

# Photodynamic therapy and anti-tumour immunity

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**Abstract** | Photodynamic therapy (PDT) uses non-toxic photosensitizers and harmless visible light in combination with oxygen to produce cytotoxic reactive oxygen species that kill malignant cells by apoptosis and/or necrosis, shut down the tumour microvasculature and stimulate the host immune system. In contrast to surgery, radiotherapy and chemotherapy that are mostly immunosuppressive, PDT causes acute inflammation, expression of heat-shock proteins, invasion and infiltration of the tumour by leukocytes, and might increase the presentation of tumour-derived antigens to T cells.

## Neutropaenia

A reduction in numbers of circulating neutrophils that predisposes to infection.

## Erysipelas

A skin disease caused by *Streptococcus pyogenes*.

## Antigen

A macromolecule (usually a protein or polysaccharide) that is perceived as foreign and stimulates an immune response.

## Major histocompatibility complex

Cell membrane proteins that bind short peptides and are recognized by T-cell receptors.

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The principle of photodynamic therapy (PDT) was first proposed over 100 years ago<sup>1</sup>. A recent review in *Nature Reviews Cancer* by Rakesh Jain and colleagues described some of the historical milestones in the development of PDT as a cancer treatment<sup>2</sup>. Many of the photosensitizers (PSs) that have been studied since PDT was first proposed are based on a porphyrin-like nucleus<sup>3</sup>. PSs function as catalysts when they absorb visible light and then convert molecular oxygen to a range of highly reactive oxygen species (ROS). The ROS that are produced during PDT have been shown to destroy tumours by multifactorial mechanisms<sup>4,5</sup> (FIG. 1). PDT has a direct effect on cancer cells, producing cell death by necrosis and/or apoptosis<sup>6</sup>. PDT also has an effect on the tumour vasculature, whereby illumination and ROS production causes the shutdown of vessels and subsequently deprives the tumour of oxygen and nutrients<sup>7,8</sup>. Finally, PDT also has a significant effect on the immune system<sup>9–11</sup>, which can be either immunostimulatory or immunosuppressive.

Most of the commonly used cancer therapies are immunosuppressive. Chemotherapy and ionizing radiation delivered at doses sufficient to destroy tumours are known to be toxic to the bone marrow, which is the source of all cells of the immune system, and neutropaenia and other forms of myelosuppression are often the dose-limiting toxicity of these therapies. However, it should be noted that low doses of either ionizing radiation<sup>12,13</sup> or chemotherapy<sup>14</sup> can have immunostimulatory effects, including the induction of heat-shock proteins<sup>15</sup>. Less well known is the fact that major surgery can also have an immunosuppressive effect that leads to a significant diminution of lymphocyte and natural killer (NK) cell function<sup>16</sup>. The ideal cancer therapy would not only destroy the primary tumour, but at the same time trigger the immune system to recognize, track down and destroy

any remaining tumour cells, be they at or near the site of the primary tumour or distant micrometastases. PDT, in common with some other local cancer therapies such as cryotherapy<sup>17</sup> and hyperthermia<sup>18</sup>, might have these desirable properties.

The importance of the immune system in the host response against cancer has been studied for many years, but immunotherapy is only accepted as a treatment option in a few cases. More than 700 cases of spontaneous regression in advanced tumours in patients have been reported<sup>19</sup>, including malignant melanoma, **hepatocellular carcinoma**, lung metastases after destruction of the primary renal cell carcinoma and **Hodgkin disease**. Moreover, such spontaneous regressions normally occur following an infection.

Cancer immunotherapy (even if unrecognized as such) has a long history. The Egyptians noted that surgical opening of the tumour site could produce tumour regression, one would assume through the generation of infection and activation of the immune system<sup>20</sup>. Over 100 years ago a surgeon from New York, William Coley<sup>21</sup>, discovered that some infections could produce tumour regression, and he created a 'vaccine' based initially on erysipelas-causing bacteria<sup>22</sup>. The bacillus Calmette-Guerin (BCG) vaccine derived from *Mycobacterium bovis* has been used to prevent tuberculosis since 1921, and has been applied for immunostimulation in neoplasia since the 1960s. The most effective use of this treatment is for superficial bladder cancer<sup>23</sup>.

Since these early studies, groundbreaking discoveries in immunology have identified the roles of lymphocyte classes and subclasses<sup>24</sup>, dendritic cells and antigen presentation<sup>25</sup>, interleukins (IL) and other cytokines<sup>26</sup>, and tumour-associated antigens and major histocompatibility complex (MHC) molecules<sup>27</sup> in mediating the anti-tumour

**At a glance**

- Photodynamic therapy (PDT) uses non-toxic dyes and harmless visible light in combination with oxygen to produce highly reactive oxygen species that kill cells.
- In addition to destroying tumour tissue by a process that can produce cellular necrosis and the expression of stress proteins, PDT produces an acute inflammation, and attracts leukocytes to treated tumours.
- PDT might increase the immunogenicity of dead tumour cells by exposing or creating new antigens, and by inducing heat-shock proteins that increase the efficiency of antigen cross-presentation to form more effective tumour-specific cytotoxic T cells.
- The pro-inflammatory effects of PDT might increase dendritic-cell migration, antigen uptake and maturation.
- PDT can produce tumour cures and long-lasting tumour-specific immunity (memory), as has been shown by the rejection of tumours on rechallenge in certain mouse and rat models.
- PDT has been combined with a range of immunostimulatory therapies, including microbial adjuvants, to increase the anti-tumour immunity produced by PDT alone.
- There are only a few reports of the immunostimulatory effects of PDT in humans, but increasing recognition of the effect should lead to further work and possibly to improved patient outcome.

**Innate immune response**

The immediately available non-specific defence against invading pathogens, which consists of cellular (neutrophils, macrophages and natural killer cells) and non-cellular (complement and antibacterial peptides) arms.

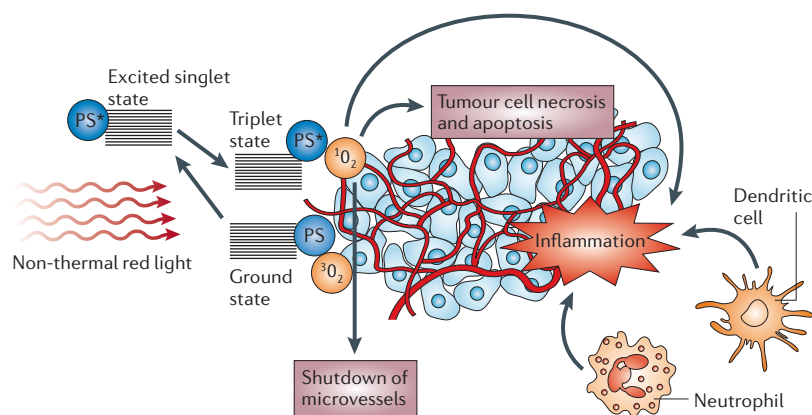
**Adaptive immune response**

An antigen-specific defence that develops with time, which consists of cellular (cytotoxic and helper T-lymphocytes) and humoral (B-lymphocytes and antibody) arms.

immune response. However, most cancers avoid or escape immune control<sup>28,29</sup>, and death from metastatic cancer is still the most likely result. In this Review we discuss the effect of PDT on the anti-tumour immune response, and the role of PDT in stimulating and suppressing both the innate immune response and adaptive immune response. We also summarize the available data on combinations of PDT with other immunostimulatory therapies.

