Priority Brief

Cancer Immunology Research

Photodynamic-Immune Checkpoint Therapy Eradicates Local and Distant Tumors by CD8$^+$ T Cells

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Abstract

Photodynamic therapy (PDT) is a clinically applied tumor ablation method that reduces tumor burden and may induce T-cell responses, providing a therapeutic option for mutated tumors. In this study, we applied PDT in two mouse tumor models and assessed its effect on outgrowth of PDT-treated and distant untreated tumors. PDT of established tumors resulted in complete tumor eradication in most mice, which were then protected against tumor rechallenge. Correspondingly, the therapeutic effect was abrogated upon systemic depletion of CD8$^+$ T cells, indicating PDT-induced tumor antigen cross-presentation and T-cell activation. In a double-tumor model, PDT of primary tumors induced enhanced infiltration of untreated distant tumors by CD8$^+$ T cells, which significantly delayed their outgrowth. Combination therapy of PDT and CTLA-4–blocking antibodies significantly improved therapeutic efficacy and survival of double-tumor–bearing mice. These results show that local tumor ablation by PDT induces CD8$^+$ T-cell responses crucial for systemic tumor eradication, which can be further enhanced by combination with immune checkpoint blockade. This combination of two clinically applied therapies may be a treatment strategy for advanced cancer without previous knowledge of tumor-specific antigens. *Cancer Immunol Res; 5(10); 832–8. ©2017 AACR.*

Introduction

Clinically apparent cancers often evade immune eradication and progress, despite the presence of antitumor T-cell responses (1). Immune evasion of tumors can be achieved through the formation of an immunosuppressive tumor microenvironment, including chronic exposure of T cells to cognate antigen. Based on the expression of immune checkpoint molecules such as PD-1 and CTLA-4 on functionally impaired tumor-infiltrating T cells, preclinical and clinical studies using PD-1 or CTLA-4–blocking antibodies have shown impressive results (2). However, there is considerable variation in individual responsiveness to immune checkpoint blockade. Some patients show durable tumor regression, whereas others fail to respond to therapy (3–5). Combination strategies may improve clinical outcome, either by blocking multiple immune checkpoints or by combining immunotherapy with tumor-ablating therapies such as chemotherapy, radiotherapy, or photodynamic therapy (PDT; refs. 6, 7). PDT is a strongly localized and nonmutagenic method of tumor-cell killing, minimizing adverse effects of therapy (8). PDT for cancer consists of localized activation of a light-sensitive photosensitizer by exposure of the tumor to visible light. Besides merely reducing tumor burden, the resulting massive tumor-cell death triggers strong acute inflammation involving the influx of neutrophils and macrophages (9). Dying tumor cells can serve as a source of tumor antigen and immunogenic factors able to induce or enhance tumor-specific T-cell responses, which may strongly enhance the therapeutic effect (10, 11). In a previous study, we showed that combination of PDT and specific peptide vaccination induced CD8$^+$ T-cell responses against tumor antigens resulting in the clearance of both treated and distant untreated tumors (12). Therefore, the therapeutic potential of PDT may be superior in immunogenic tumor models in which tumor-specific T cells are present but unable to clear the tumor, mimicking a common clinical situation. In this study, we apply Bremachlorin-based PDT to MC38 and CT26 tumors, two mutated mouse tumors syngeneic to the C57BL/6 and BALB/c mouse strains, respectively. PDT treatment of established tumors resulted in complete tumor clearance dependent on CD8$^+$ T cells, which could also control outgrowth of distant untreated tumors. In a double-tumor setting, PDT of primary tumors combined with systemic CTLA-4 blockade significantly reduced the tumor burden in both MC38 and CT26 tumor models. Our findings show the immunogenic and therapeutic potential of PDT in mutated tumors and a potent combination treatment for advanced cancer.

