

Systemic antitumor protection by vascular-targeted photodynamic therapy involves cellular and humoral immunity

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Received: 28 February 2008 / Accepted: 17 April 2008
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Abstract Vascular-targeted photodynamic therapy (VTP) takes advantage of intravascular excitation of a photosensitizer (PS) to produce cytotoxic reactive oxygen species (ROS). These ROS are potent mediators of vascular damage inducing rapid local thrombus formation, vascular occlusion, and tissue hypoxia. This light-controlled process is used for the eradication of solid tumors with Pd-bacteriochlorophyll derivatives (Bchl) as PS. Unlike classical photodynamic therapy (PDT), cancer cells are not the primary target for VTP but instead are destroyed by treatment-induced oxygen deprivation. VTP initiates acute local

inflammation inside the illuminated area accompanied by massive tumor tissue death. Consequently, in the present study, we addressed the possibility of immune response induction by the treatment that may be considered as an integral part of the mechanism of VTP-mediated tumor eradication. The effect of VTP on the host immune system was investigated using WST11, which is now in phase II clinical trials for age-related macular degeneration and intended to be evaluated for cancer therapy. We found that a functional immune system is essential for successful VTP. Long-lasting systemic antitumor immunity was induced by VTP involving both cellular and humoral components. The antitumor effect was cross-protective against mismatched tumors, suggesting VTP-mediated production of overlapping tumor antigens, possibly from endothelial origin. Based on our findings we suggest that local VTP might be utilized in combination with other anticancer therapies (e.g., immunotherapy) for the enhancement of host antitumor immunity in the treatment of both local and disseminated disease.

Y.S. and A.S are the incumbents of the Tillie and Charles Lubin Professorial Chair in Biochemical Endocrinology, and the Robert and Yaddele Sklare Professorial Chair in Biochemistry, respectively. S.J. is the incumbent of the Pauline Recanati Career Development Chair. D.P. in partial fulfillment of her PhD Thesis requirements at the Feinberg graduate school of the Weizmann Institute of Science.

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Keywords Bacteriochlorophyll · Cancer ·
Immune response · Immunotherapy ·
Photodynamic therapy · Vascular targeting

Introduction

Photodynamic therapy (PDT) utilizes light-dependent activation of a non-toxic photosensitizer (PS) in the presence of molecular oxygen leading to the formation of cytotoxic reactive oxygen species (ROS). Classical PDT (first and second generation) relies on the selective accumulation of the PS in the tumor cells. The majority of malignant cells are directly ablated by ROS generated upon

photosensitization [9, 30]. Vascular-targeted PDT (VTP) with Pd-bacteriochlorophyll derivatives (Bchl) differs from classical PDT in many aspects. In contrast to PDT, the intravenously (i.v.) injected Bchl-derived PS do not extravasate and remain constrained in the circulation, thus ROS generated locally upon illumination remain confined to the vascular compartment of the tumor [15, 31, 51], resulting in severe vascular damage and acute blood stasis. As a result, tumor cells become deprived from oxygen and nutrient supply, leading to the elimination of the tumor [27, 36]. The PS used in the present study, WST11, is now in phase II clinical trials for the treatment of age-related macular degeneration, a pathological neovascularization of the macula leading to blindness of the patients [45] and is intended to be evaluated for cancer treatment.

In past decades, it was realized that a functional immune system is crucial for long-term survival following PDT. Photosensitization triggers innate immunity with subsequent development of adaptive responses [6, 26, 33]. An acute inflammatory reaction develops during the first hours/days following the treatment. Tumor cells undergoing necrotic or apoptotic death release their antigens into the surrounding inflammatory milieu, encouraging antigen uptake by professional antigen presenting cells (APC), as demonstrated *in vitro* [19], and antigen presentation in a context that strongly promotes formation of specific antitumor immunity. Nevertheless, diverse PS demonstrated different effects on the immune system with some of them being rather immunosuppressive [20, 29, 32]. Additionally, in a study by Henderson et al. [17] it was demonstrated that the most effective treatment regimen caused a very mild inflammatory response. It therefore seems that the contribution of the immune system to photodynamic tumor ablation is dependent upon the PS and treated tumor, as well as on treatment protocol. Importantly, a recently published case report provided the first evidence of immune system contribution to PDT-mediated tumor eradication in the clinical setup [44]. The patient, treated with chlorine e6-based PDT for skin angiosarcoma of the upper limbs, showed regression of several untreated tumor foci in both the treated and untreated limbs. Additionally, massive T cell infiltration was found in biopsies of untreated tumors. These exciting observations suggest that the characterization of the immune system's contribution to PDT could harbor potential to improve treatment protocols for the patient's benefit.

In light of the above, it was of interest to investigate the impact of intravascular photosensitization on host immunity based on the different PS, targets, and the treatment strategies involved in VTP. Moreover, since hypoxic tumor regions can escape death by PDT/VTP, the involvement of the immune system, if significant, might be important for handling minimal residual disease as well as eradicating distant metastatic foci.

In the course of the present study, we demonstrate that local WST11-VTP induces effective and long-lasting systemic antitumor immunity. Furthermore, in contrast to previous studies [14, 25, 39], the immune protection induced by WST11-VTP was cross-reactive, protecting mice from mismatched tumors. We also show that both humoral and cellular immune arms appear to be involved in VTP-mediated immune response.

Materials and methods

Cell lines

CT26 mouse colon carcinoma and 4T1 mouse mammary carcinoma cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM glutamine, 100 µg/ml penicillin and 100 U/ml streptomycin.

Animals

Female, 6- to -8 week-old BALB/c, BALB/nude, and NOD/scid mice were purchased from Harlan Laboratories. Diphtheria toxin (DTx) receptor (DTR) transgenic mice (FVB-Tg Itgax-DTR/GFP 57Lan/J) of BALB/c background were bred in the institute facility [21]. Heterozygous transgenic males were intercrossed with female BALB/c mice. Selection of transgenic offspring was performed by PCR (described under DTR-chimera preparation). All animals were housed and handled in the animal facility according to the guidelines of the Weizmann Institute of Science Animal Care and Use Committee. At the end of the experiments or in the case of animal distress, mice were euthanized by cervical dislocation or by inhaling CO₂.

Photosensitizer

In vitro studies. WST11, a water-soluble Bchl derivative (Steba-Biotech, Rehovot, Israel) was dissolved in culture medium at final concentration as indicated in the specific experiments.

In vivo studies. WST11 was dissolved in phosphate buffered saline (PBS) and i.v. injected at doses indicated in the specific experiments.

Light sources

In vitro studies. A home built 755 ± 5 nm LED (light emitting diode) array set at a fluency rate of 2.5 mW/cm² through a Perspex stage.

In vivo studies. A 4 W diode laser (755 nm) (CERA-MOPTEC, Bonn, Germany) was used at the energy indicated in the specific experiments.

Tumor models

Single-cell CT26 and 4T1 suspensions were prepared by scraping monolayers in exponential growth phase under saline. For local tumor implantation, 2×10^6 washed cells in 50 μ l PBS were subcutaneously (s.c.) injected to the hind leg of the mice. Tumors were considered suitable for the treatment 10–14 days after injection when reaching a diameter of 6–8 mm.

For lung metastases assay 2×10^5 of CT26 cells in 200 μ l PBS were injected i.v. into the mouse tail vein. After 2 weeks, mice were sacrificed and lungs were weighed and processed for histological examination. For each experiment, the following control groups were used: positive control group-mice received only i.v. injection of tumor cells, naïve control group-mice were not manipulated.

VTP protocol

Tumor-bearing mice were anaesthetized by intra-peritoneal (i.p.) injection of a mixture of ketamine (Rhone Merieux, Lyon, France) and 2% xylazine (Vitamed, Israel) (85/15%, v/v). The tumor area was illuminated immediately following i.v. injection of WST11 (9 mg/kg body weight) (1 cm Φ , 30 J/cm², fluence rate 100 mW/cm², 5 min) [31]. Following illumination, the mice were placed back in the cage. After VTP, the animals were injected with 2.5 mg/kg finadyne solution (containing 2.5 mg/ml flunixin, Schering-Plough, UK) for analgesia.

DTR-bone marrow chimera preparation and dendritic cell (DC) depletion protocol

DTR transgenic mice screening was done by PCR using the following primers: DTR1 5'-gcc acc atg aag ctg ctg ccg-3', DTR2 5'-tca gtg gga att agt cat gcc-3'. DTR transgenic bone marrow was obtained by flushing tibia, femur and hips with PBS. Ten-week-old BALB/c female mice received a single lethal dose of 800 Rad total body irradiation and the following day were reconstituted with DTR transgenic bone marrow (i.v. injection of $4\text{--}5 \times 10^6$ cells/mouse in 200 μ l PBS). Mice were allowed 8 weeks rest before further use. For local DC depletion, mice were injected peri-tumorally with 200 ng DTx (D-2918; Sigma-Aldrich) dissolved in PBS starting 24 h before VTP and depletion was maintained for 14 days after VTP by daily injections. For systemic DC depletion, DTx was injected i.p. (16 ng/g body weight) starting

48 h before VTP and maintaining the depletion for 14 days by every 2 days injections. Depletion was verified by flow cytometry.

