PhD thesis

Induction of immunological changes induced by photodynamic therapy (PDT) for cancer

submitted by

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ABSTRACT

Photodynamic therapy (PDT) is a clinically approved procedure for treatment of cancer and certain non-malignant diseases. PDT consists of systemic or topical administration of a photosensitizer (PS) or a PS precursor (prodrug) such as aminolevulinic acid, followed by irradiation of the diseased area with light of wavelengths corresponding to the absorbance band of the PS. When the PS is activated to its excited state by the light, it can react with the surrounding environment and transfer energy to the molecular tissue oxygen, triggering a photochemical reaction and causing cell death. Besides causing direct cytotoxic effects on illuminated cancer cells, PDT is known to cause damage to the tumor vasculature and to induce the release of pro-inflammatory mediators. Previous studies in mouse models and patients have demonstrated that PDT is capable of affecting both innate and adaptive arms of the immune system. It has been shown that besides stimulating tumor-specific cytotoxic T-cells capable to destroy distant untreated tumor cells, PDT can lead to development of anti-tumor memory immunity that potentially prevents the recurrence of cancer. Moreover, several lines of evidence suggest that PDT may also interfere with the immune-suppressive regulatory T cells (Treg). In the present work we thoroughly investigated the intricate immune profile of PDT in both preclinical and clinical studies, involving (1) a colon adenocarcinoma CT26 wild-type tumor mouse model, (2) patients suffering from esophageal squamous cell carcinoma (ESCC) treated with porfimer sodium (Photofrin) and Laser and (3) patients with actinic keratoses (AK), treated with the porphyrin precursor methyl aminolevulinate and red LED light. Our results from the animal model suggested that PDT did not cause any long-term effect on the levels of Treg in the spleen or lymph nodes. However, Treg cells depletion via administration of cyclophosphamide (CY) prior PDT potentiated anti-tumor immunity, leading to increased long-term survival and development of memory immunity. The results obtained from the clinical part of the study indicated that PDT abrogated the suppressive capacity of peripheral Treg from ESCC patients, whereas their Treg levels seemed to be unaffected. Differently, in AK patients neither the level nor the function of peripheral Treg was altered after treatment. Together, this confirms the significant role of the immune system in the response to PDT and enriches the understanding of the role of Treg in PDT-mediated immunological responses. However, the ultimate immunologic outcome upon PDT may depend on the type of cancer and body site treated and vary reliant on the photosensitizer, waveband(s) and/or light

doses applied. These results also disclose potential far-reaching clinical implications for the improvement of treatment strategies in patients with cancer (e.g. combining PDT with appropriately designed anti-Treg therapy).

ZUSAMMENFASSUNG

Die photodynamische Therapie (PDT) ist ein klinisch zugelassenes Verfahren zur Behandlung von Krebs und bestimmten nicht-malignen Erkrankungen. PDT besteht aus systemischer oder topischer Verabreichung eines Photosensibilisators (PS) oder eines PS-Vorläufers (Prodrug) wie Aminolävulinsäure mit nachfolgender Bestrahlung des erkrankten Bereichs. Die Wellenlängen des verwendeten Lichtes sind dabei auf die Absorptionseigenschaften des PS abgestimmt ist. Wenn der PS durch die Einwirkung des Lichtes in den angeregten Zustand übergeführt wird, kann dieser mit der Umgebung reagieren und Energie auf den molekularen Sauerstoff im Gewebe übertragen, ein Prozess, der zu zu einer photochemischen Reaktion und zum Zelltod führt. Neben der direkten zytotoxischen Wirkung auf die bestrahlten Krebszellen schädigt die PDT auch die tumorversorgenden Gefäßen und führt zur Freisetzung bestimmter entzündungsfördernder Mediatoren. Studien in Mausmodellen und bei Patienten zeigten, dass die PDT in der Lage ist sowohl den Schenkel der angeborenen als auch der erworbenen Immunantwort zu beeinflussen. Neben Stimulierung tumorspezifischer zytotoxischer T-Zellen, die auch ferne Tumorzellen zerstören können, kann die PDT auch zur Ausbildung eines immunologische Gedächtnisses führen, welches das Wiederauftreten des Krebses verhindern kann. Außerdem hat eine Reihe von Arbeiten gezeigt, dass die PDT immunsuppressive regulatorische T-Zellen (Treg) beeinflusst. Im Rahmen der vorliegenden Arbeit untersuchten wir das komplexe Immunprofil der PDT in präklinischen Experimenten und klinischen Studien. Diese beinhalteten Untersuchungen in (1) einem Kolon-Adenokarzinom CT26-Wildtyp-Tumor-Mausmodell, (2) eine klinische Studie bei Patienten mit Plattenepithelkarzinom des Ösophagus (PEKÖ), die mit Porfimer Natrium (Photofrin®) und Laser behandelt wurden und (3) eine Studie bei Patienten mit aktinischen Keratosen (AK), deren Behandlung mit Porphyrin-Vorläufer Methyl-Aminolävulinat und rotem LED-Licht erfolgte. Unsere Ergebnisse aus dem Tiermodell wiesen darauf hin, dass die PDT keinen langfristigen Einfluss auf die Anzahl der Treg in Milz und Lymphknoten hatte. Jedoch verstärkte die Verabreichung von Cyclophosphamid (CY) vor der PDT die Anti-Tumor-Immunität und verbesserte das langfristige Überleben verbunden mit der Entwicklung eines immunologische Gedächtnisses. Die Ergebnisse aus dem klinischen Teil der Arbeit zeigten, dass die PDT die immunsuppressive Funktion peripherer Treg von Patienten mit PEKÖ aufhob, während deren Anzahl unbeeinflusst blieb. Bei Patienten mit

AK hingegen änderte sich nach PDT weder die Funktion, noch die Anzahl der peripheren Treg. Diese Ergebnisse bestätigen die wichtige Rolle des Immunsystems bei den Auswirkungen einer PDT und verbessern das Verständnis der Rolle von Treg bei den durch die Behandlung vermittelten Immunantworten. Je nach Art des zu behandelten Krebses, des PS, der Körperstelle, der gewählten Wellenlänge und/oder der Lichtdosis kann die immunologische Wirkung nach PDT variieren. Diese Ergebnisse eröffnen auch das Potenzial für die Verbesserung und Weiterentwicklung der Behandlungsstrategien bei Patienten mit Krebs, beispielsweise durch die Kombination von PDT mit entsprechend gestalteter Anti-Treg-Therapie.

1. INTRODUCTION

1.1 Basic components of PDT

PDT is a therapeutic procedure that consists of three components: a photosensitizer (PS), light of appropriate wavelength to excite the PS and molecular oxygen (Dougherty et al., 1998, Dolmans et al., 2003). None of these three components is toxic alone, but when combined together they initiate a photochemical reaction that culminates in the generation of highly reactive oxygen species (ROS) (Ochsner, 1997) (Figure 1). When the PS is illuminated by the light, it is converted from its electronic ground state (S_0) into its short-lived excited single state (S_1). From there, it can return to its ground state by either emission of fluorescence or heat dissipation, or it can be converted to the long-lived PS triplet state (T_1) through intersystem crossing. The T_1 triplet state PS is capable of exchanging energy with the surrounding microenvironment, hence triggering photochemical reactions with the molecular oxygen ($^3O_2$) present in the tissue. The molecular oxygen is then converted into highly reactive singlet oxygen ($^1O_2$), which in turn generates ROS and eventually destroy the vital cells in the close proximity (Ogilby, 2010).

Overall, the anti-tumor effects of PDT derive from three inter-related mechanisms (Figure 1): *direct cytotoxic effects* via induction of apoptosis and/or necrosis, *damage of the tumor microvessels* and induction of a *robust inflammatory response,* which can trigger the development of a systemic immunity. Each of these mechanisms largely depends on the type and dose of PS, the modality of PS administration, the interval between PS administration and the light dosimetry. Most likely, also the type of tumor and tissue oxygen concentration may be crucial variables.

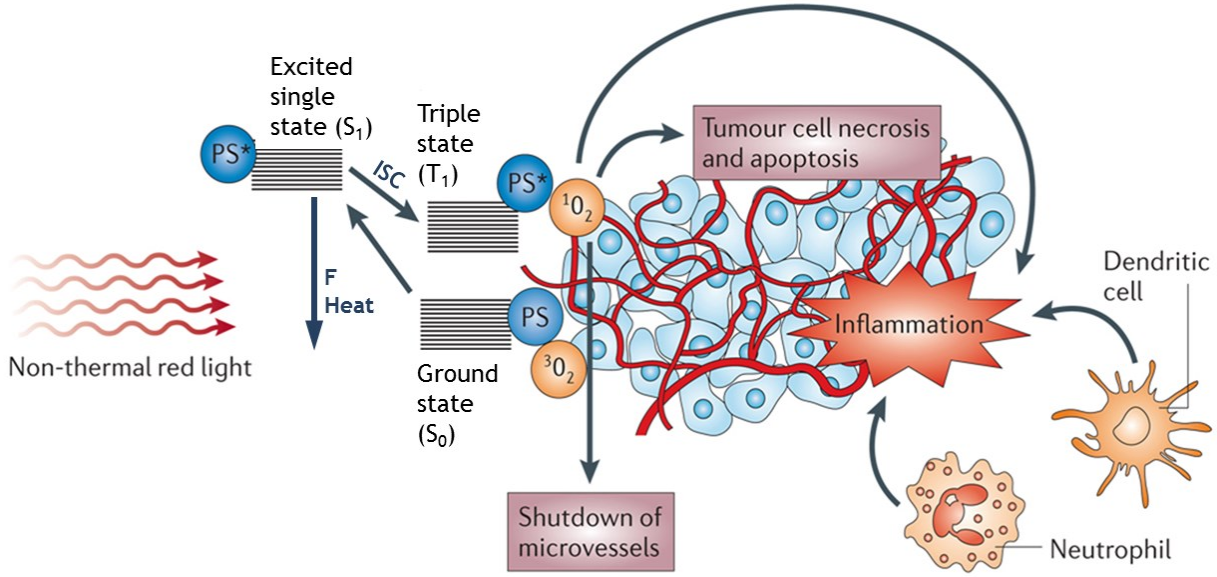

Figure 1: *The photosensitizer (PS) absorbs the light and it is converted to its short-lived excited singlet state (S_1). From there it can lose energy by fluorescence (F), internal conversion to heat or it can undergo intersystem crossing (ISC) to long-lived PS Triple state (T_1) that can trigger a photochemical reaction and lead to local production of reactive singlet oxygen ($^1O_2$). $^1O_2$ can directly kill tumour cells by the induction of necrosis and/or apoptosis, cause destruction of tumour vasculature and produce an acute inflammatory response that attracts host innate immune cells such as dendritic cells and neutrophils. Figure adapted from (Castano et al., 2006).*

1.2 Photosensitizers and light sources

Most of the PSs used in PDT are based on a tetrapyrrole structure, similar to the protoporphyrin contained in hemoglobin (Agostinis et al., 2011). They have an absorption peak between 600 and 800 nanometers (nm) (red to deep red), since lower wavelengths of light would not penetrate efficiently through the tissue and light at longer wavelengths than 800 nm would not have sufficient energy to initiate a photochemical reaction and generate a substantial yield of ROS (Agostinis et al., 2011) (Figure 2). Ideal PSs should selectively localize to the malignant tumor, have a rapid clearance from normal tissues, strong phototoxicity, but no toxicity in the dark. The first PS tested in a large series of patients was a water-soluble mixture of porphyrins, called hematoporphyrin derivative (HPD). Porfimer sodium (a purified form of HPD) became later known as Photofrin and it is still the most employed PS although it presents some disadvantages such as the long-lasting

skin photosensitivity and a low absorbance at 630 nm (its maximum peak of absorbance is at around 400 nm).

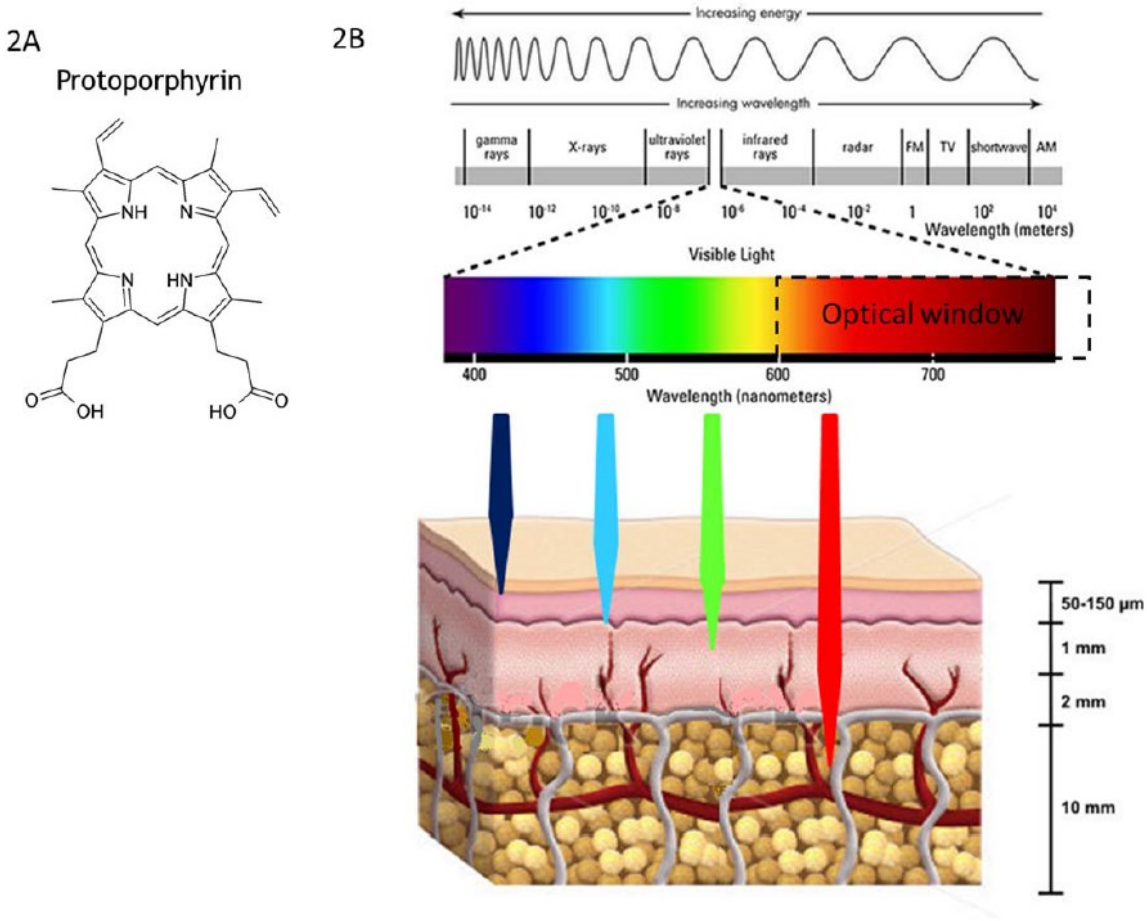

***Figure 2**: Structure of protophorphyrin (2A); electromagnetic spectrum and light propagation through the tissue (2B). Blue light cannot penetrate efficiently through the tissue, whereas red light can penetrate more deeply. The region between 600 and 1200 nm is called "optical window of tissue". However the light at wavelengths longer than 800 nm cannot initiate a photochemical reaction as it has insufficient energy to generate $^1O_2$.*

In the last years, several second-generation PSs have been developed and tested in clinical trials for cancer PDT. Besides the phorphyrin-based PSs, also chlorophyll-based PSs (e.g. chlorins, bacteriochlorins) and dyes (e.g. phtalocyanine) have been developed. Kennedy et al (Kennedy et al., 1990) introduced the new concept of PDT with endogenous porphyrins, based on 5-aminolevulinic acid (ALA) as a precursor of the PS protoporphyrin IX (De Rosa and Bentley, 2000). The administration of exogenous ALA leds to photosentization through endogenous protoporphyrin IX, produced by the tumor cells themselves. The

approval of ALA-PDT by the US FDA in 2000 marked a successful and historic event in the field of PDT. Since then, topical ALA or its later on approved methyl ester (methyl aminolevulinate; MAL) have become the most common used substances in PDT, in particular in dermatology. They have been approved as prodrugs to photosensitize for PDT of non-melanoma skin cancers, including basal cell carcinoma, Morbus Bowen, and actinic keratoses. Currently, they are widely used among other agents as first line treatment in those conditions.

Depending on the PS used and on the type of disease (size of lesions, localization, tissue characteristics), different light sources can be employed for the treatment (Brancaleon and Moseley, 2002). Historically, large and complex lasers have been used to carry out PDT treatment, but nowadays there is a wide range of laser and non-lasers sources that can be used. In particular, diode laser represented a major breakthrough in the widespread clinical use of PDT as they are small, have automated dosimetry and are simple to operate. Lasers are normally coupled with optical fibers and are ideal for endoscopic PDT (e.g. in the digestive tract). Moreover, there are commercially available elastic balloons covered on the inside with a strongly scattering material and by inflating them, they adapt to the shape of the hollow organ in which they are inserted, allowing direct contact to the tumor and a homogenous light distubution. Light-emitting diodes (LEDs) constitute convenient alternative light sources. The major advantages of the use of LED in PDT are their potential of a relatively narrow spectral bandwidth (5-10 nm) and thigh irradiance. Furthermore, they are inexpensive, versatile and are ideal for the irradiation of large and/or bending anatomic areas (Brancaleon and Moseley, 2002).

1.3 PDT and immune regulation

The ideal therapy for cancer should be able to selectively destroy the tumor cells at the primary site and at the same time trigger the immune system to recognize any remaining or recurring tumor cells. In comparison to other unspecific and/or immunosuppressive cancer therapies such as chemotherapy, ionizing radiation and surgery, PDT might have these desirable properties. The current knowledge of the innate and adaptive immune responses induced by PDT against tumors, as well as the impact of Treg in PDT-mediated anti-tumor

immunity have been summarized in a review article published in World Journal of Immunology 2014 and included in the Appendix (Addendum 1).

Eleonora Reginato, Peter Wolf, Michael R Hamblin

Immune response after photodynamic therapy increases anti-cancer and anti-bacterial effects

World Journal of Immunology 2014 March 27; 4(1): 1-11

1.4 PDT for esophageal squamous cell carcinoma

Esophageal cancer is one of the least studied cancers, though it constitutes a major health problem being the sixth leading cause of death from cancer worldwide (Enzinger and Mayer, 2003). Esophageal cancer is classified in two main subtypes, each with distinct etiological and pathological characteristics: esophageal squamous cell carcinoma (ESCC) and esophageal adenocarcinoma (EAD) (Enzinger and Mayer, 2003). ESCC is the most frequent subtype. It arises from the squamous epithelial cells that line the upper part of the esophagus. Its pathogenesis has been associated mainly to tobacco smoking (De Stefani et al., 1993, Brown et al., 2001) and heavy alcohol use (Brown et al., 2001); in addition, other risk factors such as age (most of the patients are over 60 years old), sex (the disease is more common in men) and heredity have been recognized (Enzinger and Mayer, 2003, Ellis et al., 1994, Risk et al., 1999). EAD instead arises from glandular cells that are present at the junction of the esophagus and stomach and it is often associated with a history of gastroesophageal reflux disease (Lagergren et al., 1999).

