mechanisms, which will in turn potentially improve long-term survival of PDT treated patients.

2. PDT induces anti-tumor immunity

In contrast to surgery, radiotherapy, and chemotherapy, which are mostly immunosuppressive, PDT causes acute inflammation, expression of heat-shock proteins, and invasion and infiltration of the tumor by leukocytes, and may increase the presentation of tumor-derived antigens to T cells [35]. The mechanism of PDT action on tumors including immune-responses reported by Hamblin *et al.* is shown in Figure 1 [35]. In the case of necrosis, cytosolic constituents spill into the extracellular space through the damaged plasma membrane and provoke a robust inflammatory response. By contrast, these products are safely isolated by the intact membranes that initially persist in apoptotic cells, which are ultimately phagocytosed by macrophages. The acute inflammation caused by PDT-induced necrosis might potentiate immunity by attracting host leukocytes into the tumor and increasing antigen presentation (Figure 2) [35].

Korbelik *et al.* reported many results regarding the PDT-associated host response and its role in determining the therapeutic outcome. Numerous preclinical and clinical studies have demonstrated

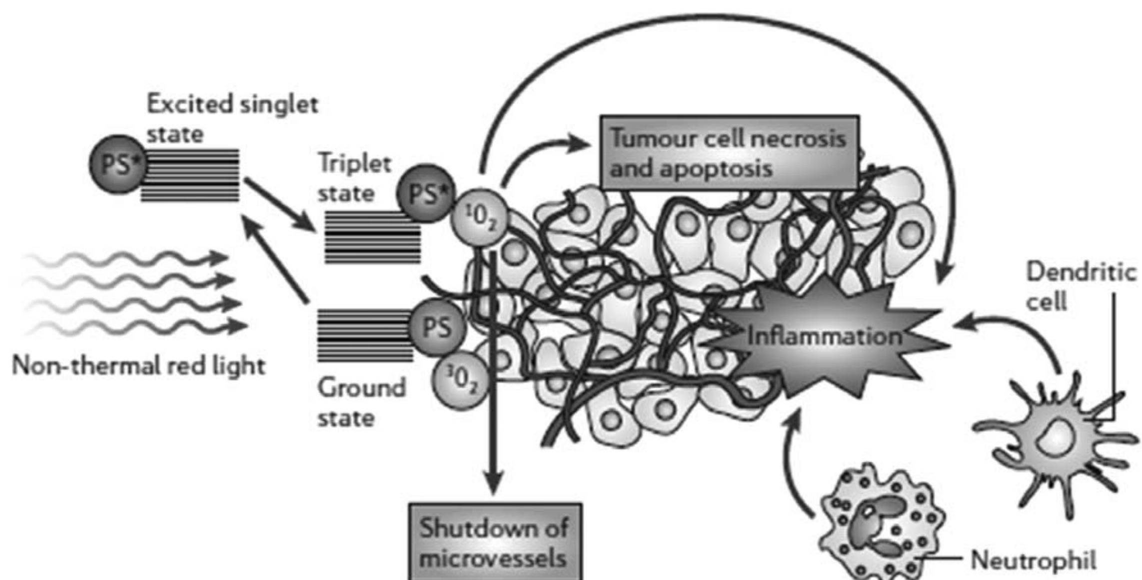

Figure 1. The mechanism of PDT action on tumors. Reactive singlet oxygen ($^1O_2$) can directly kill tumor cells by inducing necrosis and/or apoptosis, destroy tumor vasculature, and produce an acute inflammatory response that attracts leukocytes such as dendritic cells and neutrophils. PS: photosensitizer. PS*: excited PS. Adapted with permission from Nature Publishing Group: Castano, A. P., Mroz, P. and Hamblin, M. R. Nat. Rev. Cancer, 6, 535, Copyright (2006).