Materials and Methods

Mice and tumor cell lines

C57BL/6 mice were obtained from Harlan Laboratories—ENVIGO (the Netherlands), and BALB/c mice were purchased

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from Charles River (France) and housed under specified pathogen-free conditions in the animal facility of the Leiden University Medical Center. All animal experimentations were approved by and according to guidelines of the Dutch Animal Ethical Committee. MC38 and CT26 cells (kindly provided by Mario Colombo) were cultured as described elsewhere (13). Cell lines were mycoplasma and MAP-tested before the start of experiments, and cultured for 2 weeks (3–5 passages) before inoculation. For tumor inoculation, 5×10^5 tumor cells in 100 μL PBS were injected subcutaneously in the right flank or both flanks of the mice. In double-tumor experiments, the largest tumor on day 8 was designated primary tumor. Tumor volume was measured 3 times per week by caliper and calculated as length × width × height. Survival curves are based on the moment of sacrificing the mice upon reaching the maximally allowed tumor volume of 2,000 mm^3.

PDT

Tumors were treated 8 days after inoculation at an average tumor diameter of 5 mm. PDT was performed as described previously (12). In short, Bremachlorin photosensitizer (20 mg/kg; RadaPharma International) was injected intravenously. After 6 hours, the tumors were irradiated for 1,000 seconds at 116 mW/cm^2 (total energy 116 J/cm^2) using a 662 nm Milon Lakhta laser.

Anti–CTLA-4 antibody treatment

Antagonistic CTLA-4–blocking antibody (clone 9D9; BioXCell) was administered intraperitoneally on days 7, 10, and 14 after tumor inoculation, using 200 μg dissolved in 200 μL PBS per treatment.

T-cell depletion

Depleting CD8 antibody (clone 2.43) and depleting CD4 antibody (clone GK1.5) were produced in-house using hybridomas. To deplete T cells, mice received an intraperitoneal injection of 50 μg depleting antibody the day before treatment, followed by additional injections of 50 μg antibody when periodical screening (every 5 days from day 10 after initial injection) showed return of the targeted T-cell population in systemic blood. All control mice received similar amounts of isotype control rat immunoglobulin G in parallel.

Flow cytometry

Ex vivo tumor analysis was performed as described elsewhere (13). Tumors were harvested 6 days after PDT, incubated with Liberase TL (Roche), cut into small pieces, and mashed on 70 μm cell strainers (BD) to create single-cell suspensions. Cells were then stained with the viability dye 7-AAD (Invitrogen) and the cell surface markers CD45.2 (clone 104; BioLegend), CD3ε (clone 145-2C11; BioLegend), CD8α (clone 53-6.7; BioLegend), CD69 (clone H1.2F3; BioLegend), and 4-1BB (clone 1AH2; BD), and measured on a LSRII cytometer (BD) analyzed with FlowJo software (Tree Star).

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 6.0 software. Data are shown as the mean ± SEM for each group, and comparison of groups was performed by two-tailed Student *t* test or Mann–Whitney *U* test depending on normality of data distribution. Survival curves were compared using the Log-rank Mantel–Cox test. Statistical differences were considered significant at $P < 0.05$.