Histology

Lungs and tumors were excised from euthanized animals and fixed in formaldehyde (4%) or Bouin's fixative at room temperature for 24–48 h. Paraffin-embedded sections were prepared and stained with hematoxyline/eosin (H&E) under standard conditions. Slides were examined under light microscope (Nicon Eclipsed E600 equipped with Nikon Digital Camera DMX1200F).

Immunohistochemistry

Slices 4 μ m thick from tumors fixed with Bouin's fixative were deparaffinized by consecutive immersions in xylene, 100, 95, 70, 50% ethanol, and double distilled water. Intrinsic peroxidase activity was inactivated by immersing the slides in 3% H₂O₂ and 1% HCl in methanol/ PBS (1:1). Sections were blocked with 5% bovine serum albumin (BSA) in PBS. For T cell staining, antigen retrieval was done by boiling (10 min, 10% efficiency) in 1 mM EDTA and then rat α -human CD3 antibodies (Serotec, Raleigh, NC, USA) were applied in PBS containing 1% BSA. After washing in PBS, biotinylated α -rat antibodies were added (Vector Laboratories, Burlingame, CA, USA) and the binding was visualized with avidin-biotin peroxidase complex (Vectastain Elite ABC kit; Vector Laboratories) according to the manufacturer's instructions. For macrophage staining, antigen was retrieved with 0.1% trypsin containing 0.1% CaCl₂ in 20 mM Tris buffer. Rat α -mouse F4/80 antibodies (Serotec) were applied in 1% BSA and 0.1% triton in PBS. Universal immuno-peroxidase polymer for mouse tissue (Histofine 414311F, Nichirei Corp., Tokyo, Japan) was used according to manufacturer's instructions. For B-cell staining, antigen retrieval by boiling (10 min, 10% efficiency) with 10 mM sodium citrate was performed. Biotinylated rat α -mouse CD45R antibodies (PharMingen, San Diego, CA, USA) were applied and ABC kit was added. Di-amino-benzidine (DAB) reagent (Vector Laboratories) was used for visualization. All slides were counterstained with hematoxylin-Mayer (Finkelman) and examined by light microscopy.

Neutrophil infiltration

Specific neutrophil esterase activity was detected in deparaffinized slides using naphthol AS-D chloro-acetate esterase kit (Sigma, St. Louis, MO, USA) according to the manufacturer's instructions. Sections were then examined by light microscopy.

Splenocyte isolation

Single-cell suspensions were obtained by passing spleens through a stainless steel grid (mesh size 100 μm). Cells were harvested by centrifugation, depleted of erythrocytes by treatment with lysis solution (155 mM NH_4Cl , 10 mM KHCO_3 , 1 mM EDTA, pH 7.3) and washed with PBS.

Adhesion assay

Single-cell suspensions of naïve or stimulated splenocytes were counted and incubated in the dark with vital fluorescent dyes [1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) 5 μM or 4-(4-(dihexadecylamino)-styryl-*N*-methylpyridinium iodide (Di-Asp) 10 μM , 30 min, RT, Molecular Probes] to enable tracking, then centrifuged in order to remove excess dye and used immediately. Equal numbers of naïve and VTP-sensitized splenocytes were mixed and incubated with cultured tumor cells for 2 h at 37°C in 24-well plates. Wells were then washed extensively to remove unbound cells and examined under a fluorescent microscope (Nikon Eclipse TE 2000-U equipped with Nikon LH-M100c-1 camera). Ten images were acquired for each group using respective excitation/emission filter pairs 510–560/590 and 450–480/505–550 nm for DiI and Di-Asp. Area covered by splenocytes was calculated and analyzed with ImageJ software. Statistical analysis was performed using student's *t* test.

Cytotoxicity assay

The lytic activity of splenocytes was assessed in a standard 4 h cytotoxicity assay. Splenocytes were isolated from naïve mice or mice 2 weeks after VTP and incubated with cultured CT26 cells at an effector/target ratio of 50:1. After 4 h, cytotoxicity was tested by colorimetric lactate dehydrogenase (LDH) release assay using LDH kit (Sigma) according to manufacturer's instructions. The percent of specific lysis was calculated according to formula:

$$\% \text{ Specific_Lysis} = \left[\frac{(\text{Experimental_Release}) - (\text{Spontaneous_Release})}{(\text{Maximum_Release}) - (\text{Spontaneous_Release})} \times 100 \right]$$

centrifugation at 4,000 rpm for 20 min. If not used immediately, the serum was stored at -20°C .

IgG binding

CT26 cells were plated in 96-well plates and incubated for 24 h. Cells were washed with fresh medium and 50 μl of serum diluted 1:5 with assay buffer [Tris-HCl buffer, 50 mMol, pH 7.7, containing BSA (5 g/l), NaCl (9 g/l) and Tween-20 (0.1 ml/l)] added. After 2 h of incubation at 37°C, cells were washed and blocked with 25 μl normal mouse serum (Sigma, St. Louis, MO, USA). Horse α -mouse IgG conjugated to horseradish peroxidase (Vector, Laboratories) was added for 1 h. After washing with PBS, color was developed with 3,3',5,5'-tetramethylbenzidine (TMB) solution (Zymed) and read at 650 nm on a Victor 1,420 multi-label counter (Perkin-Elmer).

Flow cytometry

Mouse lymph nodes and spleens were disaggregated in PBS. For isolation of DCs, tissue was subjected to enzymatic degradation with 1 mg/ml collagenase D (Roche) for 30 min at 37°C. Single-cell suspensions were blocked with rat- α -mouse CD16/32 and stained with appropriate antibodies. Blocking α -CD16/32, FITC-conjugated rat- α -mouse CD4, PE-conjugated hamster- α -mouse CD3 and APC-conjugated rat- α -mouse CD8 antibodies were purchased from eBioscience (San Diego, CA, USA). APC-conjugated hamster- α -mouse CD11c were purchased from BioLegend (San Diego, CA, USA). Single-cell suspensions were prepared in flow cytometry buffer (PBS, 1% FBS, 2 mM EDTA, and 0.05% sodium azide). Cells were analyzed by multicolor flow cytometry using a FACSort cytometer (Becton-Dickinson, Mountain View, CA) and CellQuest software (Becton-Dickinson). A live lymphocyte gate was set according to forward and side scatter parameters. For analysis of T lymphocytes $1-2 \times 10^4$ cells were acquired per sample and for DCs $2-5 \times 10^5$ cells were acquired.

Animal bleeding and serum isolation

Mice were bled peri-orbitally. Whole blood was allowed to clot at room temperature and serum was separated by

In vitro $\text{IFN}\gamma$ production assay

Splenocytes were isolated from VTP cured mice (3 months after treatment) or naïve BALB/c mice as described above

and stimulated with cultured CT26 cells that were previously subjected to a lethal dose of WST11 and light (10 μ M, 10 min illumination). After 2 h of stimulation, cytokine secretion was blocked by brefeldin A (3 μ g/ml, eBioscience) for 4 h. Cells were stained for CD4 and CD8 surface markers, permeabilized with permeabilization solution (0.1% saponin, 5% fetal calf serum, and 0.1% azide in PBS) and intracellular staining for IFN γ was performed for 30 min. Rat- α -mouse IFN γ antibodies were purchased from eBioscience. Cells were washed and analyzed by flow cytometry.

Adoptive transfer experiments

Mice cured from CT26 tumors or naïve mice were stimulated by i.p. injection of 10^5 CT26 cells subjected to a lethal dose of WST11 and light. After 2 days, CD8 $^+$ and CD4 $^+$ cells were purified from mouse spleens using magnetic beads (BD Bioscience) and immediately injected i.v. to naïve BALB/c mice (9×10^6 cells/mouse) that received lymphodepleting irradiation (300 Rad) a week earlier. Cell purity was determined by flow cytometry and found to be >95 and >91% for CD8 $^+$ and CD4 $^+$ cells, respectively. Two days after adoptive transfer, mice were challenged i.v. with live CT26 cells (2×10^5 cells/mouse). After 2 weeks, challenge mice were sacrificed, the lungs were excised and weighed.

Statistical analysis of tumor infiltration

At least four fields were acquired per section and analyzed with Image Pro Plus or ImageJ software. The areas covered by neutrophils or CD3 $^+$ were measured and compared using student's *t* test. At least three mice were tested for each time point.