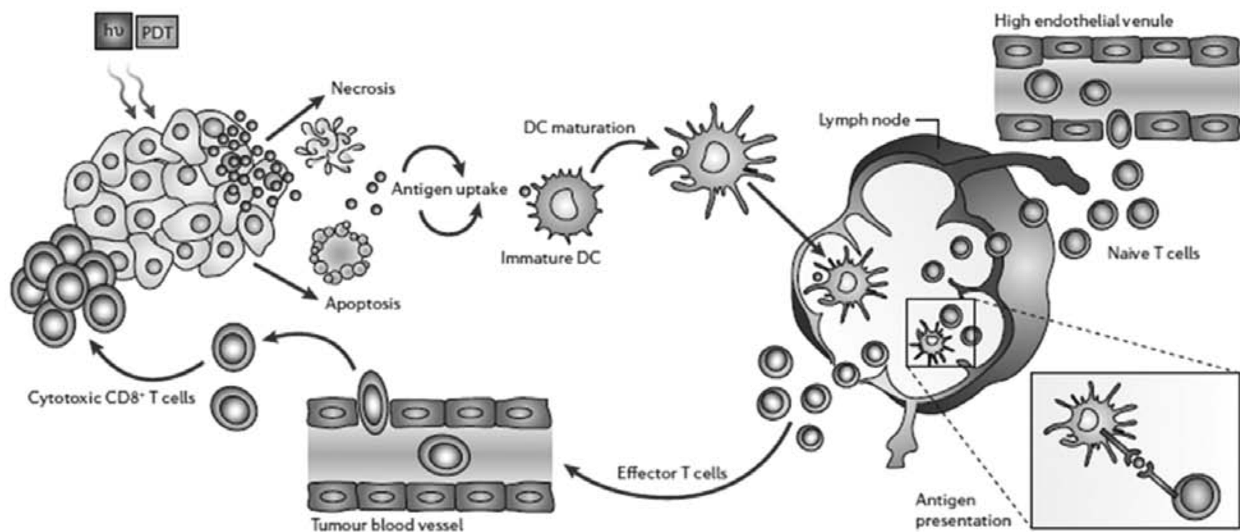

Figure 2. PDT induces activation of antigen-specific T cells. When light (hν) is delivered to a photosensitizer-loaded tumor, it induces both apoptotic and necrotic cell death. These cells are phagocytosed by dendritic cells (DCs) that accumulated in response to the acute inflammatory response triggered by PDT. DCs mature after stimulation by cytokines, which are released at the site of inflammation, and home to the regional lymph nodes, where they present antigens to the T lymphocytes. Activated T lymphocytes become effector T cells, which are attracted by chemokines to migrate into the tumor and kill cancer cells. Adapted with permission from Nature Publishing Group: Castano, A. P., Mroz, P. and Hamblin, M. R. Nat. Rev. Cancer, 6, 535, Copyright (2006).

that PDT can influence the host immune response as well as the therapeutic outcome (Table 1). The precise mechanisms that lead to potentiation versus suppression are unclear, but it appears that the effect of PDT on the immune system is dependent upon the treatment regimen, the area treated, and the type of photosensitizer used.

Fluence rate has a large impact on the host immune response to PDT. In PDT against angiosarcoma, lower fluence rate PDT results in better outcome; furthermore, the fluence rate per se, rather than the total light dose, is the more crucial determinant of the treatment outcome [38]. Specifically, PDT at lower fluence rates appears to activate the body's immune response against untreated lesions. Consistent with this, in treatment of basal cell carcinoma, immune reactivity following PDT is inversely correlated with treatment area and light dose [39]. However, in other studies, higher-energy doses resulted in large increases in reactive oxygen intermediates (ROI) and production of factors that could contribute to complete eradication of tumors; furthermore, higher doses were associated with decreased activation of NF-κB and IL-1β production that could inhibit tumor progression [40].

To the extent that the effect of PDT on anti-tumor immunity is regimen-dependent, it is tightly linked to the degree and nature of inflammation induced by PDT [41]. Although the precise mechanism underlying PDT-regulated adaptive anti-tumor immunity remains unclear, a growing body of literature has characterized the effects of PDT on various branches of the innate and adaptive immune system. Compared to surgery, radiotherapy, and chemotherapy, which are mostly immunosuppressive, PDT tends to activate the immune system. For example, immune recognition of Hip1 increased in patients whose basal cell carcinoma lesions were treated with PDT, and this increase in reactivity was significantly greater than reactivity observed in patients whose lesions were surgically removed [39]. PDT causes acute inflammation, expression of HSPs, and invasion and infiltration of the tumor by leukocytes, and may increase the presentation of tumor-derived antigens to T cells [35]. In the case of necrosis, cytosolic constituents spill into the extracellular space through the damaged plasma membrane and provoke a robust inflammatory response. By contrast, these products are safely isolated by the

Table 1. Recent (2002-2012) literatures regarding immune responses after PDT.