Although the standard treatment of esophageal cancer focuses on the triad of surgery, chemotherapy and radiotherapy, their effectiveness is unsatisfactory and the patients' 5-year follow-up outcome remains poor. In the last years other therapeutic strategies for the treatment of esophageal cancer have been developed, among them photodynamic therapy (PDT) (Moghissi, 2012). In case of esophageal cancer, the PS injected intravenously and PDT is performed endoscopically, under general anaesthesia (Figure 4). Photofrin-mediated PDT for esophageal carcinoma was first approved by the US Food and Drug

Administration in 1995 for the palliation of symptoms and the reduction of partial or complete esophageal obstruction caused by the cancer (Dougherty, 2002).

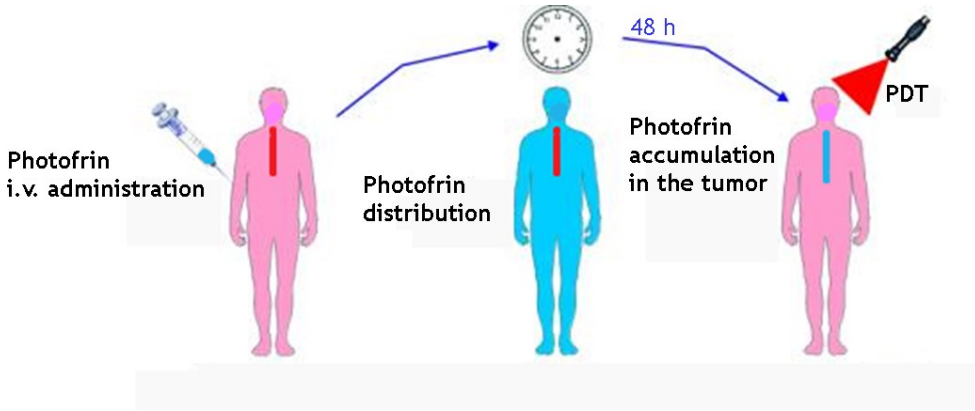

***Figure 3**: The first step of PDT for ESCC is the intravenous administration of 1mg/kg bodyweight of Photofrin (represented in blu). After 48 hours of systemic distribution, photofrin selectively accumulates in the malignant cells of the esophagus (represented in red). Forty-eight hours after photosensitization, the laser treatment is done endoscopically, under general anaesthesia.*

After decades of clinical practice, PDT is known as a minimally invasive, feasible and efficient endoscopic treatment option for treatment of early unresectable ESCC, palliation of locally advanced disease or as salvage when other treatment options have failed (Lindenmann et al., 2011, Yano et al., 2011, Wu et al., 2013).

1.5 PDT for actinic keratosis

Actinic keratosis (AK) is a very common skin condition occurring on sun-exposed body sites. It is considered today as in situ squamous cell carcinoma of the skin, manifested by intraepidermal proliferation of neoplastic keratinocytes (Madan et al., 2010). In approximately 10% of the cases, AK lesions can expand more deeply to the dermis and thus progress to invasive squamous cell carcinoma (SCC) (Glogau, 2000). Currently,

topical photodynamic therapy (PDT) mediated with 5-aminolevulenic acid (ALA) or its methyl ester (MAL) (Dirschka et al., 2012, Dirschka et al., 2013) is among other agents one of the most common treatment modalities for AK. The main advantages of topical PDT are that this method is non-invasive, highly effective, can be applied multiple times and gives excellent cosmetic outcomes compared to surgery (Ericson et al., 2008) or cryotherapy. The overall results of several randomized clinical trials demonstrated that PDT of AK leads to complete response rates in the range of 70% to 90%, with good to excellent cosmetic outcomes in more than 90% of the patients (Agostinis et al., 2011).

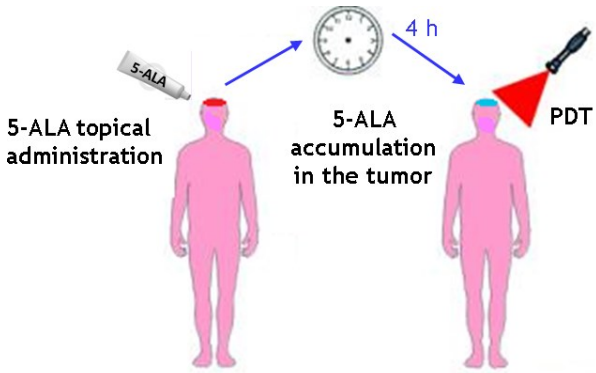

Figure 4*: The first step of PDT for AK is the topical application of ALA or methyl-ALA (MAL) to the lesion. The medication is applied for 3 hours (MAL) or 4 hours (ALA), then the remnant cream is carefully wiped off and the lesions are illuminated with red light.*

2. MAJOR AIMS OF THE THESIS WORK

The main goal of the study was to investigate the immunological events linked to PDT for cancer. In particular we focused our investigations on the reciprocal effects between PDT and Treg in both preclinical and clinical settings. In **thesis part 1** we investigated the immunological effects of PDT alone or combined with Treg depletion (by administration of cyclophosphamide) in the CT26 wild-type colon adenocarcinoma mouse tumor model. For the clinical part of the thesis study, we examined the effects of PDT on levels and function of Treg in patients suffering from ESCC (**thesis part 2**) and AK (**thesis part 3**).

AIMS

Thesis part 1
To investigate the effects of PDT on Treg cells in a mouse tumor model

Thesis part 2
To study the immunological changes induced by PDT in patients with esophageal carcinoma, focusing on Treg

Thesis part 3
To study the effects of PDT on Treg level and function in patients with actinic keratoses.

3. THESIS PART 1

3.1 Background

- PDT can lead to development of antigen-specific immune response
- Low-dose of CY (50 mg/kg) selectively depletes Treg in mice
- PDT mediated immunity can be potentiated by Treg depletion
- CT26 wild-type colon adenocarcinoma express the gp70 self-antigen
- PDT of CT26 wild-type tumors leads only to local regression, followed by recurrence

3.2 Questions

- Does PDT cause any effect on systemic Treg in spleen and lymph nodes?
- Does the combination of PDT + CY foster antitumor immunity against CT26 wild-type tumors?
- Do PDT + CY lead to development of memory immunity?

3.3 Results

- The combination of 50 mg/kg CY with PDT leads to tumor eradication and survival advantage
- CY combined with PDT abrogates the increase in Treg induced by PDT alone
- PDT combined with CY, but not PDT alone, decreases TGF-beta to a baseline level
- Treg suppresses the reactivation of the long-term anti-tumor memory immunity generated by PDT plus CY

These results here above summarized have been published in British Journal of Cancer 2013 and included in the Appendix (Addendum 2).

Eleonora Reginato, Pawel Mroz, Hoon Chung, Masayoshi Kawakubo, Peter Wolf and Michael Hamblin

Photodynamic therapy plus regulatory T-cell depletion produces immunity against a mouse tumour that expresses a self-antigen

British Journal of Cancer 2013; 109, 2167-2174.

The first three authors contributed equally to this work.

4. THESIS PART 2

4.1 Background

- PDT for ESCC has considerable beneficial effects resulting in significantly improved and prolonged survival
- While PDT-mediated effects on host´s immune system have been thoroughly investigated in animal studies, clinical evidence of PDT's immunological effects is limited

4.2 Questions

- Does PDT of ESCC affect level and/or function of circulating Treg?
- Is there any change between before and after PDT in the level of tumor infiltrating Treg?
- Are systemic levels of IL-6, IL-8, IL-10 and TGF-β cytokines up- or down-regulated after PDT?
- Does PDT affect peripheral blood cell levels?

4.3 Results

- PDT does not affect peripheral or tumor-infiltrating Treg levels
- PDT inhibits immunosuppressive function of peripheral Treg
- IL-6 is significantly up-regulated at 7 days after PDT
- PDT affects peripheral leukocyte levels. This increase is mostly due to elevation of granulocyte and monocyte numbers

These results here above summarized have been published in Photochemical & Photobiological Sciences 2014 and included in the Appendix (Addendum 3).

Eleonora Reginato, Jörg Lindenmann, Cord Langner, Nina Schweintzger, Isabella Bambach, Freyja Smolle-Jüttner, Peter Wolf

Photodynamic therapy downregulates the function of regulatory T cells in patients with esophageal squamous cell carcinoma

Photochemical & Photobiological Sciences 2014, DOI:10.1039/c4pp00186a

5. THESIS PART 3

5.1 Background

- **Topical ALA-PDT is a first-line therapy for the treatment of AK**
- **Efficacy of clinical PDT for AK may be depend on patients´ ability to mount an adequate immune response (better responses to PDT in immune-competent patients vs immune-suppressed transplant patients)**

5.2 Questions

- **Does PDT of AK affect level and/or function of circulating Treg?**
- **Is IL-6 up- or down-regulated after PDT?**

5.3 Results

- **PDT does not affect level, not function of systemic Treg level**
- **IL-6 concentration is not changed after PDT**

These results here above summarized will be submitted for publication in the form of a letter to the Editor. The draft of the letter is included in the Appendix (Addendum 4).

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APPENDIX

Addendum1

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REVIEW

Immune response after photodynamic therapy increases anti-cancer and anti-bacterial effects

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Abstract

Photodynamic therapy (PDT) is a clinically approved procedure for treatment of cancer and infections. PDT involves systemic or topical administration of a photosensitizer (PS), followed by irradiation of the diseased area with light of a wavelength corresponding to an absorbance band of the PS. In the presence of oxygen, a photochemical reaction is initiated, leading to the generation of reactive oxygen species and cell death. Besides causing direct cytotoxic effects on illuminated tumor cells, PDT is known to cause damage to the tumor vasculature and induce the release of pro-inflammatory molecules. Pre-clinical and clinical studies have demonstrated that PDT is capable of affecting both the innate and adaptive arms of the immune system. Immune stimulatory properties of PDT may increase its beneficial effects giving the therapy wider potential to become more extensively used in clinical practice. Besides stimulating tumor-specific cytotoxic T-cells capable to destroy distant untreated tumor cells, PDT leads to development of anti-tumor memory immunity that can potentially prevent the recurrence of cancer. The immunological effects of PDT make the therapy more effective also when used for treatment of bacterial infections, due to an augmented infiltration of neutrophils into the infected regions that seems to potentiate the outcome of the treatment.

Key words: Photodynamic therapy; Anti-tumor immunity; T-cell activation; Damage-associated molecular patterns; Inflammatory cells

Core tip: The immune stimulatory properties of photodynamic therapy (PDT) make this therapy one of the most promising therapeutic procedures for the management of cancer lesions and microbial infections. This review will focus on the current knowledge of the innate and adaptive immune responses induced by PDT against tumors and pathogens.

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INTRODUCTION

The ideal therapy for cancer should be able to selectively destroy the tumor cells at the primary site and at the same time trigger the immune system to recognize any remaining or recurring cancer cells. Compared to other unspecific and/or immunosuppressive cancer therapies

such as chemotherapy, ionizing radiation and surgery, photodynamic therapy (PDT) might have these desirable properties. PDT is a procedure that consists of three components: A photosensitizer (PS), light of appropriate wavelength to excite the PS and molecular oxygen[1,2]. None of these three components is individually toxic, but when combined together they initiate a photochemical reaction that culminates in the generation of highly reactive oxygen species (ROS)[3]. Most of the PSs used in PDT are based on a tetrapyrrole structure, similar to that of the protoporphyrin contained in hemoglobin[4]. They have an absorption peak between 600 and 800 nm (red to deep red), since light at lower wavelengths would not penetrate efficiently through the tissue and light at longer wavelengths than 800 nm would not have sufficient energy to initiate a photochemical reaction and generate a substantial yield of ROS[4].

The ROS produced during PDT can directly kill tumor cells by induction of necrosis and/or apoptosis[5] and damage the tumor vasculature, leading to depletion of oxygen and nutrients in the tumor[6,7]. As a result of this traumatic insult to the tumor and its microenvironment, a strong acute inflammatory reaction is provoked at the targeted site[1]. The acute inflammatory response following PDT causes infiltration of host innate immune cells that carry out the removal of damaged cells. Acute inflammation also seems to be implicated in the development of adaptive anti-tumor immunity[1]. In particular, the efficacy of PDT in some models has been shown to be dependent upon such induction of anti-tumor immunity. Early studies showed that while PDT of EMT6 tumors exhibited curative effects and long-term tumor control in Balb/c mice, the long-term protection from tumors was lost when PDT was performed in either *scid* (which lack T and B cells), or nude (which lack T cells) immune-compromised mice. However, when the *scid* mice were reconstituted with splenic T cells or bone marrow cells from Balb/c mice, the curative effect of PDT was restored[8,9].

While the immune stimulatory effects of PDT have been widely studied, although not completely understood in cancer models, much effort still has to be done to understand these effects of PDT in microbial infections. Tanaka *et al*[10] discovered that the therapeutic effect of PDT in a mouse model of bacterial arthritis was dependent on the attraction and accumulation of neutrophils into the infected region and could also produce a protective effect if carried out before infection. This review will focus on the current knowledge of the beneficial immunological effects of PDT for cancer and bacterial infections. A list of notable publications that show that PDT can activate different constituents of the immune system is provided in Table 1.

DAMAGE-ASSOCIATED MOLECULAR PATTERNS

After the traumatic insult to the tumor induced by PDT, one of the first events occurring at the treatment site is the generation of "danger" signals, so called damage-associated molecular patterns (DAMPs) or cell death-associated molecular patterns (CDAMPs) that serve as warning signals in innate immunity[11-14]. DAMPs play a similar role to that of pathogen-associated molecular patterns, but instead of being associated with pathogenic microbes, they are associated with host tissue damage. DAMPs are endogenous intracellular molecules normally "hidden" within living cells, but upon exposure or secretion from dying and/or damaged cells, they acquire immune-stimulatory properties. DAMPs are thought to be the key mediators of the immunogenicity of tumor cells killed by PDT *via* necrosis or apoptosis. They constitute alarm signals warning that "self-altered" antigens were released from dying cells; the immune system recognizes them and triggers a vigorous immunological response. It is generally accepted that while necrotic cells are pro-inflammatory and immunogenic, some forms of apoptotic cells are efficiently engulfed and disposed of by macrophages and other phagocytic cells, therefore they should not induce inflammation and are unlikely to stimulate the immune system[15,16]. However, it has been reported that under certain circumstances, other forms of apoptotic cells such as tumor cells undergoing apoptosis by some particular cancer therapies can effectively generate an immune response[17,18]. In this case the process is defined as "immunogenic apoptosis" *vs* the conventional "non-immunogenic apoptosis"[16,19,20].

It is conceivable that while the physiological programmed cell death is non-inflammatory and non-immunogenic, some cancer therapies (such as particular forms of chemotherapy and PDT) cause tumor damage, and produce an immunogenic form of apoptosis characterized by release of DAMPs and enhancement of inflammation.

The release of DAMPs after PDT has been investigated in some studies[11,12,21]. Korbelik *et al*[22] found that squamous cell carcinoma Ⅶ (SCCⅦ) cancer cells treated by *in vitro* photofrin-PDT expose on the surface heat shock proteins (HSPs) such as HSP60, HSP70 and glucose-regulated protein 94 (GRP94) and release HSP70 to the extracellular space. Interestingly, when PDT was applied in *in vivo* settings, they found a different spectrum of DAMPs exposed on the surface of treated SCCⅦ cells. While HSP70 was still exposed, HSP60 and GRP94 were no longer detected and replaced by GRP78 on the surface of PDT-treated SCCⅦ cancer cells. This indicated for the first time that the DAMPs associated with PDT can differ in the same cancer cells between *in vitro* and *in vitro* settings[22].

It is worth mentioning also that the spectra of DAMPs exposed and/or released after PDT correlate with the sub-cellular localization patterns of the PS, where the ROS-based stress is originated. For instance, PSs targeting the endoplasmatic reticulum (*e.g.*, hypericin) are known to cause surface exposure of calreticulin (CRT); conversely, Photofrin (whose localization is mostly associated with lipid membranes)-PDT, has been linked primarily to surface exposure of HSP70[23,24].

Table 1 Milestone studies on effects of photodynamic therapy affecting the immune system

Immune components	Immunomodulatory effect of PDT	Ref.
Pro-inflammatory cytokines	Production of pro-inflammatory cytokines after PDT *in vivo*	[26]
Macrophages	First evidence of cytokine production by PDT-treated macrophages *in vivo*	[107]
Dendritic cells	DCs can efficiently phagocytose PDT-treated tumor cells in *in vivo* experiments. Immature DCs administered in combination with PDT produce effective antitumor response *in vivo*	[38,108]
NKs	Role of NKs in immune response after PDT, control of distant untreated tumors	[43]
Neutrophils	Evidences that neutrophils have a crucial role in the PDT response *in vivo*	[30,109]
Memory immunity	First demonstration that a specific antitumor memory immunity is induced after PDT: resistance to tumor rechallenge in animals cured by PDT	[110]
T lymphocytes, memory immunity	Essential role of host T lymphocytes in immune response after PDT: curative effect of PDT in immune-competent Balb/c mice, but not in immune-suppressed *scid* mice. Adoptive transfer of splenocytes from PDT-cured mice to *scid* mice confers resistance to tumor rechallenge	[8,111]
Treg	Evidences for the role of Treg in inhibiting the immune response after PDT	[77]
Patient lymphocytes	First demonstration that an antigen-specific immune response can be observed after PDT	[65]

PDT: Photodynamic therapy; DCs: Dendritic cells; NKs: Natural killer cells; Treg: T regulatory cells.

Table 2 Damage-associated molecular pattern molecules that may be released or exposed on the outer leaflet of dying tumor cells after photodynamic therapy

DAMP	Function	Ref.
HSP60, HSP70, HSP90, gp96, GRP94, GRP78	Molecular chaperones that normally reside in intracellular regions/organelles, but under stress they are exposed on the damaged cell surface and prime immunomodulatory processes	[11,21,22,112]
Calreticulin	Calcium binding protein located in intracellular regions/organelles (mostly in ER), but under stress its presence on the PM is augmented. On the PM it acts as "danger signal" and increases the immunogenicity of the dying cells	[11,112]
ATP	High-energy molecule, normally intracellular, but can be released by necrotic and apoptotic cells under particular stresses. Extracellular ATP has the ability to help in chemoattraction of immune cells	[12,112]
Phosphatidylserine	When cells are damaged/dying, phosphatidylserine is transposed from the inner to the outer leaflet and acts as an "eat me" signal by interacting with multiple immune cells receptors, mediating efficient phagocytosis and anti-inflammatory responses	[112,113]
High mobility group box-1	Nuclear chromatin-binding protein; it has prominent cytokine-line properties and when released by dying cells tends to stimulate immune cells to produce various pro-inflammatory cytokines	[11,112]
Calgranulin family members (S100A8, S100A9, S100A12)	Calcium-binding proteins; when released by necrotic cells they act as "find me" signals attracting various immune cells and interacting with immune cell receptor (TLR4/RAGE) to induce the secretion of pro-inflammatory cytokines	[11,112,114]
Cross-linked dimer of ribosomal protein S19	Constituent of small ribosomal subunit; when released by necrotic cells it acts as a chemotactic factor for attracting various immune cells	[11,112]

DAMPs: Damage-associated molecular patterns; ER: Endoplasmatic reticulum; PM: Plasma membrane; HSP: Heat shock protein; GRP: Glucose-regulated protein; ATP: Adenosine triphosphate; TLR4: Toll-like receptor 4; RAGE: Receptor for advanced glycation end-products.

