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## Photodynamic Therapy for Cancer and Activation of Immune Response

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### ABSTRACT

Anti-tumor immunity is stimulated after PDT for cancer due to the acute inflammatory response, exposure and presentation of tumor-specific antigens, and induction of heat-shock proteins and other danger signals. Nevertheless effective, powerful tumor-specific immune response in both animal models and also in patients treated with PDT for cancer, is the exception rather than the rule. Research in our laboratory and also in others is geared towards identifying reasons for this sub-optimal immune response and discovering ways of maximizing it. Reasons why the immune response after PDT is less than optimal include the fact that tumor-antigens are considered to be self-like and poorly immunogenic, the tumor-mediated induction of CD4+CD25+foxP3+ regulatory T-cells (T-regs), that are able to inhibit both the priming and the effector phases of the cytotoxic CD8 T-cell anti-tumor response and the defects in dendritic cell maturation, activation and antigen-presentation that may also occur. Alternatively-activated macrophages (M2) have also been implicated. Strategies to overcome these immune escape mechanisms employed by different tumors include combination regimens using PDT and immunostimulating treatments such as products obtained from pathogenic microorganisms against which mammals have evolved recognition systems such as PAMPs and toll-like receptors (TLR). This paper will cover the use of CpG oligonucleotides (a TLR9 agonist found in bacterial DNA) to reverse dendritic cell dysfunction and methods to remove the immune suppressor effects of T-regs that are under active study.

**Keywords:** photodynamic therapy, anti-tumor immunity, regulatory T-cells, CpG oligonucleotides, low dose cyclophosphamide,

### 1. PDT AND ANTI-TUMOR IMMUNITY

PDT consists of an administration of a non-toxic dye or photosensitizer (PS) followed after a suitable interval with illumination of the tumor with light (hv) with a wavelength to match the absorption spectrum of the PS. This PS molecule absorbs energy in to give the first excited singlet state and can then undergo a transition to a long-lived excited triplet state that allows energy transfer to the ground state of molecular oxygen (a triplet) to produce the highly cytotoxic singlet oxygen ( $^1O_2^*$ ) (Type II reaction), or alternatively undergo electron transfer to form radical anions that are scavenged by oxygen (Type I reaction) with the formation of superoxide radical anion ( $O_2^{\cdot-}$ ) (Fig 1.). The mechanisms that operate to allow PDT to destroy tumors are multifactorial. There is a direct effect of PDT towards cancer cells producing cell death by necrosis and/or apoptosis [1]. There is also an effect against the tumor vasculature whereby illumination produces the shutdown of vessels that subsequently deprives the tumor of oxygen and nutrients [1]. The balance between these two mechanisms depends on many factors among which are the chemical structures of the PS (lipophilicity, amphiphilicity and protein binding) and the drug light time interval between injection and illumination. Finally, PDT also has a significant effect on the immune system [2], which can be either immunostimulatory or in some cases immunosuppressive.

PDT is an anti-cancer modality that can efficiently destroy local tumors in the context of acute local inflammation. PDT can increase DC maturation and differentiation and leads to generation of tumor specific CD8 T cells that can destroy distant deposits of untreated tumor. Successful PDT of tumors growing in immunocompetent syngeneic mice can in some cases cause a long-term memory anti-tumor immunity as demonstrated by a resistance to a rechallenge with the tumor from which they were cured, but not a different syngeneic tumor [3-5]. This effect is not observed when the same tumors are grown in immunosuppressed nude or SCID mice [6]. Splenocytes adoptively

transferred from immunocompetent mice cured of tumors by PDT can restore the curative effect of PDT in immunosuppressed animals, and demonstrate specific lysis of tumor cells growing in vitro in a classical CTL assay. Tumor cells killed with PDT in vitro are more effective as tumor vaccines than the same cells killed by other methods [7]. PDT has effects on cancer cells that make immune activation more likely in an in vivo tumor treated with PDT. PDT can induce strong expression of heat shock proteins (especially HSP70) [8-10] that has been shown to potentiate immune recognition of tumors. PDT can cause activation of the transcription factors nuclear factor kappa B (NFκB) [11] and activator protein (AP)-1 [12] leading to production of a large variety of inflammatory mediators including eicosanoids, interleukins (IL) 1, 6, 8 and 10. Neutrophils are an important cell type for the PDT response [13] and if mice are depleted of neutrophils before PDT, the curative effect is lost [14]. PDT has been shown to induce both a systemic neutrophilia and a strong and prolonged tumor infiltration by neutrophils. In addition tumor infiltration by dendritic cell (DC), macrophages and mast cells has been observed [15]. Complement activation is also observed both in the tumor and serum after PDT [16]. Nevertheless, it is clear that although PDT has the potential to stimulate a systemic anti-tumor immune response in animal models of cancer, this favorable result is not always observed. The explanation for this observation may be due to variations in the immunogenicity of different syngeneic mouse tumors, the presence of immune suppression caused by Tregs or the existence of DC dysfunction caused by the tumor.