Observations	Authors	Reference
Immune response against angiosarcoma following lower fluence rate clinical PDT. Lower fluence rate PDT results in better outcome and also indicate that the fluence rate, rather than the total light dose, is a more crucial determinant of the treatment outcome.	Thong, P. S. *et al.*	[38]
Immune recognition of Hip1 increased in patients whose basal cell carcinoma lesions were treated with PDT. Patients with superficial lesions exhibited greater enhancement of reactivity compared with patients with nodular lesions. Immune reactivity following PDT was inversely correlated with treatment area and light dose.	Kabingu, E. *et al.*	[39]
At the higher energy doses, there was a large increase in reactive oxygen intermediates (ROI) and TNF-alpha release and decreased levels of NF-kappaB p50 and p65, IL-1beta production and NO release. The decrease in NF-kappaB p50 and p65 and IL-1beta levels could inhibit tumor progression.	Kawczyk-Krupka, A. *et al.*	[40]
The effect of PDT on the generation of anti-tumor immunity is regimen-dependent and was tightly linked to the degree and nature of inflammation induced by PDT. This review article focused on the current knowledge of immune regulation by PDT.	Brackett, C. M. *et al.*	[41]
Neutrophils were inflammatory and immune effectors in PDT-treated mouse SCCVII tumors. IL-1beta activity was critical for the therapeutic outcome, since its neutralisation diminished the cure rates of PDT-treated tumours.	Sun, J. *et al.*	[42]
PDT induced neutrophil migration into the treated tumour, which was associated with a transient, local increase in the expression of the chemokines macrophage inflammatory protein (MIP)-2 and KC.	Gollnick, S. O. *et al.*	[43]
Tumor cells dealing with non-lethal PDT were found to significantly upregulate a number of immune genes, which included the chemokine genes CXCL2, CXCL3 and IL8/CXCL8 as well as the genes for IL6 and its receptor IL6R, which can stimulate proinflammatory reactions, while IL6 and IL6R can also enhance tumor growth.	Kammerer, R. *et al.*	[44]
Although therapeutic-PDT could not demonstrate direct bacterial killing, neutrophils were accumulated into the infectious joint space after PDT and MRSA arthritis was reduced. With the preconditioning preventive-PDT regimen, neutrophils were quickly accumulated into the joint immediately after bacterial inoculation and bacterial growth was suppressed and the establishment of infection was inhibited.	Tanaka, M. *et al.*	[46]
Using 5-aminolevulinic acid as a pro-drug, kinetically favorable biologic conversion to the photosensitive protoporphyrin IX, appropriate trafficking of syngeneic bone marrow-derived DCs injected into PDT-treated tumors, and improved survival over either modality alone.	Sur, B. W. *et al.*	[58]
Macrophages coincubated with PDT-treated SCCVII cells displayed elevated levels of both HSP70 and GRP94 on their surface and were stimulated to produce tumor necrosis factor (TNF) alpha, whose production was inhibited by the presence of antibodies against either HSP70, Toll-like receptors 2 and 4, or specific NF-kappaB inhibitor in the coincubation medium.	Korbelik, M. *et al.*	[59]

Table 1 continued..

A sudden appearance of a large number of PDT vaccine cells elicits host responses for securing their optimized clearance, which in addition to producing seminal acute phase reactants includes the engagement of glucocorticoid hormones.	Korbelik, M. *et al.*	[60]
PDT tumor lysate-pulsed DC (PDT-DC) inhibited the growth of mammary EMT6 tumors to a greater extent than freeze/thawed tumor lysate-pulsed DC (FT-DC) or PDT tumor lysates.	Jung, N. C.	[61]
PDT treatment resulted in the induction of apoptotic and necrotic cell death and expression of HSP27, HSP60, HSP72/73, HSP90, HO-1, and GRP78 in C-26 cells. Immature DCs cocultured with PDT-treated C-26 cells efficiently engulfed killed tumor cells, acquired functional features of maturation, and produced substantial amounts of IL-12.	Jalili, A. *et al.*	[62]
PDT-generated lysates were able to activate DCs to express IL-12. PDT effects on tumor cells alone were sufficient to generate an antitumor immune response, indicating that the direct tumor effects of PDT played an important role in enhancing that host antitumor immune response.	Gollnick, S. O. *et al.*	[63]
PDT-generated tumor cell lysate induces IL-1alpha, IL-1beta, and IL-6 secretion from DCs, suggesting PDT enhanced anti-tumor immunity is due in part to increased DC activation.	Kushibiki, T. *et al.*	[64]
Elimination of IL-6 had no effect on innate cell mobilization into the treated tumor bed or tumor draining lymph node and did not affect primary antitumor T-cell activation by PDT.	Brackett, C. M. *et al.*	[74]
Photoreaction by a cell surface-bound photosensitizer was 10-fold more effective than photosensitizers localised to mitochondria or lysosomes. High-dose intracellular, but not cell surface, photoreaction inactivated IL-1 and reduced fibroblast stimulation.	Tracy, E. C. *et al.*	[77]
In vitro PDT significantly induces the MHC class I-related molecules, MICA, in Colo205 cells, but had no effect on MHC class I molecule expression. PDT also induced expression of NKG2D ligands (NKG2DL) following *in vivo* HPPH-PDT of a murine tumor.	Belicha-Villanueva, A. *et al.*	[78]
Surface expression of heat shock proteins and complement opsonization were the two unique features of PDT-treated cells securing avid immune recognition of vaccinated tumor and the development of a strong and effective antitumor adaptive immune response.	Korbelik, M. *et al.*	[79]
Complement genes C3, C5, and C9 become up-regulated in tumors treated by PDT, but not in the host's liver. Tumor-localized up-regulation of these genes can be largely attributed to monocytes/macrophages invading the treated lesion after PDT.	Stott, B. *et al.*	[80]
The lymphocytes that were isolated from PDT-treated mice were able to induce anti-tumor immunity in nude mice. The anti-glioma immunity fostered by PDT was inhibited in complement C3 knockout mice and the nude mice indicate the requirement of the activities of complement C3 and T cells.	Li, F. *et al.*	[81]
The immunohistochemical pattern was dominated by dense $CD4^+$ T lymphocytes infiltrating the superficial dermis, accompanied by an accumulation of Langerhans cells. Simultaneously, CD8 began to increase in the lesions of responding patients, and Langerhans cells seemed to migrate towards the dermis.	Giomi, B. *et al.*	[82]

Table 1 continued..