Further investigations on cellular and molecular mechanisms are certainly required to establish in more detail the correlations between DAMPs and PDT. However, the most important examples of DAMPs which are produced after PDT reported so far are HSPs, CRT, adenosine triphosphate and other mediators[21,22,25]. Table 2 lists the DAMPs that have been reported to be produced after PDT.

INFLAMMATION AND INNATE IMMUNE RESPONSES IN ANTI-CANCER PDT

The PDT-induced oxidative stress and traumatic insult to the tumor microenvironment are known to stimulate the release or expression of various proinflammatory mediators [tumor necrosis factor (TNF)-α, interleukin (IL)-6, IL-1, complement proteins, HSPs and arachidonic acid metabolites] from the treated site[26]. Moreover, as mentioned above, immunogenic DAMPs are released after PDT and they can be detected by the innate immune cells that are programmed to detect microbial invasion[27]. For these reasons, innate immune cells such as monocytes or macrophages, neutrophils and dendritic cells (DCs) are recruited to the treated site and infiltrate in large numbers to attack what is expected to be a microbial invasion but turns out to be damaged tumor cells[28]. The primary function of the inflammatory cells is to neutralize the DAMPs by engulfing and eliminating the cellular debris as well as compromised tissue components. This promotes local healing with restoration of normal tissue function. At the onset of PDT-induced inflammation, the tumor vasculature undergoes significant changes and become permeable for blood proteins and pro-adhesive for inflammatory cells *via* over-expression of adhesion molecules (Intracellular Adhesion Molecule 1, Vascular Cell Adhesion Molecule 1, selectins)[27], thus favoring the

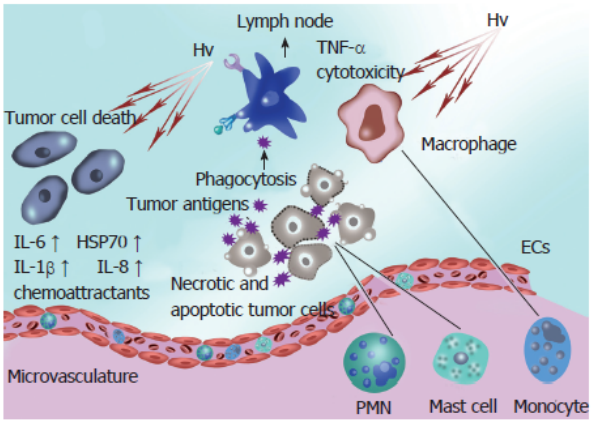

Figure 1 Innate immune responses in anti-cancer photodynamic therapy. Photodynamic therapy of tumors leads to the development of local inflammation mediated by the release of danger signals and cytokines. Various cells of the immune system infiltrate into the treated area. ECs: Endothelial cells; HSP70: Heat-shock protein; Hv: Light; PMNs: Polymorphonuclear neutrophils; TNF: Tumor necrosis factor; IL-6: Interleukin-6. Original figure based upon Ref. [115] and Ref. [116].

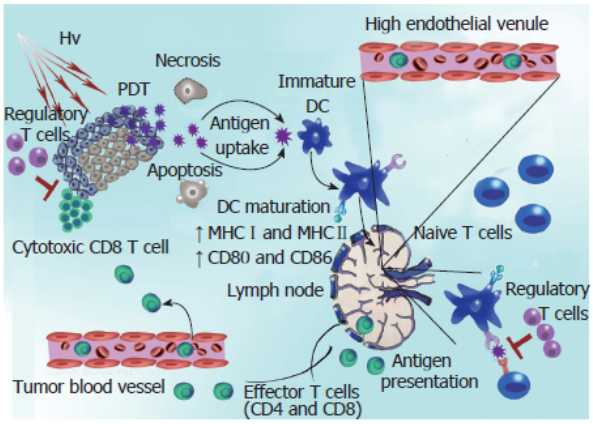

Figure 2 Stimulation of adaptive anti-tumor immunity by photodynamic therapy. PDT-treated tumor cells release the antigens, which are phagocytosed by DCs and presented to naive T cells in the tumor draining lymph node. Activated effector T cells return in circulation and migrate to the tumor. Regulatory T cells seem to inhibit the immune responses after PDT. DCs: Dendritic cells; Hv: Light; MHC I : Major histocompatibility class I ; PDT: Photodynamic therapy. Original figure based upon Ref. [115] and Ref. [116].

massive infiltration of the immune cells into the tumor.

The inflammatory cells are known to be necessary to achieve efficacious PDT, as several studies have shown that their depletion (or inhibition of their activity) diminishes the therapeutic effect of the treatment[9,29,30]. Among all the cytokines involved in the PDT-induced inflammatory process, IL-1β and IL-6 seem to play the most important role[26,31] and conversely, IL-10 and transforming growth factor (TGF)-β seem to hamper PDT-effects as their blockade remarkably improves the cure rates after PDT[27]. Also, blocking the function of various adhesion molecules can affect the efficacy of PDT[26,32]. Figure 1 shows the important cells and mediators that are activated in the tumor environment after PDT of a tumor.

Although PDT is a local treatment, its effect is not limited to the local site, but it can induce a potent acute phase response with systemic consequences[33]. Studies in mouse models have shown that PDT leads to drastic rise in serum levels of acute phase reactants such as serum amyloid P components (SAP), C-reactive protein (CRP) and mannose-binding lectin A (MBL-A)[34]. SAP and CRP belong to the pentaxin family proteins and are involved in acute immunological responses[35]. They are specialized in facilitating the phagocytosis and removal of dying cells such as those killed in PDT-treated tumors. SAP production and release is a hallmark acute phase reactant response in mice, but in humans CRP is a more important acute phase reactant than SAP and PDT dose-dependent up-regulation of CRP has been demonstrated in human lung tumor A549 cells[35]. MBL-A is another important acute phase reactant with functional attributes similar to SAP[36].

Furthermore, a rapid increase in peripheral neutrophils is observed immediately after PDT and it is still present 24 h later, that is correlated with the influx of neutrophils into the treated tumors[37].

ADAPTIVE IMMUNE RESPONSES IN ANTI-CANCER PDT

The PDT-induced local and systemic inflammatory responses can enhance the development of an adaptive immune response capable of protecting the host organism in an antigen-specific manner, owing to immunological memory. It can be asked what is mediating the crosstalk between the innate and adaptive arms of the immune system after PDT. It has been realized that PDT enhancement of adaptive anti-tumor immunity involves the activation of DCs. DC are stimulated by the recognition of DAMPs/CDAMPs released and/or exposed by dying tumor cells[38]. One of the best characterized DAMPs induced by PDT is HSP70, which is released after PDT and forms stable chaperone complexes with cytoplasmic tumor antigens. Thereafter, the HSP-antigen complexes bind to the danger signal receptors, Toll-like receptors 2 and 4[39] on the surface of DCs, which are most potent antigen presenting cells (APCs). In the absence of inflammation DCs remain in an immature state, but when tissue inflammation and release of DAMPs occur, they mature and migrate in large numbers to the draining lymph nodes. The transition to the mature state of DC involves the upregulation of surface major histocompatibility class I and II molecules (MHC I and MHC II) and of the costimulatory molecules CD80 and CD86. These changes allow the DCs to express peptide-MHC complexes at the cell surface and prime efficiently $CD4^+$ T helper cells and $CD8^+$ cytotoxic T lymphocytes (CTLs) and hence to initiate an adaptive immune response. Figure 2 shows the process by which DCs engulf tumor antigens, become activated, traffic to lymph nodes where antigen specific T-cells proliferate and then return to attack remaining tumor cells.

The generation of $CD8^+$ effector and memory T cell

induction is generally, but not always dependent on $CD4^+$ helper T cells[40-42]. Kabingu *et al*[43] showed in fact that $CD8^+$ T cell-mediated immunity is independent of $CD4^+$ T cells and depends instead on natural killer cells. Also other studies suggest that $CD8^+$ cells play the most critical role in PDT mediated anti-tumor immunity, as in the absence of their activation and/or tumor infiltration the efficacy of PDT is reduced[9,43]. Furthermore, it has been shown that adoptive transfer of bare $CD8^+$ T cells to immunocompromised *scid* mice can significantly restore PDT efficacy[8].

The adaptive immunity is not provided only by antigen-specific T cells, but also by B cells. B cells produce antigen-specific immunoglobulins, mounting the so called humoral immune response. So far there is only one study showing that the activation of humoral immunity is implicated in the PDT-induced systemic antitumor protection, as seen by (1) increased serum IgG titers after PDT; (2) production of antibodies against existing antigens; and (3) marked B-cell infiltration in the tumor rim 24 h after PDT[44]. Nonetheless, the importance of the humoral components to the tumor eradication process remains unclear and needs further investigations.

ROLE OF TUMOR ANTIGENS IN THE ANTI-TUMOR IMMUNE RESPONSE

Tumor antigens (TAs) represent a sort of "bait" for the immune system, since they activate DCs and allow the antigen-specific CTLs to recognize and destroy the tumor cells. Some TAs have been well defined in murine and human tumors[45] and are generally classified in three distinct groups: (1) Antigens encoded by cancer-testis genes expressed in various tumors, but not in normal tissues, such as the mouse gene *P1A* and human genes of the melanoma antigen (MAGE)-type, B MAGE and G antigen families[46-51]; (2) Differentiation antigens of the melanocytic lineage, which are present on most melanomas but also on normal melanocytes (*i.e.*, melanoma antigen recognized by T-cells 1, gp100)[51-53]; and (3) Antigens that result from tumor-specific mutations in genes which are expressed in all tissues (*i.e.*, p53, p16) or come from viruses (*i.e.*, Epstein-Barr virus, Hepatitis B virus)[54-58]. Successful immunotherapeutic strategies targeting the TAs have been developed in preclinical studies and early-phase clinical trials[59,60], and our group was the first to realize the importance of TAs expression in PDT anti-tumor immunity.

We showed that a vascular PDT regimen was able to produce 100% of long term cures and rejection of re-challenge when tumors were induced in C3H mice with green fluorescent protein-expressing radiation-induced fibrosarcoma cells, but not with their wild-type counterpart[61]. The same effect was observed when we used a pair of equally lethal Balb/c colon adenocarcinomas: The antigen negative CT26 wild-type and the CT26.CL25 transduced with *lacZ* gene, and thus expressing the tumor antigen β-galactosidase[62]. We could further show that PDT of antigen positive tumors, but not of antigen negative tumors could trigger a highly potent antigen-specific systemic immune response capable to induce regression of distant untreated tumors. Recently we employed the P1A antigen positive mouse mastocytoma P815 wild-type and P1A antigen negative P1.204 (P815 derived) tumor models to study the antigen-specific PDT-induced antitumor immunity[63]. This model is clinically more relevant than others as the P1A is a naturally occurring murine cancer antigen, homologue of the human MAGE-type antigen[64]. We found that tumor cures, significantly higher survival and rejection of tumor rechallenge were obtained with P815, but not with P1.204 tumors that lack the antigen.

The role of the TAs in PDT anti-tumor immunity has been recently investigated also in the clinical setting. In a study published by Kabingu *et al*[65] in 2009, they demonstrated for the first time the enhancement of systemic immune reactivity to a basal cell carcinoma (BCC) associated TA (Hedgehog-interacting protein 1) following PDT in patients. These novel findings in patients are important as they are supporting the results in preclinical models, but more effort needs be done in clinical trials to elucidate the PDT-induced systemic immune responses to tumor antigen.

IMPACT OF T REGULATORY CELLS IN THE ANTI-TUMOR IMMUNE RESPONSE

In addition to directly stimulating anti-tumor immunity by triggering DCs and T cells activation, PDT may also interfere with immune-suppressive T cells. The main class of T cells suppressing the immune response consists of $CD4^+CD25^+FoxP3^+$ T regulatory cells (Treg)[66]. The involvement of Treg in both autoimmune disease[67] and cancer[68] has been extensively described in mice and humans. Treg are thought to mediate their immunosuppressive effects by multiple mechanisms[69]. Treg express the protein receptor cytotoxic T-lymphocyte antigen 4 (CTLA-4), which is similar to the T-cell costimulator protein CD28. CTLA-4 binds with much higher affinity to B7-1 and B7-2 costimulatory molecules on APCs compared to the equivalent molecule CD28 and transmits inhibitory signals, rather than stimulatory[70].

Treg are generally classified into two main subpopulations: Natural Treg and induced Treg[71]; the former are found in the thymus and thought to have T-cell receptors that recognize self-antigens, therefore important in the prevention of autoimmune disease, the latter can be induced and differentiate in the periphery, *i.e.*, upon influence by TGF-β in the tumor microenvironment[72]. Several studies have shown that Treg inhibit the generation of immune responses against tumors[71], but on the other hand, their depletion *in vivo* facilitates tumor eradication and enhances-anti-tumor immunity[73-75]. A summary of the features of Treg is provided in Table 3.

Our research group was the first to investigate the potential relationship between PDT and Treg and we realized that Treg play an important and negative role in PDT anti-tumor immunity. We observed that if Treg are

Table 3 Common features of T regulatory cells

Features of Treg	Ref.
Phenotypic and functional specialization	[66-68]
Treg are $CD4^+CD25^+FoxP3^+$ immunosuppressive T cells. They are important for the maintenance of the immune homeostasis and involved in both autoimmune disease and cancer	[71,72]
Cells subpopulations	
Treg are generally classified into nTreg and iTreg. The former are found in the thymus and thought to have T-cell receptors that recognizes self-antigens, therefore important in the prevention of autoimmune disease, the latter can be induced and differentiate in the periphery, *i.e.*, upon influence by TGF-β in the tumor microenvironment	[69,70]
Immunosuppressive mechanisms	
Treg are thought to mediate their immunosuppressive effects by multiple mechanisms, among which	
Secretion of immunosuppressive cytokines	
High affinity binding of his CTLA-4 receptor to B7-1 and B7-2 costimulatory molecules on antigen presenting cells and transmission of inhibitory signals	
Role of Treg in anti-tumor immunity	[71,73-75]
Treg are known to inhibit the generation of immune responses against tumors. Treg depletion *in vivo* facilitates tumor eradication and enhances-anti-tumor immunity	

nTreg: Natural Treg; iTreg: Induced Treg; CTLA-4: Cytotoxic T-lymphocyte antigen 4; Treg: T regulatory cells; TGF: Transforming growth factor.

depleted by low-dose cyclophosphamide (CY) (a traditional cytotoxic cancer drug that at low doses selectively depletes Treg[76]) prior to PDT, the anti-tumor immune responses are potentiated and a memory immunity is generated against metastatic J774 tumors[77]. This effect was not seen when PDT and CY treatments were given separately or when PDT was combined with high-dose CY that destroyed all T-cells not just Treg. Another recently completed study involving the colon adenocarcinoma CT26 wild-type tumor model revealed that the combination of PDT with low-dose CY produced a dramatic improvement in long-term survival, compared with either treatment alone and led the development of immune response to the mouse cancer shared/auto-antigen gp70[78]. Moreover, this combination treatment activated a long-lasting immune memory, that could however be uncovered only when Treg were depleted again by CY before rechallenge. These new findings are important, because they emphasizes that one of the most effective approaches for optimally improving anti-cancer PDT would be by restraining host's regulatory immune cell populations.

CLINICAL EVIDENCE FOR THE IMPACT OF THE IMMUNE SYSTEM IN ANTI-CANCER PDT EFFECTS

The first clinical use of PDT for cancer in modern times dates back to the beginning of the 20th century when Von Tappeiner *et al*[79] used eosin as topical PS combined to sunlight to treat facial BCC. That first trial was successful as out of 6 patients, 4 showed complete tumor resolution. Many years later, in the 1970s, hematoporphyrin derivative (HPD) and light were administered to the tumor area of patients with bladder cancer[80] and resulted in positive outcomes. In the same decade Dougherty *et al*[81] tested for the first time HPD-PDT in a large series of patients with skin tumors reporting striking results: Complete or partial responses were observed in 111 out of 113 patients.

Since then, over 200 clinical trials for PDT as treatment for a large variety of tumors have been carried out. Some clinical studies have demonstrated that PDT efficacy seems to depend on antitumor immunity also in patients. Dragieva *et al*[82] published a study comparing the efficacy of PDT for actinic keratosis and Bowen's disease in immune-competent patients *vs* immune-suppressed transplant patients. The two groups of patients showed comparable initial response, however the immune-suppressed patients had an increased propensity to develop new lesions after the treatment. It has also been shown that patients with vulval intraepithelial neoplasia (VIN) expressing MHC I molecules on the tumor cells were more likely to respond to aminolevulinic acid-PDT compared to patients whose tumors had down-regulated MHC I molecules[83]. MHC I recognition is critical for activation of $CD8^+$ T cells and the down-regulation of MHC I molecules is one of the mecha-nisms used by tumors to evade immune recognition in general and PDT-induced immunity in particular. VIN patients who did not respond to PDT had significantly lower $CD8^+$ T cell infiltration into the treated tumors compared with responders, confirming the important role of $CD8^+$ CTLs in PDT efficacy. The first clinical case of systemic PDT-immune response observed in patients has been published in 2007: PDT of multifocal angiosarcoma of the head and neck located on the right upper limb of a patient, resulted in a spontaneous regression of the untreated distant tumors on the contralateral left upper limb, accompanied by increased immune cell infiltration[84]. Two years later Kabingu *et al*[65] found that PDT treatment of BCC lesions enhanced the reactivity of patients lymphocytes against Hip1, a known BCC-associated TA, as seen by increased secretion of IFN-γ by patients lymphocytes following incubation with the TA derived peptide.