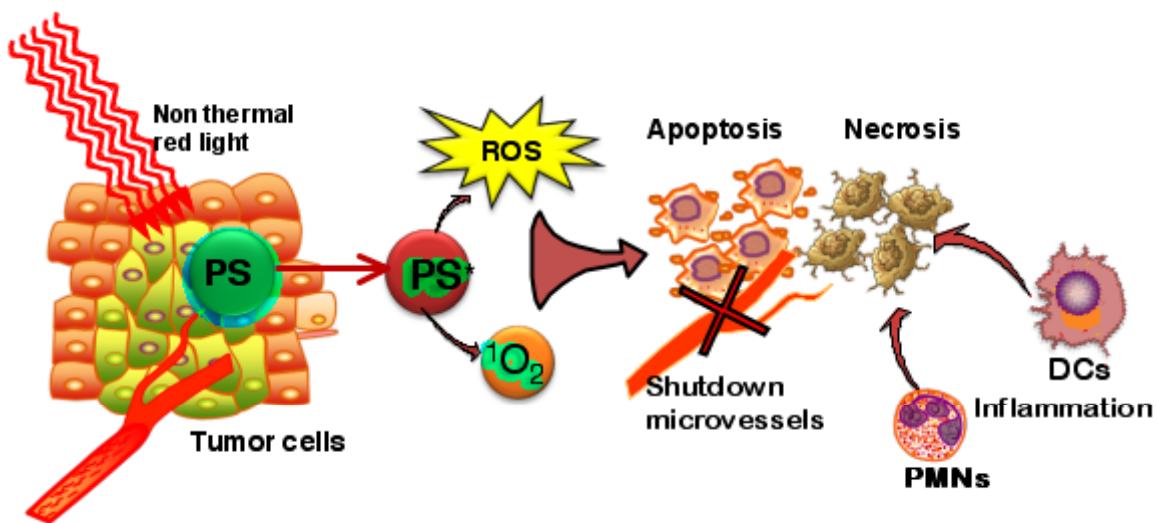


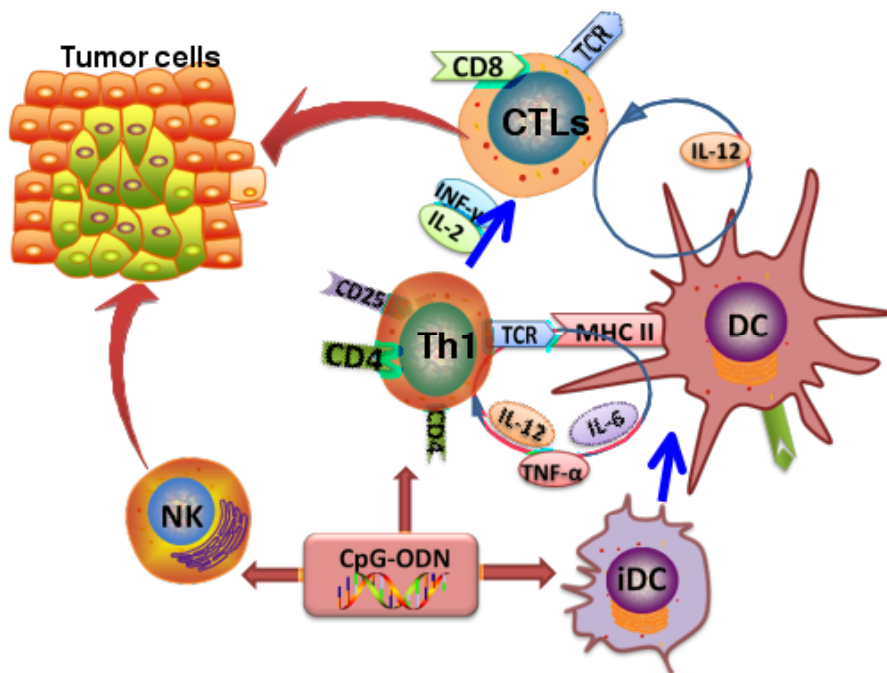
Figure 1. Schematic illustrating how PDT of cancer can stimulate the immune response.