The immunosuppressive effects of phthalocyanine photodynamic therapy in mice were mediated by $CD4^+$ and $CD8^+$ T cells and can be adoptively transferred to naive recipients. PDT-induced immunosuppression could be adoptively transferred with spleen cells from PDT treated donor mice to syngenic naive recipients and was mediated primarily by T cells, although macrophages were also found to play a role.	Yusuf, N. *et al.*	[83]
PDT was capable of destroying distant, untreated, established, antigen-expressing tumors in 70% of the mice. The remaining 30% escaped destruction due to loss of expression of tumor antigen.	Mroz, P. *et al.*	[84]
Photodynamic-therapy activated immune response against distant untreated tumours in recurrent angiosarcoma.	Thong, P. S. *et al.*	[85]
$CD8^+$ T cell-mediated control of distant tumors following local PDT was independent of $CD4^+$ T cells and dependent on natural killer (NK) cells. Local PDT treatment led to enhanced anti-tumour immune memory that was evident 40 days after tumour treatment and was independent of $CD4^+$ T cells.	Kabingu, E. *et al.*	[86]
NK cells, T cells or macrophages in nonilluminated liver tumors in mTHPC-treated rats did not increase significantly when compared with tumors in rats without mTHPC treatment. No antitumor effect of a systemic immune response was present, as measured by the effect of PDT on growth of distant tumors and the number of T lymphocytes, NK cells and macrophages in these tumors.	van Duijnhoven, F. H. *et al.*	[87]
Cancer vaccine generated by PDT is therefore of considerable interest, particularly as it is becoming increasingly clear that it holds unique prospects for optimally presenting tumor antigens and because of emerging indications that its efficacy can be further potentiated by continued development.	Korbelik, M.	[90]
While the introduction of PDT vaccines into the clinics and testing on patients is still in a very early phase, much work can still be done on further improvement of the potency of PDT vaccines.	Korbelik, M.	[91]
The combination Navelbine+PDT+immune lymphocytes demonstrated a significant synergistic antitumor effect while the chemotherapy treatment with low dose of the drug is uneffective.	Canti, G. *et al.*	[92]
Pre-clinical and clinical studies demonstrated that PDT can induce various host immune responses. Clinical data also were shown that improved clinical outcomes can be obtained through the sequential use of PDT and immunomodulation.	Wang, X. L. *et al.*	[93]
Two different immunotherapy agents, gamma-interferon and antibody blocking inhibitory FcgammaRIIB receptor, were both found to be highly effective in potentiating the curative effect of SL052-PDT with SCCVII tumors.	Korbelik, M. *et al.*	[94]
This review article covered the combination approaches using immunostimulants including various microbial preparations that activate Toll-like receptors and other receptors for pathogen-associated molecular patterns, cytokines growth factors, and approaches that target regulatory T-cells.	St Denis, T. G. *et al.*	[95]

Table 1 continued..

This review article mainly discusses the effects exerted by PDT on cancer cells, immune cells as well as tumour microenvironment in terms of anti-tumour immunity.	Garg, A. D. *et al.*	[96]
Antitumor immunity promoted by vascular occluding therapy: lessons from vascular-targeted photodynamic therapy (VTP). VTP is about to enter phase III clinical trials for the therapy of prostate cancer and the potential involvement of the immune system may contribute an interesting aspect for the understanding and future development of this treatment modality.	Preise, D. *et al.*	[97]

intact membranes that initially persist in apoptotic cells, which are ultimately phagocytosed by macrophages. The acute inflammation caused by PDT-induced necrosis might potentiate immunity by attracting host leukocytes into the tumor and increasing antigen presentation [35].