**Effects of PDT on tumour cells**

Many effects of PDT on cancer cells that are grown in tissue culture have been reported that, if replicated *in vivo*, would make activation of the immune system probable after PDT treatment in patients. The combination of PSs with their activating light causes an unusual mixture of



**Figure 1 | The mechanism of action on tumours in photodynamic therapy.**

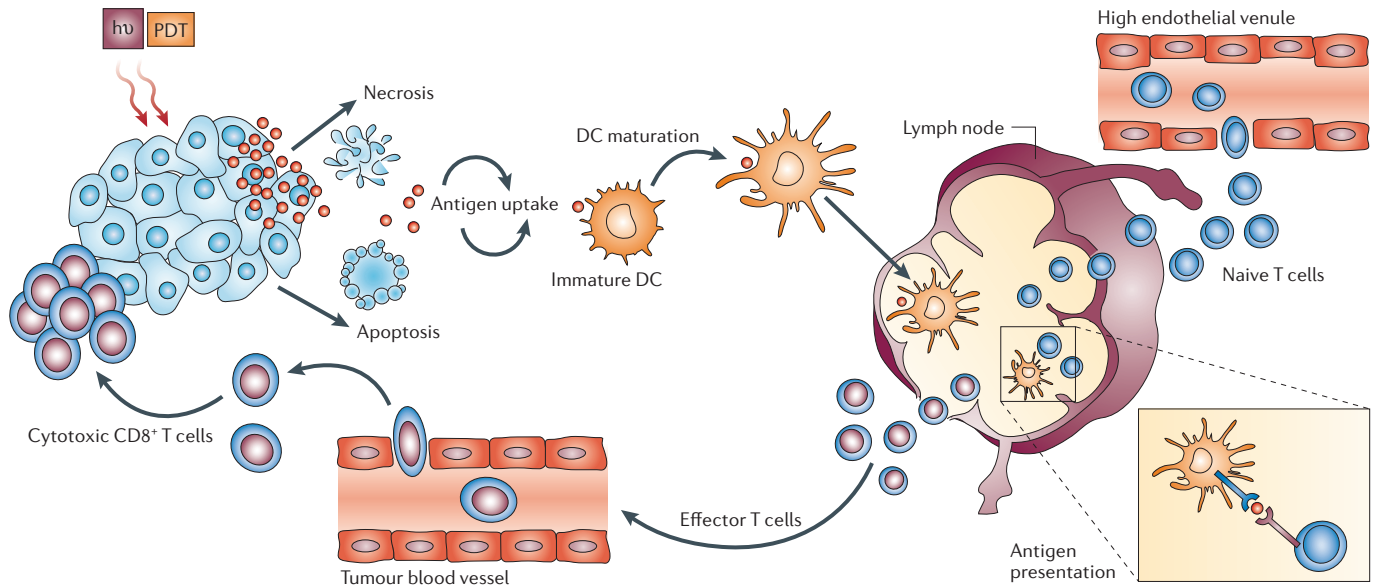
The photosensitizer (PS) absorbs light and an electron moves to the first short-lived excited singlet state. This is followed by intersystem crossing, in which the excited electron changes its spin and produces a longer-lived triplet state. The PS triplet transfers energy to ground-state triplet oxygen, which produces reactive singlet oxygen (<sup>1</sup>O<sub>2</sub>). <sup>1</sup>O<sub>2</sub> can directly kill tumour cells by the induction of necrosis and/or apoptosis, can cause destruction of tumour vasculature and produces an acute inflammatory response that attracts leukocytes such as dendritic cells and neutrophils.

apoptotic and necrotic cell death<sup>6</sup>, which is different from most conventional cytotoxic agents that usually only trigger apoptotic cell death. The balance between apoptosis and necrosis after PDT *in vitro* depends on several parameters, including the total PDT dose (PDT dose is the product of PS concentration and light fluence), the intracellular localization of the PS, the fluence rate, the oxygen concentration and the cell type<sup>4</sup>. There is an extensive body of literature that examines the pathways of apoptosis that are induced after PDT in both normal and tumour cells in tissue culture, such as signalling pathways<sup>30,31</sup>, mitochondrial events<sup>4</sup> and mediators of apoptosis<sup>32</sup>. The occurrence of apoptosis after PDT in tumours *in vivo* has also been shown<sup>33-35</sup>, but there have been no studies looking at *in vivo* clearance mechanisms of apoptotic cells in tumours after PDT.

Many studies have examined the relationship between the mode of tumour cell death (by methods other than PDT) and the efficiency of induction of the immune response, both *in vitro* and *in vivo*<sup>36,37</sup>. Although some reports show that apoptotic tumour cells are more effective than necrotic tumour cells at inducing an immune response<sup>38,39</sup>, there are other reports that show that modes of cancer therapy that predominantly induce necrosis are actually better at activating the immune system than methods that predominantly induce apoptosis<sup>40,41</sup>. In the case of necrosis, cytosolic constituents spill into the extracellular space through the damaged plasma membrane and provoke a robust inflammatory response. These products are safely isolated by the intact membranes that initially persist in apoptotic cells, which are phagocytosed by macrophages. The acute inflammation that is caused by PDT-induced necrosis might potentiate immunity by attracting host leukocytes into the tumour and increasing antigen presentation (FIG. 2).

One of the most important cellular factors induced by PDT and released from necrotic tumour cells is extracellular heat-shock protein 70 (HSP70) (FIG. 3). HSP70 is effectively induced after stress and, when it remains intracellular, it chaperones unfolded proteins and prevents cell death by inhibiting the aggregation of cellular proteins<sup>42</sup>. These properties not only enable intracellular HSP70 to inhibit tumour cell death by apoptosis, but also promote the formation of stable complexes with cytoplasmic tumour 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 (APCs) and stimulate an anti-tumour immune response<sup>43</sup>. Extracellular HSP70 binds to high-affinity receptors on the surface of the APCs, which leads to the activation and maturation of dendritic cells (DCs), a process that enables the cross-presentation of the peptide antigen cargo of HSP70 by the APCs to CD8<sup>+</sup> cytotoxic T cells<sup>44</sup>.