Results

Tumor ablation by PDT depended on $CD8^+$ T cells

We applied PDT using the photosensitizer Bremachlorin to C57BL/6 mice bearing established subcutaneous MC38 tumors and followed tumor outgrowth. Whereas tumors in untreated mice grew progressively, a single PDT treatment caused strong and durable tumor regression in all mice (Fig. 1A, solid lines). In less immunogenic models, PDT was only effective when combined with specific induction of antitumor $CD8^+$ T cells (12). Therefore, we analyzed whether the clearance of MC38 tumors by PDT monotherapy was mediated by $CD8^+$ T cells by depleting them systemically. In the absence of $CD8^+$ T cells, initial tumor regression upon PDT remained intact, but tumors eventually grew out. This suggests that tumor ablation by PDT works independently of $CD8^+$ T cells, whereas $CD8^+$ T cells are crucial in subsequent tumor clearance and prevention of tumor regrowth (Fig. 1A and Supplementary Fig. S1). Consequently, PDT treatment cleared MC38 tumors in the majority of mice, resulting in significantly improved long-term survival, which was fully abrogated when $CD8^+$ T cells were depleted (Fig. 1B). Untreated tumors also grew faster in the absence of $CD8^+$ T cells, suggesting that growth control of untreated tumors is mediated by $CD8^+$ T cells. Conversely, depletion of $CD4^+$ T cells resulted in slower growth of untreated tumors and enhanced clearance of PDT-treated tumors (Supplementary Fig. S1), which may suggest a suppressive role of $CD4^+$ regulatory T cells. All PDT-treated tumor-free mice were protected against developing new tumors when new MC38 tumor cells were injected in the contralateral flank 50 days after tumor clearance, suggesting the formation of immunological memory (Supplementary Fig. S2). In summary, a single PDT treatment could fully eradicate established tumors involving $CD8^+$ T-cell responses.

Local PDT-induced systemic T-cell responses inhibiting distant tumor growth

The induction of $CD8^+$ T-cell responses and their involvement in tumor clearance suggested that T cells may circulate systemically and target untreated tumors growing at distant sites. We inoculated mice with MC38 tumor cells in both flanks and treated the largest tumor with PDT, following the outgrowth of both tumors over time. Also, in the double-tumor setting, PDT-treated tumors regressed and were cleared (Fig. 2A). Untreated tumors grew significantly slower if the contralateral tumor received PDT treatment, an effect that was completely abrogated when $CD8^+$ T cells were systemically depleted (Fig. 2B). Individual tumor outgrowth curves are shown in Supplementary Fig. S3A–S3D. Local PDT caused clearance of treated tumors and delayed the growth of distant tumors, dramatically prolonging survival of double-tumor–bearing mice (Fig. 2C). Analysis of distant tumors 6 days after PDT of contralateral primary tumors showed an increased infiltration of activated $CD8^+$ T cells compared with untreated mice, suggesting that $CD8^+$ T cells directly mediate the abscopal effect of local PDT

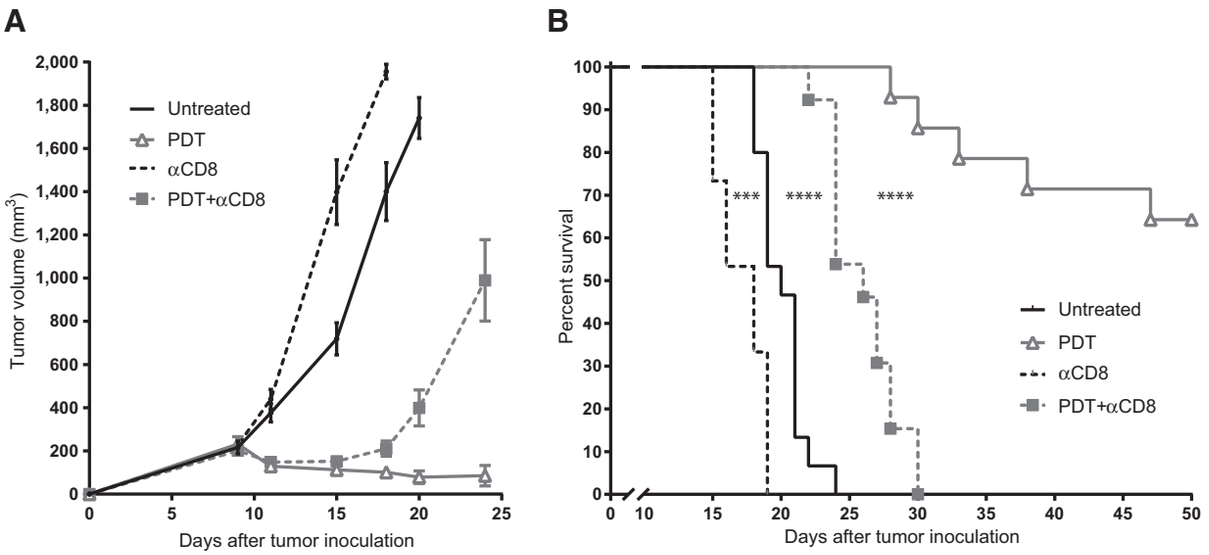

Figure 1.