Results

A functional immune system is necessary for effective eradication of local tumors by WST11–VTP

To investigate the role of adaptive immunity on VTP, we grafted wild type and immuno-deficient mice with mouse CT26 colon carcinoma cells and subjected the developed tumors to WST11–VTP with drug and light doses that gave the highest cure rates as determined in preliminary studies (not shown). We found that while immuno-competent wildtype BALB/c mice responded well to the treatment with high cure rates (above 70%), BALB/nude and NOD/scid mice that exhibit defects in adaptive immunity responded very poorly (18.7 and 11%, respectively) (Fig. 1). This demonstrates the necessity of the host

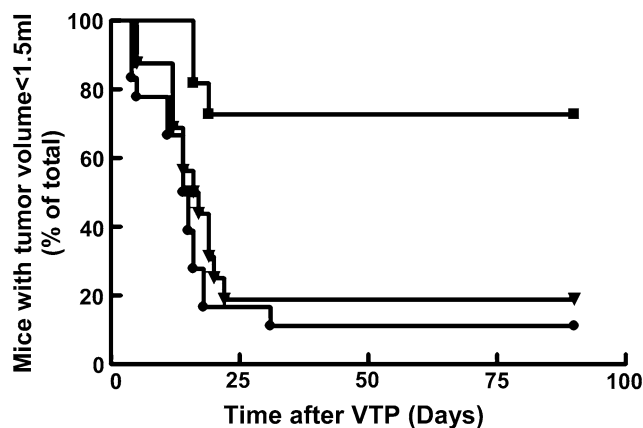


Fig. 1 Effect of host immune system on WST11–VTP efficacy. Three mouse strains with normal (BALB/c; squares, $n = 11$), T-cell deficient (BALB/nude; triangles, $n = 16$) or T- and B-cell deficient (NOD/scid; circles, $n = 18$) immune system and bearing CT26 s.c. tumors were subjected to WST11–VTP and monitored for tumor response ($*P < 0.004$ by student's *t* test)

immune system for effective VTP. Notably, nude mice lack functional T-cells, while Scid mice lack both B and T lymphocytes; however, there was no significant difference in cure rates of the two strains, implying that cell-mediated immunity has a dominant effect over humoral immunity in the acute antitumor action of VTP.

WST11–VTP induces long-lasting systemic heterologous antitumor protection

Next, BALB/c mice bearing s.c. CT26 tumors were subjected to WST11–VTP. After 2 weeks, the animals were challenged by i.v. injection of viable CT26 cells, and lung metastases development was examined 2 weeks after challenge. Mice exposed to VTP were found completely protected from metastases (as verified by histological examination), while positive control mice (that received the challenge only) developed multiple tumor foci in the lungs (Fig. 2a, b). This antitumor protection lasted 3 months posttreatment (Fig. 2c) and even upto 1 year later (data not shown). These results indicate that despite the local nature of the treatment and the fact that tumor cells are not the primary targets, VTP induces systemic and long-lasting antitumor protection.

We also found that when the primary tumor was mouse 4T1 mammary carcinoma, WST11–VTP conferred protection to BALB/c mice against subsequent challenge with CT26 cells as verified 2 weeks and 3 months posttreatment (Fig. 2d, e). The observed cross-protection phenomenon could potentially be attributed to several mechanisms: (1) antigenic similarity between the tumor types tested, (2) determinant spreading [18, 37] initiated by VTP-induced oxidative modification of proteins and lipids, and (3) the

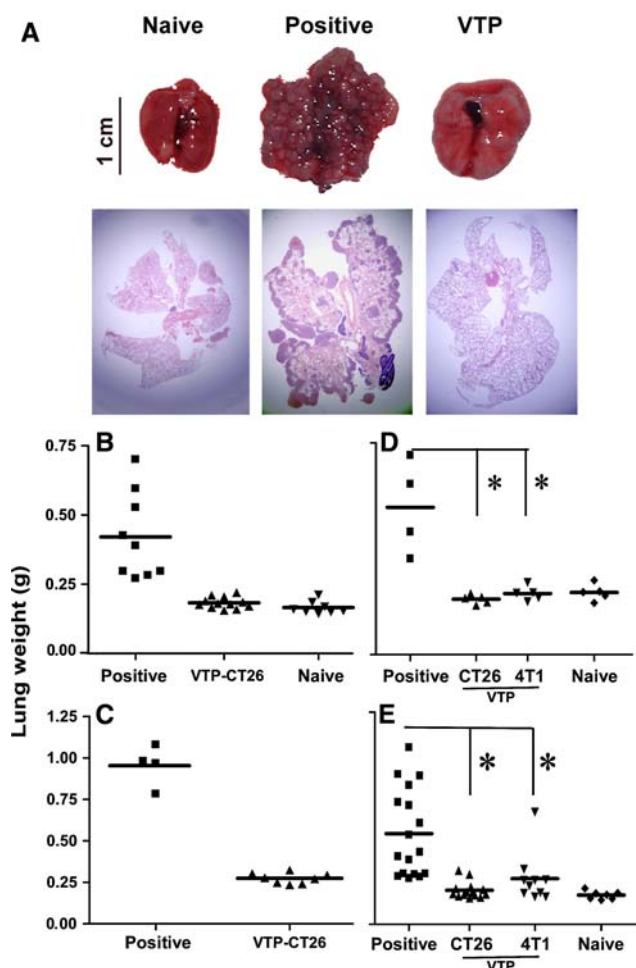


Fig. 2 WST11-VTP induced systemic anti-tumor protection. BALB/c mice bearing s.c. CT26 tumors were subjected to VTP and challenged i.v. with viable CT26 cells. Lungs were excised 2 weeks after challenge and weighed. **a** Lung micrographs and respective histological images, **b** homologous protection 2 weeks, **c** three months after VTP, **d** cross-protection 2 weeks and **e** three months after VTP. Positive control-naïve age-matched mice i.v. injected with CT26 cells. Representatives of 2–3 experiments are shown ($*P < 0.008$ by student's *t* test)

mounting of specific cellular and/or humoral responses against endothelial cell-derived antigens that might interfere with neo-vascularization upon tumor re-challenge.

An acute inflammatory response is initiated immediately after WST11-VTP

To gain further insight into the protection phenomenon, we performed an in depth characterization of the local VTP response. VTP of s.c. tumors induces acute wounding in the treated site that is resolved within a month after treatment, leaving minimal scarring. We investigated the nature and kinetics of acute inflammatory phase initiated upon vascular photosensitization. A rapid and massive neutrophil infiltration was observed immediately after illumination.

Significant numbers of neutrophils were detected in the tumor rim and the interface between the tumor and the surrounding skin culminating 1 h postVTP (Fig. 3). No neutrophils were observed in the tumor core. The number of infiltrating neutrophils then dropped dramatically to non-detectable levels 24 h posttreatment. Additionally, a substantial recruitment of lymphocytes into the tumor was observed 24 h postVTP, as confirmed by tumor immunostaining for the pan T-cell marker CD3 (Fig. 4d). Most infiltrated T-cells were located in the tumor rim, similarly to the neutrophils. We noted almost complete absence of CD3⁺ cells shortly after the illumination (Fig. 4b). Statistical analysis showed significant differences between tumor T-cell content 1 h posttreatment compared to infiltration both before and 24 h postVTP ($P < 1.5 \times 10^{-7}$ and $P < 1.5 \times 10^{-6}$, respectively). Closer examination of the slides under large magnification revealed some CD3⁺ cells inside and in close proximity to the blood vessels (Fig. 4c) as a first sign of T-cell infiltration initiated by VTP. T-cell infiltration was almost completely resolved by 48 h after treatment ($P < 5.7 \times 10^{-6}$ compared to 24 h posttreatment) (Fig. 4e) with only a few CD3⁺ cells seen in the areas still containing live tumor cells (Fig. 4f). Possibly, these lymphocytes recognize tumor antigens exposed by VTP and are involved in antitumor cytotoxic lymphocyte (CTL) responses.

Cellular antitumor activity and T-cell-mediated immune memory is induced upon WST11-VTP

The results presented in Figs. 2, and 3 demonstrate rapid recruitment of different immune cells into the treated tumor area. We next decided to test VTP-mediated stimulation of the effector cell activity and formation of immune memory. In an in vitro adhesion assay, we found that sensitized splenocytes isolated from VTP-treated mice adhered better to cultured tumor cells compared to splenocytes isolated from naïve mice (Fig. 5a). There was a significant difference in adhesion of CT26-sensitized splenocytes to naïve cultured 4T1 cells compared to naïve cultured CT26 cells. However, interestingly, this difference was abolished by photosensitization of tumor cells in culture prior to the addition of splenocytes. We hypothesize that the photodynamic treatment produces overlapping tumor antigens by means of oxidative modification and/or determinant spreading. Importantly this finding is in agreement with the results obtained with these tumor cells in vivo (Fig. 2d, e).

