The use of light in cancer immunotherapy

Kleinovink, E.J.W.

Chapter 3

Photodynamic-immune checkpoint therapy eradicates local and distant tumors by CD8 T cells

Jan Willem Kleinovink, Marieke F. Fransen, Clemens W. Löwik, Ferry Ossendorp

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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, forming an attractive 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 the majority of mice, which were then protected against tumor re-challenge. 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 novel treatment strategy for advanced cancer without previous knowledge of tumor-specific antigens.

Introduction

Clinically apparent cancers often evade immune eradication and progress despite the presence of anti-tumor T cell responses (1). Immune evasion of tumors can be achieved through the formation of 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, as some patients show durable tumor regression while 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) (6,7). PDT is an attractive approach due to its strongly localized and non-mutagenic nature of tumor cell killing, minimizing adverse effects of therapy (8). PDT of 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). Moreover, dying tumor cells can serve as 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 curative potential 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 curative 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 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 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. For tumor inoculation, 500,000 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 2000 mm3.

Photodynamic Therapy

Tumors were treated 8 days after inoculation, both at an average tumor diameter of 5 mm. Photodynamic Therapy was performed as described previously (12). In short, 20 mg/kg Bremachlorin photosensitizer (RadaPharma International) was injected intravenously, followed after 6 hours by irradiation of the tumor for 1000 seconds at 116 mW/cm² (total energy 116 J/cm²) 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 antibodies the day before treatment, followed by additional injections of 50 µg antibody when periodical screening showed return of the targeted T cell population in systemic blood. All control mice received in parallel similar amounts of isotype control rat immunoglobulin G.

Flow cytometry

Ex vivo tumor analysis was performed as described elsewhere (13). In short, single-cell suspensions of harvested tumors were stained with cell-surface markers, 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's t-test or Mann-Whitney U test depending on normality of data distribution. Survival curves were compared using the LogRank Mantel-Cox test. Statistical differences were considered significant at $p < 0.05$.

Results

Curative tumor ablation by Photodynamic Therapy (PDT) depends on CD8 T cells

We applied PDT using the photosensitizer Bremachlorin to C57BL/6 mice bearing established subcutaneous MC38 tumors and followed tumor outgrowth. Strikingly, whereas tumors in untreated mice grew out progressively, a single PDT treatment caused strong and durable tumor regression in all mice (**Figure 1a**, solid lines). As we previously observed in less immunogenic models that PDT was only curative when combined with specific induction of anti-tumor CD8 T cells, we analyzed whether the clearance of MC38 tumors by PDT monotherapy was mediated by CD8 T cells by injecting antibodies depleting CD8 T cells systemically (12). 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 (**Figure 1a** and **Supplementary Figure S1**).