Tumor ablation by PDT depends on $CD8^+$ T cells. Tumor outgrowth curves (**A**) and survival curves (**B**) of mice bearing subcutaneous MC38 tumors treated by PDT in the presence or absence of $CD8^+$ T cells, plus corresponding control groups. PDT was given on day 8 by injection of Bremachlorin photosensitizer followed by tumor illumination 6 hours later. $CD8^+$ T cells were depleted by antibodies injected periodically from day 7 until mice were sacrificed or tumor-free. Survival is defined by the time until tumor size reached the maximally allowed volume of 2,000 mm^3 according to local legislation. For survival curves, the log-rank test was used to determine significance: ***, $P < 0.001$ and ****, $P < 0.0001$. Pooled data of 2 independent experiments, 13 to 15 mice per group.

(Supplementary Fig. S3E and S3F). Thus, these data indicate that local PDT triggers a $CD8^+$ T-cell–dependent effect on untreated distant tumors.

Combined local PDT and systemic CTLA-4 blockade in double-tumor models

Local PDT treatment slowed down the growth of distant tumors via $CD8^+$ T cells but did not fully clear them. We, therefore, analyzed whether enhancing the PDT-induced $CD8^+$ T-cell response by immune checkpoint blockade would enable double-tumor eradication. We treated double MC38 tumor–bearing mice with PDT of one tumor and provided systemic CTLA-4 blockade during the treatment phase. Whereas local PDT affected the primary tumor more strongly than the untreated secondary tumor, systemic CTLA-4 blockade caused a more pronounced growth delay of the smaller secondary tumors (Fig. 3A and B). Combination treatment with PDT and CTLA-4 blockade combined the strong respective effects of each treatment on both tumors and reduced total tumor burden compared with either monotherapy (Fig. 3C). We also analyzed the combination of PDT and CTLA-4 blockade in the more aggressive CT26 tumor model in BALB/c mice. Both PDT and CTLA-4 monotherapies were less efficient in delaying the growth of primary or secondary CT26 tumors compared with their effects on MC38 tumors. However, combination treatment significantly reduced CT26 tumor burden compared with either single treatment (Fig. 3D–F). Comparison of the effects of each treatment on primary and secondary MC38 or CT26 tumors is provided in Supplementary Fig. S4. This treatment strategy provides an efficient combination of local tumor-destructive therapy with systemic immunomodulation in two independent tumor models.

Long-term survival after combined PDT and CTLA-4 blockade depended on $CD8^+$ T cells

Both PDT and CTLA-4 blockade as monotherapies significantly reduced MC38 tumor burden and increased survival time of double MC38 tumor–bearing mice (Fig. 4A). The significantly lower tumor burden after combined treatment by PDT and CTLA-4 blockade resulted in a significantly further extended survival of all mice and clearance of both tumors in 20% of the mice (Fig. 4A). A depletion experiment of $CD8^+$ T cells showed that the enhanced efficacy of combined PDT and CTLA-4 blockade is dependent on the systemic presence of $CD8^+$ T cells, as the combined treatment effect is fully lost in the absence of $CD8^+$ T cells, reducing survival to the level of untreated mice (Fig. 4B).