The population of leukocytes that invades PDT-treated tumors includes neutrophils, which have been documented as inflammatory and immune effectors in photodynamic therapy–treated mouse SCCVII tumors; in that model, IL-1β activity was critical for the therapeutic outcome, because its neutralization diminished the cure rates of PDT-treated tumors [42]. Neutrophil migration into treated tumors is associated with a transient, local increase in the expression of the chemokines macrophage inflammatory protein (MIP)-2 and KC; a similar increase was detected in functional expression of adhesion molecules, e.g., E-selectin and intracellular adhesion molecule (ICAM)-1, and in both local and systemic expression of IL-6 [43]. In response to non-lethal PDT, tumors cells significantly upregulate a number of immune-related genes, including the genes encoding the chemokines CXCL2, CXCL3, and IL8/CXCL8 as well as IL6 and its receptor IL6R [44]. These chemokines can stimulate proinflammatory reactions, and IL6 and IL6R can also enhance tumor growth. Neutrophils also regulate the enhancement of antitumor immunity by PDT: by augmenting T-cell proliferation and/or survival, tumor-infiltrating neutrophils play an essential role in establishment of antitumor immunity following PDT, suggesting a mechanism by which neutrophils might affect antitumor immunity following other inflammation-inducing cancer therapies [45]. Neutrophils are also involved in the response to PDT in contexts other than cancer therapy: in a study of MRSA, although therapeutic PDT could not demonstrate direct bacterial killing, neutrophils accumulated in the infected joint space after PDT, and MRSA arthritis was reduced. With a preventive PDT preconditioning regimen, neutrophils quickly accumulated into the joint immediately after bacterial inoculation, suppressing bacterial growth and the establishment of infection [46].

For the generation of anti-tumor immunity, antigen presentation is crucial, and DCs are the most potent antigen-presenting cells. DCs pulsed with tumor-derived peptides, proteins, genes, or lysates, as well as DCs fused with cancer cells, have been studied as therapeutic cancer vaccines [47, 48, 49, 50, 51, 52, 53, 54, 55]. Although the methods involved are complex and costly to implement, promising results have been obtained in clinical trials in patients with advanced malignancies. These trials have shown DC-based vaccination to be well tolerated and capable of inducing tumor-specific T-cell responses and regression of metastatic disease. It is clear that induction of antitumor immunity after PDT is dependent upon induction of inflammation [56]. Mature DCs are critical for activation of tumor-specific $CD8^+$ T cells and the induction of antitumor immunity [57]. In response to PDT, DCs are activated [43] and migrate to tumor-draining lymph nodes, where they are thought to stimulate T-cell activation [43, 58]. Antigen presenting cells (APCs) isolated from PDT-treated mice exhibited an enhanced ability to stimulate T-cell proliferation and IFN-gamma secretion, suggesting that PDT resulted in increased APC activity [59]. PDT-mediated enhancement of antitumor immunity is believed to be due, at least in part, to stimulation

of DCs by dead and dying tumor cells, suggesting that *in vitro* PDT–treated tumor cells may act as effective antitumor vaccines. A sudden appearance of a large number of PDT vaccine cells elicits host responses for securing their optimized clearance, which in addition to producing seminal acute phase reactants includes the engagement of glucocorticoid hormones. It is becoming increasingly clear that a consummate execution of this process of PDT vaccine cell removal is critical for tumor antigen recognition and the attainment of potent antitumor immune response [60].

DC activation by material derived from PDT-treated cells positively affects treatment outcomes. For example, using 5-aminolevulinic acid as a pro-drug, kinetically favorable biologic conversion to the photosensitive protoporphyrin IX, appropriate trafficking of syngeneic bone marrow–derived DCs injected into PDT-treated tumors within 15 min of completion of therapy, and improved survival over either modality alone [58]. Likewise, PDT tumor lysate–pulsed DC (PDT-DC) inhibited the growth of mammary EMT6 tumors to a greater extent than freeze/thawed tumor lysate–pulsed DC (FT-DC) or PDT tumor lysates; PDT-DC also showed significant anti-tumor effects against fully established (i.e., late-stage) solid tumors [61].

Mechanistic studies have shown that incubation of immature DCs with PDT-treated tumor cells leads to enhanced DC maturation and activation and an increased ability to stimulate T cells. PDT treatment resulted in the induction of apoptotic and necrotic cell death and expression of HSP27, HSP60, HSP72/73, HSP90, HO-1, and GRP78 in C-26 cells; immature DCs co-cultured with PDT-treated C-26 cells efficiently engulfed killed tumor cells, acquired functional features of maturation, and produced substantial amounts of IL-12 [62]. Consistent with this, lysates from PDT-treated cells were able to activate DCs to express IL-12, and PDT effects on tumor cells alone were sufficient to generate an antitumor immune response, indicating that the direct tumor effects of PDT played an important role in enhancing the host antitumor immune response [63].