## 2. PDT AND CpG OLIGONUCLEOTIDES

The two well-established maturation states for DC include the "immature" and "mature" states [17]. Immature, conventional DC display a phenotype reflecting their specialized function as antigen-capturing cells. They are highly endocytic, able to acquire fluid-phase antigens by macropinocytosis, take up protein or antigen-antibody immune complexes by receptor-mediated endocytosis, and ingest entire cells by phagocytosis. They express relatively low levels of surface MHC-I and MHC-II gene products and costimulatory molecules such as CD80 and CD86. Although immature DC can capture antigens, they are unable to process and present them efficiently to T cells. By comparison, freshly isolated, steady-state plasmacytoid DC are more weakly endocytic than conventional DC and poor T cell stimulators. They express MHC-I but not MHC-II and have a weak costimulator expression. However, conventional and plasmacytoid, immature DC have been described as inducers of T cell tolerance [18]. Mature DC are immunogenic in that they express cell surface molecules important for T cell activation. The phenotypic changes commonly associated with DC maturation make DC potent activators of T cell immunity. The outcome of T cell stimulation as tolerance or immunity depends on whether mature DC have been activated. Previous reports have used the terms "maturation" and "activation" interchangeably. However, maturation and activation appear to be two

distinct processes. Activation is a process, dependent on additional stimuli including "danger signals". It is proposed therefore that resting or steady-state, mature DC induce a state of tolerance, and activated, mature DC induce a state of immunity. It is also feasible that mature, resting DC, which are tolerogenic, can also be activated to form immunogenic DC. Under conditions of infection or inflammation, DC encounter activating signals that may mature and activate DC simultaneously, making them immunogenic. DC activators or danger signals include proinflammatory cytokines and bacterial or viral products such as LPS, CpG motifs, and double-stranded RNA [19]. These factors may induce the maturation and activation of DC, allowing DC to present antigens to T cells. Activated DC can be distinguished from resting, mature DC by expression of higher levels of MHC and costimulatory molecules or by production of cytokines such as interleukin (IL)-12 and interferon-, in the case of plasmacytoid DC. Bacterial DNA and immunostimulatory synthetic CpG-oligodeoxynucleotides (ODN) act as adjuvants for Th1 responses and cytotoxic T cell responses to proteinaceous antigens. CpG-ODN cause simultaneous maturation of immature DC and activation of mature DC to produce cytokines. These events are associated with the acquisition of professional antigen-presenting cell (APC) function [20]. In parallel both DC subsets are activated to produce large amounts of IL-12, IL-6 and TNF- $\alpha$ . CpG-ODN-activated DC display professional APC function in allogeneic mixed lymphocyte reaction.

PDT has been shown to induce tumor infiltration by dendritic cell (DC) and there have been efforts to potentiate this response in order to facilitate better antigen presentation. In this regard we have used a potent DC activating agent, an oligonucleotide (ODN) that contains a non-methylated CpG motif and acts as an agonist of toll like receptor (TLR) 9. TLR activation is a danger signal to notify the immune system of the presence of invading pathogens. CpG-ODN (but not scrambled non-CpG ODN) increased bone-marrow DC activation after exposure to PDT-killed tumor cells, and significantly increased tumor response to PDT and mouse survival after peri-tumoral administration. We conclude that CpG may be a valuable immunoadjuvant to PDT especially for tumors that produce DC dysfunction [21, 22].



**Figure 2.** Schematic illustrating how CpG oligonucleotides can stimulate dendritic cells, natural killer cells and Th1 helper lymphocytes and potentiate PDT