Discussion

In this study, we show that PDT of mouse colon carcinoma tumors mediated strong tumor ablation and eradication by $CD8^+$ T cells, which also delayed distant tumor growth. This provides evidence that local tumor ablation can lead to systemically active T-cell responses, likely by enhanced cross-presentation of tumor antigens by local dendritic cells and the immunostimulatory effects of PDT-induced cell death (10). Our data add to a growing body of evidence that local tumor destruction can delay the growth of identical tumors growing in other sites of the body, and stress the induction of systemic immune responses as the crucial

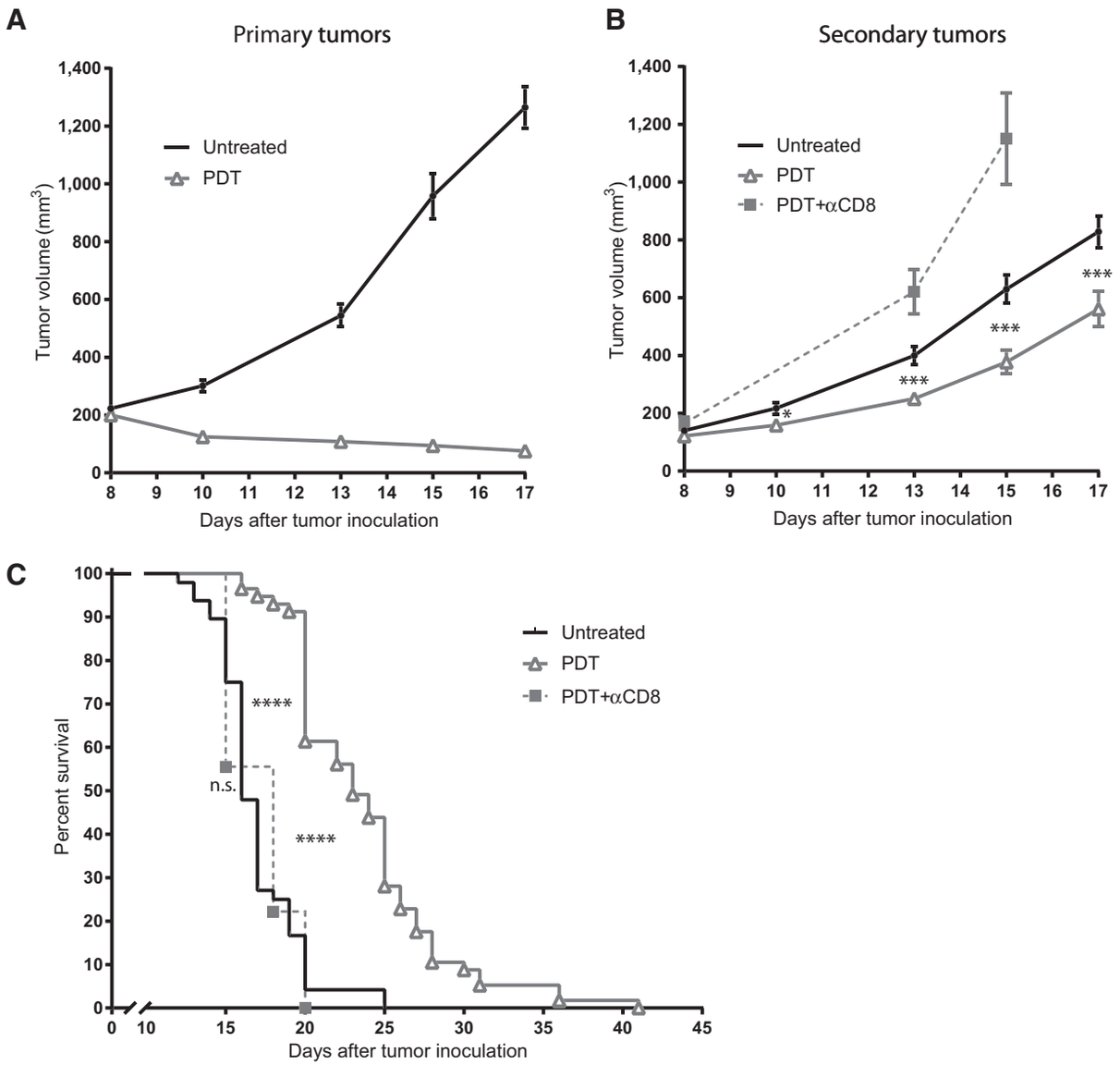

Figure 2.