Levels of HSP70 mRNA were increased with PDT mediated by three PSs (mono-L-aspartyl chlorin e6, tin etiopurpurin and Photofrin), but only mono-L-aspartyl chlorin e6 and tin etiopurpurin increased HSP70 protein levels in mouse tumour cells *in vitro* and *in vivo*<sup>45</sup>. Foster and co-workers<sup>46</sup> used fluorescence imaging to show that the PS m-tetrahydroxyphenylchlorin (mTHPC)



**Figure 2 | Photodynamic therapy induces activation of antigen-specific T cells.** When light (hv) is delivered to a photosensitizer (PS)-loaded tumour it induces both apoptotic and necrotic cell death. These cells are phagocytosed by dendritic cells (DCs) that have accumulated owing to the acute inflammatory response which is triggered by photodynamic therapy (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 and, attracted by chemokines, migrate to the tumour and kill the tumour cells.

mediated the induction of HSP70 in EMT6 cells that had undergone PDT. These cells were stably transfected with a plasmid that contained the gene which encodes green fluorescent protein (GFP) under the control of an HSP70 promoter, and they could see increased GFP expression after PDT in both an *in vitro* and *in vivo* tumour model. Verwanger *et al.* used a cDNA microarray to find the highest expression level of various genes after PDT *in vitro*. HSP70 showed the highest increase in expression (12-fold), followed by the immediate early genes *FOS* and *JUN*<sup>47</sup>. In addition, a recent paper<sup>43</sup> reported that 15–25% of total cellular HSP70 became exposed at the cell surface almost instantly after the treatment of cells with Photofrin-based PDT, and a large proportion of this was released within 1 hour of PDT at a cytotoxic dose. In addition to HSP70, there have been reports that PDT induces the expression of other heat-shock protein family members such as HSP47 (REF. 48) and HSP60 (REF. 49), as well as other stress-inducible proteins such as glucose-regulated protein 78 (GRP78) (REF. 50), GRP94 (REF. 43) and haem oxygenase<sup>51</sup>. The release of HSP-bound tumour antigens that can easily be taken up by APC from PDT-induced necrotic tumour cells might explain the efficiency of PDT in stimulating an immune response against tumours.

**Effects of PDT on immune cells**

There are reports based on data from *in vitro* studies that PDT can have an effect on monocyte/macrophage and lymphocyte cell lineages. Lymphocytes are usually easily killed by PDT<sup>52</sup>, and activated lymphocytes are especially susceptible<sup>53</sup>. This finding has led to PDT being proposed as a treatment for graft versus host disease<sup>54</sup>, some forms of

autoimmune disease<sup>55</sup> and cutaneous T-cell lymphoma<sup>56</sup>. On the other hand, macrophages can be activated by low, sublethal doses of PDT<sup>57</sup>. Reports show that PDT-treated macrophages secrete tumour-necrosis factor- $\alpha$  (TNF $\alpha$ )<sup>58</sup>. When a mixture of macrophages and lymphocytes undergoes PDT, lysophosphatidyl choline is released from lymphocytes and this molecule induces the expression of  $\beta$ -galactosidase in B lymphocytes and, together with NEU1 sialidase from T lymphocytes, these enzymes modify the vitamin D3 binding protein in bovine serum to yield a potent macrophage-activating factor (MAF)<sup>59,60</sup>. The production of this MAF also occurs in mice, where it is derived from the analogous vitamin D3 binding protein in mouse serum<sup>60</sup>. Evidence also indicates that macrophages can show preferential cytotoxicity to tumour cells that have been treated with a sublethal dose of PDT<sup>61</sup>. Another report<sup>62</sup> showed that although the tumoricidal function of peritoneal macrophages that were removed from mice after PDT was unaltered, there was a reduction in NK cell function.

**Cytokine release and inflammation after PDT**

PDT produces an acute inflammatory response whether it is delivered to normal tissue or to tumours (FIG. 3). Inflammatory cytokines and chemokines have been detected in the serum of mice that have received PDT directed at a subcutaneous tumour or to an area of normal skin. These include IL6 in particular and macrophage inflammatory protein 1 (MIP1) and MIP2 (REF. 63). Increased levels of IL1 $\beta$ , IL6, IL8 and IL10 were detected in patients after surgery and PDT for mesothelioma<sup>64</sup>. The sources of these inflammatory mediators can be many of the various cell types that are present

**Fluence**

The light energy delivered per unit area (J cm<sup>-2</sup>).

**Fluence rate**

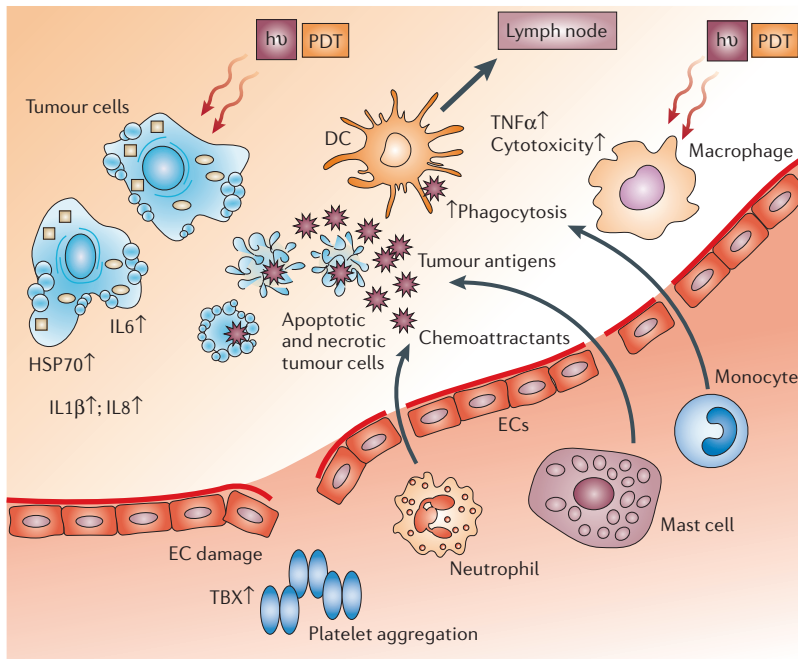
The rate at which light energy is delivered per unit area (W cm<sup>-2</sup>).

**Antigen-presenting cells**

Phagocytic cells such as dendritic cells, macrophages and B cells, which take up foreign antigens, and present them through major histocompatibility complex class II and express co-stimulatory molecules to ensure an effective T-cell response.