PDT FOR INFECTIONS

Although PDT was discovered in the field of microbiology over 100 years ago[85], up to now PDT has been

studied and applied mainly as anticancer treatment. The discovery of antibiotics in 1940s revolutionized the treatment of infectious disease, limiting the development of other potential alternative anti-microbial treatments like PDT. However, the recent worldwide increase of resistance to antibiotics has strongly enhanced the interest in alternative therapeutic strategies for the treatment of infections. PDT is capable of killing a large variety of pathogens such as bacteria, parasitic protozoa, fungi, yeasts and viruses. Furthermore, PDT does not induce resistance itself and it is a non-invasive method. PDT is more effective in inactivating Gram (+) bacteria compared to Gram (-) due to the different structure of the cell walls[86]. The membrane of Gram (+) bacteria is surrounded by a permeable layer of peptidoglycan and lipoteichoic acid that allows the PS to pass through it[87]. Gram (-) species have an inner cytoplasmic membrane and an outer membrane, which are separated by a peptidoglycan-containing periplasm. The outer membrane constitutes a permeability barrier between the cell and its environment, limiting the PS penetration. Fungal cell walls have a moderately thick layer of chitin and β-glucan that result in a barrier with moderate permeability. Several *in vitro* and *in vivo* studies have been carried out to verify the efficacy of PDT for viral infections, soft tissues infections, oral and dental infections produced by different strains of bacteria. PDT has been shown to work efficiently against *Escherichia coli* and *Pseudomonas aeruginosa* in excisional wounds[88,89] and against *Acinetobacter baumannii* and *Staphylococcus aureus* in burn infections[90,91].

There are reports of PDT on its effects on certain species of fungus, including both filamentous fungi (*Trichophyton*[92] and *Aspergillus*[93]) and yeasts (*Saccharomyces*[94] and *Candida albicans*[95,96]). Also several types of virus have been tested for the affection by PDT, including herpes viruses HSV-1[97] (PDT by methylene blue and light), enveloped RNA viruses from two different families, Semliki Forest Virus (*Togaviridae*) and vesicular stomatitis virus (*Rhabdoviridae*) (PDT by buckminsterfullerene and light)[98] and others[99].

Some clinical trials for PDT have been carried out for dental, gastric and dermatological infections such as acne as well as rosacea, a condition in which microbes may play a role in the pathogenesis[100].

IMMUNE RESPONSES IN ANTI-BACTERIAL PDT

While the immune stimulating effects of PDT have been widely studied in cancer models, little is known about the immunological effects of PDT in bacterial infections. A recent study published by Tanaka *et al*[10] convincingly demonstrated for the first time that *in vivo* PDT can stimulate an innate immune response. They used a mouse model of bacterial arthritis (*Staphylococcus aureus* infection in the knee joint) and observed a strong infiltration of neutrophils in the PDT-treated area. In order to investigate the role of neutrophils in the PDT-mediated bacteria inactivation, they administered anti-GR-1 (anti-neutrophil) antibody as well as antibodies to several pro-inflammatory mediators. The administration of such antibodies resulted in loss of the therapeutic effect of PDT. This suggests that not only killing of bacteria, but also attraction and accumulation of neutrophils into the infected regions were required mechanisms to achieve PDT-mediated clearance of bacterial infections. Additionally, PDT was tested also as a preventive therapeutic approach and delivered prior to the bacterial inoculation into the knee. PDT-mediated infiltration of neutrophils prevented the subsequent inoculation of bacteria from establishing the infection and again, such an effect was abrogated when antibodies against GR-1 and proinflammatory mediators were administered. To the best of our knowledge, this is the first demonstration of a protective innate immune response against a microbial pathogen being induced by PDT. It is well known that bacterial phagocytosis by innate immune cells such as neutrophils, plays a crucial role in the elimination of invading bacteria and, therefore, malfunction of the phagocytic immune system renders the host more susceptible to bacterial infections. Hence, it would be desirable to apply an antimicrobial PDT regimen that causes direct photoinactivation of bacteria, but at the same time that can minimize the damage to the host's neutrophils.

As described above, evidence indicated that PDT of cancer triggers the activation of both innate and adaptive arms of the immune system, while the early results from the bacterial infection models suggest that PDT is capable of stimulating (at least) the innate immune system. The biggest difference, however, could be in the stimulation of T- and B-cell-mediated adaptive immune responses. As antibodies produced by B cells are generally the most effective component of the immune response against bacterial infection, B cells are expected to be the main actors in the post-PDT immune response towards bacteria. However, to the best of our knowledge, nothing is known yet about humoral responses induced by PDT against bacterial infection.

On the other side, while the involvement of B cells in PDT-induced antitumor immunity still needs more investigation, it is widely accepted that the activation of T cell responses play a pivotal role in PDT-mediated immunity towards treated tumors.

CONCLUSION

Several studies in pre-clinical and clinical settings have demonstrated that PDT is capable of pronouncedly activating both the innate and adaptive arms of the immune system. Such effects on the immune system appear to be PDT regimen dependent and strictly linked to the degree of inflammation induced by PDT.

It has been speculated that PDT regimens causing a high degree of acute inflammation are better at immune activation compared to those in which the acute inflammation is lower. However, increase in inflammatory

mediators could promote tumor cell growth in certain circumstances[101]. Moreover, PDT has been linked also to immunosuppressive effects. Such immunosuppressive effects have been established in model of suppression of induction of contact hypersensitivity (*i.e.*, afferent immune response), which involves the application of a hapten to the skin, followed by re-challenge[102], and suppression of delayed-type hypersensitivity (Mantoux) reactions (*i.e.*, efferent immune response) for instance in healthy Mantoux-positive volunteers[103,104]. In particular, such immunosuppressive responses seem to be dependent on the rate of light delivery[105] and anatomic site of PDT[106].

Further studies using a better targeted and dose-controlled PDT treatment would help to expand the knowledge on the activation/suppression of the immune system and the possibilities to improve it in clinical practice.

The proven ability of PDT to trigger inflammation and improve the anti-tumor immune response could be successfully employed in tandem with other treatment modalities, to combat cancer and to achieve long-term tumor control. Nevertheless, up to now PDT remains clinically underutilized. We must realize that with all probability it will take several years of further investigations and clinical trials before the use of PDT becomes a clinically accepted standard practice in cancer patients.

The innate immune responses seem to be of crucial importance also in the relatively new field of PDT as anti-microbial treatment. The activation of neutrophils after PDT, their mobilization from the bone marrow and their attraction to the site of inflammation appear to be important mechanisms, significantly potentiating the antibacterial effects, *e.g.*, in bacterial arthritis mouse models. However, it still remains to be elucidated whether the activation of the host neutrophils is applicable also to other infection models, with other classes of pathogens and/or using different PS. Many years of intense research will be required providing answers to these intriguing questions.

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Addendum 2

FULL PAPER

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Keywords: photodynamic therapy; regulatory T cells; TGF β; CT26 tumour; anti-tumour immunity

Photodynamic therapy plus regulatory T-cell depletion produces immunity against a mouse tumour that expresses a self-antigen

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Background: Photodynamic therapy (PDT) can lead to development of antigen-specific immune response and PDT-mediated immunity can be potentiated by T regulatory cell (Treg) depletion. We investigated whether the combination of PDT with cyclophosphamide (CY) could foster immunity against wild-type tumours expressing self-antigen (gp70).

Methods: Mice with CT26 tumours were treated with PDT alone or in combination with low-dose CY. T regulatory cell numbers and transforming growth factor-β (TGF-β) levels were measured at several time points after treatment. Mice cured by PDT + CY were rechallenged with CT26 and monitored for long-term survival.

Results: Photodynamic therapy + CY led to complete tumour regression and long-term survival in 90% of treated mice while the absolute numbers of Treg decreased after PDT + CY and the TGF-β levels were reduced to a level comparable to naïve mice. Sixty-five percent of the mice treated with PDT + CY that survived over 90 days tumour free rejected the rechallenge with the same tumour when a second dose of CY was administered before rechallenge but not without.

Conclusion: Administration of CY before PDT led to depletion of Treg and potentiated PDT-mediated immunity, leading to long-term survival and development of memory immunity that was only uncovered by second Treg depletion.

Photodynamic therapy (PDT) is an effective anti-cancer treatment that involves the administration of a photosensitiser dye (PS), followed by visible light irradiation of the tumour (Dougherty *et al*, 1998; Dolmans *et al*, 2003; Agostinis *et al*, 2011). The light activation of the PS triggers a photochemical reaction that culminates in the production of highly reactive single oxygen ($^1O_2$) and/or reactive oxygen species that cause immediate cell damage (Henderson and Dougherty, 1992). Additionally, PDT leads to destruction of tumour vessels and the induction of acute inflammatory responses (Jalili *et al*, 2004; Castano *et al*, 2006). Photodynamic therapy has been approved as a treatment modality by US Food and Drug Administration for the use in oesophageal and bronchial cancer and also for pre-invasive and invasive malignant conditions of oral cavity, stomach, bladder, breast and skin (actinic keratosis) (Pass, 1993; Dougherty, 2002; Huang, 2005; Agostinis *et al*, 2011). One interesting aspect of PDT is its ability to stimulate a systemic immune response against a locally treated tumour (Castano *et al*, 2006). In fact, PDT has been shown to effectively stimulate both the innate and the adaptive immune systems of the host (Korbelik, 1996; Dougherty *et al*, 1998; Korbelik *et al*, 2005) by triggering the release of various pro-inflammatory and acute-phase response mediators (Cecic and Korbelik, 2002; Korbelik *et al*, 2008; Garg *et al*, 2011) that lead to infiltration of the treated site by a large number of neutrophils,

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dendritic cells and other inflammatory cells (Krosl *et al*, 1995; Cecic *et al*, 2006; Gollnick *et al*, 2006).

It has been suggested that tumour development and progression may be strongly dependent on the immune system ability to recognise and destroy malignant cells. However, several lines of evidence suggest that tumours can escape immune surveillance by decreasing or losing the expression of tumour antigens or MHC molecules or by inducing highly immunosuppressive tumour microenvironment by producing high levels of immunosuppressive cytokines like transforming growth factor-β (TGF-β) or IL-10 that in turn promote the development and proliferation of $CD4^+CD25^+FoxP3^+$ T regulatory cells (Tregs) and immature dendritic cells (Curiel *et al*, 2004). In healthy individuals, Treg has an important role in maintaining immune homeostasis and tolerance to self-antigens, and preventing autoimmune diseases (Woo *et al*, 2001; Golgher *et al*, 2002; Sakaguchi *et al*, 2008); while in cancer patients the fine balance between Treg and effector T cells is usually distorted and the unrestrained expansion of Treg may foster cancer progression (Valzasina *et al*, 2006). This immunosuppressive tumour environment presents therefore a therapeutic challenge, but several reports have indicated that the depletion of Treg *in vivo* facilitates tumour eradication and enhances anti-tumour immunity (Shimizu *et al*, 1999; Golgher *et al*, 2002; Tanaka *et al*, 2002). More recently, the anti-Treg antibody, ipilimumab that targets CTLA-4 has been approved by the FDA and the European Medicine Agency (EMEA) for the treatment of metastatic malignant melanoma (Hodi *et al*, 2010).

In this report, we further investigate a potential relationship between PDT and Treg. There are limited reports that suggest that PDT on its own may have some immunosuppressive effects (Mroz and Hamblin, 2011), but its effects on Treg have not been thoroughly elucidated. We have previously shown that if Treg is depleted by low-dose cyclophosphamide (CY, 50 mg kg^{-1}) (a traditional cytotoxic cancer drug that at low doses selectively depletes Tregs in mice; Lutsiak *et al*, 2005), the PDT-induced anti-tumour immune responses are potentiated and a memory immunity is generated against metastatic J774 tumours (Castano *et al*, 2008). This effect was not observed when PDT was combined with high-dose CY (150 mg kg^{-1}). We also showed (Mroz *et al*, 2010) that PDT of CT26 tumours expressing a model tumour antigen (Chen *et al*, 1996) led to long-term survival and spontaneous regression of remote, untreated antigen-positive tumours. However, PDT of CT26 wild-type tumours led only to local regression followed by recurrence, despite the fact that several reports showed that CT26 tumours also express tumour antigens, in particular a single peptide epitope known as AH-1, a non-mutated nonamer derived from the envelope protein (gp70) of an endogeneous ecotropic murine leukaemia provirus (Huang *et al*, 1996). Recently, a paper by McWilliams *et al* (2008) described the expression of *gp70* mRNA in several tissues of BALB/c mice resulting in immunologic tolerance that affects anti-tumour immunity. In view of these reports, it is possible that gp70 antigen in CT26 tumours behaves like a self-antigen and can serve as a model of cancer shared/auto-antigen.

In this report, we hypothesised that combination of 50 mg kg^{-1} CY leading to previously shown depletion of Treg with PDT may result in uncovering a PDT-mediated immune response to tumours expressing a model shared tumour antigen gp70.

We also investigated whether the potentiation of PDT by CY is related to the levels of Treg in the spleen and lymph nodes (LNs) and/or to the secretion of the immunosuppressive cytokine, TGF-β, in the serum.

MATERIALS AND METHODS

Cell lines. CT26 wild-type cell line (ATCC, Manassas, VA, USA) were cultured in RPMI medium with L-glutamine and $NaHCO_3$ supplemented with 10% heat inactivated fetal bovine serum, penicillin (100 U ml^{-1}) and streptomycin (100 μg ml^{-1}) (all from Sigma, St Louis, MO, USA) at 37 °C in 5% CO_2 in 75 cm^2 flasks (Falcon; Invitrogen, Carlsbad, CA, USA).

Mouse tumour model. BALB/c mice (6–8 weeks old) were purchased from Charles River Laboratories (Boston, MA, USA). All experiments were carried out according to a protocol approved by the Subcommittee on Research Animal Care (IACUC) at MGH and were in accord with NIH guidelines. Mice were inoculated with 350 000 cells subcutaneously into the depilated right thigh. Two orthogonal dimensions (a and b) of the tumour were measured 2–3 times a week with vernier calipers. Tumour volumes were calculated as follows, volume $= 4\pi/3 \times [(a+b)/4]^3$. When tumours reached a diameter of 5–7 mm (10 days after inoculation) PDT was performed.

Photosensitiser and light source. Liposomal benzoporphyrin derivative mono acid ring A (BPD) was a generous gift from QLT Inc. (Vancouver, BC, Canada) and was prepared by diluting the powder to a concentration of 0.3 mg ml^{-1} in sterile 5% dextrose. A 1 W 690-nm diode laser (B&W Tek Inc., Newark, DE, USA) was coupled into a 0.8-mm diameter fibre.

PDT and CY treatment. Tumour-bearing mice were anaesthetised with intraperitoneal (i.p.) injection of 87.5 mg kg^{-1} of ketamine and 12.5 mg kg^{-1} of xylazine and BPD (1 mg kg^{-1} in 5% dextrose solution) was administered i.v. via the supraocular plexus. Control mice received 5% dextrose only. Fifteen minutes after BPD injection, illumination was performed. We measured the light power with a power meter (THORLABS GmbH, Dachau, Germany; PM 100D) and calculated the correct distance between the optic fibre and the tumour, to use 100 mW cm^{-2} fluence rate in the exposed area. A total fluence of 120 J cm^{-2} was delivered at a fluence rate of 100 mW cm^{-2}. The mice were killed when any of the tumour diameters exceeded 1.5 cm or tumour volume reached 1500 mm^3. In certain groups of mice, 50 mg of CY was administered via i.p. injection 2 days before PDT.

Surgical operation. When tumours reached 5–7 mm in diameter tumour was excised under general anaesthesia with i.p. injection of 87.5 mg kg^{-1} of ketamine and 12.5 mg kg^{-1} of xylazine. After the surgical operation, mouse was killed at five different time points (0, 1, 4, 10 and 14 days).

Rechallenge. Mice surviving 90 days after PDT were subsequently rechallenged with 350 000 cells of CT26 in the left thigh and monitored for another 60 days. Naïve control mice were inoculated with the same sample of cells as a control for tumorigenicity. A group of mice received 50 mg kg^{-1} of CY via i.p. injection 2 days before rechallenge

Flow cytometry analysis of Treg. There were 18 mice per group killed in groups of 3 mice per time point. Spleen and inguinal LNs were harvested at six different time points (-2 days, 0 h, 1, 4, 10 and 14 days). Both organs were homogenised separately by gently pressing them through the 70-μm cell strainer, using a syringe plunger to make single-cell suspensions. Cells were counted by trypan blue exclusion on a haemocytometer after red blood cell lysis. In all, 1×10^6 of either splenocytes or lymphocytes from LNs was washed and resuspended in staining buffer (eBioscience, San Diego, CA, USA). The cells were incubated with rat anti-mouse CD16/CD32 (FcRγIII/II) mAb (eBioscience) to minimise the binding to the Fc receptor by the staining antibodies.

This was followed by labelling with fluorescent dye conjugated primary antibodies against the markers CD4 (RM4-5, FITC conjugated; eBioscience) and CD25 (PC61.5, phycoerythrin conjugated; eBioscience). After washing and fixation/permeabilisation, the cells were incubated with primary conjugated anti-Foxp3 antibody (FJK-16s). An FACSAria flow cytometer was used for data acquisition (Becton Dickinson, San Antonio, TX, USA). In all, 50 000 events were counted in the case of splenocytes and 20 000 for the lymphocytes. All data were analysed with either FACSAria or FlowJo (TreeStar, Ashland, OR, UAS) software. Isotype controls were used to set proper regional gates.

ELISA for TGF-β. Blood samples were drawn from the aorta and were centrifuged at 6000 r.p.m. for 20 min to extract serum. All samples of sera were stored in the $-80\,^{\circ}C$ degree freezer until ELISA was performed. ELISA for TGF-β (R&D Systems, Minneapolis, MN, USA; DuoSet) was performed according to the manufacturer's instructions. Briefly, 20 μl of serum was incubated with 20 μl of 2.5 N acetic acid/10 M urea for 10 min to activate TGF-β. After 10 min of incubation, the samples were neutralised by adding 20 μl of 2.7 N NaOH/1 M HEPES (at this stage the serum is diluted 1 : 3). After 10 min of incubation, 30 μl of diluted samples was 15-fold further diluted with Reagent diluents (1.4% bovine serum albumin, 0.05% Tween-20 in PBS) and used for the assay.

RT–PCR. Total RNA was extracted from cultured cancer cells using the RNeasy Plus Mini kit (Qiagen, Gaithersburg, MD, USA) and reversely transcribed by using the Seniscript RT kit (Qiagen). Primers for specific murine TGF-β (sense, TGCTTCAGCTCCA CAGAGAA: antisense, TGGTTGTAGAGGGCAAGGAC) (Sieber *et al*, 2011) and murine gp70 (sense, AAGGTCCAGCGTTCT CAAAAC; antisense, AGGTGGCGTTAGCTGTTTGT) (Abe *et al*, 2012) were used with 2 μg of sample cDNA and amplified with Taq polymerase (Qiagen) using Thermal Cycler (GeneAmp PCR system 9700; Applied Biosystems, Woburn, MA, USA).

Statistics. Comparisons of Treg and TGF-β values were done by one-way ANOVA followed by Tukey *post hoc* test. Survival analysis was carried out by plotting in the ordinate the percentage of surviving mice out of the total number of mice per treatment group. According to the protocol, when the tumours reached 1500 mm^3, the mice were euthanised and therefore counted as dead. In the statistical analysis, we used the log-rank test and in all cases the significance level was set at $P<0.05$.