Local PDT induces systemic T-cell responses inhibiting distant tumor growth. Primary (**A**) and secondary (**B**) tumor outgrowth curves and (**C**) survival curves of double MC38 tumor–bearing mice in which the primary tumor was left untreated or PDT-treated in the presence or absence of $CD8^+$ T cells. PDT was given on day 8 by injection of Bremachlorin photosensitizer followed by tumor illumination 6 hours later. $CD8^+$ T cells were depleted by antibodies injected periodically from day 7 until mice were sacrificed or tumor-free. Survival is defined by the time until tumor size reached the maximally allowed volume of 2,000 mm^3 according to local legislation. The log-rank test was used to determine significance. Statistical significance of differences in secondary tumor volume (**B**) of untreated vs. PDT-treated mice was determined by *t* test (days 10 and 15) or Mann–Whitney *U* test (days 13 and 17). Statistical significance of survival differences (**C**) was determined by the log-rank test. n.s., not significant; *, $P < 0.05$ and ***, $P < 0.0001$. Pooled data of 6 independent experiments, 48–57 mice per group.

mechanism. These tumor-specific systemic effects of local therapy, also known as the abscopal effect, have been described in several localized ablation therapies (14, 15). The advent of modern immunomodulatory antibodies has triggered a range of protocols combining local tumor ablation with immune checkpoint blockade (16–18). A study combining local radiotherapy with immunomodulatory antibodies indicated that the enhanced therapeutic efficacy was mediated by tumor antigen cross-presentation by dendritic cells to $CD8^+$ T cells (19). Enhanced systemic efficacy of PDT combined with immune checkpoint blockade has been reported using experimental setups involving surgical resection of PDT-treated tumors or advanced nanocarrier systems (20–22). Here, we combined local PDT with systemic CTLA-4 blockade in two independent tumor models to improve the therapeutic outcome in double-tumor–bearing mice. The efficacy of