We further found that VTP enhanced the cytotoxic activity of sensitized splenocytes compared to naïve splenocytes as judged by a standard in vitro cytotoxicity assay (Fig. 5b), confirming the existence of a CTL response. Lymphocytes isolated from mice several months after VTP secreted IFN γ upon re-stimulation with in vitro

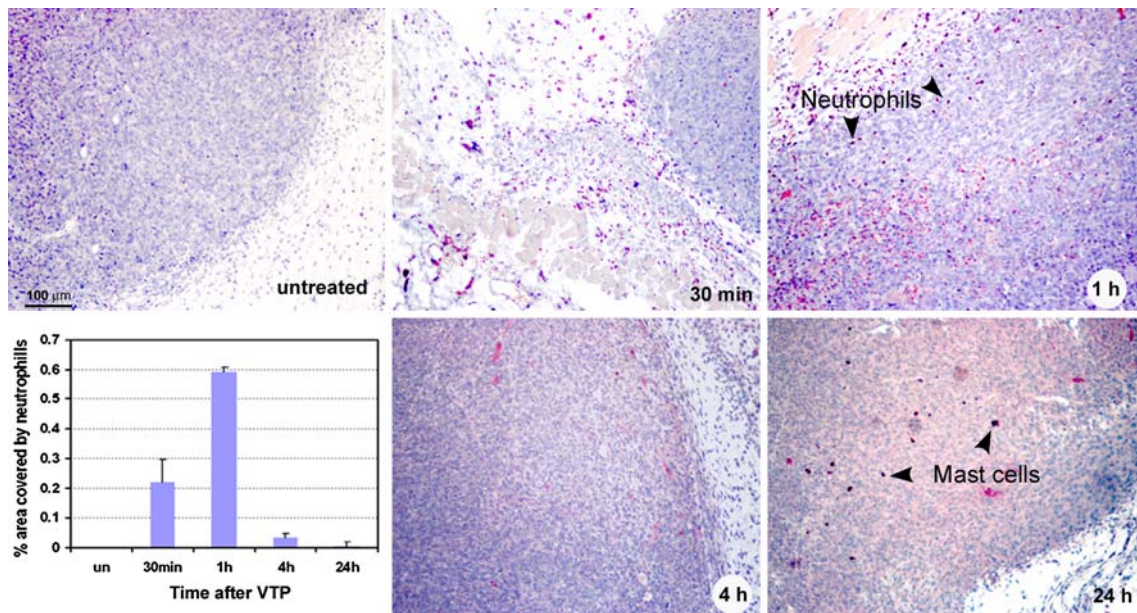


Fig. 3 Neutrophil infiltration into the s.c. CT26 tumors following WST11-VTP. Formaldehyde fixed tumors were stained for specific naphthol AS-D chloroacetate esterase activity. Analysis was done using Image Pro Plus software. Neutrophils are stained in purple.

Three mice were used for each time point. One representative experiment is shown. Each bar represents percent of area covered by neutrophils of 4–6 sequential fields taken from one slide normalized to total tumor area (mean ± SE, $n = 3$)

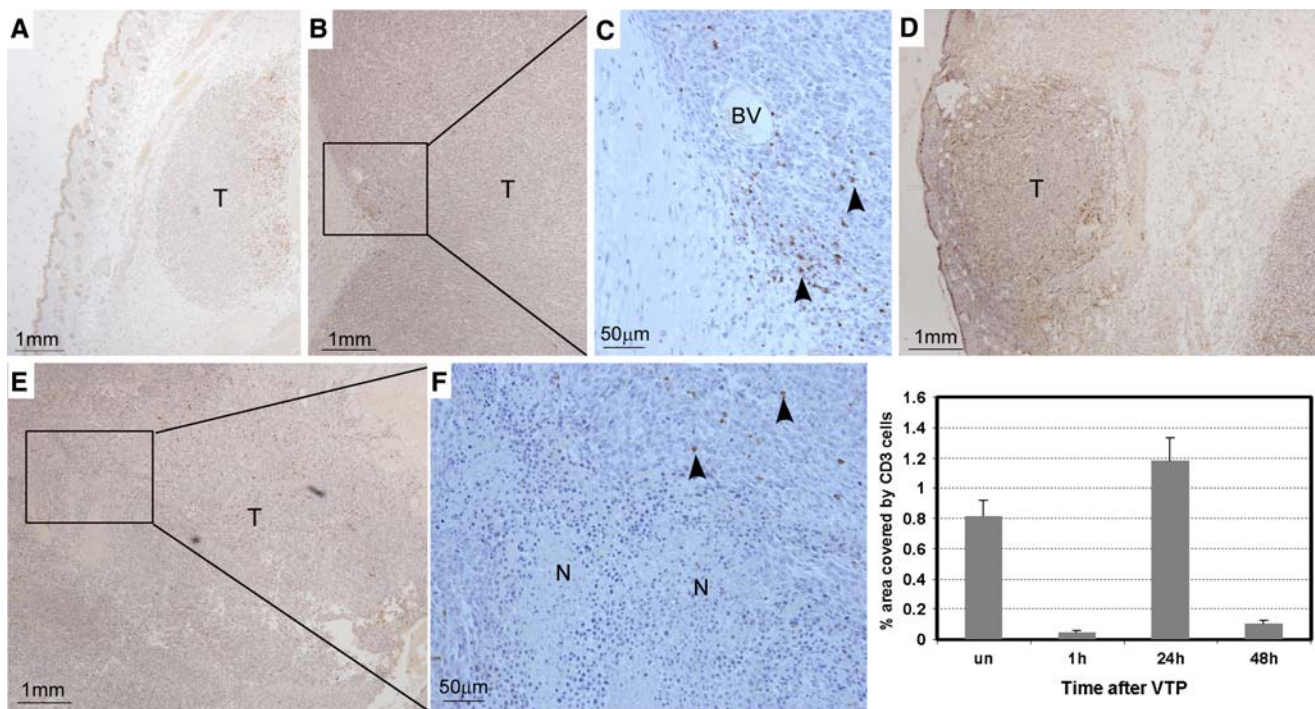


Fig. 4 CD3⁺ lymphocytes infiltration into the CT26 s.c. tumors following WST11-VTP. Tumors were excised at various times after the treatment, fixed in Bouin’s fixative and paraffin-embedded. Slices were stained with α -mouse CD3⁺ antibodies. CD3⁺ lymphocytes are stained in brown; **a** untreated tumor, **b** 1 h after VTP, **c** magnification

of the square from **b**, **d** 24 h after VTP, **e** 48 h after VTP and **f** magnification of the square from **e**. *T* tumor, *BV* blood vessel, *N* necrotic area; arrows denote CD3⁺ cells. Each bar represents percent of area covered by CD3⁺ of 4–6 sequential fields taken from one section normalized to total tumor area (mean ± SE, $n = 3$)