We have reported that PDT-generated cancer-cell lysate (from mouse Lewis Lung Carcinoma: LLC) induces secretion of IL-1α, IL-1β, and IL-6 from DCs, suggesting that PDT-enhanced anti-tumor immunity is due in part to increased DC activation [64] and the host antitumor immune response [65]. To determine whether this enhancement was at least in part a consequence of the effects of PDT on cancer cells, we tested the immunogenicity of cancer-cell lysates generated by *in vitro* PDT treatment using talaporfin sodium as a photosensitizer. IL-1α, IL-1β, and IL-6 were the most markedly increased, and TNF-α was decreased, in DC culture supernatants following this treatment (Figure 3A). These cytokines must have been secreted from DCs, because they were not detected in the cancer-cell lysates. The concentrations of other cytokines (with the exception of IL-2 and IL-12, which were below the detection limit of ELISA) were not changed relative to those of control cells. In parallel, cytokine levels were also examined in the supernatants of DC cultures treated with freeze/thaw-generated cancer cell lysates (Figure 3B). In these experiments, the levels of cytokines and growth factors secreted into the supernatant were unchanged after treatment with the freeze/thaw-generated lysates.

IL-1α, IL-1β, and TNF-α were investigated in parallel because they are recognized inducers of IL-6 and act synergistically with IL-6 to induce antitumor responses in mice [66, 67]. We confirmed the enhancement of IL-6 secretion from cells after *in vitro* PDT, described earlier by Kick *et al.* [68]. Furthermore, as suggested by Kick *et al.*, TNF-α does not seem to play a role in IL-6 induction by PDT, because the changes in IL-6 are neither preceded nor accompanied by similar changes in TNF-α. PDT induces TNF-α in murine peritoneal macrophages *in vitro* [69], and a study by Anderson *et al.* [70] demonstrated up-regulation of TNF-α in keratinocytes by *in vitro* PDT using a phthalocyanine-derived photosensitizer. The decreased levels of TNF-α observed in our study might be related to the DCs used, given that the regulatory region of the TNF-α gene exhibits polymorphism [71]. It remains to be determined whether the enhanced generation of IL-6 plays a role in the PDT-induced tumor response. Intratumoral injection of IL-6 or transduction of the IL-6 gene into cancer cells can enhance tumor immunogenicity and inhibit tumor growth in experimental murine tumor systems [67, 72, 73]. Thus, PDT may enhance local antitumor immunity

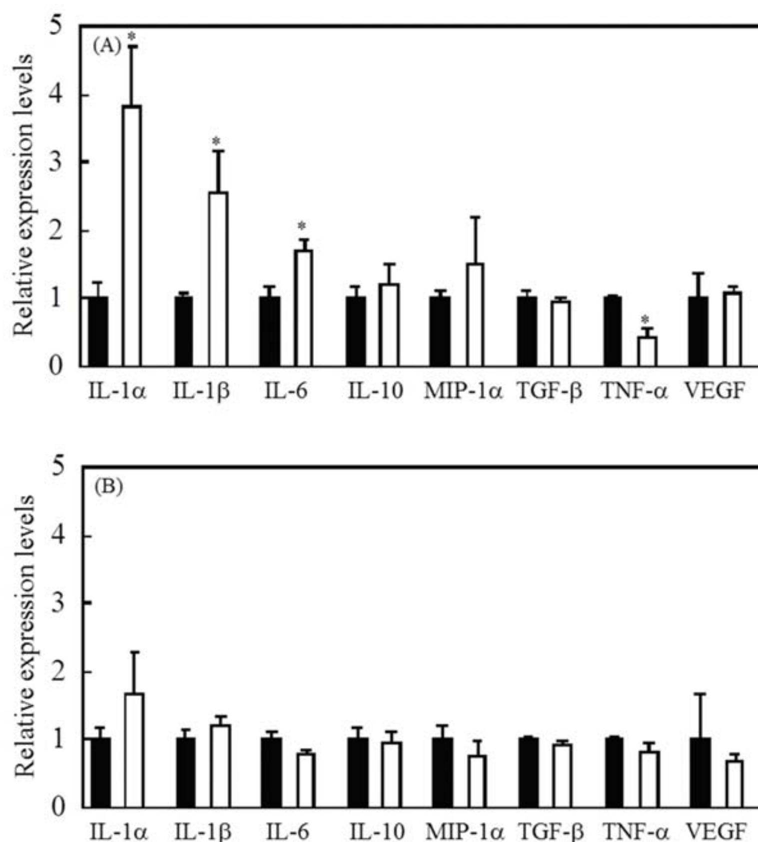

Figure 3. (A) PDT-generated cell lysates activate DCs. IL-1α, IL-1β, and IL-6 were most markedly increased, whereas TNF-α was decreased, following the addition of lysates from PDT-treated cells to DC cultures (white bars) compared with that of stationary culture media from LLC cells (black bars); talaporfin sodium (50 μg/mL) was used as a photosensitizer in the PDT. By contrast, cytokine levels did not change after addition of freeze/thaw-generated cancer-cell lysates to DC cultures (white bars) compared with that of stationary culture media from LLC cells (black bars) (B). IL-2 and IL-12 secretion levels were below the detection limits of ELISA assays. *, $p<0.01$: significant difference in levels between addition of PDT-generated lysates and addition of stationary culture media from LLC cells. Adapted with permission from e-Century Publishing Corporation: Kushibiki, T., Tajiri, T., Tomioka, Y. and Awazu, K. International journal of clinical and experimental medicine, 3, 110, Copyright (2010).