RESULTS

The combination of 50 mg kg^{-1} CY with PDT leads to tumour eradication and survival advantage. We employed a vascular BPD-PDT regimen delivered alone, or in combination with CY (50 mg kg^{-1}). Cyclophosphamide was administered on day 8 after CT26 tumour inoculation and PDT performed at day 10 (Figure 1). Photodynamic therapy produced a good local response in CT26 tumours as manifested by development of oedema followed by a circumscribed black eschar and a marked reduction in tumour size lasting until day 18 (Figure 2A). However, local tumour regrowth occurred in all treated mice and the median survival time of the PDT-treated mice was 29 days *vs* 25 days for control untreated CT26 (Figure 2B).

When mice were treated with the combination of BPD-PDT and CY, the outcome was completely different from that seen in mice treated with PDT alone. Figure 2A shows that PDT and CY together led to greater local oedema compared with PDT alone followed by tumour regression in 9 out of 10 mice with all 9 mice surviving tumour free for >90 days (Figure 2B). Mice treated with CY alone showed some tumour growth delay (Figure 2A), but the survival of this group of mice was not significantly different from

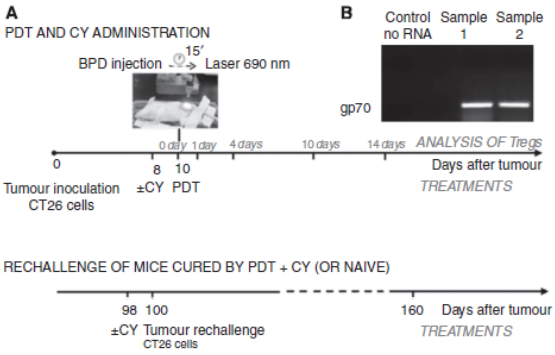

Figure 1. (A) Experimental design scheme of *in vivo* PDT, CY administration and tumour rechallenge. (B) RT–PCR expression of gp70 in CT26 cells.

control untreated mice, or treated with PDT alone (Figure 2B). These results taken together suggest that PDT combined with 50 mg kg^{-1} of CY led to a dramatic improvement in long-term survival when compared with either treatment alone.

CY combined with PDT abrogates the increase in Treg induced by PDT alone. To elucidate the immunostimulatory/immunosuppressive effects of PDT alone, and combined with 50 mg kg^{-1} of CY, we examined the absolute number of $CD4^+CD25^+$ $Foxp3^+$ Tregs in both spleen and LNs at different time points after PDT (0, 1, 4, 10 and 14 days). We included five groups of mice in the study: (A) tumour-free mice, that received PDT (PDT no cancer); (B) mice inoculated with cancer and treated with PDT (PDT cancer); (C) tumour-bearing mice that received injection of 5% dextrose and laser therapy alone (sham PDT cancer); (D) tumour-bearing mice that were treated with PDT and CY combined (PDT + CY cancer) and (E) tumour-bearing mice injected with CY alone (CY cancer). We also measured the numbers of Tregs in naïve mice (i.e., mice without cancer that had not received any kind of treatment) and the values obtained were $0.152 \pm 0.011 \times 10^6$ ($n=3$) in the spleen and $0.199 \pm 0.020 \times 10^6$ ($n=3$) in the LNs.

The absolute numbers of Treg measured from spleen and LNs in the five groups are depicted in Figure 3A and B, respectively, and the *P*-values obtained from statistical analyses are displayed in Table 1 for spleen and Table 2 for LNs.

We found that PDT alone led to a significant increase in $CD4^+CD25^+Foxp3^+$ Treg between day 0 and day 4 after treatment in the spleen and also in the LNs. At later time points, the numbers of Treg in both organs dropped back to values comparable to the naive mice. The 'PDT no cancer' group showed that Treg was highly, but not significantly increased in the spleen at day 4, and significantly increased in the LNs at day 4.

The 'sham PDT cancer' group displayed a gradual and significant raise of Treg in both the spleen (Figure 3A) and the LNs (Figure 3B), to levels much higher than in PDT-treated mice (at 14 days, $P<0.001$ in both spleen and LNs) coinciding for the progression and increase in size of tumour. In the 'CY alone' group, the absolute numbers of Treg decreased between -2 days and 1 day after PDT in both spleen (Figure 3A) and LNs (Figure 3B), confirming that CY is effectively depleting Tregs. Then, similarly to the sham PDT cancer group, Tregs increased steadily between 1 day and 14 days, in both organs, to levels higher than in naïve mice (at 14 days $P<0.001$ in both spleen and LNs).

When CY was combined with PDT, Tregs decreased in both spleen and LNs reaching a minimum at 0 day and then remained

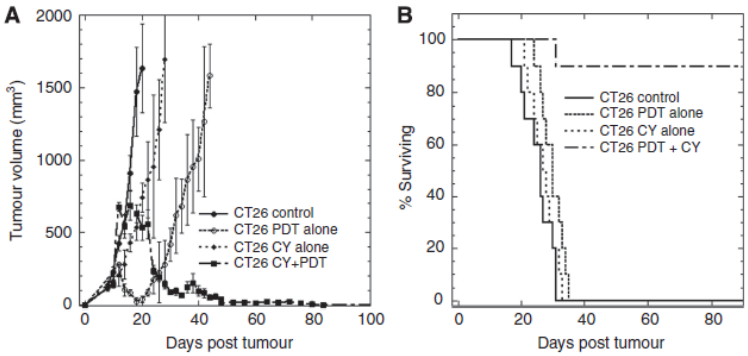

Figure 2. PDT and CY treatment (alone or combined) of CT26 tumours. (**A**) Plots of mean tumour volumes in mice bearing CT26 tumours. Points are means from 10 to 15 mice and bars are s.d. (**B**) Kaplan–Meier survival curves of the % of mice surviving after no treatment, PDT alone, CY alone or PDT plus CY. The median survival time of PDT-treated mice was 29 days *vs* 25 days for control untreated CT26 ($P=0.0304$).

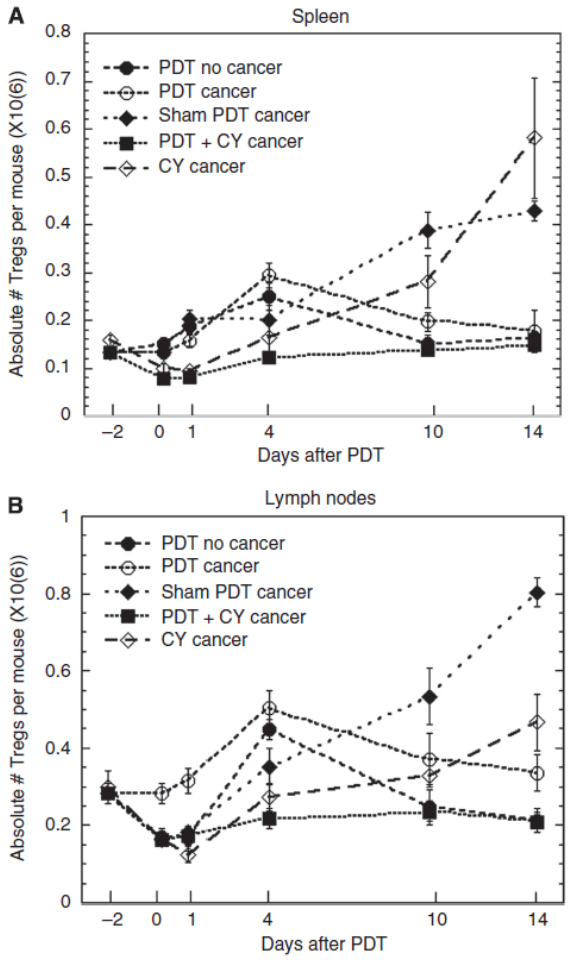

Figure 3. Quantification of Tregs in the spleens and lymph nodes. Absolute numbers of $CD4^+CD25^+Foxp3^+$ Tregs from spleens (**A**) and lymph nodes (**B**) extracted from the mouse groups: PDT no cancer, PDT cancer, sham PDT cancer, PDT + CY cancer and CY cancer. Bars are s.e.m. Statistical comparisons are shown in Table 1 (spleen) and Table 2 (lymph nodes). Significance was determined by one-way ANOVA and Tukey *post hoc* correction.

within the 'naïve' range during PDT and all the time points after the treatment

These results indicate that tumour alone as well as PDT alone may lead to an increase in Treg population, and that this unfavourable effect can be abrogated by administration of CY before PDT.

PDT combined with CY, but not PDT alone, decreases TGF-β to a baseline level. Since TGF-β is the major cytokine involved in Treg-mediated immunosuppression as well as it can lead to an increase in numbers of Treg, we measured the level of TGF-β in the serum of mice treated with PDT, PDT + CY or mice with tumour that was surgically removed. We also measured the level of TGF-β in naïve mice and the value was $1412.41 \pm 93.31\,pg\,ml^{-1}$ ($n=3$). Our results demonstrate that PDT alone led to moderate elevation of TGF-β levels in serum as compared with naïve mice while the combination of PDT + CY led to a significant decrease in TGF-β levels at day 1 (Figure 4B; Table 3) and the TGF-β levels in this group remained low throughout the entire period of the experiment.

To determine to which extent PDT is responsible for TGF-β secretion and not the tumour itself, we analysed the serum samples from mice whose tumours were surgically removed (Surgery cancer). Upon tumour resection, TGF-β decreased gradually and significantly (at 4 days $P<0.01$ and 14 days $P<0.001$ compared with 0 day), becoming comparable to the levels from naïve mice at 14 days after treatment.

Treg suppresses the reactivation of the long-term anti-tumour memory immunity generated by PDT plus CY. To investigate whether the combination of PDT and CY induces the formation of memory immunity, mice that were treated by PDT + CY and remained tumour free for 90 days were subsequently rechallenged with the same tumour cells (CT26). Naïve mice were used as a control. As shown in Figure 5, the tumours were not rejected in the PDT + CY cured mice (CT26 WT CY cured rechall) and the median survival time of PDT + CY treated rechallenged mice was 18 days *vs* 25 days for naõve mice. When the rechallenge was preceded by administration of $50\,mg\,kg^{-1}$ of CY, 65% of mice rejected the rechallenge (CT26 WT CY cured CY rechall) and remained tumour free for another 60 days of observation. When CY was given in naïve mice before tumour inoculation, all tumours progressed and all the mice were killed due to the tumour burden (median survival time = 22 days). These data show that PDT + CY leads to development of memory immunity T cells, that is however suppressed by the Treg. The administration of CY to deplete Treg again at the time of rechallenge abrogated the immunosuppressive

Table 1. *P*-values of Treg in spleen

	Time points (days)				
	0	1	4	10	14
Naive	—	—	—	—	—
A. PDT no cancer	n.s. *vs* A4	—	n.s. *vs* A10	—	—
B. PDT cancer	$P<0.001$ *vs* B4	$P<0.01$ *vs* B4 n.s. *vs* B10	$P<0.001$ *vs* D4 $P<0.01$ *vs* naive	n.s. *vs* B0 n.s. *vs* naive	n.s. *vs* naive
C. Sham PDT cancer	—	—	—	—	$P<0.001$ *vs* B14
D. PDT + CY cancer	n.s. *vs* D4	—	n.s. *vs* naive	n.s. *vs* naive	n.s. *vs* D0 n.s. *vs* naive
E. CY cancer	—	—	—	$P<0.001$ *vs* E14	$P<0.001$ *vs* B14 $P<0.001$ *vs* naive

Abbreviations: CY = cyclophosphamide; PDT = photodynamic therapy; Treg = T regulatory cells. —, comparison not applicable.

Table 2. *P*-values of Treg in lymph nodes

	Time points (days)				
	0	1	4	10	14
Naive	—	—	—	—	—
A. PDT no cancer	$P<0.01$ *vs* A4	$P<0.01$ *vs* A4	—	n.s. *vs* A1	—
B. PDT cancer	$P<0.05$ *vs* B4	—	$P<0.001$ *vs* D4 $P<0.001$ *vs* naive	n.s. *vs* B0 n.s. *vs* naive	n.s. *vs* naive
C. Sham PDT cancer	—	—	—	$P<0.001$ *vs* C0	$P<0.001$ *vs* B14
D. PDT + CY cancer	n.s. *vs* D4	—	n.s. *vs* naive	n.s. *vs* naive n.s. *vs* D0	n.s. *vs* naive
E. CY cancer	—	$P<0.001$ *vs* E14	—	—	$P<0.01$ *vs* E14 $P<0.001$ *vs* naive

Abbreviations: CY = cyclophosphamide; PDT = photodynamic therapy; Treg = T regulatory cells. —, comparison not applicable.

effects of Treg and uncovered the activity of memory T cell leading to rechallenge rejection in 65% of cases,

DISCUSSION

In a previous work, we showed that PDT combined with low-dose CY produced a dramatic improvement in survival and formation of anti-tumour immunity manifested by rejection of tumour rechallenge in mice bearing the highly metastatic J774 tumour (Castano *et al*, 2008). In the present study, we investigated whether this potentiating effect of CY on PDT-induced immunity could also be extended to other mouse tumour models. Therefore, we employed CT26 colon adenocarcinoma and found that the combination of PDT with low-dose CY produced a dramatic improvement in long-term survival, whereas treatment with PDT or 50 mg kg^{-1} of CY alone did not lead to a significant survival advantage.

The CY administration has been suggested to have a specific effect in depleting the Treg (Awwad and North, 1988; Awwad and North, 1989; Ghiringhelli *et al*, 2004) and has been used in several studies to induce tumour regression and increase the immunological responses against cancer, that could otherwise be suppressed by Treg.

To our knowledge, the effect of PDT on Treg has not been described so far. Here, we show for the first time that PDT led to a significant increase of Treg in the first few days post treatment in both spleen and LNs, reaching a peak at 4 days after PDT. In the following days, Treg numbers reduced back to levels comparable to those before the treatment.

Interestingly, this transient PDT effect on Treg levels was not related to the presence of cancer cells as PDT had the same effect in tumour-free mice. These results corroborate the hypothesis that PDT does not act only as a local therapy, capable of destroying tumours at the site of light exposure, but PDT may also have profound systemic immunological effects.

Several pre-clinical and clinical studies have shown that PDT is capable to influence the adaptive arm of the immune system in many different ways; some regimens leads to potentiation of adaptive immunity, while others produce immunosuppressive effects (Agostinis *et al*, 2011; Mroz and Hamblin, 2011). The underlying mechanism responsible for potentiation or suppression is not yet known; however, it has been suggested that they may be multifactorial and dependent on PDT treatment regimen, the area treated and the photosensitiser used. In our case, we saw that Treg reach a peak value between 0 and 4 days just in two animal groups: PDT no cancer and PDT cancer. The same two groups reach the peak of Treg at the same time points also in the LNs. We hypothesise that this early immunosuppressive effect of PDT may result from a strong inflammatory response induced by PDT at site of the treatment. The host's immune system might produce more Tregs to limit the local acute inflammation. Obviously, after day 4 the

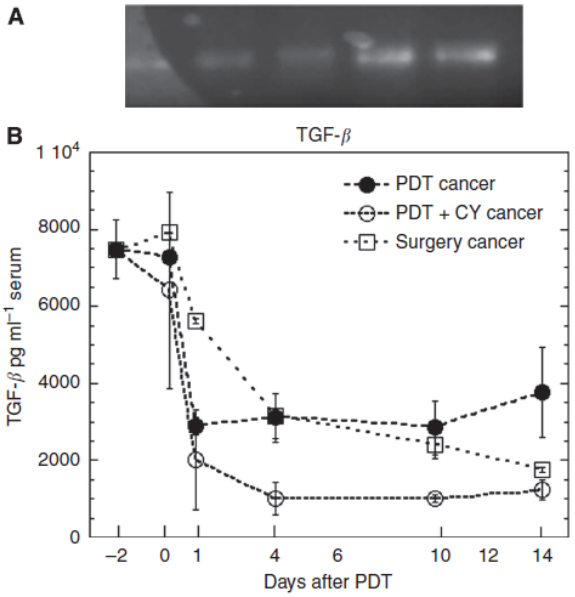

Figure 4. Detection of TGF-β. (**A**) RT–PCR analysis of TGF-β expression in CT26 cells. (**B**) Mean levels of TGF-β cytokine measured in the serum from mice at different time points after PDT, PDT + CY, or surgically operated, as well as in control naïve mice. Statistical comparisons are shown in Table 3 and significance was determined by one-way ANOVA and Tukey *post hoc* correction.

Table 3. *P*-values of TGF-β

	Time points (days)				
	0	1	4	10	14
Naive	—	—	—	—	—
A. PDT cancer	$P<0.01$ *vs* A1 $P<0.001$ *vs* naive	—	$P<0.01$ *vs* A0	—	$P<0.05$ *vs* A0
B. PDT + CY cancer	$P<0.01$ *vs* B1 $P<0.001$ *vs* naive	—	n.s. *vs* A4 $P<0.001$ *vs* B0	—	n.s. *vs* A14 $P<0.001$ *vs* B14
C. Surgery cancer	$P<0.001$ *vs* naive	—	$P<0.01$ *vs* C0	—	$P<0.001$ *vs* C0 n.s. *vs* naive

Abbreviations: CY = cyclophosphamide; PDT = photodynamic therapy; Treg = T regulatory cells. —, comparison not applicable.

withdrawal of the inflammatory response and the tumour healing restored the normal immunological conditions. This may explain why Treg was increased in the first days after PDT, even in the absence of tumour and after 4 days decreased again to the 'naïve' level.

The administration of 50 mg kg^{-1} of CY led to a considerable depletion of Treg in both spleen and LNs and nadir was reached at 2 days after CY administration (the time we selected to deliver PDT). The reduction in the absolute number of Treg was more remarkable in LNs than in spleen; the reason could be that while LNs are more pure pools of T cells, the spleen is acting mainly as a blood filter and contains a wider variety of different subsets of cells. The literature suggest three possible mechanisms of selective depletion of Treg exerted by low-dose CY: (i) inhibition of proliferation of the more rapid proliferating Tregs as compared with other classes of lymphocytes (Ercolini *et al*, 2005), (ii) induction of apoptosis and functional inhibition of Tregs (Lutsiak

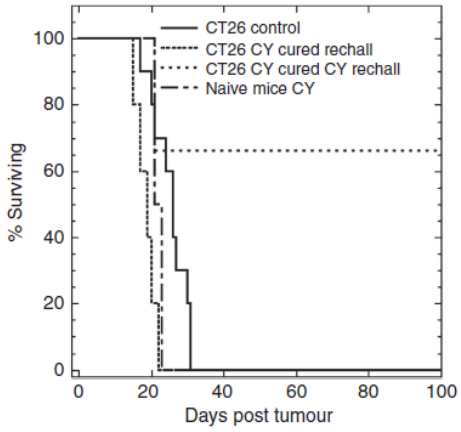

Figure 5. Tumour rechallenge of cured mice. Kaplan–Meier survival curves of the % of mice surviving after rechallenge with CT26 in the groups: naïve; naïve + CY; CT26 cured; CT26 cured + CY. The median survival time of PDT + CY rechallenged mice was 18 days *vs* 25 days for naïve mice ($P=0.0009$).

et al, 2005) and (iii) inhibition of inducible nitric oxide synthase that controls the number of Tregs (Loeffler *et al*, 2005).