### 3. PDT AND REGULATORY T-CELLS

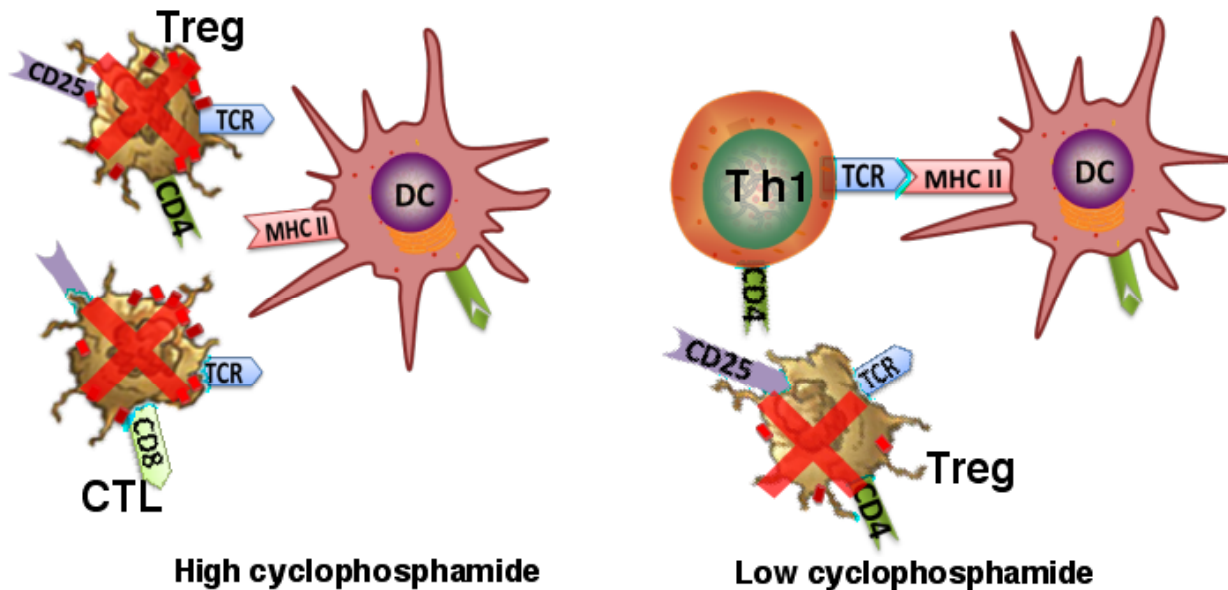
The advantage of the vast diversity of TCR created by random genetic recombination is that they have the potential to recognize an essentially infinite number of antigens. Nonetheless, because the process is random it also produces immune cells bearing receptors that recognize self-antigens (i.e., autoreactive T cells). An important mechanism of defense against autoimmunity is the permanent deletion of autoreactive T cells in the fetal thymus [23]. However, self-reactive T cells escaping thymic deletion and seeding the peripheral lymphoid organs require another strategy to curb their autoimmune potential. Regulatory T cells can be defined as a T-cell population that functionally suppresses an immune response by influencing the activity of another cell type. Regulatory T cells were initially described by Gershon et al. in the early 1970s and were called suppressor T cells [24]. There has recently been an explosion of interest in Treg in both mice and humans and many studies of their involvement in both autoimmune disease [25] and cancer [26]. Treg were initially characterized by co-expression of CD4 and high levels of CD25 (the high affinity component of the IL-2R complex ((IL-2R  $\alpha$ -chain) [27]. It was subsequently determined that most specific marker for Treg is the transcription factor Foxp3 [28], as other Treg markers (CD25, CTLA-4 and GITR) can be found on other T-cell subsets especially on activated CD4 cells [29, 30]. The transcription factor Foxp3 is specifically expressed in Treg and is required for their development [31]. CD25<sup>+</sup> regulatory T cells comprise 5-10% of CD4<sup>+</sup> T cells in naive mice and have been shown in several *in vivo* murine models to prevent the induction of autoimmune disease and inflammatory disease. Since T cells, which mediate autoimmunity, can through recognition of self-antigens also target tumor cells, it was postulated that Treg would also inhibit the generation of immune responses to tumors [32]. Depletion of these cells using anti-CD25 monoclonal antibodies has been shown to promote rejection of several transplantable murine tumor cell lines [32]. Immunization of mice with tumor cells in the absence of CD4<sup>+</sup>CD25<sup>+</sup> Treg induces immunity against a variety of different tumor cell lines [33].

Treg are divided into two main classes: (a) naturally occurring Treg found in the thymus and (b) inducible Treg found in the periphery [32]. Naturally occurring Treg are thought to have TCRs that recognize self-antigens and to play a major role in the prevention of autoimmune disease. Inducible Treg can be induced and differentiate in the periphery, such as in the tumor microenvironment. Thymus-derived Treg cells in the tumor microenvironment might clonally expand following stimulation by tumor-associated DC that frequently have an immature phenotype. Treg can also be present in a tumor as a result of conversion from CD4<sup>+</sup>CD25<sup>-</sup> T cells [34] under the influence of TGF $\beta$ , which is present at high levels in the tumor microenvironment [35]. Therefore the tumor (or tumor draining lymph nodes) might contain thymus-derived natural Treg cells, expanded and converted natural Treg cells, and locally differentiated and expanded Tr1 cells.

It is thought that Treg mediate their immunosuppressive effects by multiple pathways [36]. Treg 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, [37]. T cell activation is a dynamic process that is determined by the strength of the TCR signal; the strength of co-stimulation provided by CD28; and the magnitude of inhibitory signals generated by CTLA-4. Treg also express glucocorticoid-induced tumor necrosis factor receptor (GITR), however this appears to reduce suppressor function on binding its ligand [38]. Treg can express TGF- $\beta$  that is an immunosuppressive cytokine [39] and can induce further proliferation of Treg [40]. Recent evidence also suggests that Treg cells control T cell activation by suppression of DC activation [41]. In addition, imaging studies have suggested that Treg cells diminish the ability of DCs to form stable contacts with self-reactive T cells and thereby diminish their activation [42]. It is at present uncertain whether the Treg that inhibit anti-tumor immunity are specific for TAAs. In one report human melanoma-infiltrating Treg were cloned, and one target antigen was found to be LAGE-1, (a cancer-testis antigen) and these cloned Treg suppressed LAGE-1-specific T-cell activation [43].