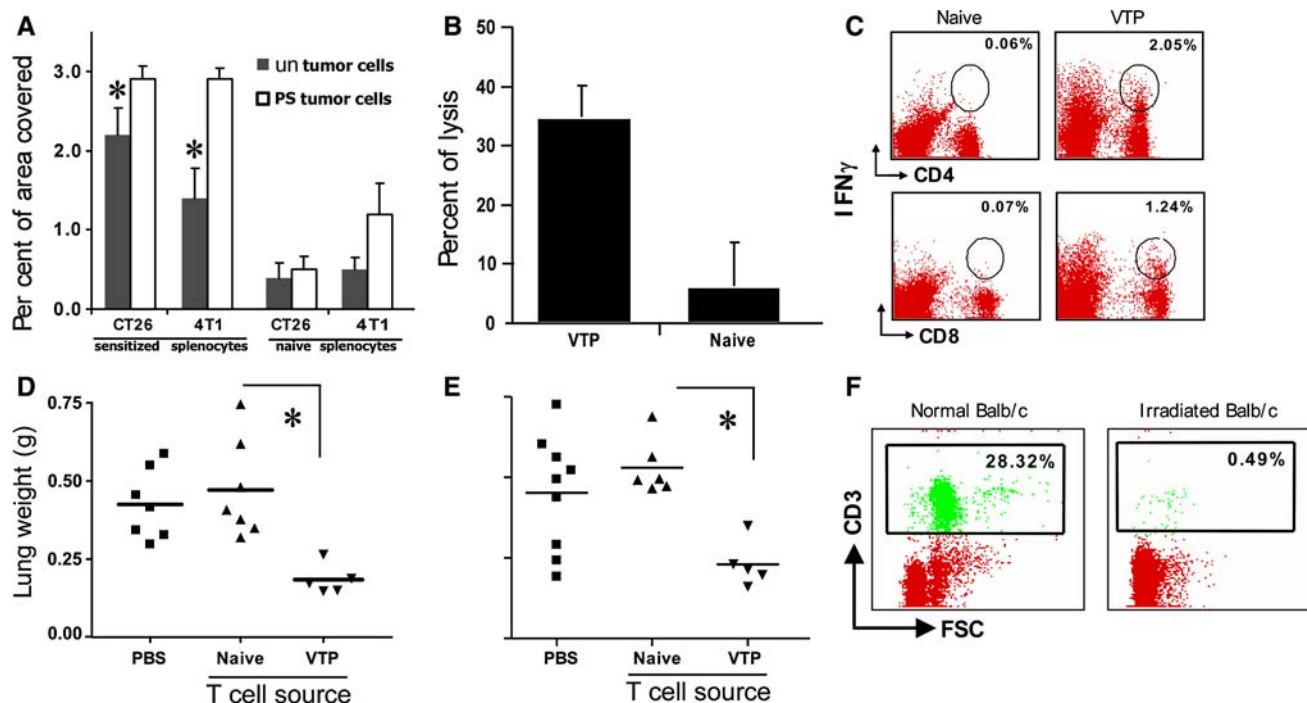


Fig. 5 Cellular immune responses induced by WST11-VTP; **a** adhesion of VTP-sensitized or naive splenocytes to cultured tumor cells. Splenocytes were stained with DiI or Di-Asp vital dyes and incubated with untreated or photodynamically pre-treated cultured CT26 or 4T1 cells. Adhesion was assessed by fluorescent microscope. Ten images were acquired for each group using appropriate filter sets and area covered by splenocytes was calculated ($P < 0.005$), **b** VTP-induced splenocyte cytotoxicity. Splenocytes were incubated with cultured CT26 cells. Specific cytotoxicity was measured by LDH release according to formula: % specific lysis = (experimental release – spontaneous release)/(maximum release – spontaneous release) \times 100. Experiments were performed in triplicates, **c** IFN γ secretion by splenic T-cells isolated from naive or cured mice upon in

vitro re-stimulation with VTP-treated CT26 cells, **d** CD8 $^+$ cells adoptive transfer ($P < 0.03$), **e** CD4 $^+$ cells adoptive transfer ($P < 0.005$). Cells were isolated from naive or cured mice and i.v. injected to lymphodepleted naive recipients that were subsequently challenged i.v. with 2×10^5 live CT26 cells and lung metastases development was assessed by lung weighing 2 weeks after challenge. Control mice were injected with PBS; representatives of 2–3 experiments are shown and **f** verification of lymphodepletion in BALB/c mice. Depletion was tested in peripheral blood of the mice irradiated with 300 Rad a week earlier. Blood was collected in the presence of anticoagulant, cells separated by gradient centrifugation and stained for flow cytometry analysis

photosensitized tumor cells (Fig. 5c), indicating the generation of a pro-inflammatory Th1 response. Moreover, adoptively transferred CD8 $^+$ and CD4 $^+$ T-cells isolated from surviving mice 3 months postVTP were able to protect naive recipients from subsequent challenge with viable cancer cells from the same type (Fig. 5d, e). Together these findings provide strong evidence that VTP induces a T-cell-mediated adaptive immune response with the formation of effective immune memory.

VTP-induced stimulation of humoral immunity

Recent studies demonstrated that in vivo antitumor antibody-dependent cell cytotoxicity (ADCC) is one of the mechanisms of antitumor immunity [22, 41, 42]. To investigate the involvement of humoral immune responses in VTP-mediated antitumor action, we utilized several approaches. Immuno-blotting of untreated or VTP pre-treated tumor lysates with serum from naive or treated mice

revealed several changes in the pattern of antigens recognized by the serum IgGs. For example, new bands were recognized by serum from treated animals in the lysate of untreated tumor cells indicating the production of novel antibodies against existing antigens (Fig. 6a-a). New bands in the lysates of treated tumors blotted with naive mouse serum indicate the formation of new antigenic epitopes or VTP-mediated exposure of those previously hidden (Fig. 6a-b). Additionally, some antigens were probably oxidatively modified preventing immune recognition as was demonstrated by bands that disappeared 24 h postVTP (Fig. 6a-c). Histological examination of VTP-treated tumors revealed that 1 h after illumination only a few B cells were found within the tumor, most of them inside or in close proximity to blood vessels similarly to T-cells, indicating B cell recruitment initiated by VTP (Fig. 6b). A marked B-cell infiltration was observed 24 h after treatment in the tumor rim with some cells distributed throughout the tumor and in the connective tissue between

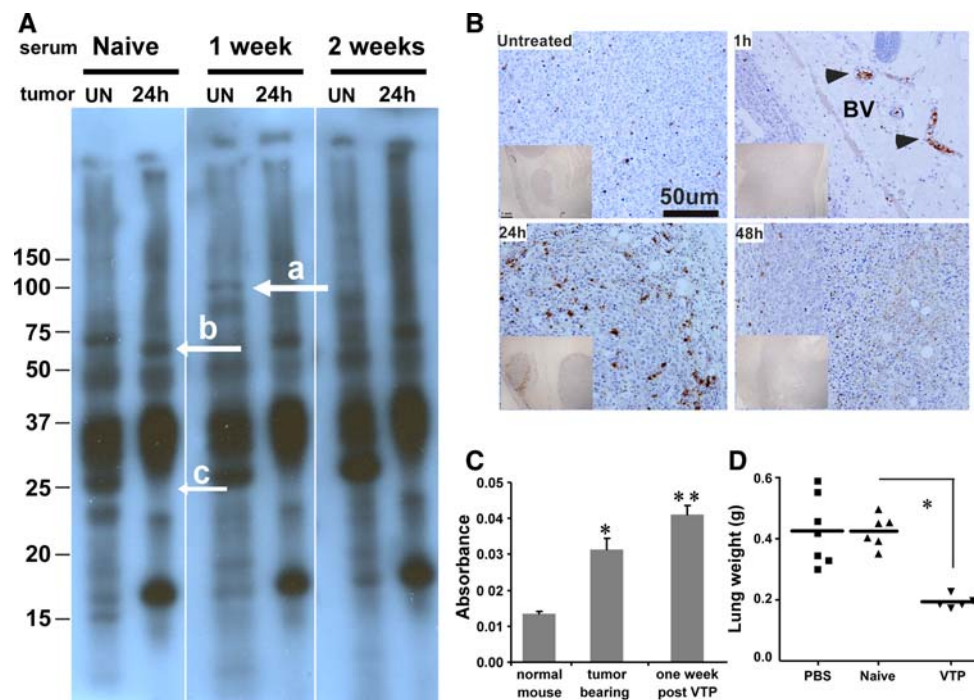


Fig. 6 Humoral responses induced by WST11-VTP; **a** sera from naïve mice or mice 1 or 2 weeks after CT26 tumor VTP was blotted against homogenates of untreated CT26 tumor or tumor isolated 24 h after VTP: **a** new Abs formed after VTP; **b** new or hidden Ag exposed by VTP; **c** Ag that disappeared after treatment; **b** CD45R⁺ cell infiltration into the CT26 s.c. tumors. Positive cells are stained in brown. *T* tumor, *BV* blood vessel, *arrows* denote CD45R⁺ cells ($n = 3$); **c** serum IgG binding to cultured CT26 cells. Serum was

obtained from naïve mice and mice-bearing CT26 s.c. tumors before and 1 week after VTP and applied on cultured CT26 cells. Binding was detected with HRP-conjugated anti-mouse IgG antibodies ($n = 3$) and **d** serum transfer; serum from naïve and cured mice was injected i.v. (500 µl/mouse) to naïve mice that were subsequently challenged with 2×10^5 live CT26 cells and lung metastases development was assessed by lung weighing 2 weeks after challenge. A representative of two experiments is shown ($P < 0.001$)

the skin and the tumor. Some of these cells could still be seen 48 h after VTP. Increased serum IgG titers were detected 1 week after VTP as judged by in vitro binding to respective tumor cells (Fig. 6c) compared with serum from naïve and untreated tumor-bearing mice. It was therefore of interest to verify the contribution of these humoral components to the tumor eradication process. Transfer of serum from mice cured from CT26 tumors protected the naïve recipients from subsequent challenge with live CT26 cells (Fig. 6d). To the best of our knowledge, this is the first demonstration that humoral immunity contributes to VTP-mediated antitumor protection.