It remains to be fully elucidated how long the effect of low-dose CY on Treg lasts for. Peng *et al* (2013) have recently found that a single dose administration of CY leads to a reduction in the number of Tregs in the tumour microenvironment in a transient manner. Eight days after CY injection they observed comparable levels of Tregs in mice treated with CY or untreated, suggesting that a repopulation of Treg occurs some days after CY administration. Here, we saw that after the initial depletion of Treg, from day 3 onwards following CY administration the number of Treg has steadily increased over time; however, this increase coincided with progression of tumour and increase in tumour volume. It was only when PDT was combined with 50 mg kg^{-1} of CY, that the increase in Treg was not seen, either in spleen or in LNs, at any time point after the therapy. This observation correlated with the PDT-mediated regression of the tumour and decrease in tumour volume.

To further investigate the relationship of Treg and tumour microenvironment in the context of PDT and CY therapy, we measured the levels of TGF-β in the serum of the mice at different time points after PDT. The TGF-β signalling has a complex role in carcinogenesis, having both tumour suppressor and oncogenic activities. It has been shown that TGF-β exerts tumour suppressor effects in normal epithelial cells and in early stage of tumour progression by inducing cell-cycle arrest, senescence and apoptosis. Conversely, during the progression of cancer the anti-proliferative effects of TGF-β are selectively lost and TGF-β induces many activities leading to growth, invasion and metastasis of cancer cells (Nagaraj and Datta, 2010). Cancer cells often escape from the growth inhibitory function of TGF-β by mutational inactivation or dysregulated expression of components in its signalling. It also known that increased levels of TGF-β lead to adverse effects on anti-tumour-immunity in multiple ways: inhibiting the proliferation and differentiation of T lymphocytes, natural killer cells, neutrophils, macrophages and B cells (Letterio and Roberts, 1998) and promoting the function of Tregs (Wan and Flavell, 2008). Moreover, Tregs mediate their suppressive activity by producing TGF-β themselves. We expected that any effect induced by PDT and/or CY on the function and level of Tregs could be reflected also in the level of TGF-β in the serum. However, it is necessary to consider that also other cell types are capable of secreting TGF-β, such as cancer cells (Elliott and Blobe, 2005), macrophages

(Wahl *et al*, 1990) and B lymphocytes (Weitzmann *et al*, 2000); therefore, the kinetics of TGF-β may not exactly reflect the kinetics of Treg numbers.

We have confirmed that indeed the CT26 cancer cells do express TGF-β (Figure 4A), a characteristic that may strongly contribute to the development of immunosuppressive microenvironment of this tumour. The fact that CT26 tumours are a significant source of TGF-β in tumour-bearing mice may explain why the TGF-β and Treg levels continued to increase unabated with tumour progression and returned to 'naïve' levels when the tumour was removed by surgery or PDT. Photodynamic therapy and PDT + CY had similar impacts on TGF-β; however, the two effects were not identical. Photodynamic therapy + CY caused a more pronounced reduction in TGF-β compared with PDT alone, decreasing the level to a range comparable to naïve mice. When PDT was applied alone, we observed a reduction in TFG-β, however, it remained significantly higher than in naïve mice.

In CT26 tumour model, the combined PDT + CY treatment led to long-term tumour regression, however, none of the mice treated by combination therapy rejected the subsequent tumour rechallenge. It was only when we administered a second dose of CY before rechallenge that 65% of mice in long-term remission after PDT + CY treatment successfully rejected the rechallenge. We attribute this effect to the fact that CT26 cells express a tumour rejection antigen gp70 (Figure 1B) that appears to behave like a shared auto-antigen. It appears that Treg can suppress the development of anti-gp70 immunity and/or reactivation of anti-gp 70 memory immunity; however, this effect can be uncovered when Treg is depleted before PDT and again before rechallenge. This observation may have far-reaching clinical implication as it suggests that in patients treated with PDT there may be developing anti-tumour immunity that could be uncovered at the time of relapse by appropriately designed anti-Treg therapy. If PDT was ever combined with low-dose CY in patients, then it may be possible to use a metronomic CY regimen (Ge *et al*, 2012) rather than a bolus administration to extend the length of time that Tregs were controlled until the possibility of tumour recurrence was no longer a real threat. In conclusion, the presented results further enrich our understanding of the effects of the role of Treg in PDT-mediated anti-tumour immune response. Further studies, however, are certainly necessary to fully understand the complicated interaction between PDT, Treg, immunosuppressive microenvironment of the tumour and the role of different types of tumour antigens to better design and exploit PDT-based immunotherapy to improve patient outcome.

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Addendum 3

Photochemical & Photobiological Sciences

PAPER

View Article Online
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Photodynamic therapy downregulates the function of regulatory T cells in patients with esophageal squamous cell carcinoma†

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Photodynamic therapy (PDT) by selective photosensitization of cancer cells and subsequent laser application results in local tumor necrosis. However, the effects of PDT on immune function, which may depend on the type of immune response, are controversial. We investigated the immunological changes induced by PDT and the effect of PDT on level and function of regulatory T cells (Treg) in patients with invasive esophageal squamous cell carcinoma (ESCC). We analyzed patient's blood samples before and after PDT. Blood CD4+CD25+CD127−FoxP3+ Treg levels were quantified by FACS, and Treg function was evaluated by coculture proliferation assays with T effector (Teff) cells. We found that PDT abrogated the suppressive capacity of peripheral Treg (Days 7 and 14, $p = 0.016$) but had no effect on Treg levels. The effect of PDT on Treg function at Day 7 was accompanied by slight but statistically significant increases in peripheral neutrophil granulocytes ($p = 0.035$) and monocytes ($p = 0.013$) and a statistically significant increase (approximately 18-fold) in serum IL-6 levels ($p = 0.008$). In conclusion, PDT abolished Treg function, possibly due to increased IL-6 levels in treated ESCC patients. This may be crucial for an improved therapeutic outcome.

Introduction

Esophageal cancer, one of the least studied cancers, is the sixth-leading cause of death from cancer worldwide.[1] Geographically, its incidence varies from approximately 10 cases per 100 000 persons in Europe and the United States to 139 cases per 100 000 persons in some areas of China.[2–4] The most common tumor subtype is esophageal squamous cell carcinoma (ESCC), which is associated mainly with tobacco smoking[5,6] and heavy alcohol use[6] but also with age >60 years, male gender, and heredity.[1,7,8] The standard of care for esophageal cancer combines surgery, chemotherapy, and radiotherapy. However, it is associated with unsatisfactory results and poor 5-year survival. One of the most successful and promising alternatives is photodynamic therapy (PDT).[9]

In PDT, a photosensitizer is first administered intravenously and then visible light is delivered to the cancerous area.[10,11] Light activation of the photosensitizer triggers a photochemical reaction resulting in the production of highly reactive oxygen species and immediate cell damage. Photofrin-mediated PDT for esophageal carcinoma was first approved by the US Food and Drug Administration in 1995 for the palliation of symptoms and the reduction of partial or complete esophageal obstruction by the cancer.[12] Since then, PDT has also been approved for the treatment of other tumors including head, neck, skin, lung, bladder, and cervical cancer.[12–15]

After decades of clinical use, PDT is now considered a feasible and efficient minimally invasive endoscopic option for treatment of early unresectable ESCC, palliation of locally advanced disease, and salvage when other treatment options have failed.[2,16,17] We and others have successfully employed PDT alone or as part of a multimodal approach to the palliative treatment of advanced esophageal cancers.[18–20] In our experience, PDT produces a statistically significant advantage in relieving stenosis, improving dysphagia, and improving mean survival.[20] When applied as initial endoluminal treatment in the palliative setting, PDT has considerable beneficial effects resulting in significantly improved and prolonged survival.[21]

The improvement in survival observed after PDT might be due to PDT's induction of immune responses. One of the most interesting aspects of PDT is its ability to stimulate both the innate and the adaptive arms of the host immune system.[10,22,23] Such immune responses are triggered by the

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release of various proinflammatory and acute-phase response mediators from the treated site.[24–26] In response to the traumatic insult to the tumor and its microenvironment, large numbers of neutrophils and other proinflammatory cells infiltrate the treated site and attack the tumor cells.[27,28] PDT also appears to activate dendritic cells (DCs) that, upon maturation, phagocytose remnants of destroyed tumor, home in on lymph nodes, and efficiently present tumor antigens to naïve lymphocytes.[29] In animal models, PDT has been shown to elicit a systemic, tumor antigen-specific immune response that can lead to regression of distant untreated tumors and inhibit cancer recurrence.[30]

Clinical evidence of PDT's immunological effects is limited,[31,32] and the preclinical evidence is controversial. It has been suggested that, under some circumstances, PDT may have an immunosuppressive effect that limits its potential as a practical cancer therapy.[33] In one recent mouse study on CT26-wild type colon adenocarcinoma, we observed an important and negative role for regulatory T cells (Treg) in PDT-induced antitumor immunity.[34] In this study we found that selectively depleting Treg with low-dose cyclophosphamide before PDT resulted in dramatically better long-term survival than did either PDT or cyclophosphamide treatment alone.[35] These results corroborated similar previous findings in the J774 tumor model.[36]

In this study, we investigated the effect of PDT on the immune system, and particularly on the immunosuppressive CD4+CD25+CD127−FoxP3+ regulatory T cells (Treg) so critical to the maintenance of immune homeostasis, in patients with ESCC.

Material and methods

Patients

Between January 2011 and December 2013, 8 patients with histologically confirmed ESSC were enrolled in the study. The main inclusion criteria were (1) fair general condition with no significantly enhanced risk for surgery, (2) absence of lymph node or distant metastases, and (3) primary tumor localization in the middle or distal third of the esophagus. The study was approved by the local ethics committee and performed according to the Helsinki Declaration. All patients provided written consent. Patient and lesion characteristics as well as treatment and clinical follow-up are described in ESI table.†

PDT

Photodynamic therapy consisted of photosensitization and laser light application. First, a photosensitizer (Photofrin; Houdon, France) was administered intravenously in the arm at a dose of 1 mg kg^{-1} body weight. Then, 48 hours later, laser light (630 nm) was applied by a KTP-Np:YAG laser with DYE-box (Laserscope; Surgical Systems, Gwent, UK) at a fluence of 80 J cm^{-2} under general anesthesia and routine cardiorespiratory monitoring. The light diffuser was maneuvered to the target lesion site in an elastic balloon catheter (Photodynamic Therapy®, Vienna, Austria) that was inflated to shape the esophageal hollow and allow homogenous light distribution across the target lesion. Up to four placements of the balloon catheter were necessary to illuminate the entire tumor surface. Light wavelength and dose at the tip of the light diffuser were controlled immediately before and after PDT.

Flow cytometry analysis of Treg

Aliquots (400 μL) of fresh blood from ESSC patients were stained for Treg phenotype (CD4+CD25+CD127−FoxP3+) with CD25 FITC (BD Biosciences, Cat. no. 345796), CD127 PE (BD Biosciences, Cat. no. 557938), and CD4 PerCP (BD Biosciences, Cat. no. 345770) using the FoxP3 Staining Buffer Set (eBioscience, Cat. no. 00-5523) according to the manufacturer's instructions. After washing, fixation, and permeabilization, the cells were incubated with primary conjugated FoxP3 APC antibody (eBioscience, Cat. no. 17-4776). All data were acquired with a FACS Calibur flow cytometer and analyzed with either CellQuest Pro (BD Biosciences) or FlowJo (TreeStar; Ashland, OR, US) software. IgG2a APC (eBioscience, Cat. no. 17-4321) isotype controls were used to set proper gates. Absolute Treg cell counts were based on the percentage of gated lymphocytes that were CD4+CD25+ CD127−Foxp3+ and the absolute number of lymphocytes per liter (G L^{-1}).

Immunohistochemical staining

Paraffin-embedded esophageal tumor biopsy specimens were sectioned and stained with monoclonal anti-human FoxP3 (clone 236A/E7, Serotec) (1 : 100). In brief, the sections were pretreated with citric acid at pH 6, incubated with primary antibody, and then incubated with goat anti-rabbit/mouse antibody (Dako REAL Detection System, HRP/AEC; DakoCytomation, Carpinteria, CA), according to the manufacturer's instructions. All specimens were evaluated by two blinded investigators who manually counted positively stained cells in three separate high power fields (HPF, 400×). The density of stained cells was determined by calculating the mean number of positively stained cells per HPF. Images were acquired with a Nikon Eclipse 80i microscope.

Treg immunosuppressive assay

Blood samples from ESSC patients were collected in 8 mL Falcon tubes containing EDTA as anticoagulant at three time points: immediately before intravenous injection of Photofrin and 7 and 14 days after PDT. For all experiments, fresh blood samples were used. Lymphocytes were isolated by placing whole blood on Ficoll Lymphoprep (Axis Schield, Cat. no. 1114545), subjecting the preparation to gradient centrifugation at 2500 rpm for 30 minutes, and then extracting and storing serum at −80 °C. Lymphocytes were then resuspended in PBS; counted by a CASY Cell counter (Schärfe System); and incubated with CD4 FITC, CD25 PE-Cy7, and CD127 PE antibodies (BD Pharmigen) for 30 minutes. Antibody-stained lymphocytes were sorted by FACS analysis (BD FACS ARIA) into Treg (CD4+CD25+CD127−) and Teff (CD4+CD25−CD127+) subsets. To analyse the proliferation and suppressive capacity of Treg in

response to polyclonal activation, Treg or Teff (10^5) were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% fetal calf serum, penicillin (100 IU mL^{-1}), streptomycin (200 µg mL^{-1}), and L-glutamine (2 mM) (all from PAA Laboratories, Pasching, Austria). Each cell subset was cultured alone or together at ratios of 1 : 1, 1 : 2, and 1 : 4 in a round-bottom 96-well plate at a final volume of 200 µL; stimulated and incubated with plate-bound anti-CD3 (5 µg mL^{-1}) and soluble CD28 (2.5 µg mL^{-1}) for 72 hours; and then incubated with 1 µCi [^{3}H]thymidine (Amersham Biosciences, Piscataway, NJ) per well for an additional 16 hours. Radioactivity was measured by a liquid scintillation counter (1450 Microbeta Trilux; PerkinElmer).

RT-PCR

Tumor biopsy specimens were collected during endoscopy and stored in RNAlater stabilization reagent (Qiagen) at 4 °C for up to 1 week. Total RNA was extracted from these specimens using the RNeasy Fibrous Tissue Minikit (Qiagen, Gaithersburg, MD, USA) and reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche). Sample DNA (1 µg) was incubated with primers for specific human FoxP3 (forward: GCTCTGCACCTTCCCAAAT; reverse: TCTCTGGAGGAGACATTGTGC) and GAPDH (RT^2 qPCR Primer Assay for Human GAPDH, Qiagen, Cat. no. PPH00150E-200) and amplified with RT^{2}SYBR Green qPCR Mastermix (Qiagen, Cat. no. 330500) using a LightCycler 480 (Roche).

ELISA for IL-6, IL-8, IL-10, and TGF-β

Blood samples from ESSC patients were collected in 8 mL Falcon tubes containing EDTA as anticoagulant at three time points: before intravenous injection of Photofrin and 7 and 14 days after PDT. Whole blood was centrifuged at 2500 rpm for 30 minutes. Serum was then extracted and stored at −80 °C until subjected to enzyme-linked immunosorbent assay (ELISA) for IL-6 (eBioscience, Cat. no. 88-7066-22), IL-8 (eBioscience, Cat. no. 88-8082), IL-10 (eBioscience, Cat. no. 88-7106), and TGF-β (eBioscience, Cat. no. 88-8350), according to the manufacturer's instructions. In brief, the ELISA plates were coated with capture antibodies and incubated overnight at 4 °C, incubated with blocking buffer for an additional 1 hour at room temperature, incubated with standards and samples as applicable (100 µL per well) overnight, and then incubated with antibodies (Avidin-HRP and Substrate Solution). Between each incubation step, the plates were washed three times. Finally, optical densities at 450 nm were detected. In the case of TGF-β, serum samples were first activated by 1 N HCl (20 µL of 1 N HCl per 100 µL of sample) for 10 minutes at room temperature and then neutralized with 1 N NaOH (20 µL of 1 N NaOH per 100 µL of sample). The final concentration of TGF-β was calculated by taking into consideration the sample dilution factor of 1.4.

Statistical analysis

Statistical analysis was done by GraphPad Prism Version 6.0b (GraphPad Software, Inc., San Diego, CA, USA) using Friedman test with Dunn post hoc correction, Wilcoxon test or one-sample *t*-test, as appropriate, to compare PDT pretreatment to posttreatment values. All data are presented as the mean ± standard error of mean. The level of significance was set at $p < 0.05$. Bonferroni correction was done to adjust the *p*-value level of significance for multiple end point testing, whenever appropriate.

Results

PDT increases peripheral Treg levels

The relative and absolute levels of peripheral Treg (Fig. 1A and 1B, respectively) in ESCC patients before and after PDT were highly heterogeneous, but showed an overall trend toward

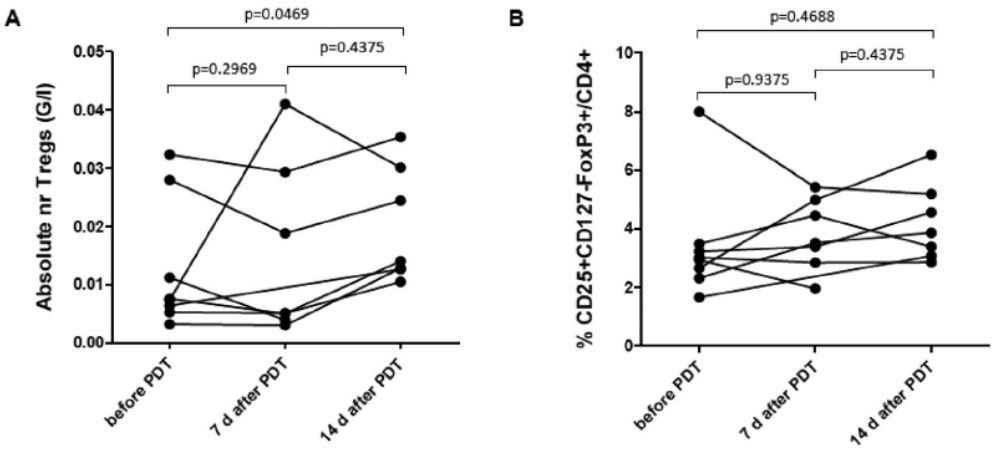

Fig. 1 Quantification of Treg in peripheral blood. Relative (A) and absolute (B) numbers of Treg were determined in blood samples collected from ESCC patients (n = 8) before and at 7 and 14 days after PDT. Comparisons of relative and absolute numbers of Treg were done by Wilcoxon test. The level of statistical significance was set at $p < 0.017$ after Bonferroni correction.