Another important question is whether Treg inhibit anti-tumor immunity primarily in the priming phase of the immune response or in the effector phase or both. Administration of anti-CD25, from either 4 days before to 1 day after (but not 2 days after) tumor inoculation, was efficient in promoting tumor regression [44] suggesting that Treg mediate their suppressive effect by inhibiting T-cell priming in lymphoid organs. By contrast, other evidence indicates that Treg reduce the effector function of TAA-specific T cells. Transfer of Treg cells reduced the therapeutic efficiency of adoptively transferred TAA-specific CD8<sup>+</sup> effector T cells in a mouse model of B16 melanoma in C57BL/6 mice [45]. Furthermore, depletion of intratumoral Treg induced regression of large established Ag104 tumors in C3H mice [46].

As long ago as the 1970s it was realized [47, 48] that treatment of mice with low dose cyclophosphamide (CY) had an additional and different effect on tumor growth that involved the host immune system rather than a direct cytotoxic effect on the tumor cells. Low dose CY in combination with adoptive transfer of splenocytes from normal mice that had been previously vaccinated against the MethA tumor, caused complete and permanent regressions of established tumors [49]. This effect was abrogated by additional adoptive transfer of splenocytes from tumor bearing mice and the conclusion was that a particular population of suppressor T-cells were sensitive to low dose CY. More recent studies have shown that low dose CY administration depletes Tregs [50] and also increases the fraction of both CD4 and CD8 T-cells with a memory phenotype and induces type I interferon production [51]. It has been proposed that the reason for the selective action of low dose CY is its preferential induction of apoptosis in Treg and reduction in their suppressor function [52] or to the ability of CY to inhibit or reduce the activity of inducible nitric oxide synthase (iNOS) [51]. There have been some published studies that have compared the anti-tumor effects of low dose CY with high dose CY in mouse models. Motoyoshi et al [53] found an equal effect of 20 mg/kg and 200 mg/kg CY on slowing the growth of a murine hepatoma MH129 in immunocompetent mice but only the high dose CY had any anti-tumor effect in nude mice that lack T-cells. Low dose CY depleted Tregs, while high dose CY depleted all classes of T-cells. Brode and others reported [54] that CY can induce autoimmune diabetes in NOD mice and that this can be prevented by adoptive transfer of islet antigen-specific CD4+CD25+FoxP3 Tregs to CY-treated recipients. Taieb and coworkers reported [55] that CY administration to tumor bearing mice reduced Tregs and also enhanced secondary CTL response induced by a tumor-vaccine.



**Figure 3.** Schematic illustrating how low dose cyclophosphamide (but not high dose cyclophosphamide) can kill T-regulatory cells and potentiate immune response after PDT.

Our recent report showed that low-dose CY (but not high dose CY) increases PDT-induced anti-tumor immunity in BALB/c mice with J774 tumors and increased survival in BALB/c mice with J774 tumors treated with low-dose CY and PDT agrees with this hypothesis [56].

#### 4. CONCLUSION

Knowledge is steadily growing concerning the interaction between the immune system and cancerous tumors in the host. Although major advances have been made in understanding the mechanisms whereby tumors evade the attack of the host immune system, these advances have not yet been translated into highly effective immunotherapy regimens that improve patient survival and outcome. We believe that the unique properties of PDT may offer the

hope of combination approaches such as PDT plus CpG or PDT plus low dose CY that can not only destroy the primary tumor but educate the host immune system to recognize, track down, and ultimately destroy tumor cells that have metastasized to distant regions of the body.