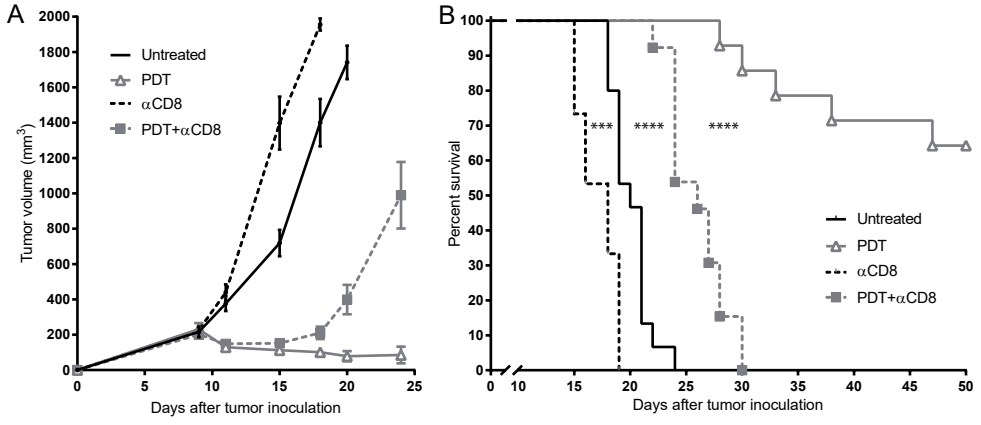

Figure 1. Curative tumor ablation by Photodynamic Therapy depends on CD8 T cells. Tumor outgrowth curves **(A)** and survival curves **(B)** of mice bearing subcutaneous MC38 tumors treated by Photodynamic Therapy (PDT) in the presence or absence of CD8 T cells, plus corresponding control groups. PDT was given on day 8 by injection of Bremachlorin photosensitizer followed after 6 hours by tumor illumination. 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 2000 mm³ according to local legislation. Survival differences were statistically significant by Log-Rank test: Untreated vs PDT $p<0.0001$, PDT vs PDT+αCD8 $p<0.0001$, Untreated vs αCD8 $p<0.001$, Untreated vs PDT+αCD8 $p<0.0001$. Pooled data of 2 independent experiments, 13-15 mice per group.

Consequently, PDT treatment cured the majority of mice bearing MC38 tumors resulting in significantly improved long-term survival, which was fully abrogated when CD8 T cells were depleted (**Figure 1b**). Interestingly, untreated tumors also grew out faster in the absence of CD8 T cells, suggesting that growth control of untreated tumors is mediated by CD8 T cells. Instead, depletion of CD4 T cells resulted in slower growth of untreated tumors and enhanced clearance of PDT-treated tumors, suggesting a suppressive role of CD4+ regulatory T cells (**Supplementary Figure S1**). All cured mice were protected against developing new tumors when new MC38 tumor cells were injected in the contralateral flank long after tumor clearance, suggesting the formation of immunological memory (**Supplementary Figure S2**). In summary, a single PDT treatment can fully eradicate established tumors involving CD8 T cell responses.

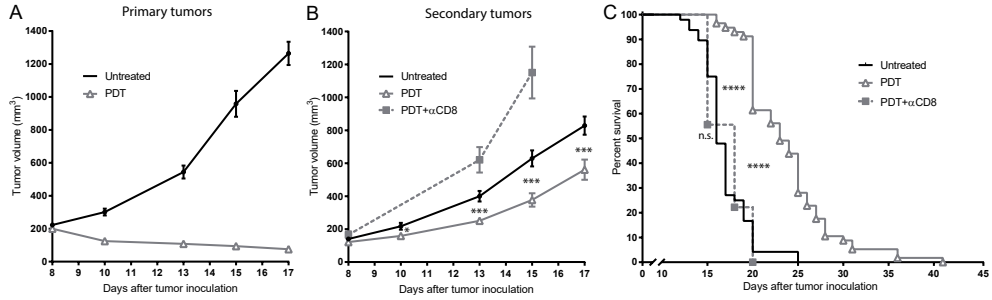

Figure 2. Local PDT induces systemic T cell responses inhibiting distant tumor growth. Primary **(A)** and secondary **(B)** tumor outgrowth curves and **(C)** survival curves of double MC38 tumor-bearing mice in which the primary tumor was left untreated or PDT-treated in the presence or absence of CD8 T cells. PDT was given on day 8 by injection of Bremachlorin photosensitizer followed after 6 hours by tumor illumination. 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 mm³ according to local legislation. 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.

Local PDT induces systemic T cell responses inhibiting distant tumor growth

The induction of CD8 T cell responses and their involvement in tumor clearance suggests 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 in time. Also in the double tumor setting, PDT-treated tumors regressed and were cleared (**Figure 2a**). Moreover, 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 (**Figure 2b**). Individual tumor outgrowth curves are shown in Supplementary Figure S3a-d. Altogether, local PDT caused clearance of treated tumors and delayed the growth of distant tumors, dramatically prolonging survival of double tumor-bearing mice (**Figure 2c**). Analysis of distant tumors 6 days after PDT of contralateral primary tumors showed an increased infiltration

of activated CD8 T cells compared to untreated mice, suggesting that CD8 T cells directly mediate the abscopal effect of local PDT (**Supplementary Figure S3e,f**). Together, these data indicate that local curative PDT triggers a CD8 T cell-dependent effect on untreated distant tumors.