**Cross-presentation**

The process by which exogenous antigens that would normally be presented by dendritic cells in the context of major histocompatibility complex (MHC) class II to CD4<sup>+</sup> T cells are also presented in the context of MHC class I to CD8<sup>+</sup> T cells.



**Figure 3 | Consequences of photodynamic therapy-induced inflammation.** Damage to endothelial cells (ECs) activates a cascade of events that lead to local inflammation, vessel dilatation and platelet aggregation. Much of this is caused by the release of thromboxane (TBX), cytokines such as interleukin 1β (IL1β), IL6 and IL8, the production of tumour-necrosis factor-α (TNFα), and infiltration of the treated tumour by cells of the immune system. Necrotic and apoptotic tumour cells express heat-shock proteins (HSPs) and provide antigens to dendritic cells (DCs) that migrate to lymph nodes. hv, light; PDT, photodynamic therapy.

in tumours. For instance, malignant cells themselves, tumour endothelial cells and tumour-infiltrating leukocytes, but not fibroblasts, have been shown to produce members of the class of inflammatory mediators known as prostaglandins<sup>65,66</sup>. The release of thromboxane from endothelial cells after PDT is partly responsible for the vascular shutdown<sup>67</sup>. This induction of acute inflammation is important in triggering the immune response, as it shares some similarities with the type of danger signal provided by the host inflammatory response to the microbial invasion of healthy tissue. The tumour environment is more aptly described as a state of chronic inflammation, as opposed to a state of acute inflammation<sup>68</sup>.

**Transcription factors and cytokine production.** The acute inflammation that is observed after PDT is likely to be caused by the expression of two transcription factors, nuclear factor κB (NFκB) and activator protein 1 (AP1). Both factors participate in the transcriptional activation of genes that encode immunoregulatory and proinflammatory proteins<sup>69</sup>, and are known to be activated by cellular oxidative stress<sup>70,71</sup>. Photofrin-mediated PDT produced NFκB translocation in murine L1210 leukaemia cells under PDT conditions that resulted in approximately 20% cell survival<sup>72</sup>. However, Kick *et al.* found that HeLa cells that were treated with Photofrin-mediated PDT showed an increase in IL6 expression caused by the activation of AP1, not NFκB<sup>73</sup>. Colon carcinoma HCT116 cells that

were treated with pyrropeophorbide, a methyl ester, and red light, led to IκB processing and two distinct waves of NFκB activation; first by promoting the internalization of surface IL1 receptors, and then by ceramide generation<sup>74,75</sup>. HL60 cells that were transfected with a construct containing 5 NFκB sites of the HIV type-1 terminal repeat, cloned upstream of the luciferase gene, showed increased luciferase activity after benzoporphyrin derivative (BPD)-mediated PDT<sup>76</sup>. NFκB activation is the most important mediator of acute inflammation, and its induction after PDT *in vitro* confirms the observation that PDT induces acute inflammation *in vivo*.

**COX2 and prostaglandin synthesis.** Expression of the enzyme cyclooxygenase 2 (COX2, also known as prostaglandin endoperoxide synthase 2 (PTGS2)) is regulated by NFκB, and produces the inflammatory mediators known as the eicosanoids (including prostaglandin E2 (PGE2) and leukotrienes). PDT was found to cause prolonged expression of COX2 in PDT-treated mouse cancer cells and tumours *in vivo*, along with increased PGE2 synthesis<sup>77</sup>. Although PGE2 is pro-inflammatory, it is usually thought to have immunosuppressive effects<sup>78</sup>. PGE2 levels were attenuated in cells that were coincubated with the COX2 inhibitor NS-398. Moreover, systemic administration of NS-398 decreased the PDT-induced expression of both PGE2 and **vascular endothelial growth factor** in BA mouse mammary tumours, and increased the number of cures. COX2 inhibitors do not sensitize cancer cells to PDT-mediated killing *per se*, but can be used to potentiate the anti-tumour effectiveness of PDT when they are given after illumination. This anti-tumour effect is probably caused by the inhibition of angiogenesis, which is necessary for tumour regrowth<sup>79</sup>. Volanti *et al.* found that COX2 expression was mainly the result of NFκB activation, but the mechanism of activation differed in two cell lines<sup>80</sup>. In T24 bladder carcinoma cells, NFκB activation occurs through a protein kinase C-α (PKCα)- and phosphatidylinositol-3-kinase (PI3K)-dependent activation of the IκB kinase complex, whereas in HeLa cells, NFκB activation is mediated by PKC- and PI3K-independent pathways. Interestingly, hypericin-mediated PDT<sup>81</sup> led to the increased expression of COX2 and PGE2, except that this time activation of the p38 mitogen activated protein kinase (MAPK) pathway was implicated (as shown by the MAPK inhibitor PD169316), as opposed to the activation of NFκB found in other systems. Overexpression of p38 MAPK also increased cellular resistance to PDT-induced apoptosis, but this effect was independent of COX2. Further work is necessary to understand the precise role of COX2 and eicosanoids in the PDT-induced immune response against tumours.

**Neutrophil recruitment and the production of IL6.** Kick *et al.*<sup>73</sup> compared IL6 mRNA production after PDT or UVB treatment. PDT-induced IL6 protein levels were higher and were detectable earlier than after treatment with UVB. PDT-induced IL6 expression was mediated by AP1, and was independent of PKC activity, NFκB or the multiple cytokine- and second messenger-responsive element in the IL6 promoter.

Using a BALB/c mouse model, Gollnick and colleagues<sup>82</sup> showed that PDT delivered to normal and tumour tissue caused marked changes in the expression of IL6 and IL10, but not TNF $\alpha$ . This group<sup>63</sup> also found that 2-[1-hexyloxyethyl]-2-devinyl pyropheophorbide-a (HPPH)-mediated PDT caused neutrophil migration into the treated tumour area owing to a transient and local increase in the expression of the chemokine MIP2 (the murine equivalent of IL8), and the increased expression of the adhesion molecule E-selectin. Although increased local and systemic expression of IL6 were found, this was not necessary for neutrophil recruitment. A subsequent report<sup>83</sup> compared the effect of a low and a high fluence (total light energy), each delivered at a low and high fluence rate, against Colo 26 murine tumours treated with HPPH. It has previously been proposed that PDT is less efficient when light is delivered at a high fluence rate because the tissue oxygen is completely used up and cannot be supplied fast enough by the microvasculature to keep up with photochemical consumption<sup>84</sup>. Oxygen-conserving low fluence rate PDT at a high fluence resulted in 70–80% tumour cures, whereas the same fluence at the oxygen-depleting high fluence rate resulted in 10–15% tumour cures. High fluence at a low fluence rate led to the ablation of blood vessels. The highest levels of inflammatory cytokines and neutrophilic infiltrates were observed when low fluence was delivered at a low fluence rate (10–20% cures). The optimally curative PDT regimen (high fluence at a low fluence rate) produced minimal inflammation. The depletion of neutrophils did not significantly change the high cure rates of that regimen, but abolished curability in the maximally inflammatory regimen. These data indicate that tumour cure can be mediated by maximizing the photochemical action of PDT, but the importance of causing inflammation and neutrophil infiltration is less clear.