by upregulating IL-6 production in DCs, although the mechanisms by which this is achieved are not yet clear. Dougherty *et al.* [72] have suggested that IL-6 may further the recruitment of tumoricidal macrophages into the tumor bed. On the other hand, Mule *et al.* [67] have shown that IL-6–mediated tumor regression could be abrogated by *in vivo* depletion of either $CD4^+$ or $CD8^+$ T-cell subsets. Although this study did not examine T-cell responses, changes in T-cell function might occur, and we are presently addressing this issue using co-culture methods. In another study, elimination of IL-6 had no effect on innate cell mobilization into the treated tumor bed or tumor-draining lymph node, and did not affect primary antitumor T-cell activation by PDT; however, IL-6 does appear to negatively regulate the generation of antitumor immune memory and PDT efficacy against murine colon and mammary carcinoma models, in a manner that may be related to regulation of Bax protein expression [74].

Luna *et al.* [75] have shown in murine RIF cells *in vitro* that the early-response proteins c-fos and c-jun are induced by Photofrin; these gene products form the AP-1 transcription factor, which induces IL-6 expression [66, 68, 76]. Gollnick *et al.* [63] reported that vaccination with lysates from PDT-treated cancer cells elicits a tumor-specific immune response, as demonstrated by protection

against subsequent tumor inoculation, induction of tumoricidal activity in the spleen, and increased numbers of IFN-γ–secreting splenic cells. These studies demonstrate that PDT can enhance the inherent immunogenicity of at least some cancer cells.

The nature of the activating factor in lysates from PDT-treated cancer cells is unknown, although there are several promising candidates. In determining the response to PDT-treated cells, surface-expressed proteins may play a crucial role: photoreaction by a cell surface-bound photosensitizer was 10-fold more effective than photosensitizers localized to mitochondria or lysosomes; furthermore, high-dose intracellular, but not cell surface, photoreaction inactivated IL-1 and reduced fibroblast stimulation [77]. In the same study, the authors showed that epithelial cells released IL-1β as the primary fibroblast-stimulatory activity under basal conditions. Intracellular IL-1α, externalized following photoreaction, accounted for most of the PDT-mediated fibroblast activation. Expression of IL-1 was subject to increase or loss during oncogenic transformation, resulting in altered alarm functions that could be mobilized by PDT. *In vitro* PDT significantly induces the MHC class I-related molecules, MICA, in Colo205 cells, but had no effect on MHC class I molecule expression. PDT also induced expression of NKG2D ligands (NKG2DL) following *in vivo* HPPH-PDT of a murine tumor. Induction of MICA corresponded to increased NK killing of PDT-treated tumor cells [78].

Macrophages co-incubated with PDT-treated SCCVII cells displayed elevated levels of both HSP70 and GRP94 on their surface and were stimulated to produce tumor necrosis factor (TNF) alpha, whose production was inhibited by the presence of antibodies against either HSP70, Toll-like receptors 2 and 4, or specific NF-κB inhibitor in the co-incubation medium [59]. Surface expression of heat shock proteins and complement opsonization were two distinctive features of PDT-treated cells that conferred avid immune recognition of vaccinated tumors and the development of a strong and effective antitumor adaptive immune response [79]. Complement genes C3, C5, and C9 become upregulated in tumors treated by PDT, but not in the host's liver; tumor-localized up-regulation of these genes can be largely attributed to monocytes/macrophages invading the treated lesion after PDT [80]. Lymphocytes isolated from PDT-treated animals were able to induce anti-tumor immunity in nude mice; however, the anti-glioma immunity fostered by PDT was inhibited in complement C3 knockout mice, indicating the requirement of the activities of complement C3 and T cells. T cells that produce cytokines, along with complement C3, may play crucial roles in mediating PDT-induced anti-glioma responses [81].

T cells play other important roles in determining the response to PDT. In a study of PDT against genital warts, the immunohistochemical pattern was dominated by dense $CD4^+$ T lymphocytes infiltrating the superficial dermis, accompanied by an accumulation of Langerhans cells. Simultaneously, CD8 began to increase in the lesions of responding patients, and Langerhans cells seemed to migrate towards the dermis. $CD68^+$ macrophages apparently did not participate in the immune inflammatory response [82]. Conversely, the immunosuppressive effects of phthalocyanine photodynamic therapy in mice were mediated by $CD4^+$ and $CD8^+$ T cells and can be adoptively transferred to naive recipients. PDT-induced immunosuppression could be adoptively transferred with spleen cells from PDT treated donor mice to syngenic naive recipients and was mediated primarily by T cells, although macrophages were also found to play a role [83].