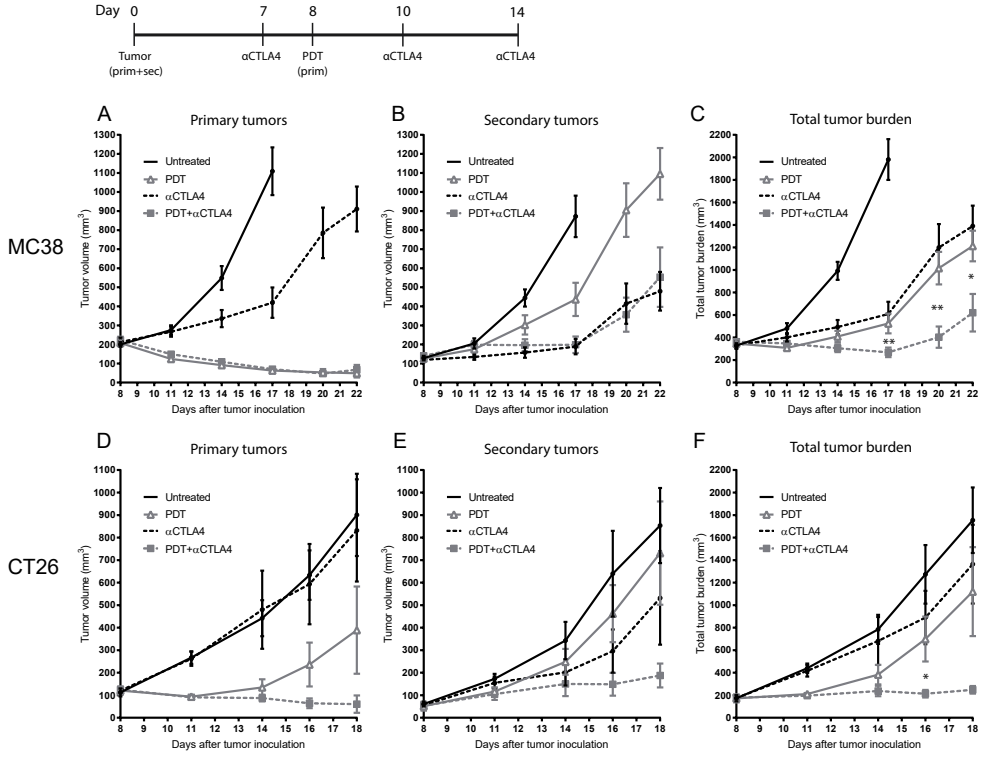

Figure 3. Efficient treatment of local and distant tumors by combined local PDT and systemic CTLA-4 blockade. Tumor growth curves. Primary tumors **(A)**, secondary tumors **(B)**, and total tumor **(C)** burden for mice bearing two MC38 tumors. Primary tumors **(D)**, secondary tumors **(E)**, and total tumor burden **(F)** for mice bearing two CT26 tumors. Mice received PDT of primary tumors on day 8, systemic CTLA-4 blocking antibody on days 7+10+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+aCTLA-4 combination therapy compared with PDT or aCTLA-4 monotherapy. * $p<0.05$ and ** $p<0.01$. Pooled data of 2 independent experiments, 14–16 mice per group.

Efficient treatment of local and distant tumors by combined local PDT and systemic CTLA-4 blockade

As local PDT treatment slowed down the growth of distant tumors via CD8 T cells but did not fully clear them, we analyzed whether enhancing the PDT-induced CD8 T cell response by immune checkpoint blockade would enable double tumor eradication. Therefore, 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 again affected the treated primary tumor much more strongly than the untreated secondary tumor, systemic CTLA-4 blockade caused a much

more pronounced growth delay of the smaller secondary tumors (**Figure 3a,b**). Combination treatment with PDT and CTLA-4 blockade combined the strong respective effects of each treatment on both tumors, and significantly reduced total tumor burden compared to either monotherapy (**Figure 3c**). Next, we also analyzed combination of PDT and CTLA-4 blockade in the more aggressively growing 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 to their effects on MC38 tumors. However, combination treatment significantly reduced CT26 tumor burden compared to either single treatment (**Figure 3d-f**). A comparison of the effects of each treatment on primary and secondary MC38 or CT26 tumors is provided in Supplementary Figure 4. This treatment strategy provides 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 depends 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 (**Figure 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 (**Figure 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 (**Figure 4b**).