Sluiter *et al.*<sup>85</sup> first observed that neutrophils adhere to the microvascular wall after PDT *in vivo*, but PDT did not stimulate the expression of P-selectin (one of the principal adhesion molecules that bind leukocytes) by endothelial cells (ECs). The ECs retracted after PDT, which enabled neutrophils to adhere to the subendothelial matrix by their  $\beta$ 2-integrin adhesion receptors, and this could be blocked by anti- $\beta$ 2-integrin antibodies<sup>86</sup>. This finding was supported by a report which showed that expression levels of the adhesion molecules intercellular adhesion molecule 1 (ICAM1) and vascular cell adhesion molecule 1 (VCAM1) were downregulated in ECs after PDT<sup>87</sup>. The administration of anti-rat neutrophil serum with PDT in rhabdomyosarcoma-bearing rats completely abrogated the normal PDT-induced retardation of tumour growth<sup>88</sup>, which shows that an influx of neutrophils is required for an effective anti-tumour response in this model. An increase in the number of peripheral-blood neutrophils was found 4 hours after PDT treatment, and lasted for 24 hours. The increase in neutrophils was preceded by an increase in serum levels of IL1 $\beta$ . Anti-GCSF (granulocyte colony-stimulating factor) antibodies decreased neutrophil numbers and decreased the

efficacy of PDT. The reasons why neutrophils are so important in producing an effective response to PDT in some (but not all) tumour models are still uncertain.

Krosi and co-workers<sup>89</sup> measured cellular populations in the murine squamous cell carcinoma VII (SCCVII) model treated with Photofrin-mediated PDT. They found a 200-fold increase in the number of neutrophils within 5 minutes of PDT, followed immediately by an increase in the levels of mast cells. Another type of myeloid cell, most likely monocytes, invaded the tumour 2 hours after PDT. Cecic *et al.*<sup>90</sup> found that pronounced neutrophilia developed rapidly after Photofrin or mTHPC-mediated PDT of mice with SCCVII or EMT6 mammary carcinomas. Neutrophilia was also observed after PDT treatment of normal dorsal skin, but not in the footpad of tumour-free mice. Complement inhibition completely prevented the development of PDT-induced neutrophilia. Complement fragments from C3 and C5a proteins can induce neutrophilia either by mobilizing bone-marrow pools or as a response to transient neutropaenia caused by the adhesion of neutrophils to the endothelium<sup>91</sup>. Korbek *et al.* went on to show that complement activation occurred after Lewis lung carcinomas (LLC) were treated with Photofrin-mediated PDT's and observed increased levels of C3 in the tumour and serum<sup>92</sup>. Increased alternative complement pathway activity in the serum was evident 1–3 days after PDT. Blocking C3a or C5a receptors in the host mice decreased the efficacy of PDT in producing LLC tumour cures. Korbek and colleagues also showed that blocking ICAM1 with monoclonal antibodies reduced the number of tumour cures<sup>93</sup>. A marked upregulation of the ICAM1 ligands CD11b and CD11c, which are found on neutrophils, was also associated with PDT-treated tumours. IL1-neutralizing antibodies diminished the number of cures of PDT-treated tumours. Neutrophils express MHC class II molecules, which suggests that they are engaged as antigen-presenting cells and involved in the development of the anti-tumour immune response. Korbek *et al.* also found that IL1 and TNF $\alpha$  both function as potent promoters of the early phase of PDT-induced neutrophilia, but do not seem to have a significant role in the advanced phase<sup>94</sup>. The data attained by blocking two other cytokines, GCSF and IL10, showed that they are important contributors to advanced-phase neutrophilia, with no apparent influence in the early phase.

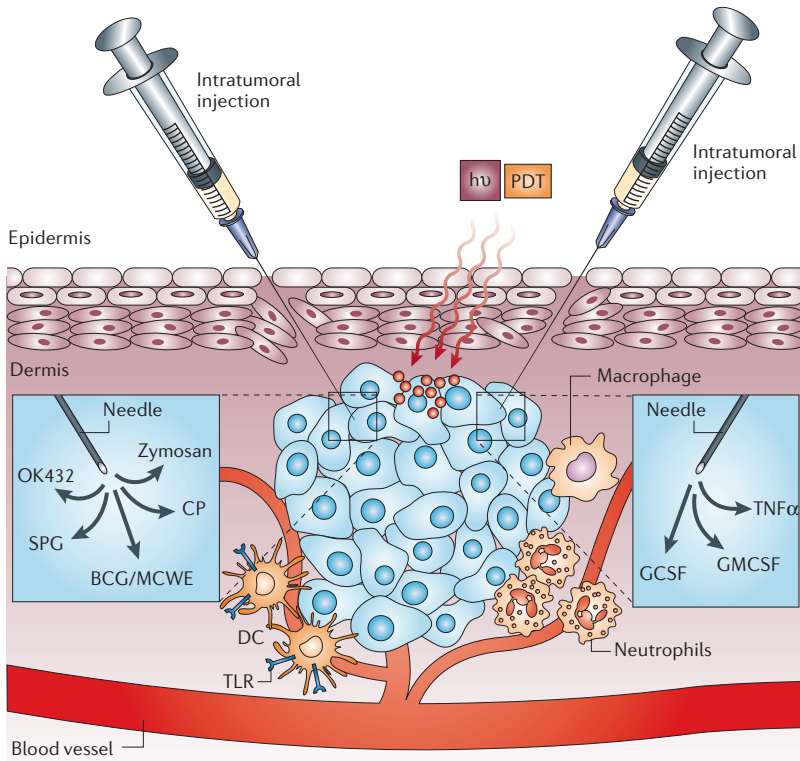
The reports described above show that the acute inflammation, which is produced by PDT, and both a systemic and tumour-localized increase in neutrophils is important in obtaining tumour cures. It is highly probable (although difficult to show) that these phenomena will also be important in the development of a memory T-cell anti-tumour immune response after PDT.