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#### REFERENCES

- [1] Castano, A. P., Demidova, T. N., and Hamblin, M. R., "Mechanisms in photodynamic therapy: part three - photosensitizer pharmacokinetics, biodistribution, tumor localization and modes of tumor destruction," *Photodiagn. Photodyn. Ther.* 2, 91-106 (2005)
- [2] Castano, A. P., Mroz, P., and Hamblin, M. R., "Photodynamic therapy and anti-tumour immunity," *Nat Rev Cancer.* 6, 535-545 (2006)
- [3] Canti, G., Lattuada, D., Nicolin, A., Taroni, P., Valentini, G., and Cubeddu, R., "Antitumor immunity induced by photodynamic therapy with aluminum disulfonated phthalocyanines and laser light," *Anti-Cancer Drugs.* 5, 443-447 (1994)
- [4] Hendrzak-Henion, J. A., Knisely, T. L., Cincotta, L., Cincotta, E., and Cincotta, A. H., "Role of the immune system in mediating the antitumor effect of benzophenothiazine photodynamic therapy," *Photochem. Photobiol.* 69, 575-581 (1999)
- [5] Korbelik, M., and Dougherty, G. J., "Photodynamic therapy-mediated immune response against subcutaneous mouse tumors," *Cancer Res.* 59, 1941-1946 (1999)
- [6] Korbelik, M., Krosi, G., Krosi, J., and Dougherty, G. J., "The role of host lymphoid populations in the response of mouse EMT6 tumor to photodynamic therapy," *Cancer Res.* 56, 5647-5652 (1996)
- [7] Gollnick, S. O., Vaughan, L., and Henderson, B. W., "Generation of effective antitumor vaccines using photodynamic therapy," *Cancer Res.* 62, 1604-1608. (2002)
- [8] Gomer, C. J., Ryter, S. W., Ferrario, A., Rucker, N., Wong, S., and Fisher, A. M., "Photodynamic therapy-mediated oxidative stress can induce expression of heat shock proteins," *Cancer Res.* 56, 2355-2360 (1996)
- [9] Mitra, S., Goren, E. M., Frelinger, J. G., and Foster, T. H., "Activation of heat shock protein 70 promoter with meso-tetrahydroxyphenyl chlorin photodynamic therapy reported by green fluorescent protein in vitro and in vivo," *Photochem. Photobiol.* 78, 615-622 (2003)
- [10] Korbelik, M., Sun, J., and Cecic, I., "Photodynamic therapy-induced cell surface expression and release of heat shock proteins: relevance for tumor response," *Cancer Res.* 65, 1018-1026 (2005)
- [11] Granville, D. J., Carthy, C. M., Jiang, H., Levy, J. G., McManus, B. M., Matroule, J. Y., Piette, J., and Hunt, D. W., "Nuclear factor-kappaB activation by the photochemotherapeutic agent verteporfin," *Blood.* 95, 256-262 (2000)
- [12] Kick, G., Messer, G., Plewig, G., Kind, P., and Goetz, A. E., "Strong and prolonged induction of c-jun and c-fos proto-oncogenes by photodynamic therapy," *Br J Cancer.* 74, 30-36 (1996)
- [13] Sun, J., Cecic, I., Parkins, C. S., and Korbelik, M., "Neutrophils as inflammatory and immune effectors in photodynamic therapy-treated mouse SCCVII tumours," *Photochem. Photobiol. Sci.* 1, 690-695 (2002)
- [14] de Vree, W. J., Essers, M. C., de Bruijn, H. S., Star, W. M., Koster, J. F., and Sluiter, W., "Evidence for an important role of neutrophils in the efficacy of photodynamic therapy in vivo," *Cancer Res.* 56, 2908-2911 (1996)
- [15] Krosi, G., Korbelik, M., and Dougherty, G. J., "Induction of immune cell infiltration into murine SCCVII tumour by photofrin-based photodynamic therapy," *Br. J. Cancer.* 71, 549-555 (1995)
- [16] Cecic, I., Serrano, K., Gyongyossy-Issa, M., and Korbelik, M., "Characteristics of complement activation in mice bearing Lewis lung carcinomas treated by photodynamic therapy," *Cancer Lett.* 225, 215-223 (2005)
- [17] Adams, S., O'Neill, D. W., and Bhardwaj, N., "Recent advances in dendritic cell biology," *J Clin Immunol.* 25, 177-188 (2005)
- [18] O'Neill, D. W., Adams, S., and Bhardwaj, N., "Manipulating dendritic cell biology for the active immunotherapy of cancer," *Blood.* 104, 2235-2246 (2004)