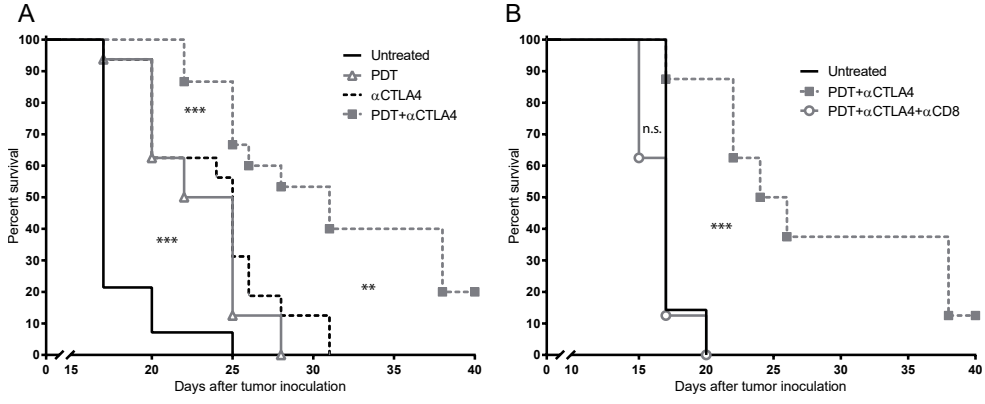

Figure 4. Long-term survival after combined PDT and CTLA-4 blockade depends on CD8 T cells. Long-term survival after combined PDT and CTLA-4 blockade depends on CD8 T cells. **(A)** Survival curves of double MC38 tumor–bearing mice receiving either PDT of primary tumors on day 8, systemic CTLA-4 blocking antibody on days 7+10+14, both therapies, or left untreated. **(B)** Survival curves of double MC38 tumor–bearing mice left untreated or receiving PDTþaCTLA-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 mm³ 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.

Discussion

In this study, we show that Photodynamic Therapy (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 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). Recent studies reported enhanced systemic efficacy of PDT combined with immune checkpoint blockade 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 immunomodulatory antibodies such as CTLA-4 blockade is often largely determined by tumor size at the start of treatment. In our double-tumor models, CTLA-4 blockade indeed affected smaller secondary tumors more strongly than the bigger primary tumors, while 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. In addition, CTLA4 blockade has been shown to specifically deplete 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 anti-tumor immune responses were observed (25). These findings, suggesting a suppressive role of regulatory T cells that dampen the PDT-induced T cell response, may further explain the superior efficacy of combined PDT and CTLA4 blockade as described in this study. We have previously reported on combination therapy of PDT and specific peptide vaccination, in which PDT-induced T cell responses were further enhanced by peptide vaccination, 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 introduce combination therapy of PDT and CTLA-4 blockade as a more broadly applicable therapeutic option without the need to have identified the antigens expressed by the tumor. Photodynamic therapy 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 undergoes clinical trials for several other types of human cancer, 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.