### PDT and anti-tumour immunity

The introduction of transplantable tumours grown in inbred mouse or rat strains that share the same MHC haplotype (syngeneic animals) and have intact immune systems, has enabled researchers to study anti-tumour immunity after PDT. Canti and colleagues<sup>95</sup> examined the effects of PDT with the PS aluminium disulphonated

### Complement

A group of proteins in serum that function with antibodies (classical pathway) or in response to microbial stimuli (alternative pathway) to achieve the destruction of foreign blood cells or bacteria.



**Figure 4 | Combination of photodynamic therapy with immunostimulants.** The intratumoral injection of various Toll-like receptor (TLR) ligands: bacillus Calmette–Guerin (BCG), Mycobacterial cell-wall extract (MCWE), OK432, zymosan, schizophyllan (SPG) or *Corynebacterium parvum* (CP), effectively activates dendritic cells (DCs) and increases antigen presentation and local inflammation. The injection of various cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and tumour-necrosis factor- $\alpha$  (TNF $\alpha$ ), results in increased infiltration by macrophages, activation of neutrophils, and direct destruction of tumour vessels, respectively. hv, light; PDT, photodynamic therapy.

phthalocyanine on the anti-tumour immune response 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, whereas immunosuppressed surviving animals and animals cured by surgery died after tumour rechallenge. Different syngeneic murine leukaemias were not rejected.

Korbelik *et al.*<sup>96</sup> reported that Photofrin-based PDT cured 100% of EMT6 mammary sarcomas in syngeneic BALB/c mice, but no long-term cures were observed in non-obese diabetic (NOD), severe combined immunodeficient (SCID) or nude mice. The adoptive transfer of splenic T-lymphocytes from naive BALB/c mice into SCID mice before PDT postponed the recurrence of treated tumours, whereas adoptive transfer carried out immediately or 7 days after PDT had no benefit. Adoptive transfer of non-adherent splenocytes (a mixture of CD4<sup>+</sup> and CD8<sup>+</sup> T cells with some B cells, NK cells and monocytes) from normal mice cured of EMT6 tumours by PDT 5 weeks previously, fully restored the curative effect of PDT on EMT6 tumours that were growing in SCID mice. Splenocytes obtained from donors that were cured by X-rays were much less effective. The depletion

**Toll-like receptors**  
First discovered in *Drosophila*, these represent a conserved set of pattern-recognition molecules that are triggered by motifs present on bacteria, viruses and fungi to initiate signalling which attracts and activates immune cells.

of specific T-cell populations from donor splenocytes indicated that CD8<sup>+</sup> cytotoxic T-lymphocytes had the most curative effect, whereas CD4<sup>+</sup> helper T cells played a supportive role<sup>97</sup>. Analogous studies were performed by a different group<sup>98</sup> using PDT with the PS 2-iodo-5-ethylamino-9-diethylaminobenzo[a]-phenothiazinium chloride.

A recent report<sup>99</sup> showed that BPD-mediated PDT of RIF1 tumours (a poorly immunogenic murine sarcoma) in wild-type C3H/HeN mice leads to initial tumour disappearance but not to permanent cures because of local recurrence. By contrast, when the tumours were genetically engineered to express GFP from jellyfish, 100% cures and long-term resistance to rechallenge was obtained after PDT. PDT (but not surgical removal) induced immune recognition of the foreign GFP as a model tumour antigen. As additional tumour-rejection antigens are identified in mouse tumour cell lines<sup>100</sup>, a more rational approach can be taken to studying the factors that govern the relative strength of anti-tumour immune responses stimulated by PDT for different tumours and PDT regimens.

**PDT-produced cancer vaccines**

A related approach that takes advantage of the immunostimulatory effects of PDT is the preparation of cancer vaccines using *in vitro* PDT of cell cultures. Gollnick *et al.*<sup>101</sup> compared the cancer-vaccine potential of PDT-generated cell lysates (EMT6 and P815 tumour cells) with lysates generated by UV or ionizing radiation. PDT-generated vaccines were tumour-specific, induced a cytotoxic T-cell response and, unlike the other methods, did not require the co-administration of an adjuvant to be effective. PDT-generated lysates were able to induce phenotypic DC maturation and IL12 expression. Korbelik and Sun<sup>102</sup> produced a vaccine by treating SCCVII cells with BPD-mediated PDT and later with a lethal X-ray dose, and showed that these cells, when injected peritumorally into mice with established SCCVII tumours, produced a significant therapeutic effect, including growth retardation, tumour regression and cures. Importantly, vaccine cells that were retrieved from the treatment site at 1 hour after injection were intermixed with dendritic cells (DCs), HSP70 was expressed on their surface and they were opsonized by complement C3. This observation verifies some of the earlier findings in mouse models and *in vitro*.

**PDT combined with other therapies**

Reports of PDT combined with other immunostimulatory agents or strategies can be divided into three broad classes (FIG. 4).

**PDT and microbial adjuvants.** First, agents that are derived from microbial stimulators of innate immunity can be injected into the tumour or surrounding area before, during or after PDT (FIG. 4). Their role is to activate Toll-like receptors (TLRs) or similar pattern-recognition molecules that are present on macrophages and dendritic cells<sup>103</sup>. So far, 13 TLR family members have been identified on monocytes and macrophages,

dendritic cells, mast cells and some epithelial cells<sup>104–106</sup>. Their principal function is thought to be as detectors of danger signals; early warning systems of imminent infection. The activation of TLR pathways can induce NFκB and, consequently, the expression of several genes involved in the activation of the immune system that are also important for the anti-tumour immune response<sup>107</sup>. These findings gave rise to the hypothesis that combination therapy that involves the administration of immunoadjuvants (often potential TLR ligands) and different PDT regimens might prove effective.