- [19] Ferrantini, M., Capone, I., and Belardelli, F., "Dendritic cells and cytokines in immune rejection of cancer," *Cytokine Growth Factor Rev.* 19, 93-107 (2008)
- [20] Sparwasser, T., Vabulas, R. M., Villmow, B., Lipford, G. B., and Wagner, H., "Bacterial CpG-DNA activates dendritic cells in vivo: T helper cell-independent cytotoxic T cell responses to soluble proteins," *Eur J Immunol.* 30, 3591-3597 (2000)
- [21] Mroz, P., Castano, A. P., and Hamblin, M. R. Stimulation of dendritic cells enhances immune response after photodynamic therapy. *In: Biophotonics and Immune Responses IV*, San Jose, CA, 2009, pp. doi: 10.1117/1112.809630.
- [22] Castano, A. P., and Hamblin, M. R. Enhancing photodynamic therapy of a metastatic mouse breast cancer by immune stimulation. *In: Biophotonics and Immune Responses*, San Jose, 2006, pp. 608703.
- [23] Clayton, L. K., Ghendler, Y., Mizoguchi, E., Patch, R. J., Ocain, T. D., Orth, K., Bhan, A. K., Dixit, V. M., and Reinherz, E. L., "T-cell receptor ligation by peptide/MHC induces activation of a caspase in immature thymocytes: the molecular basis of negative selection," *Embo J.* 16, 2282-2293 (1997)
- [24] Curiel, T. J., Coukos, G., Zou, L., Alvarez, X., Cheng, P., Mottram, P., Evdemon-Hogan, M., Conejo-Garcia, J. R., Zhang, L., Burow, M., Zhu, Y., Wei, S., Kryczek, I., Daniel, B., Gordon, A., Myers, L., Lackner, A., Disis, M. L., Knutson, K. L., Chen, L., and Zou, W., "Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival," *Nat Med.* 10, 942-949 (2004)
- [25] Dejaco, C., Duftner, C., Grubeck-Loebenstien, B., and Schirmer, M., "Imbalance of regulatory T cells in human autoimmune diseases," *Immunology.* 117, 289-300 (2006)
- [26] Yamaguchi, T., and Sakaguchi, S., "Regulatory T cells in immune surveillance and treatment of cancer," *Semin Cancer Biol.* 16, 115-123 (2006)
- [27] Antony, P. A., and Restifo, N. P., "CD4+CD25+ T regulatory cells, immunotherapy of cancer, and interleukin-2," *J Immunother.* 28, 120-128 (2005)
- [28] Fontenot, J. D., and Rudensky, A. Y., "A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3," *Nat Immunol.* 6, 331-337 (2005)
- [29] Scotto, L., Naiyer, A. J., Galluzzo, S., Rossi, P., Manavalan, J. S., Kim-Schulze, S., Fang, J., Favera, R. D., Cortesini, R., and Suci-Foca, N., "Overlap between molecular markers expressed by naturally occurring CD4+CD25+ regulatory T cells and antigen specific CD4+CD25+ and CD8+CD28- T suppressor cells," *Hum Immunol.* 65, 1297-1306 (2004)
- [30] Albers, A. E., Ferris, R. L., Kim, G. G., Chikamatsu, K., DeLeo, A. B., and Whiteside, T. L., "Immune responses to p53 in patients with cancer: enrichment in tetramer+ p53 peptide-specific T cells and regulatory T cells at tumor sites," *Cancer Immunol Immunother.* 54, 1072-1081 (2005)
- [31] Ziegler, S. F., "FOXP3: of mice and men," *Annu Rev Immunol.* 24, 209-226 (2006)
- [32] Zou, W., "Regulatory T cells, tumour immunity and immunotherapy," *Nat Rev Immunol.* 6, 295-307 (2006)
- [33] Jones, E., Golgher, D., Simon, A. K., Dahm-Vicker, M., Screaton, G., Elliott, T., and Gallimore, A., "The influence of CD25+ cells on the generation of immunity to tumour cell lines in mice," *Novartis Found Symp.* 256, 149-152; discussion 152-147, 259-169 (2004)
- [34] Curotto de Lafaille, M. A., Lino, A. C., Kutchukhidze, N., and Lafaille, J. J., "CD25- T cells generate CD25+Foxp3+ regulatory T cells by peripheral expansion," *J Immunol.* 173, 7259-7268 (2004)
- [35] Chen, W., Jin, W., Hardegen, N., Lei, K. J., Li, L., Marinos, N., McGrady, G., and Wahl, S. M., "Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3," *J Exp Med.* 198, 1875-1886 (2003)
- [36] Bluestone, J. A., and Tang, Q., "How do CD4+CD25+ regulatory T cells control autoimmunity?," *Curr Opin Immunol.* 17, 638-642 (2005)
- [37] Tang, Q., Boden, E. K., Henriksen, K. J., Bour-Jordan, H., Bi, M., and Bluestone, J. A., "Distinct roles of CTLA-4 and TGF-beta in CD4+CD25+ regulatory T cell function," *Eur J Immunol.* 34, 2996-3005 (2004)
- [38] Stephens, G. L., McHugh, R. S., Whitters, M. J., Young, D. A., Luxenberg, D., Carreno, B. M., Collins, M., and Shevach, E. M., "Engagement of glucocorticoid-induced TNFR family-related receptor on effector T cells by its ligand mediates resistance to suppression by CD4+CD25+ T cells," *J Immunol.* 173, 5008-5020 (2004)
- [39] Marie, J. C., Letterio, J. J., Gavin, M., and Rudensky, A. Y., "TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells," *J Exp Med.* 201, 1061-1067 (2005)