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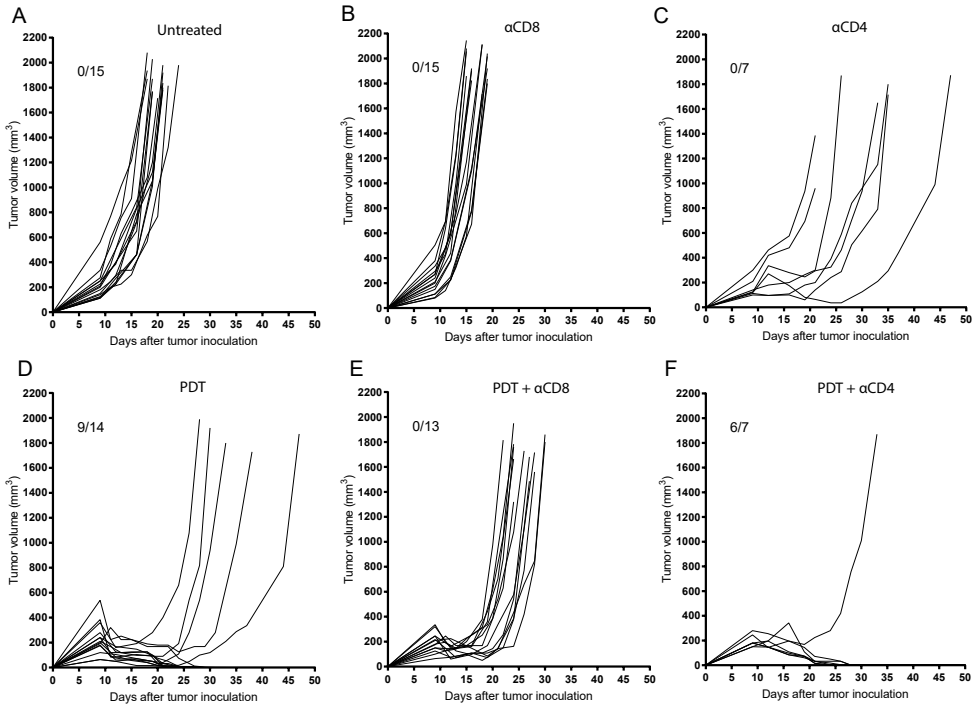
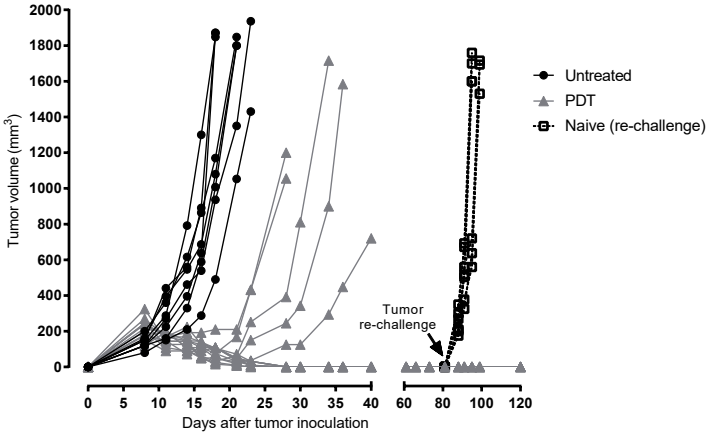
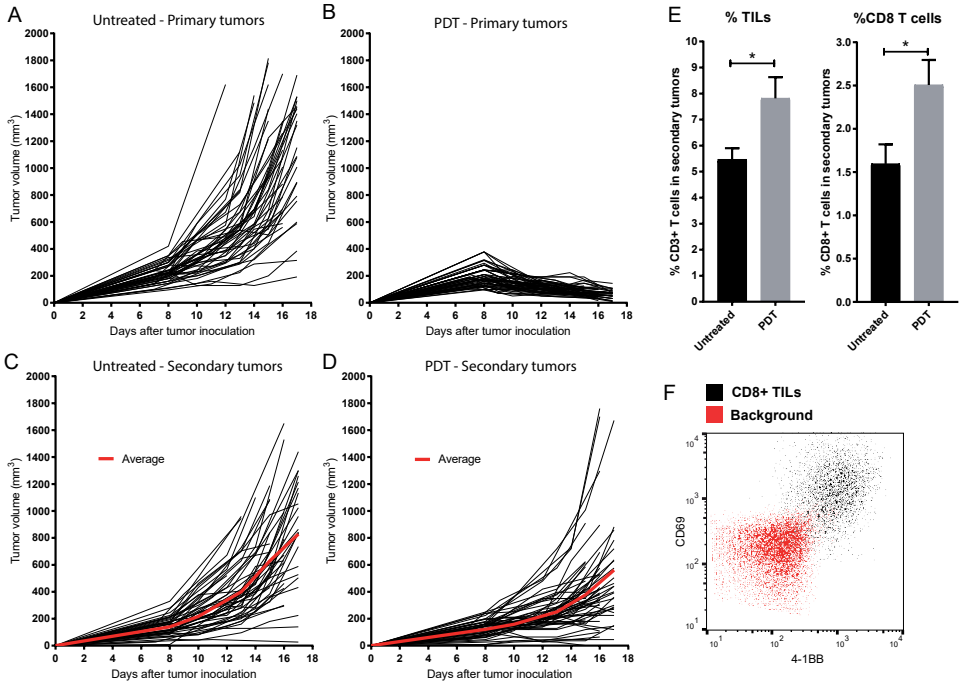
Supplementary Information