Dendritic cells are necessary for VTP-mediated eradication of local tumors

Next we investigated the contribution of DCs to the VTP-triggered immune response. Lethally irradiated BALB/c mice were reconstituted with bone marrow of mice expressing a diphtheria toxin (DTx) receptor transgene under the CD11c promoter, enabling selective depletion of DCs upon DTx injection [21, 40] (Fig. 7a). In this model only transgenic cells are sensitive to DTx-mediated

depletion, while no effect was observed upon DTx injection to the wildtype mice. After a 2 month recovery period, the mice were s.c. implanted with CT26 tumors and subjected to WST11-VTP with or without DC depletion. Two depletion protocols were tested: (1) local depletion by peritumoral DTx injections starting 24 h before the treatment and continuing with daily injections for 14 consecutive days and (2) systemic depletion by i.p. injection of DTx starting 48 h before VTP followed by injections every 2 days for 14 days posttreatment. We found that both protocols resulted in significantly higher tumor recurrence rates compared to VTP alone (Fig. 7b, c). No effect on tumor growth was observed in mice that received DTx without VTP and the animals were sacrificed shortly thereafter due to tumor progression. This is the first direct evidence supporting DC contribution to tumor destruction by VTP.

Discussion

There is increasing recognition that the immune system can effectively contribute to the eradication of solid tumors.

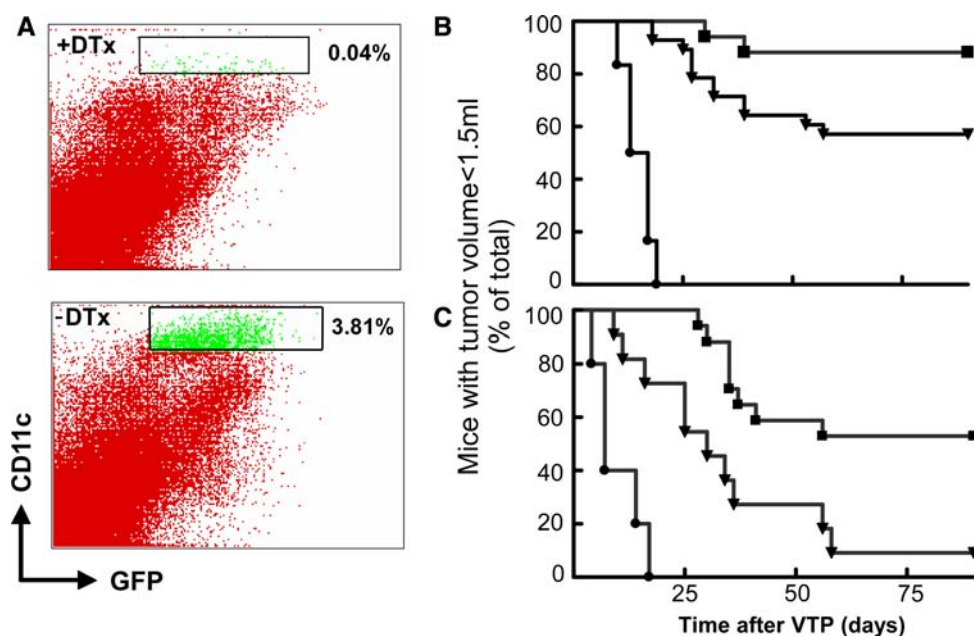


Fig. 7 Effect of DCs depletion on WST11-VTP. DTR bone marrow chimera mice bearing CT26 s.c. tumors were depleted from DCs and subjected to WST11-VTP; **a** effect of systemic DTx injection on splenic DC population, **b** local DC depletion was initiated by peritumoral DTx injection 24 h before VTP and maintained by daily injections for 14 days ($P < 0.04$), **c** systemic DC depletion was

initiated by i.p. DTx injection 48 h before VTP and maintained every 2 days by injection for 14 days ($P < 0.004$). Group received VTP only (squares, $n = 17$), group received VTP with depletion (triangles, local $n = 28$; systemic $n = 11$), group received DTx only (circles, local $n = 6$; systemic $n = 5$)

The major disadvantage of chemo- and radiotherapy is that these modalities suppress host immunity preventing its beneficial consequence on the treatment outcome. Therefore, any anticancer modality that preserves or even enhances host immune responses will be particularly valuable in the fight against this fatal disease. Vascular-targeted photodynamic therapy (VTP) relies on intravascular photosensitization without direct ablation of tumor cells [15, 31, 36]. In previous reports, we demonstrated the benefit of targeting the non-malignant tumor vasculature, by effective eradication of drug-resistant tumors [36]. In the present study, we tested the hypothesis that an acute VTP-mediated inflammatory response, together with the rapid necrotic death of a large tumor mass, might provide a perfect milieu for effective antitumor sensitization of the host immune system. The results presented here provide first evidence for immune system involvement in systemic antitumor protection induced by VTP with the Bchl derivative WST11. Data available in the current literature relates to first generation photosensitizers, whose action is based on selective PS accumulation within tumor cells. It was shown in various experimental settings that the immune effect of PDT is dependent on multiple factors such as photosensitizer type, treatment regimen, and specific tumor model and can vary from effective pro-inflammatory responses to systemic immunosuppression [10, 11, 13, 25, 50].

In our study we found that mice lacking a functional immune system are less responsive to WST11-VTP than immunocompetent BALB/c mice (Fig. 1), demonstrating that an immune reaction is crucial for successful eradication of local tumors by VTP, similarly to other PDT protocols [13, 23, 24]. Moreover, VTP of local mouse CT26 colon tumors grown in BALB/c mice induced systemic protection against a second tumor challenge in cured mice (Fig. 2a–c). Surprisingly, in contrast to PDT with hematoporphyrin-based and other photosensitizers that always induced tumor-specific immunity [14, 25, 39], protection induced by WST11-VTP was found to be non-tumor-specific. Namely, mice cured from local mouse 4T1 mammary tumors resisted the challenge with CT26 cells (Fig. 2d, e). We hypothesize that several mechanisms can be responsible for this cross-protection phenomenon. First, it was previously shown that some tumors share antigenic epitopes that may result in cross-reactivity [38]. Second, cross-protection could be attributed to the determinant spreading phenomenon in which T-cell responses, initiated by a single antigenic epitope, may evolve into multi-epitopic responses [5, 37]. In case of VTP, the large amount of potentially antigenic molecules that is released from dying tumor cells can provide a vast reservoir of such epitopes. Finally, the most interesting possibility relies on the anti-vascular nature of our treatment modality. It was shown that tumor vessels express unique antigens that distinguish

it from the normal vasculature [43, 46]. Moreover, it was demonstrated that targeting such antigens, for example vimentin, with specific antibodies inhibited angiogenesis and markedly reduced tumor growth. This was also accompanied by reduced microvessel density, implying that the antiangiogenic character of the treatment is responsible for the observed effect. In another study vaccination with tumor endothelial marker 8 (TEM8) combined with xenogeneic tumor antigens induced effective immunity against tumor challenge [12]. In this study, TEM8 had no activity as a single agent but significantly enhanced the antitumor immunity for the second antigen, demonstrating effect of adjuvant rather than immunologic target. Since in VTP the photosensitization process is confined to the vascular compartment of the tumor, it is likely that photodamaged endothelial cells may provide an additional significant source of such specific antigens that could augment immune responses. When modified by VTP-induced ROS, some of those putative antigens might even become sufficiently immunogenic to be recognized by the immune system. These antigens are host derived and as such are expected to be at least in part shared by different tumors as documented previously [43], forming a basis for mismatched tumor protection. The specific adaptive immune memory induced against these antigens upon VTP may inhibit the development of new tumors by interfering with their angiogenesis. Several studies report that oxidative modifications such as nitration of proteins [16, 34, 35] or formation of aldehyde adducts of phospholipids and lipoproteins [49] are capable of breaking immune tolerance to the modified entities. It was even demonstrated that, in case of 4-hydroxynonenal (HNE) adduction, the induced immune cells also mediated responses against parental unmodified proteins [48], which is particularly important for eradication of distant metastases. We found previously that VTP with Bchl derivatives induces lipid peroxidation with formation of HNE adducts [27, 36] as well as protein nitration (Gal, Preise unpublished); therefore, these VTP-induced modifications could be partly responsible for treatment-enhanced tumor immunogenicity. It is known that classical PDT also induces different degrees of vascular damage (depending on PS and drug-to-light interval) [10]. Nevertheless, the highest sensitizer concentration is found in the tumor cells and not in the vasculature, thus the contribution of vasculature-related factors on immune stimulation is probably minor and not expected to have a significant impact on the final outcome.