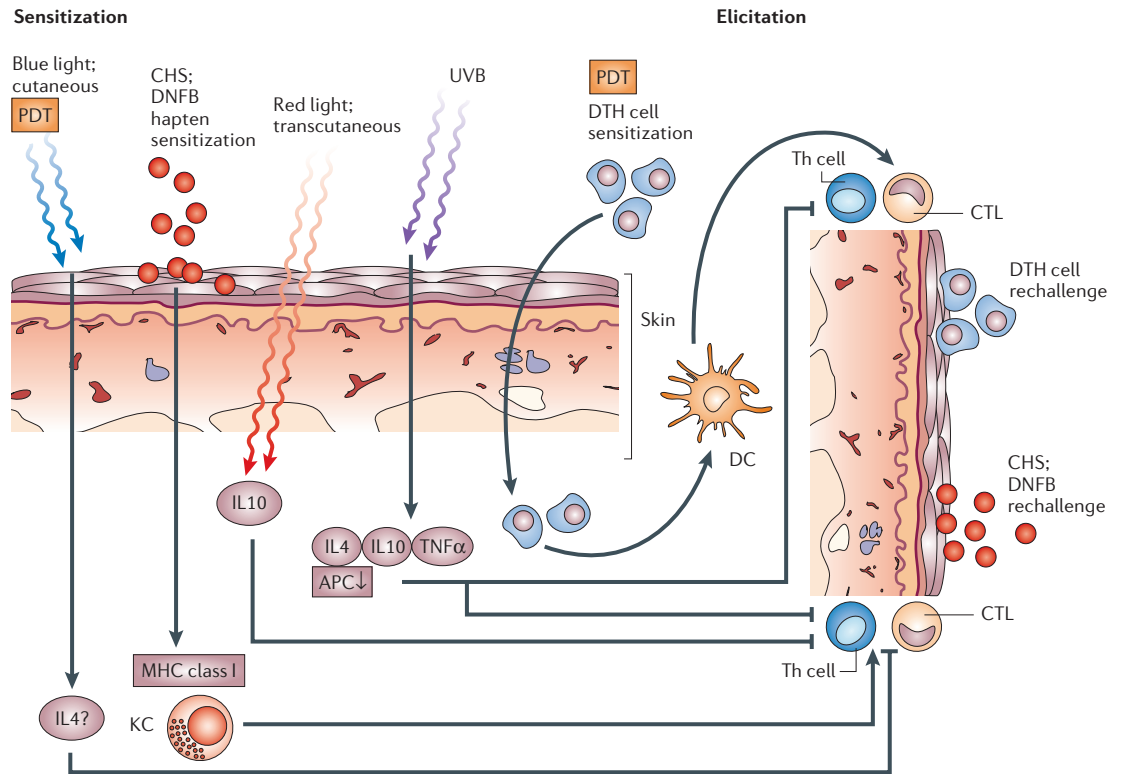
Myers *et al.*<sup>108</sup> combined haematoporphyrin derivative (HPD)-mediated PDT with a killed preparation of *Corynebacterium parvum* (CP, now *Propionibacterium acnes*) in a mouse model of subcutaneous bladder cancer. Giving a high dose of CP after PDT was shown to have a significantly greater effect than CP treatment before PDT. Subcutaneous mouse EMT6 tumours were treated with a single dose of BCG in combination with PDT using six PSs<sup>109</sup>. Regardless of the PS used, BCG significantly increased the number of cured tumours and the number of memory T cells in tumour-draining lymph nodes compared with PDT alone. PDT was combined with a single dose of *Mycobacterium* cell-wall extract immediately after light exposure<sup>110</sup>, and produced significantly more long-term cures of EMT6 tumours in BALB/c mice and more tumour-infiltrating leukocytes at 22 hours after PDT. OK432 is a preparation derived from killed streptococcal bacteria, and increased the tumour-free time in mice with NRS1 squamous cell carcinomas when it was injected intratumorally 3 hours before HPD-mediated PDT. OK432 injected immediately after PDT, or OK432 alone had little effect<sup>111</sup>. The intratumoral injection of OK432 also potentiates PDT-induced anti-tumour immunity against EMT6 tumours (A.P.C., P.M. and M.R.H., unpublished observations). Schizophyllan (SPG) is an example of a β-D-glucan fungal polysaccharide, which are thought to be potent inducers of humoral and cell-mediated immunity by the macrophage dectin-1 receptor<sup>112</sup>, as well as TLRs<sup>113</sup>. The tumour cure rate increased threefold when SPG was given intramuscularly before the Photofrin-mediated PDT of mice with SCCVII, whereas SPG given after PDT had little effect<sup>114</sup>. A report from Chen and colleagues<sup>115</sup> showed that a preparation of glycosylated chitosan derived from shrimp shells injected intratumorally increased the curative effects of Photofrin-mediated PDT on EMT6 tumours and Line1 lung tumours. The receptors that are responsible for mediating the effects of glycosylated chitosan are unknown.

Korbelik's group has observed the activation of the complement system during PDT<sup>92</sup>, and has proposed this as an additional mechanism of anti-tumour response. Tumour-localized treatment with zymosan, an alternative complement pathway activator, and TLR2 and TLR6 ligands, reduced the number of recurrent tumours after PDT<sup>116</sup>. However, a similar treatment with heat-aggregated γ-globulin (a classical complement pathway activator) had no significant effect as a PDT adjuvant. Systemic complement activation with streptokinase treatment had no detectable effect on complement deposition at the

tumour site without PDT, but it augmented the complement activity in PDT-treated tumours. Photofrin-mediated PDT was tested against SSCVII tumours in combination with serum vitamin D3 binding protein-derived macrophage-activating factor (DBPMAF)<sup>117</sup>. DBPMAF markedly improved the outcome of PDT, but as a single agent had no significant effect on the growth of SCCVII tumours.

**PDT and cytokine therapy.** Another class of combination therapies concerns the administration of cytokines such as TNFα, which was shown by Bellnier<sup>118</sup> to potentiate Photofrin-mediated PDT of murine SMT-F adenocarcinoma after a single dose of intravenously administered recombinant human material. Localized tumour treatment with GCSF in combination with Photofrin-mediated PDT resulted in a significant reduction of tumour growth and an increase in the length of survival of BALB/c mice bearing two types of tumour: colo 26 tumours and Lewis lung carcinomas<sup>119</sup> (FIG. 4). Moreover, 33% of colo 26 tumour-bearing mice were completely cured after combined therapy, and developed a specific and long-lasting immunity. Krosli *et al.*<sup>120</sup> repeatedly injected lethally irradiated SCCVII cells that were genetically engineered to produce GM-CSF and showed augmented anti-tumour effectiveness of Photofrin- and BPD-based PDT in mice with SCCVII. The treatment with GM-CSF resulted in higher cytotoxic activity of tumour-associated macrophages against SCCVII cells.