- [40] Ghiringhelli, F., Puig, P. E., Roux, S., Parcellier, A., Schmitt, E., Solary, E., Kroemer, G., Martin, F., Chauffert, B., and Zitvogel, L., "Tumor cells convert immature myeloid dendritic cells into TGF-beta-secreting cells inducing CD4+CD25+ regulatory T cell proliferation," *J Exp Med.* 202, 919-929 (2005)
- [41] Veldhoen, M., Moncrieffe, H., Hocking, R. J., Atkins, C. J., and Stockinger, B., "Modulation of dendritic cell function by naive and regulatory CD4+ T cells," *J Immunol.* 176, 6202-6210 (2006)
- [42] Rudensky, A. Y., and Campbell, D. J., "In vivo sites and cellular mechanisms of T reg cell-mediated suppression," *J Exp Med.* 203, 489-492 (2006)
- [43] Wang, H. Y., Lee, D. A., Peng, G., Guo, Z., Li, Y., Kiniwa, Y., Shevach, E. M., and Wang, R. F., "Tumor-specific human CD4+ regulatory T cells and their ligands: implications for immunotherapy," *Immunity.* 20, 107-118 (2004)
- [44] Onizuka, S., Tawara, I., Shimizu, J., Sakaguchi, S., Fujita, T., and Nakayama, E., "Tumor rejection by in vivo administration of anti-CD25 (interleukin-2 receptor alpha) monoclonal antibody," *Cancer Res.* 59, 3128-3133 (1999)
- [45] Antony, P. A., Piccirillo, C. A., Akpınarlı, A., Finkelstein, S. E., Speiss, P. J., Surman, D. R., Palmer, D. C., Chan, C. C., Klebanoff, C. A., Overwijk, W. W., Rosenberg, S. A., and Restifo, N. P., "CD8+ T cell immunity against a tumor/self-antigen is augmented by CD4+ T helper cells and hindered by naturally occurring T regulatory cells," *J Immunol.* 174, 2591-2601 (2005)
- [46] Yu, P., Lee, Y., Liu, W., Krausz, T., Chong, A., Schreiber, H., and Fu, Y. X., "Intratumor depletion of CD4+ cells unmasks tumor immunogenicity leading to the rejection of late-stage tumors," *J Exp Med.* 201, 779-791 (2005)
- [47] Rollinghoff, M., Starzinski-Powitz, A., Pfizenmaier, K., and Wagner, H., "Cyclophosphamide-sensitive T lymphocytes suppress the in vivo generation of antigen-specific cytotoxic T lymphocytes," *J Exp Med.* 145, 455-459 (1977)
- [48] Glaser, M., "Regulation of specific cell-mediated cytotoxic response against SV40-induced tumor associated antigens by depletion of suppressor T cells with cyclophosphamide in mice," *J Exp Med.* 149, 774-779 (1979)
- [49] Berendt, M. J., and North, R. J., "T-cell-mediated suppression of anti-tumor immunity. An explanation for progressive growth of an immunogenic tumor," *J. Exp. Med.* 151, 69-80 (1980)
- [50] Ghiringhelli, F., Larmonier, N., Schmitt, E., Parcellier, A., Cathelin, D., Garrido, C., Chauffert, B., Solary, E., Bonnotte, B., and Martin, F., "CD4+CD25+ regulatory T cells suppress tumor immunity but are sensitive to cyclophosphamide which allows immunotherapy of established tumors to be curative," *Eur J Immunol.* 34, 336-344 (2004)
- [51] Loeffler, M., Kruger, J. A., and Reisfeld, R. A., "Immunostimulatory effects of low-dose cyclophosphamide are controlled by inducible nitric oxide synthase," *Cancer Res.* 65, 5027-5030 (2005)
- [52] Lutsiak, M. E., Semnani, R. T., De Pascalis, R., Kashmiri, S. V., Schlom, J., and Sabzevari, H., "Inhibition of CD4(+)25+ T regulatory cell function implicated in enhanced immune response by low-dose cyclophosphamide," *Blood.* 105, 2862-2868 (2005)
- [53] Motoyoshi, Y., Kaminoda, K., Saitoh, O., Hamasaki, K., Nakao, K., Ishii, N., Nagayama, Y., and Eguchi, K., "Different mechanisms for anti-tumor effects of low- and high-dose cyclophosphamide," *Oncol Rep.* 16, 141-146 (2006)
- [54] Brode, S., Raine, T., Zacccone, P., and Cooke, A., "Cyclophosphamide-induced type-1 diabetes in the NOD mouse is associated with a reduction of CD4+CD25+Foxp3+ regulatory T cells," *J Immunol.* 177, 6603-6612 (2006)
- [55] Taieb, J., Chaput, N., Scharz, N., Roux, S., Novault, S., Menard, C., Ghiringhelli, F., Terme, M., Carpentier, A. F., Darrasse-Jeze, G., Lemonnier, F., and Zitvogel, L., "Chemoimmunotherapy of tumors: cyclophosphamide synergizes with exosome based vaccines," *J Immunol.* 176, 2722-2729 (2006)
- [56] Castano, A. P., Mroz, P., Wu, M. X., and Hamblin, M. R., "Photodynamic therapy plus low-dose cyclophosphamide generates antitumor immunity in a mouse model," *Proc Natl Acad Sci U S A.* 105, 5495-5500 (2008)