Acute inflammatory reactions manifested by neutrophil tumor infiltration started immediately upon treatment completion, culminating 1 h postVTP with a drop in cell numbers within the tumor area (Fig. 3). These findings are inline with other reports [24, 28] but the infiltration kinetics are faster than reported by those authors that found

maximal neutrophil recruitment 24 h posttreatment. In the wound healing process, the neutrophils progressively accumulate in the damaged site for a few days after the injury (maximal infiltration around 12–24 h postinjury and then their numbers progressively decline) [2, 8]. The consequence of such rapid neutrophil clearance from VTP-treated area might be a prolonged exposure of tumor-derived antigens (which are not removed fast enough) to immune cells participating in the activation and propagation of adaptive immune response (like DCs that cross-prime tumor specific T-cells) leading to the formation of effective antitumor memory.

T-cells are known mediators of adaptive immune responses. We found that existing T-cells in the tumor before treatment (Fig. 4a) could not be detected already 1 h postVTP (Fig. 4b). In agreement with data showing that immune cells are highly susceptible to direct photodynamic action [4], these T-cells are probably destroyed by the treatment. This might be beneficial to the final treatment outcome as multiple studies show that these tumor infiltrating lymphocytes suppress antitumor functions, many of them are actually regulatory T-cells inhibiting pro-inflammatory immune responses [1]. By removing this tumor-imposed suppression, VTP may open an opportunity for effective pro-inflammatory Th1 immune polarization by lymphocytes recruited upon photosensitization. Such newly infiltrating T-cells can be seen at the proximity of tumor feeding blood vessels shortly after VTP (Fig. 4c). Taken together, the recruitment of neutrophils and T lymphocytes into the illuminated area denote a strong and rapid inflammatory reaction, initiated shortly after VTP and practically resolved several days after treatment. This is much faster than in wound- or bacteria-induced inflammation, where infiltrate accumulates for several days and stays for up to 2 weeks [8]. The reason could be the rapid occlusion of tumor feeding blood vessels, precluding further accumulation of immune cells.

It is worth mentioning that two papers reported that PDT protocols inducing maximal inflammatory response are actually less effective in local tumor eradication [17, 28]. In the study by Kousis et al. [28] a less inflammatory regimen was highly efficient in inhibiting the tumor growth, but did not induce durable immune responses. Consequently, the authors proposed combination of inflammation-inducing PDT protocol with additional agents enabling efficient control of the treated tumor. In contrast, in our work, the VTP protocol applied induced the highest cure rates in our experimental set up and provided long-lasting systemic protection from repetitive tumor challenges.

The role of humoral immunity in PDT/VTP was only occasionally addressed. PDT with *meta*-tetra(hydroxyphenyl)bacteriochlorin (*m*THPBC) of rat CC531 colon

adenocarcinoma tumors failed to induce anti-CC531 antibodies [47]. Chen et al. [7] reported some new bands in western blots of tumors probed with sera from rats cured by Photofrin-PDT combined with the immune-adjuvant glycated chitosan suggesting that combined treatment resulted in the development of specific anti-tumor antibodies. In our study we also found different protein patterns when tumors were probed with sera from naïve or successively cured mice (Fig. 6a). The comparison between untreated tumors and tumors 24 h after VTP demonstrates that there are antigens that normally escape the immune system but become exposed upon treatment, as they were recognized by antibodies from naïve mice. Additionally, serum obtained from treated animals contained novel antibodies recognizing antigens in the untreated tumor, suggesting that VTP converted these antigens to more immunogenic. Overall IgG binding to cultured tumor cells revealed increased titers in the treated animals (Fig. 6c). Taken together these findings suggest that VTP enables more effective immune probing of the tumor cells by exposing tumor antigens to immune cells. Our results obtained from the serum transfers provide first evidence that humoral immunity is sufficiently stimulated by VTP to induce antitumor protection in naïve mice (Fig. 6). The remaining question is concerning the nature of soluble factors that mediate the observed effect and the exact mechanism by which tumor rejection is achieved. In the preliminary experiment we found that serum transfer into lympho-depleted mice failed to protect the recipients from subsequent tumor challenge (unpublished data) suggesting ADCC is responsible for the observed protection.

Exposure of DCs to photosensitized tumor cells in culture reportedly induces their phenotypical and functional maturation [14]. DCs represent a crucial link between innate and adaptive immunity [3]. As professional APC they are unrivaled in potential to induce primary immune responses, thus permitting the establishment of immunological memory [3]. Following tissue damage and upon antigen uptake, DCs migrate to the regional lymph nodes and initiate specific adaptive immune responses. Consequently, the combination of PDT with ATX-S10 Na(II), a hydrophilic chlorin photosensitizer [39] and Photofrin [19] with intra-tumoral injection of immature DCs was shown to be beneficial for tumor eradication. We utilized a transgenic mouse model in which selective depletion of DCs can be achieved (Fig. 7a) [21]. Using these mice we demonstrate that local and systemic depletion of DCs during VTP and for short period after the treatment greatly reduces VTP effectiveness (Fig. 7b, c). This suggests that these cells actively participate in the acute phase of VTP-mediated stimulation of immune responses affecting the final treatment outcome.

Taken together, the observations presented in this study demonstrate that the host immune response elicited by tumor WST11-VTP is an integral component of the therapeutic effect of this modality. WST11-VTP induces long-lasting highly efficient antitumor protection. VTP activates cellular and humoral immune arms and possibly the final outcome is achieved by the contribution of both. Further research is needed in order to better understand the mechanism of VTP-induced antitumor immunity with emphasis on the nature of antigens that are involved and immune cells that participate in the process. This information may enable the improvement of current anticancer immunotherapy protocols. Moreover, novel approaches combining additional immune stimulation with VTP may further augment the efficiency of the treatment of local tumors and possibly to provide an adjuvant effect in the treatment of disseminated disease.

Acknowledgments The authors wish to thank Prof. L. Eisenbach and Dr. E. Tzeoval for valuable discussions and technical help, to Dr. Ori Brenner for histopathology work, to Ester Shai, for her technical help. Study supported by STEBA-BIOTECH (France).

References

1. Adam JK, Odhav B, Bhoola KD (2003) Immune responses in cancer. *Pharmacol Ther* 99:113–132
2. Agaiby AD, Dyson M (1999) Immuno-inflammatory cell dynamics during cutaneous wound healing. *J Anat* 195(Pt 4):531–542
3. Banchereau J, Briere F, Caux C, Davoust J, Lebecque S, Liu YJ, Pulendran B, Palucka K (2000) Immunobiology of dendritic cells. *Annu Rev Immunol* 18:767–811
4. Boumedine RS, Roy DC (2005) Elimination of alloreactive T cells using photodynamic therapy. *Cytotherapy* 7:134–143
5. Butterfield LH, Ribas A, Dissette VB, Amarnani SN, Vu HT, Oseguera D, Wang HJ, Elashoff RM, McBride WH, Mukherji B, Cochran AJ, Glaspy JA, Economou JS (2003) Determinant spreading associated with clinical response in dendritic cell-based immunotherapy for malignant melanoma. *Clin Cancer Res* 9:998–1008
6. Castano AP, Mroz P, Hamblin MR (2006) Photodynamic therapy and anti-tumour immunity. *Nat Rev Cancer* 6:535–545
7. Chen WR, Huang Z, Korbely M, Nordquist RE, Liu H (2006) Photoimmunotherapy for cancer treatment. *J Environ Pathol Toxicol Oncol* 25:281–291
8. Diegelmann RF, Evans MC (2004) Wound healing: an overview of acute, fibrotic and delayed healing. *Front Biosci* 9:283–289
9. Dolmans DE, Fukumura D, Jain RK (2003) Photodynamic therapy for cancer. *Nat Rev Cancer* 3:380–387
10. Dougherty TJ, Gomer CJ, Henderson BW, Jori G, Kessel D, Korbely M, Moan J, Peng Q (1998) Photodynamic therapy. *J Natl Cancer Inst* 90:889–905
11. Elmets CA, Bowen KD (1986) Immunological suppression in mice treated with hematoporphyrin derivative photoradiation. *Cancer Res* 46:1608–1611
12. Felicetti P, Mennecozzi M, Barucca A, Montgomery S, Orlandi F, Manova K, Houghton AN, Gregor PD, Concetti A, Venanzi