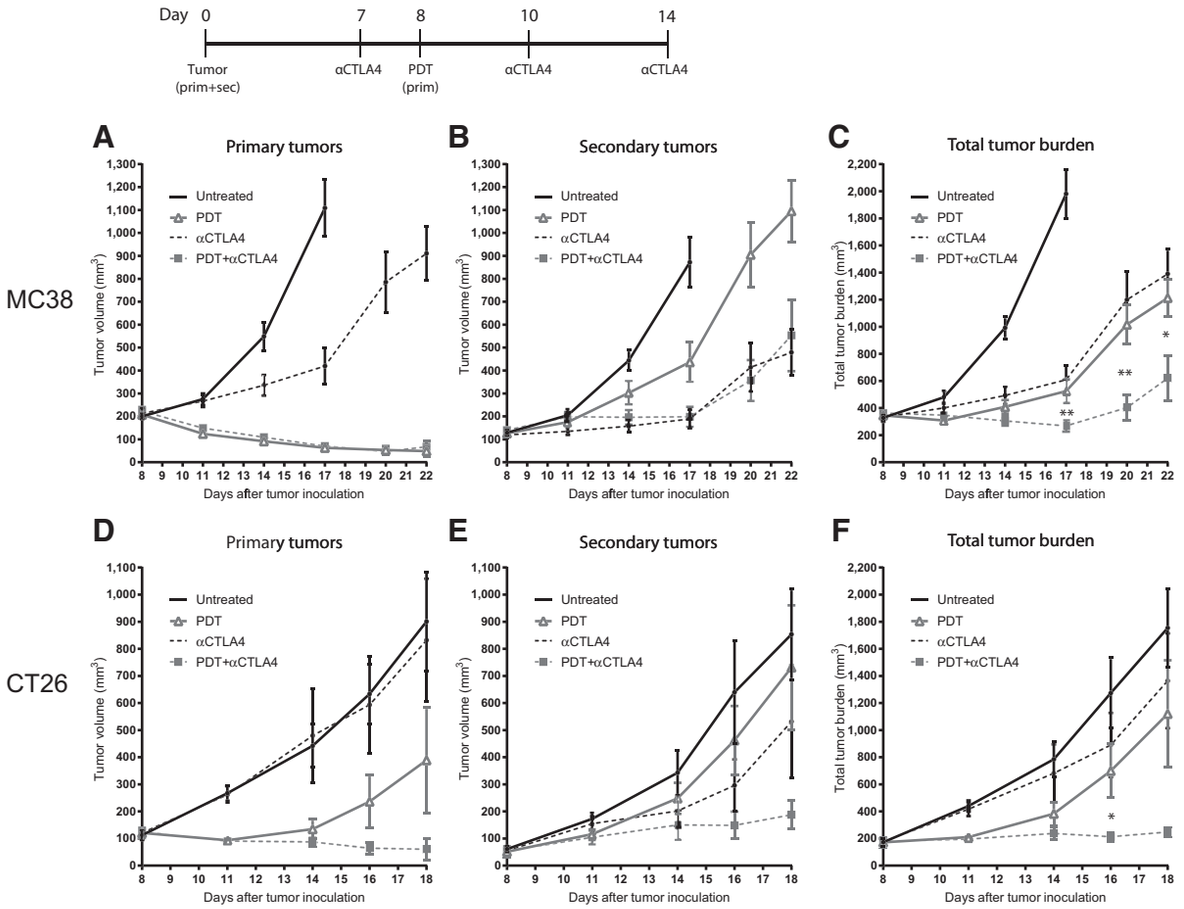

Figure 3.

Efficient treatment of local and distant tumors by combined local PDT and systemic CTLA-4 blockade. Tumor growth curves. Primary tumors (**A**), secondary tumors (**B**), and total tumor (**C**) burden for mice bearing two MC38 tumors. Primary tumors (**D**), secondary tumors (**E**), and total tumor burden (**F**) for mice bearing two CT26 tumors. Mice received either PDT of primary tumors on day 8, systemic CTLA-4–blocking antibody on days 7, 10, and 14, both therapies, or were left untreated. Mann–Whitney U test (MC38 model) and *t* test (CT26 model) were used to determine statistical significance of differences in total tumor burden of mice receiving PDT+αCTLA-4 combination therapy compared with PDT or αCTLA-4 monotherapy. *, $P < 0.05$ and **, $P < 0.01$. Pooled data of 2 independent experiments, 14–16 mice per group.

immunomodulatory antibodies such as CTLA-4 blockade is often largely determined by tumor size at the start of treatment. In our double-tumor model, CTLA-4 blockade indeed affected smaller secondary tumors more strongly than bigger primary tumors, whereas PDT obviously affected the PDT-treated tumor more strongly. The increased efficacy of combination therapy in our double-tumor experiments may, therefore, be explained by the combining the strengths of each individual treatment. CTLA-4 blockade specifically depletes tumor-infiltrating regulatory T cells in several tumor models, including MC38 and CT26, favoring the subsequent expansion of intratumoral effector $CD8^+$ T cells (23, 24). In a preclinical study using depletion of regulatory T cells by low-dose cyclophosphamide in the context of PDT treatment, increased antitumor immune responses were observed (25). These findings suggest a suppressive role of regulatory T cells that dampen the PDT-induced T-cell responses that may further explain the superior efficacy of combined PDT and CTLA-4 blockade as described in this study. Combination therapy of PDT and specific peptide vaccination enhances PDT-induced T-cell responses, allowing eradication of local and distant tumors (12). Combinations of PDT with specific immunotherapy allow efficient treatment of tumors of which the antigenic profile is known, such as human papillomavirus (HPV)–induced gynecological and head/neck tumors expressing known HPV antigens. Here, we introduced combination therapy of PDT and CTLA-4 blockade as a more broadly applicable therapeutic option without the need to identify the antigens expressed by the tumor. PDT is already clinically applied in the treatment of various tumors, including HPV-induced cancer, skin tumors, and gastrointestinal malignancies. CTLA-4 blockade has been approved for use in melanoma, and clinical trials for several other types of human cancer are underway, based on promising results in preclinical studies. Combinations of PDT and CTLA-4 blockade can, therefore, be smoothly introduced into clinical practice and may be applied to a wide variety of human cancer types.