**Regulatory T cells and adoptive cellular therapies.** A third group of PDT combination therapies includes interventions that are designed to alter or augment the cellular arm of the anti-tumour immune response. There is a growing realization that CD4<sup>+</sup>CD25<sup>+</sup> T-regulatory cells have an important function in suppressing the immune response against multiple targets, and these cells are depleted by a low dose of cyclophosphamide (CY), therefore potentiating immunity<sup>121</sup>, whereas a high dose of CY is immunosuppressive<sup>122</sup>. Low-dose CY combined with BPD-mediated PDT, using a short drug to light interval that predominantly targeted the tumour blood vessels, led to a significant number of long-term J774 reticulum cell sarcoma cures and resistance to tumour rechallenge, whereas each treatment alone led to 100% death from progressive tumours or metastasis<sup>123</sup>. The examination of splenocytes recovered from tumour-bearing mice after low-dose CY showed that CD4<sup>+</sup>CD25<sup>+</sup> T cells were reduced in number, and the splenocytes secreted significantly less transforming growth factor-β (TGFβ). TGFβ is an important immunosuppressive cytokine that is secreted by T-regulatory cells, and also stimulates T-regulatory cells<sup>124,125</sup>. Golab and colleagues<sup>126</sup> showed that the injection of immature dendritic cells into tumours that were treated with Photofrin-mediated PDT resulted in effective homing to regional and peripheral lymph nodes and stimulation of CTLs and NK cells. The combination treatment produced the best tumour response and some resistance to a tumour rechallenge. A recent paper<sup>127</sup>



**Figure 5 | Mechanism of photodynamic therapy-induced immune suppression.** Contact hypersensitivity (CHS) is induced by the application of a hapten, such as dinitrofluorobenzene (DNFB), to the skin, and is mediated by the expression of the major histocompatibility complex (MHC) class I on keratinocytes (KC). A subsequent rechallenge with DNFB elicits an inflammatory response caused by the cytotoxic T cells. Delayed type hypersensitivity (DTH) is induced by the injection of cellular antigens such as foreign proteins, and is mediated by MHC class II expressed by dendritic cells (DCs) that are recognized by T-helper (CD4<sup>+</sup>) lymphocytes. CHS is suppressed by photodynamic therapy (PDT) using blue light that does not penetrate the skin and red light that does penetrate. Only by using red light does the suppression of CHS depend on the secretion of interleukin 10 (IL10). DTH is not suppressed by PDT, whereas ultraviolet light (UVB) suppresses both CHS and DTH. These differences might explain the paradoxical observation that PDT can both simultaneously stimulate and suppress parts of the immune system, whereas UVB is only found to be immunosuppressive. APC, antigen-presenting cells; CTL, cytotoxic T cells; Th, T-helper cells.

studied the combination of intratumoral dendritic cells and PDT mediated by the chlorin-type PS ATX-S10 Na(II) against CT26 tumours in BALB/c mice. The combination therapy produced tumour cures that were not seen with either treatment alone. Furthermore, when mice bearing two tumours had only one treated with the combination of PDT and dendritic cells, the contralateral untreated tumour underwent regression. The presence of tumour-specific lymphocytes was shown by chromium-release CTL assay and by IFN $\gamma$  production. Korblick and Sun<sup>128</sup> used adoptive transfer of a human NK cell line that was genetically altered to produce IL2 combined with mTHPC-mediated PDT of subcutaneous human squamous cell carcinoma growing in SCID mice. Peritumoral or intravenous injection of cells immediately after PDT produced an improvement in the outcome of PDT, which was not seen with a cell line that did not produce IL2.

**Immunosuppressive effects of PDT**

Paradoxically, considering the discussions above, there are also several reports that PDT can induce various

forms of immunosuppression<sup>129</sup>. These have nearly all been concerned with the suppression of the contact hypersensitivity (CHS) reaction in mice<sup>130</sup>. This involves application of a hapten such as dinitrofluorobenzene to skin, followed by a rechallenge at a distant site, and can be suppressed for up to 28 days after PDT (FIG. 5). It seems that this suppression involves systemic IL10 release in cases where the PDT illumination penetrates the skin (red light)<sup>131</sup>, but is independent of IL10 when the PDT is confined to the skin layers (blue light)<sup>132</sup>. In contrast to UVB irradiation that suppresses both CHS and delayed type hypersensitivity (DTH) responses, PDT does not suppress DTH<sup>133</sup>. One difference between CHS and DTH is that CHS is thought to be an MHC-class-I-mediated process, whereas DTH is mediated by MHC class II, (REF. 134). As dendritic cells present antigens derived from destroyed tumour cells by MHC class II it could be argued that this difference explains why PDT-induced immunosuppression does not abrogate anti-tumour immunity. MHC class I molecules usually present endogenous molecules to CD8<sup>+</sup> T cells, whereas MHC class II molecules present exogenous molecules

**Hapten**  
A small reactive molecule that can bind to host proteins and stimulate an immune response.

**Delayed type hypersensitivity**  
A delayed-onset, cytokine-induced localized inflammatory reaction characterized by a large influx of macrophages.



to CD4<sup>+</sup> T cells. It is likely that the efficient induction of immune response against tumours requires the priming of CD4<sup>+</sup> T cells by MHC class II molecules and CD8<sup>+</sup> T cells by MHC class I molecules around the time of treatment, followed by the recognition of antigens presented on MHC class I molecules at the effector stage.

### Clinical studies and future outlook

Considering the number of patients (several thousand) that have been treated with PDT for various cancers over the previous three decades, there have been remarkably few studies that have even attempted to determine the effects of PDT on the human immune system, or to detect anti-tumour immunity after patients were treated. Scattered reports exist about the measurement of PDT-induced cytokine expression in patients<sup>64</sup>, and there are anecdotal reports about the unexpectedly long survival of patients who were treated with PDT for recurrent cancer<sup>135</sup>. There have been two reports about effects on the immune system in PDT of human papilloma virus lesions in patients<sup>136,137</sup>. A systematic study designed to detect the possible immune recognition of tumour cells after PDT for patients with cancer is long overdue. A recent meeting abstract (S.O. Gollnick, personal communication) reported that patients who were treated with PDT for basal

cell carcinoma (BCC) demonstrated a significant increase (50–130%) in the numbers of peripheral-blood T cells that produced IFN $\gamma$  when they recognized the sonic hedgehog ligand, hedgehog interacting protein (HIP1). HIP1 is not mutated in BCC, and has been shown to function as a tumour-associated antigen. For other cancers, measuring PDT-induced immune response might involve tumour-reactive serum antibodies or tumour-specific CD8<sup>+</sup> or CD4<sup>+</sup> T cells after PDT, but would involve taking tumour biopsy samples before PDT.

As we learn more it should be possible to understand how PDT can influence the precise cellular aspects of anti-tumour immunity. For instance, T-regulatory cells might be specifically inactivated by IL6 (REF. 138), a cytokine that is abundantly produced after PDT<sup>82</sup>. One point to be considered in the design of future clinical trials is suggested by the report from Henderson *et al.*<sup>83</sup>, which is referred to above. It is entirely possible that the optimal PDT regimen for producing local tumour cures will be different from the optimal PDT regimen for producing inflammation and stimulating immune response. Time will determine whether PDT-induced anti-tumour immunity is a clinically useful phenomenon that could benefit patients and potentially save lives, or whether it is a curiosity only applicable to mice and rats in the laboratory.

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#### Competing interests statement

The authors declare no competing financial interests.

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