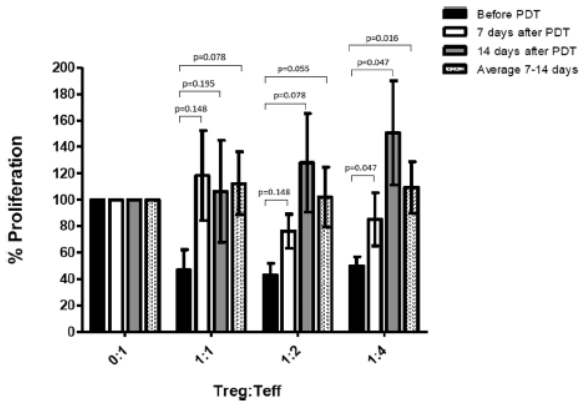

Fig. 2 Evaluation of immunosuppressive function of peripheral Treg. Treg and Teff were isolated from peripheral blood collected from ESCC patients (n = 8) and cocoltured at ratios of 0 : 1 (control), 1 : 1, 1 : 2, and 1 : 4. Black, white, and grey bars indicate values of proliferation observed before and at Days 7 and 14 after PDT, respectively. The pattern filled bars represent mean values at Day 7 and 14 after PDT. Teff proliferation was considered to be 100%. Proliferation values were compared by Wilcoxon test. The level of statistical significance was set at $p < 0.017$ after Bonferroni correction.

increased levels at 7 and 14 days after PDT. Mean relative Treg levels were 3.407 ± 0.640% before, 3.791 ± 0.428% 7 days after, and 4.204 ± 0.460% 14 days after PDT. Mean absolute Treg counts were 0.013 ± 0.004 (G L^{-1}) before, 0.015 ± 0.005 (G L^{-1}) 7 days after, and 0.020 ± 0.003 (G L^{-1}) 14 days after PDT.

PDT inhibits immunosuppressive function of peripheral Treg

The immunosuppressive function of Treg was analyzed by coculturing Treg and Teff cells in different dilution ratios (1 : 1, 1 : 2, 1 : 4) and culturing Teff cells alone (0 : 1) as a control (Fig. 2). Treg immunosuppressive activity was pronounced before PDT, even at the highest coculture ratio (1 : 4), and totally abrogated after PDT (Days 7 and 14) regardless of the coculture ratio.

PDT does not affect tumor-infiltrating Treg levels

To evaluate the local effect of PDT on Treg directly at the site of treatment, tumor-infiltrating Treg were quantified by immunohistochemical staining of biopsy specimens with FoxP3 as a surrogate marker. Interestingly, FoxP3+ Treg are known to accumulate around ESCCs, and FoxP3 overexpression in ESCC is known to be associated with poor prognosis.[37] Variable amounts of tumor infiltrating Treg were observed before PDT, the cell density being lower in some patients (Fig. 3A, upper panels) and higher in others (Fig. 3A, lower panels). There was an apparent trend toward immediate but transient reduction in tumor-infiltrating FoxP3+ Treg numbers (Fig. 3B), as shown by mean FoxP3+ Treg counts of 77.2 ± 9.7 cells per HPF before PDT and 50.7 ± 11 cells per HPF immediately after PDT. Thereafter, Treg counts completely recovered to baseline levels by Days 7 and 14.

Real-time RT-PCR analysis of *FoxP3* mRNA in ESCC biopsy specimens revealed heterogeneity in expression levels before and after PDT within individual patients (Fig. 3C), However, mean *FoxP3* mRNA levels remained nearly constant in the samples at all time points analyzed (Fig. 3D).

PDT affects systemic leukocyte levels

PDT slightly increased the absolute number of circulating leukocytes (ESI Fig. A†). This increase was mostly due to increases in granulocyte and monocyte numbers (ESI Fig. B–D†). The most significant increase was in monocytes 7 days after PDT (p = 0.0131). Overall, however, no significant change was seen in either absolute peripheral lymphocyte counts or systemic CD4+ T cell counts after PDT (ESI Fig. E and F†).

PDT differentially affects systemic IL-6, IL-8, IL-10 and TGF-β expression

Previous studies showed that PDT can significantly affect the expression of certain cytokines *in vitro* and *in vivo*,[38–41] suggesting that such changes may be mechanistically involved in the observed effects of PDT on host immune responses. Therefore, mean serum concentrations of IL-6, IL-8, IL-10, and TGF-β were measured before and after PDT. On average, IL-6 expression was statistically significantly upregulated by approximately 18-fold at 7 days (p = 0.0078) and 11-fold at 14 days after PDT (p = 0.109) (Fig. 4). This corresponded to increases in mean IL-6 concentrations from 0.37 ± 0.22 pg mL^{-1} before PDT to 6.73 ± 4.46 pg mL^{-1} at 7 days and 4.11 ± 2.41 pg mL^{-1} at 14 days after PDT. In contrast, concentrations of IL-8, IL-10, and TGF-β showed large interindividual, statistically nonsignificant differences before and after PDT. For IL-8, mean concentrations ranged from 8.54 ± 3.72 pg mL^{-1} before PDT to 7.33 ± 5.32 pg mL^{-1} at 7 days and 5.68 ± 1.53 pg ml^{-1} at 14 days after PDT; for IL-10, from 27.34 ± 8.01 pg mL^{-1} to 34.18 ± 10.90 and 27.43 ± 7.36 pg mL^{-1}, respectively; and for TGF-β, from 1.52 ± 0.93 pg mL^{-1} to 3.93 ± 1.93 and 1.10 ± 0.51 pg mL^{-1}, respectively.

Discussion

In this study, we observed that PDT downregulated the immunosuppressive function of peripheral Treg (Fig. 2). It has long been known that Treg hinder the generation of protective antitumor immunity, thereby inhibiting Teff and promoting cancer progression. Therefore, PDT's ability to abrogate immunosuppressive Treg function may be of crucial importance for the immune system in triggering efficacious antitumor immune responses, at least in patients with ESCC. While Treg play an important role in maintaining tolerance to self-antigens and preventing autoimmune diseases in healthy individuals,[42,43] that role may be disrupted in cancer patients. This disruption of the fine balance between Treg and Teff may in turn lead to unrestrained expansion of Treg, massive infiltration into tumor, and inhibition of antitumor Teff[44] and ultimately to cancer progression.[45] However, several alternative

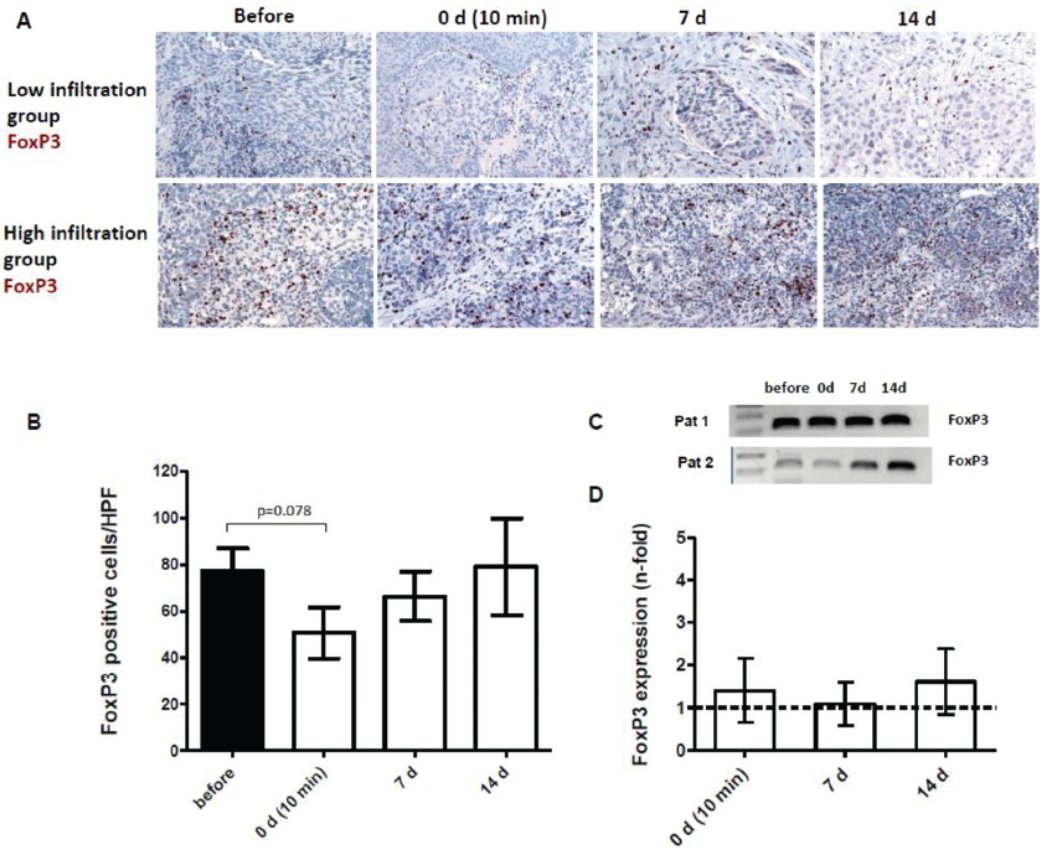

Fig. 3 FoxP3 expression in tumor tissue of ESCC. (A) FoxP3 protein expression was detected by immunohistochemical staining. Examples of highly variable protein expression and low- and high-density of FoxP3+ Treg staining from different patients at various time points are shown in the upper and lower panels, respectively. Original magnification: ×200. (B) FoxP3+ cell counts per high power field (HPF) were determined and compared by Wilcoxon test. (C) RT-PCR products were subjected to gel electrophoresis and in two patients showed heterogeneity in *FoxP3* expression levels. (D) RNA was extracted from ESCC biopsies, and *FoxP3* gene expression was quantified by RT-PCR; values were normalized to *FoxP3* expression before PDT (considered as 1-fold) and compared by one-sample *t*-test.

lines of evidence suggest that depleting Treg *in vivo* may in fact facilitate tumor eradication and enhance antitumor immunity.[46–48]

In contrast to the observed downregulation of peripheral Treg function, their levels did not change significantly after PDT in our study (Fig. 1). This is consistent with results obtained in a CT26-wild type colon adenocarcinoma mouse model *in vivo*,[34] in which we observed comparable Treg levels before and 7 and 14 days after PDT despite transient increases in Treg in spleen and lymph nodes in the first few days after treatment.

PDT induces a traumatic insult to the tumor microenvironment, causing immediate cell death at the site of the treatment. These PDT-mediated cytotoxic events are most likely the cause of the transient reduction in the number of Treg infiltrating into the tumor area immediately after PDT (Fig. 3B). The number of tumor-infiltrating Treg was, however, fully restored to baseline levels at the later time points. Also, PDT appeared to cause more pronounced damage to FoxP3 protein (Fig. 3B) than to *FoxP3* mRNA expression, which showed no alteration immediately after PDT (Fig. 3D).

While we observed no change in absolute lymphocyte numbers after PDT, we did observe statistically significant increases in neutrophil (granulocyte and monocyte) numbers (ESI Fig.†). This is not unexpected since neutrophils are known to be first responders to microbial infections as well as cancer and granulocytes and monocytes are both known to play a key role in PDT-induced anti-tumor immunity *in vivo*.[49–51] Moreover, monocytes and macrophages are known to contribute substantially to PDT's antitumor effects. In particular, the tumoricidal activity of these non-specific immune effector cells has been found to be potentiated by PDT *in vivo* and *in vitro*.[52]

Because Treg suppressor activity is strictly dependent on the synergistic action of cytokines such as IL-6, IL-8, IL-10, and TGF-β,[53] we analysed their concentrations in patients' serum samples and found IL-6 to be highly upregulated after PDT (Fig. 4). The literature suggests that Toll-like receptors (TLRs)

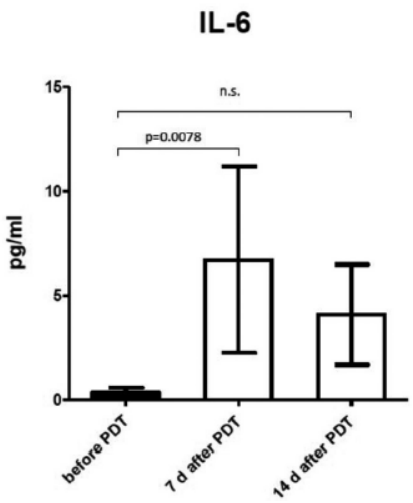

Fig. 4 Measurement of IL-6. IL-6 was detected by ELISA in serum samples collected from ESCC patients (n = 8) before and at 7 and 14 days after PDT. Concentration values were compared by Wilcoxon test.

present on the membrane of DCs are capable of modulating Treg function.[54] These receptors are ordinarily committed to detecting infection by recognizing conserved pathogen-associated molecular patterns (PAMPs).[55] In particular, *Liu et al.*[56] demonstrated that when TLRs are activated *in vivo* by a synthetic bacterial lipoprotein (BLP, a TLR2 agonist), the Treg population undergoes a transient loss of suppressive activity. Similarly, Pasare *et al.*[57] reported that microbial induction of the Toll pathway blocked the suppressive effect of Treg, allowing activation of pathogen-specific adaptive immune responses, and that this block of suppressor activity depended in part on IL-6.

Activation of TLRs by released "danger" signals, or cell death-associated molecular patterns (DAMPs), is a thoroughly described key component of PDT-elicited host response.[25,58–60] DAMPs play a role similar to that of PAMPs, but instead of being associated with pathogenic microbes, they are associated with host tissue damage. In view of these reports, we hypothesized that the activation of TLRs by PDT-generated DAMPs may cause a transient loss of Treg immunosuppressive function. This hypothesis is strengthened by the previous finding in an *in vivo* mouse model that expression of IL-6 (but not IL-10), which is involved in the inhibition of Treg suppressive function, was enhanced in tumor and normal tissue after PDT.[41] Our hypothesis that IL-6 is involved in PDT-induced loss of Treg function is supported by results we recently obtained (unpublished data) from patients with actinic keratosis who received PDT with topical aminolevulinic acid or methyl aminolevulinate.[61–63] In contrast to ESCC patients in the present study, those patients showed no change in serum IL-6 concentrations or peripheral Treg function after PDT.

However, it must be remembered that PDT-elicited host response ultimately depends on the balance between activated inflammatory/effector cells and the concomitant negative regulatory mechanisms that come into play after their transient loss of function is recovered. This balance is important for two reasons. First, it limits the potential for autoimmunity due to overactivated Teff. Second, it controls the intensity and duration of PDT-induced immunological responses.

In conclusion, the present results enrich the understanding of the immunological events linked to clinical PDT especially in ESCC. However, further investigations (*e.g.*, in different treatment settings) are warranted to elucidate the complicated immune profile elicited by PDT and translate it into improved therapy for patients with ESCC.

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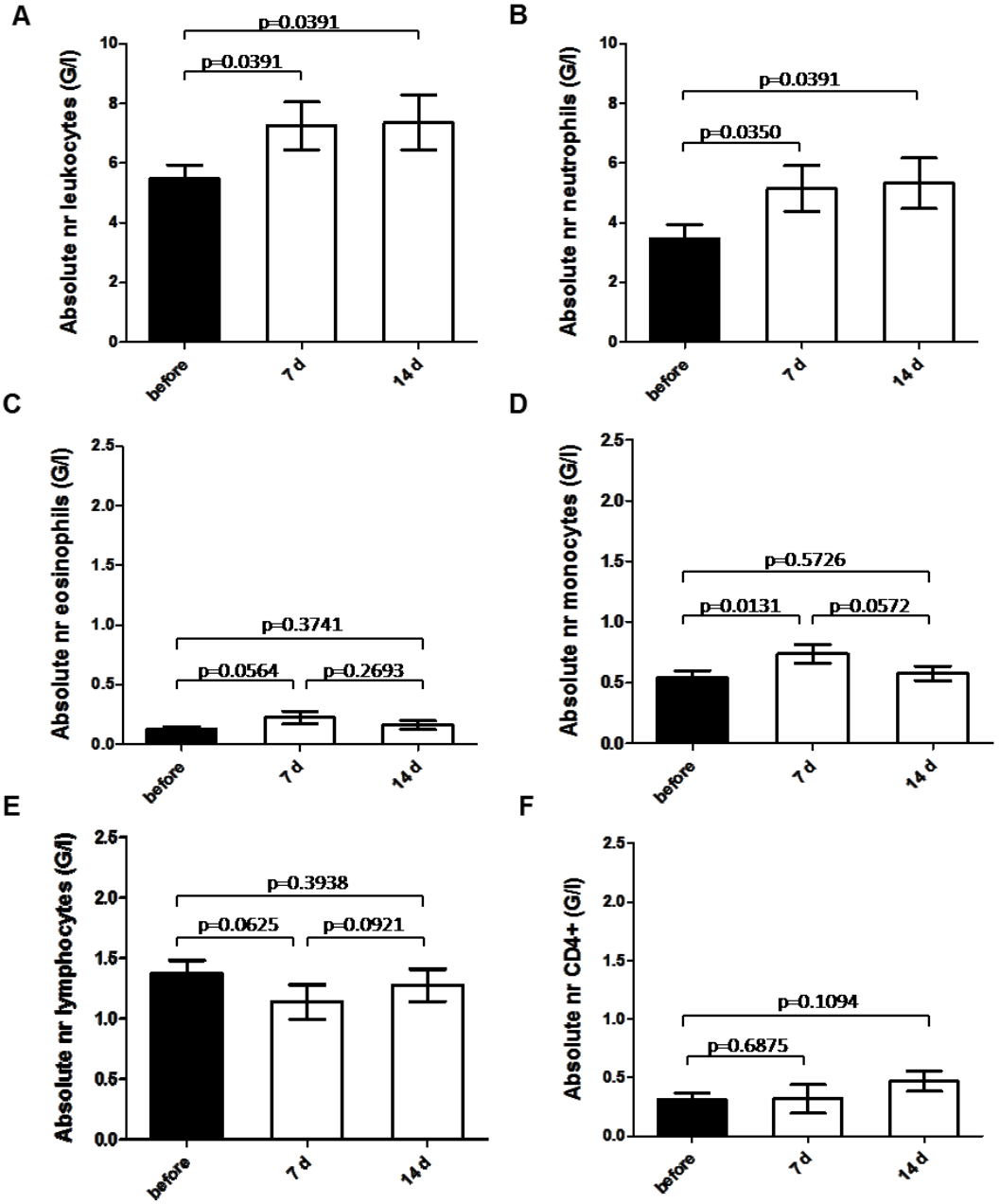
Supplementary Figure: Quantification of leukoytes in peripheral blood. (A) Overall leukocytes, (B) neutrophils, (C) eosinophils, (D) monocytes, (E) lymphocytes, and (F) CD4+ cells. Absolute numbers were determined in blood samples collected from ESCC patients (n=8) before and at 7 and 14 days after PDT. Comparisons of numbers were done by Friedman test with Dunn post hoc correction and exact p-values were determined by Wilcoxon test.