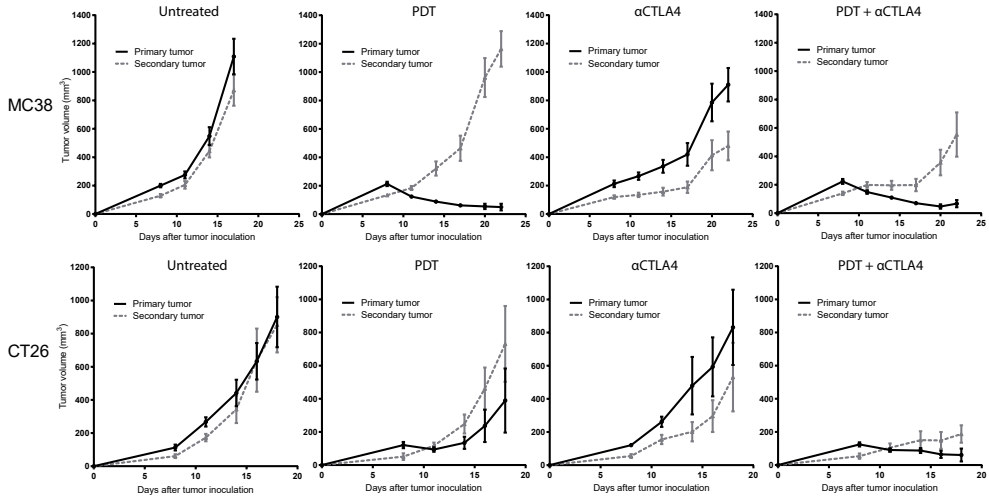
Supplementary Figure S1. Curative tumor ablation by Photodynamic Therapy depends on CD8 T cells. Tumor outgrowth curves of individual mice bearing subcutaneous MC38 tumors treated by Photodynamic Therapy (PDT) in the presence of T cells or with CD8 or CD4 T cell depletion (αCD8, αCD4). PDT was given on day 8 by injection of Bremachlorin photosensitizer followed after 6 hours by tumor illumination. T cells were depleted by antibodies injection on day 7 to assure complete absence of T cells until mice were sacrificed or tumor-free. The number of cured mice is indicated by fractions.



Supplementary Figure S2. Mice cured by PDT are protected against tumor rechallenge. Tumor outgrowth curves of untreated and PDT-treated MC38 tumor-bearing mice. PDT-cured mice (8 out of 13) and naïve control mice were injected with new MC38 tumor cells on day 80 and tumor outgrowth was monitored. All 8 mice remained tumor-free.



Supplementary Figure S3. Local PDT inhibits distant tumor growth. (A-D) Outgrowth curves of primary and secondary tumors of individual double MC38 tumor-bearing mice receiving PDT or left untreated. PDT was given on day 8 by injection of Bremachlorin photosensitizer followed after 6 hours by primary tumor illumination. Group average tumor volume of secondary tumors is indicated by the red line. Pooled data of 6 independent experiments, 48-57 mice per group. **(E)** Ex vivo analysis of secondary tumors by flow cytometry, showing percentage of total CD3+ tumor-infiltrating lymphocytes (TILs) or CD8 T cells from total CD45+ cells in the tumor. Asterisks indicate statistically significant differences ($p<0.05$) between untreated and PDT-treated mice. **(F)** Tumor-infiltrating CD8 T cells have an activated phenotype. Representative flow cytometry plot showing 4-1BB and CD69 activation marker expression.



Supplementary Figure S4. Responses of primary and secondary tumors to therapy. Group average tumor outgrowth curves of primary and secondary MC38 tumors (upper graphs) or CT26 tumors (lower graphs). Mice received PDT of primary tumors on day 8, systemic CTLA4-blocking antibody on days 7, 10 and 14, both therapies, or were left untreated. Pooled data of 2 independent experiments, 14-16 mice per group.