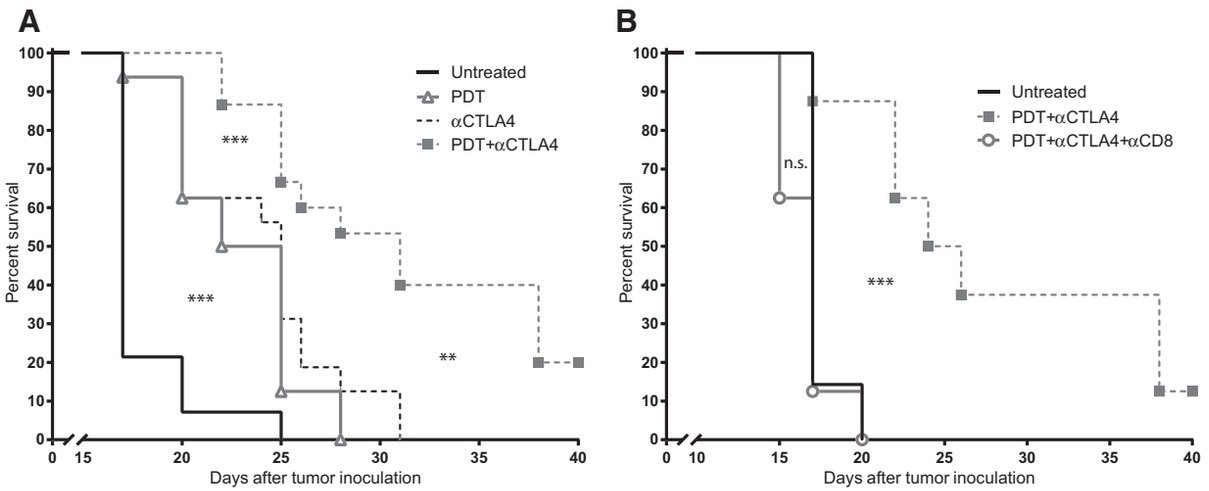

Figure 4.

Long-term survival after combined PDT and CTLA-4 blockade depends on $CD8^+$ T cells. **A,** Survival curves of double MC38 tumor–bearing mice receiving either PDT of primary tumors on day 8, systemic CTLA-4–blocking antibody on days 7, 10, and 14, both therapies, or left untreated. **B,** Survival curves of double MC38 tumor–bearing mice left untreated or receiving PDT+αCTLA-4 combination therapy with or without $CD8^+$ T-cell depletion on day 7. Survival is defined by the time until tumor size reached the maximally allowed volume of 2,000 mm^3 according to local legislation. The log-rank test was used to determine significance. **, $P < 0.01$ and ***, $P < 0.001$. Pooled data of 2 independent experiments, 14–16 mice per group.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Development of methodology: J.W. Kleinovink, M.F. Fransen, F. Ossendorp

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.W. Kleinovink, M.F. Fransen

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J.W. Kleinovink, M.F. Fransen

Writing, review, and/or revision of the manuscript: J.W. Kleinovink, M.F. Fransen, C.W. Löwik, F. Ossendorp

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J.W. Kleinovink

Study supervision: F. Ossendorp

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