Supplementary Table.

Patient characteristics and follow-up results										
Patient nr	Age (years)	Sex	Tumor stage (a)	Location of tumor	Tumor stenosis (%)	Tumor length (cm)	Presence of tumor necrosis after PDT (as determined by clinical and/or histologic examination)	Esophagectomy post PDT	Postoperation tumor histology (b)	Follow-up
N1	79	M	n.a.	Middle third	75	5	Yes	No	Lost in follow-up	Patient refused further treatment. Died 23 months after PDT
N2	63	F	n.a.	Middle third	75	6	Yes (c)	No	n.a.	Patient was unfit for surgery due to cachexia. Received endoluminal brachytherapy and external mediastinal radiation. Endoscopic esophageal stent implantation was done; stent penetration into the trachea caused patient's death 12 months after PDT
N3	70	M	IIA	Middle third	75	10	Yes	Yes	SCC G2 pT3 N0 R0 M0	Patient had tumor recurrence in the hypopharynx. Died 7 months after PDT duet to hypopharynx tumor progression
N4	73	M	IIA	Middle third	50	6	Yes	Yes	SCC G3 pT3 N0 M0 R0 V1	Alive, 23 months after PDT
N5	62	F	I	Middle third	25	2	Yes	Yes	SCC G3 pT1b N0 R0 M0	Alive, 15 months after PDT. Strong suspicion of mediastinal lymph node metastases and pulmonary metastases
N6	62	M	IIIC	Middle third	75	6	Yes	Yes	SCC G3 pT4 N2 L0 V1 R2	Alive, 15 months after PDT. Patient exhibited liver metastasis; received palliative chemotherapy and external mediastinal radiation
N7	63	M	I	Middle third	25	5	Yes (c)	Yes	SCC pG2 T2 N0 M0 R0	Alive, 14 months after PDT
N8	60	M	IIIB	Distal third	50	3	Yes	Yes	SCC G2 pT3 N1 N0 R0 M0	Alive, 13 months after PDT

(a) Tumor stage according to TNM (tumor, node, metastasis) staging system [1]
(b) Classifications according to [2]
(c) Maximal necrosis compared to other cases
n.a.: not available

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Addendum 4

Methyl aminolevulinate photodynamic therapy of actinic keratoses does not affect level and function of patients' peripheral regulatory T cells

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Key words: PDT - immune response - regulatory T cells - actinic keratosis

Background

Topical photodynamic therapy (PDT) mediated with 5-aminolevulinic acid (ALA) or its methyl ester (methyl aminolevulinate; MAL) is widely used to treat actinic keratosis ((Dirschka et al., 2012), s1-s4). The main advantages of topical PDT are that it is non-invasive, highly effective and gives excellent cosmetic outcomes compared to surgery. However, besides inducing direct phototoxic effects to the illuminated neoplastic cells, PDT can also affect the activation of both innate and adaptive arms of host's immune system by triggering the release of various pro-inflammatory and acute-phase response mediators from the treated site (review in s5). Such acute inflammatory response provoked by PDT causes infiltration of host innate immune cells, which carry out the removal of damaged cells. Furthermore, the PDT-induced acute inflammation may also lead to the development of adaptive anti-tumor immunity. For instance, a recent experiment in mice has indicated that ALA PDT may induce specific anti-tumor responses in cutaneous squamous cell carcinomas (Wang et al., 2013). However, there is concern that under certain conditions, ALA PDT could exhibit immunosuppressive effects (similar to UV radiation) ((Schwarz et al., 2011), s6-s8), thus limiting its potential as a therapy for cancer (Wolf et al., 1997). For example, PDT has been shown to suppress the induction of contact or delayed type hypersensitivity in mice and humans (reviewed and cited in s5). In particular, studies by Damian's group ((Frost et al., 2011, Matthews and Damian, 2010), s9) has suggested that topical ALA or MAL PDT exhibit immunosuppressive properties in humans that can be influenced by reducing the rate of light delivery or the administration of nicotinamide. Our recent work in a mouse tumor model has shown that administration of cyclophosphamide before PDT led to depletion of regulatory T cells (Treg) and potentiated PDT-mediated immunity, leading to long-term survival and development of memory immunity (Reginato et al., 2013).

Questions addressed

Preclinical and clinical studies have demonstrated that PDT is capable of affecting the innate and adaptive arms of the immune system (s10-s12). In this study we asked whether PDT can affect the systemic level und function of Treg (Singh et al., 2012) in patients with multiple AK photosensitized with MAL and exposed to visible red light.

Experimental design

Between June and November 2011 five patients (four men and one woman; age range, 58 to 74 years) suffering from AK were enrolled in the study. The patients were treated with standard methyl aminolevulinate (methyl 5-amino-4-oxopentanoat; Metvix™) 160mg/g cream for multiple AK on scalp, face and/or hands. The cream was applied to the AK lesions and 1 cm of surrounding skin, and three hours later, the lesions were illuminated with the LED source Aktilite® CL 128 (Photocure, Oslo, Norway), which has a narrow emission spectrum around 630 nm. All patients received the recommended light dose of 37J cm^{-2}. The patients were from a study approved by the local ethics committee and performed according to the Helsinki Declaration.

Blood samples from AK patients were collected in 8-ml falcon tubes supplemented with EDTA as anticoagulant at two time points, i.e. immediately before topical application of MAL and at 7 or 14 days after PDT. For flow cytometry analysis of Treg, aliquots of 400 µl of fresh blood were stained with CD25 FITC (BD, Cat. No. 345796), CD127 PE (BD, Cat. No. 557938), CD4 PerCP (BD, Cat. No. 345770) and FoxP3 APC antibody (eBioscience, Cat. No. 17-4776) using the FoxP3 Staining Buffer Set (eBioscience, Cat. No. 00-5523), according to the manufacturer's instructions. All data were acquired with a FACS Calibur flow cytometer and analysed with Flow Jo (TreeStar, Ashland, OR, US) software. All plots were gated on CD4+.

For Treg immunosuppressive assay, lymphocytes were isolated from the blood using Ficoll Lymphoprep (Axis-schield, Cat. No. 1114545), stained with CD4 FITC, CD25 PE-Cy7 and CD127 antibodies (BD Pharmigen) and sorted by FACS (BD FACS ARIA) in Treg (CD4+CD25+CD127-) and Teff (CD4+CD25-CD127+). Each cell subset was cultured either alone or together at 1:1, 1:2, 1:4 ratios and immunosuppressive assay was performed as described by Singh *et al.* (s13).

Results

We evaluated the relative number of Treg in the peripheral blood stream of AK patients before and one or two weeks after a single session of MAL PDT. As depicted in Figure 1A and B, we observed inter-individual differences in the course Treg frequency but overall, there was no statistically significant difference between pre-PDT and post-PDT levels (Figure 1B). We also analysed the immunosuppressive function of Treg by co-culturing

the cells together with Teff at different ratios. The results indicated that although MAL PDT did cause ambivalent effects in treated patients (i.e. up- or down-regulation of Treg function) they were not statistically different (data not shown).

Conclusions

The data presented in this study indicate that MAL PDT does not significantly affect the level and function of Treg in patients with AK. At first glance, this seems to be contradictory to other work from our laboratory in patients suffering from esophageal squamous cell carcinoma (ESCC) where treatment with Laser-guided Photofrin-PDT downregulated the immunosuppressive function of peripheral Treg (Reginato et al., 2014). However, the observed differences could have multiple explanations: (i) while in ESCC patients Photofrin, used as a photosensitizer, was administered systemically (i.v.), in AK patients, the photosensitzer MAL was applied topically; (ii) the PDT set-up for the two groups was different in ways that in ESCC patients the tumor was illuminated 48h after PS administration, whereas in AK patients, the lesions were illuminated 3h after photosensitization; (iii) PDT light dose and fluence rate administered for the treatment of ESCC and AK were different (80 J cm^{-2} vs. 37 J cm^{-2}; laser of 1.2-1.5 W cm^{-2} vs. LED light of 68 mW cm^{-2}, respectively); (iv) the anatomical location of the lesions was different (s14); and (v) above all, the type of tumor and stage in the two patient groups differed.

Nonetheless, our results are quite consistent with what we had observed in ESCC patients in whom the frequency of systemic Treg was not significantly affected by PDT in the AK patients as well (Figure 1). Moreover, when we looked for IL-6 levels in the serum of the AK patients before and after MAL PDT, we found no significant differences (data not shown). This is in contrast to our findings in the ESCC patients in whom systemic PDT did significantly upregulated IL-6 serum levels and downregulated the function of peripheral Treg (Reginato et al., 2014). Together, this indicates a crucial role of IL-6 in affecting the function of Treg upon PDT.

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Author contribution

PW and AG-W designed the experiments; ER performed most of the experiments; ER and PW, analyzed and interpreted the data and wrote the paper; all authors revised and approved the final manuscript version.

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Conflict of interests

The authors state no conflict of interest.

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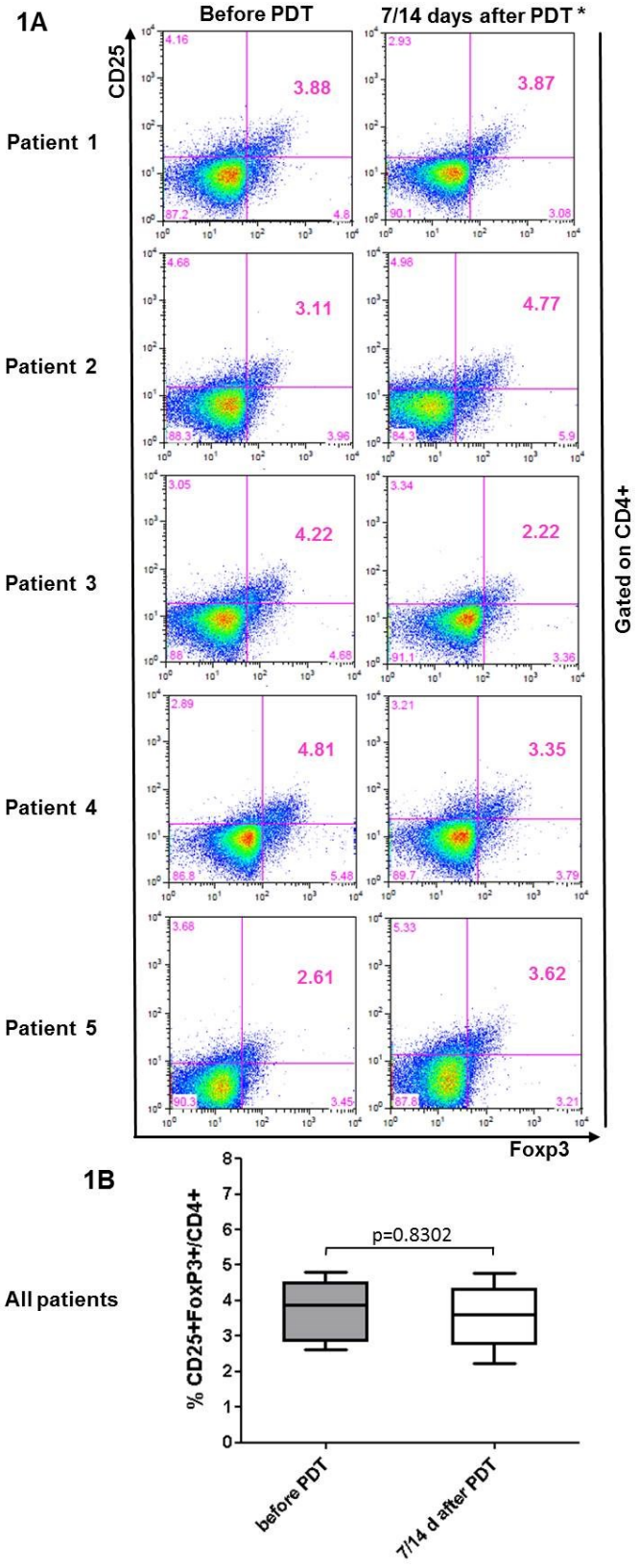
Figure 1: Quantification of regulatory T cell in the peripheral blood. (1A) CD4+CD25+FoxP4+ Treg measured in the blood of AK patients (n=5) before and at 7 (patients 2 to 5*) or 14 days after PDT (patient 1*). Comparison of Treg percentages was done by two-tailed Student paired T-test (1B).



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Declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organisations that have contributed to the research for this thesis. The acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the guidelines of "Good Scientific Practice".

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WORK EXPERIENCE

Graduate student (10/2010–08/2014)
Medical University of Graz, Dept of Dermatology, Graz, Austria (Prof. Wolf)
Study of the immunological changes induced by photodynamic therapy in esophageal carcinoma patients and in tumor mouse models.

- Developed flow cytometry-based and cell-based assays to assess the phenotypic and functional characteristics of T lymphocytes from patients and from mice. Focused on the immunosuppressive CD4+CD25+FoxP3+ regulatory T cells
- Performed ELISA assays to detect the levels of cytokines and chemokines in serum from patients and from mice
- Analyzed proteins from tumor biopsies: SDS-PAGE protein separation and western blotting
- Isolated DNA and RNA from blood and tumor biopsies, studied mRNA level expression using RT-PCR

Visiting graduate student (02/2013–08/2013)
Harvard Medical School, Massachusetts General Hospital, Wellman Center for Photomedicine, Boston, MA (Prof. Hamblin)
Study of the humoral immunological response induced by photodynamic therapy in tumor mouse models.

- Cultured murine cancer cell lines and injected in mice to generate *in vivo* mouse tumor models
- Performed *in vivo* photodynamic therapy: treatment of tumor bearing mice mediated by intravenous administration of photosensitive molecule, followed by exposure to laser light
- Conducted *in vivo* imaging, to detect tumor cells expressing the fluorescent protein GFP
- Developed ELISA assays to detect tumor-specific Abs titers in serum samples

Exchange student (08/2009–06/2010)
Stockholm University, Dept of Genetics, Microbiology and Toxicology, Stockholm, Sweden (Prof. Aro)
Studied the role of gonococcal infection in preventing cellular apoptosis.

- Used microbiology techniques such as infection of cells and bacterial adhesion binding assay
- Applied flow cytometry assays to detect apoptotic and viable cells
- Isolated RNA from cells and analyzed the expression of genes involved in apoptosis regulation using RT-PCR

Undergraduate internship (03/2008–06/2008)
University of Padova, Dept of Biology, Padova, Italy (Prof. Beltramini)

- Molecular biology techniques such as primer design and protein cloning in *E.coli*, gel electrophoresis, PCR

EDUCATION

Medical University of Graz, Dept of Dermatology, Graz, Austria **(10/2010–08/2014)**
PhD in Molecular medicine
Thesis: "Induction of immunological changes by photodynamic therapy (PDT) for cancer"

University of Padova, Padova, Italy **(10/2008–07/2010)**
Master of Science in Molecular biology, GPA of 108/110
Thesis: "*Neisseria gonorrhoeae* prevents apoptosis in staurosporine treated Me-180 cervical epithelial cells"

University of Padova, Padova, Italy **(10/2005–09/2008)**
Bachelor of Science in Molecular biology, GPA of 105/110
Thesis: "Cloning of human superoxide dismutase 2 (SOD2)"

AWARDS

- **Fellowship** to attend the 15th European Society for Photobiology (ESP) Congress in Liège, Belgium, June 2013
- **Best poster prize** award at the Doctoral Day of the Medical University of Graz, Austria, December 2012

SKILLS AND KNOW-HOW

- Highly advanced knowledge of the main bio-lab procedures:
  - DNA and RNA purification from cells and tissues
  - Gel electrophoresis, PCR, RT-PCR (Light Cycler480, Roche)
  - ELISA, Western blot, Immunohistochemistry, Flow cytometry (FACS Calibur, BD Bioscience) and softwares (Cellquest Pro, FlowJo)
  - Isolation of T lymphocytes from human and mice and development of *in vitro* assays. Culture of human and murine cells. Handling of mice, experience with tumor mouse models
- Planning of results oriented experiments tailored to the goals of the project and management of the workload within the deadlines. Management of the instrument maintenance and safe lab practices (BSL requirements).
- Guidance of new students in their initial training on laboratory procedures. Presentation of group achievements at international conferences (see presentations section).
- Excellent use of most common desktop applications: MS Office 2013, EndNote, GraphPad Prism

LANGUAGES

Strong verbal and written communication skills in Italian (native speaker), English (fluent), German (level B1)

PUBLICATIONS

1. "Photodynamic therapy downregulates the function of regulatory T cells in patients with esophageal squamous cell carcinoma". **Reginato E**., Lindenmann J., Langner C., Schweintzger N., Bambach I., Smolle-Jüttner F., Wolf P.; *Photochemical & Photobiological Sciences,* July **2014**. Manuscript accepted for publication.
2. "Immune response after photodynamic therapy increases anti-cancer and anti-bacterial effects". **Reginato E**., Wolf P., Hamblin M.R.; *World Journal of Immunology*, March **2014**, 4(1):1-11
3. "Photodynamic therapy plus regulatory T-cell depletion produces immunity against a mouse tumor expressing a self-antigen". **Reginato E**., Mroz P., Chung H., Kawakubo M., Wolf P., Hamblin M.R.; *British Journal of Cancer*; October **2013**, 109(8):2167-74

PRESENTATIONS

1. "Photodynamic therapy (PDT) and immune regulation". Reginato E., et al.; *Austrian Society of Dermatology and Venereology (ÖGDV) annual meeting*. November **2013**. Vienna, Austria. Oral presentation
2. "Effect of photodynamic therapy (PDT) on regulatory T cells". Reginato E., et al.; 15th *European Society for Photobiology Congress*. September **2013**, Liège, Belgium. Poster presentation
3. "Photodynamic therapy (PDT) downregulates the function of regulatory T cells in patients with esophageal squamous cell carcinoma". Reginato E., et al.; *IID* (*International Investigative Dermatology) meeting*. May **2013**, Edinburgh, United Kingdom. Poster presentation
4. "Effect of photodynamic therapy (PDT) on immune regulation". Reginato E., et al.; *9th International Symposium on Photodynamic Therapy and Photodiagnosis in Clinical Practice*. October **2012**, Bressanone, Italy. Oral presentation
5. "Effect of photodynamic therapy (PDT) on immune regulation". Reginato E., et al.; *42nd ESDR (European Society for Dermatological Research) meeting.* September **2012**, Venice, Italy. Poster presentation
6. "Induction of immunological changes by photodynamic therapy (PDT) for esophageal carcinoma". Reginato E., et al.; *European Society for photobiology (ESP) Photobiology School*. June **2012**, Bressanone, Italy. Poster presentation