The implications of PDT-induced antitumor immunity and efficacious PDT-generated vaccines are significant, and raise the exciting possibility of using PDT in the treatment of metastatic disease or as an adjuvant in combination with other anti-cancer therapeutic modalities. Several preclinical studies have demonstrated that PDT can control the growth of tumors present outside the treatment field, although other studies have failed to demonstrate control of distant disease following PDT. In one study, PDT was capable of destroying distant, untreated, established, antigen-expressing tumors in 70% of mice, while the remaining 30% escaped destruction due to loss of expression of tumor antigen; these PDT anti-tumor effects were completely abrogated in the absence of the adaptive immune response [84]. In another study,

photodynamic-therapy activated the immune response against distant untreated tumors in recurrent angiosarcoma [85]. $CD8^+$ T cell-mediated control of distant tumors following local photodynamic therapy was independent of $CD4^+$ T cells and dependent on natural killer (NK) cells. Local PDT treatment led to enhanced anti-tumor immune memory that was evident 40 days after tumor treatment and was independent of $CD4^+$ T cells. $CD8^+$ T cell control of the growth of lung tumors present outside the treatment field following PDT was dependent upon the presence of NK cells [86]. NK cells, T cells or macrophages in non-illuminated liver tumors in mTHPC-treated rats did not increase significantly when compared with tumors in rats without mTHPC treatment. No antitumor effect of a systemic immune response was present, as measured by the effect of PDT on growth of distant tumors and the number of T lymphocytes, NK cells, and macrophages in these tumors [87].

Although some studies have focused on the use of genetically engineered cancer vaccines or tumor-associated antigen-primed DCs [88, 89], there is no convincing evidence that these vaccines have an overwhelming advantage over crude vaccines [89]. Although not all tumors are amenable to PDT, either because of size or location, the finding that PDT-generated cancer-cell lysates can act as effective antitumor vaccines has potentially broad clinical implications. Cancer vaccines generated by PDT are therefore of considerable interest, particularly as it is becoming increasingly clear that this approach holds unique prospects for optimally presenting tumor antigens and because of emerging indications that its efficacy can be further potentiated by continued development [90]. While the introduction of PDT vaccines into the clinics and testing on patients is still in a very early phase, much work can still be done on further improvement of the potency of PDT vaccines. Considerable advances can be expected by identifying the most effective adjuvants to be used with PDT vaccines, which will most likely be different with different types of cancerous lesions [91].

One could conceive of an adjuvant use for PDT vaccines in conjunction with other cancer modalities that do not enhance the host antitumor immune response, such as surgery and/or chemotherapy. The combination of navelbine, PDT, and immune lymphocytes demonstrated a significant synergistic antitumor effect, even though chemotherapy treatment with a low dose of the drug alone was ineffective; the same positive results were obtained with the combination of cisplatin, PDT and immune lymphocytes [92]. In addition to combinations with chemotherapy, PDT might also be used in combination with immunomodulation approaches. Recent clinical data also were shown that improved clinical outcomes can be obtained through the sequential use of PDT and immunomodulation [93]. Two different immunotherapy agents, γ-interferon and antibody blocking inhibitory FcγRIIB receptor, were both found to be highly effective in potentiating the curative effect of SL052-PDT with SCCVII tumors: combining SL052-PDT with FcγRIIB-blocking antibody treatment caused a further increase in the number of cells in tumor-draining lymph nodes and in degranulating $CD8^+$ cells, suggesting the amplification of the immune response induced by PDT. Vaccines consisting of SCCVII cells treated with SL052-PDT *in vitro* were effective in reducing growth of established subcutaneous SCCVII tumors [94]. Still other studies have investigated the effects of combined approaches using immunostimulants including various microbial preparations that activate Toll-like receptors and other receptors for pathogen-associated molecular patterns, cytokines growth factors, and approaches that target regulatory T-cells [95].

PDT is unique among other approved therapeutic procedures in generating a microenvironment suitable for development of systemic anti-tumor immunity. Furthermore, recent studies have described the emergence of certain promising modalities based on PDT, such photoimmunotherapy [96]. A variant of conventional PDT, vascular-targeted photodynamic therapy (VTP), is about to enter phase III clinical trials for the therapy of prostate cancer; the potential involvement of the immune system may contribute an interesting aspect for the understanding and future development of this treatment modality [97].

3. CONCLUSIONS

The rising interest in PDT as a promising anticancer treatment is demonstrated by the

growing body of literature on the photodynamic mechanism. Understanding the immunological aspects of PDT will certainly prove crucial to the development of new therapeutic modalities in PDT aimed at increasing the efficiency of cancer-cell killing. Moreover, a better knowledge of the way cancer cells die following PDT will contribute to a better understanding of the impact that different cell-death modalities have on the innate and adaptive immune responses, as well as on therapeutic outcome. The conjunction of imaging technologies, drug-delivery technologies, and a detailed understanding of PDT-related molecular mechanisms will provide an important source for new applications of PDT and for the development of individualized treatments.

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