- FM (2007) Tumor endothelial marker 8 enhances tumor immunity in conjunction with immunization against differentiation Ag. *Cytotherapy* 9:23–34
13. Gollnick SO, Owczarczak B, Maier P (2006) Photodynamic therapy and anti-tumor immunity. *Laser Surg Med* 38:509–515
 14. Gollnick SO, Vaughan L, Henderson BW (2002) Generation of effective antitumor vaccines using photodynamic therapy. *Cancer Res* 62:1604–1608
 15. Gross S, Gilead A, Scherz A, Neeman M, Salomon Y (2003) Monitoring photodynamic therapy of solid tumors online by BOLD-contrast MRI. *Nat Med* 9:1327–1331
 16. Habib Moinuddin S, Ali R (2006) Peroxynitrite modified DNA: a better antigen for systemic lupus erythematosus anti-DNA auto-antibodies. *Biotechnol Appl Biochem* 43(Pt 2):65–70
 17. Henderson BW, Gollnick SO, Snyder JW, Busch TM, Kousis PC, Cheney RT, Morgan J (2004) Choice of oxygen-conserving treatment regimen determines the inflammatory response and outcome of photodynamic therapy of tumors. *Cancer Res* 64:2120–2126
 18. Houghton AN, Guevara-Patino JA (2004) Immune recognition of self in immunity against cancer. *J Clin Invest* 114:468–471
 19. Jalili A, Makowski M, Switaj T, Nowis D, Wilczynski GM, Wilczek E, Chorazy-Massalska M, Radzikowska A, Maslinski W, Bialy L, Sienko J, Sieron A, Adamek M, Basak G, Mroz P, Krasnodebski IW, Jakobisiak M, Golab J (2004) Effective photoimmunotherapy of murine colon carcinoma induced by the combination of photodynamic therapy and dendritic cells. *Clin Cancer Res* 10:4498–4508
 20. Jolles CJ, Ott MJ, Straight RC, Lynch DH (1988) Systemic immunosuppression induced by peritoneal photodynamic therapy. *Am J Obstet Gynecol* 158:1446–1453
 21. Jung S, Unutmaz D, Wong P, Sano G, De los Santos K, Sparwasser T, Wu S, Vuthoori S, Ko K, Zavala F, Pamer EG, Littman DR, Lang RA (2002) In vivo depletion of CD11c(+) dendritic cells abrogates priming of CD8(+) T cells by exogenous cell-associated antigens. *Immunity* 17:211–220
 22. Kasprzyk PG, Song SU, Di Fiore PP, King CR (1992) Therapy of an animal model of human gastric cancer using a combination of anti-erbB-2 monoclonal antibodies. *Cancer Res* 52:2771–2776
 23. Korbelik M (2006) PDT-associated host response and its role in the therapy outcome. *Laser Surg Med* 38:500–508
 24. Korbelik M, Cecic I (1999) Contribution of myeloid and lymphoid host cells to the curative outcome of mouse sarcoma treatment by photodynamic therapy. *Cancer Lett* 137:91–98
 25. Korbelik M, Dougherty GJ (1999) Photodynamic therapy-mediated immune response against subcutaneous mouse tumors. *Cancer Res* 59:1941–1946
 26. Korbelik M, Sun J, Cecic I (2005) Photodynamic therapy-induced cell surface expression and release of heat shock proteins: relevance for tumor response. *Cancer Res* 65:1018–1026
 27. Koudinova NV, Pinthus JH, Brandis A, Brenner O, Bendel P, Ramon J, Eshhar Z, Scherz A, Salomon Y (2003) Photodynamic therapy with Pd-Bacteriopheophorbide (TOOKAD): successful in vivo treatment of human prostatic small cell carcinoma xenografts. *Int J Cancer* 104:782–789
 28. Kousis PC, Henderson BW, Maier PG, Gollnick SO (2007) Photodynamic therapy enhancement of antitumor immunity is regulated by neutrophils. *Cancer Res* 67:10501–10510
 29. Lynch DH, Haddad S, King VJ, Ott MJ, Straight RC, Jolles CJ (1989) Systemic immunosuppression induced by photodynamic therapy (PDT) is adoptively transferred by macrophages. *Photochem Photobiol* 49:453–458
 30. Macdonald IJ, Dougherty GJ (2001) Basic principles of photodynamic therapy. *J Porphyrins Phthalocyanines* 5:105–129
 31. Mazor O, Brandis A, Plaks V, Neumark E, Rosenbach-Belkin V, Salomon Y, Scherz A (2005) WST11, a novel water-soluble bacteriochlorophyll derivative; cellular uptake, pharmacokinetics, biodistribution and vascular-targeted photodynamic activity using melanoma tumors as a model. *Photochem Photobiol* 81:342–351
 32. Musser DA, Fiel RJ (1991) Cutaneous photosensitizing and immunosuppressive effects of a series of tumor localizing porphyrins. *Photochem Photobiol* 53:119–123
 33. Nowis D (2005) The influence of photodynamic therapy on the immune response. *Photodiagn Photodyn Ther* 2:283–298
 34. Ohmori H, Kanayama N (2005) Immunogenicity of an inflammation-associated product, tyrosine nitrated self-proteins. *Autoimmun Rev* 4:224–229
 35. Ohmori H, Oka M, Nishikawa Y, Shigemitsu H, Takeuchi M, Magari M, Kanayama N (2005) Immunogenicity of autologous IgG bearing the inflammation-associated marker 3-nitrotyrosine. *Immunol Lett* 96:47–54
 36. Preise D, Mazor O, Koudinova N, Liscovitch M, Scherz A, Salomon Y (2003) Bypass of tumor drug resistance by antivascular therapy. *Neoplasia* 5:475–480
 37. Ribas A, Timmerman JM, Butterfield LH, Economou JS (2003) Determinant spreading and tumor responses after peptide-based cancer immunotherapy. *Trends Immunol* 24:58–61
 38. Rodeberg DA, Erskine C, Celis E (2007) In vitro induction of immune responses to shared tumor-associated antigens in rhabdomyosarcoma. *J Pediatr Surg* 42:1396–1402
 39. Saji H, Song W, Furumoto K, Kato H, Engleman EG (2006) Systemic antitumor effect of intratumoral injection of dendritic cells in combination with local photodynamic therapy. *Clin Cancer Res* 12:2568–2574
 40. Sapozhnikov A, Fischer JA, Zaft T, Krauthgamer R, Dzionek A, Jung S (2007) Organ-dependent in vivo priming of naive CD4+, but not CD8+, T cells by plasmacytoid dendritic cells. *J Exp Med* 204:1923–1933
 41. Schreiber GJ, Hellstrom KE, Hellstrom I (1992) An unmodified anticarcinoma antibody, BR96, localizes to and inhibits the outgrowth of human tumors in nude mice. *Cancer Res* 52:3262–3266
 42. Sondel PM, Hank JA (2001) Antibody-directed, effector cell-mediated tumor destruction. *Hematol Oncol Clin North Am* 15:703–721
 43. St Croix B, Rago C, Velculescu V, Traverso G, Romans KE, Montgomery E, Lal A, Riggins GJ, Lengauer C, Vogelstein B, Kinzler KW (2000) Genes expressed in human tumor endothelium. *Science* 289:1197–1202
 44. Thong PS, Ong KW, Goh NS, Kho KW, Manivasager V, Bhuvanewari R, Olivo M, Soo KC (2007) Photodynamic-therapy-activated immune response against distant untreated tumours in recurrent angiosarcoma. *Lancet Oncol* 8:950–952
 45. Valamanesh F, Berdugo M, Bejjani RA, Savoldelli M, Jeanny JC, Brun PH, Blanc D, Ben-Ezra D, Behar-Cohen F (2004) Reduced collateral effect of photodynamic therapy (PDT) using a new water soluble photosensitizing agent. *Investr Ophthalmol Vis Sci* 45:
 46. van Beijnum JR, Dings RP, van der Linden E, Zwaans BM, Ramaekers FC, Mayo KH, Griffioen AW (2006) Gene expression of tumor angiogenesis dissected: specific targeting of colon cancer angiogenic vasculature. *Blood* 108:2339–2348
 47. van Duijnhoven FH, Tollenaar RA, Terpstra OT, Kuppen PJ (2005) Locoregional therapies of liver metastases in a rat CC531 coloncarcinoma model results in increased resistance to tumour rechallenge. *Clin Exp Metastasis* 22:247–253
 48. Wuttge DM, Bruzelius M, Stemme S (1999) T-cell recognition of lipid peroxidation products breaks tolerance to self proteins. *Immunology* 98:273–279
 49. Yla-Herttuala S, Palinski W, Butler SW, Picard S, Steinberg D, Witztum JL (1994) Rabbit and human atherosclerotic lesions

- contain IgG that recognizes epitopes of oxidized LDL. *Arterioscler Thromb* 14:32–40
50. Yusuf N, Katiyar SK, Elmets CA (2008) The immunosuppressive effects of phthalocyanine photodynamic therapy in mice are mediated by CD4(+) and CD8(+) T cells and can be adoptively transferred to naive recipients. *Photochem Photobiol* 84:366–370
51. Zilberstein J, Schreiber S, Bloemers MC, Bendel P, Neeman M, Schechtman E, Kohen F, Scherz A, Salomon Y (2001) Antivascular treatment of solid melanoma tumors with bacteriochlorophyll-serine-based photodynamic therapy. *Photochem Photobiol* 73:257–266