The use of light in cancer immunotherapy

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Chapter 2

Combination of Photodynamic Therapy and specific immunotherapy efficiently eradicates established tumors

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Abstract

Purpose: The efficacy of immunotherapy against advanced cancer may be improved by combination strategies. Photodynamic therapy (PDT) is a local tumor ablation method based on localized activation of a photosensitizer, leading to oxygen radical-induced tumor cell death. PDT can enhance antitumor immune responses by release of antigen and danger signals, supporting combination protocols of PDT with immunotherapy.

Experimental Design: We investigated the local and systemic immune effects of PDT after treatment of established tumors. In two independent aggressive mouse tumor models, TC-1 and RMA, we combined PDT with therapeutic vaccination using synthetic long peptides (SLP) containing epitopes from tumor antigens.

Results: PDT of established tumors using the photosensitizer Bremachlorin resulted in significant delay of tumor outgrowth. Combination treatment of PDT with therapeutic SLP vaccination cured one third of mice. Importantly, all cured mice were fully protected against subsequent tumor rechallenge, and combination treatment of primary tumors led to eradication of distant secondary tumors, indicating the induction of a systemic antitumor immune response. Indeed, PDT by itself induced a significant CD8 T-cell response against the tumor, which was increased when combined with SLP vaccination and essential for the therapeutic effect of combination therapy.

Conclusions: We show that immunotherapy can be efficiently combined with PDT to eradicate established tumors, based on strong local tumor ablation and the induction of a robust systemic immune response. These results suggest combination of active immunotherapy with tumor ablation by PDT as a feasible novel treatment strategy for advanced cancer.

Translational relevance

Cancer immunotherapy has shown promising results although a significant proportion of patients respond poorly or relapse at a later stage, therefore more potent combination therapies are required. Tumor ablation by Photodynamic Therapy (PDT) can strongly reduce tumor mass and induce the release of tumor antigen and pro-inflammatory mediators, therefore being an attractive option for combination with immunotherapy. In this preclinical study, we show that tumor-specific immunotherapy by synthetic long peptide (SLP) vaccination can be efficiently combined with PDT, leading to eradication of established tumors based on strong local tumor ablation and the induction of a CD8 T cell response. PDT and SLP vaccination are independently already applied in the clinic, allowing a swift translation for potentially a large group of cancer patients.

Introduction

A major challenge in medical oncology is the development of efficient treatment options for advanced cancer, which currently are limited. The clinical situation of advanced primary tumors with possible metastases asks for therapeutic protocols that combine a strong anti-tumor effect to eradicate known tumors with the induction of a systemic anti-tumor immune response to eliminate distant metastases. As the immune system can strongly and specifically attack targets based on the principle of antigen-specificity, cancer immunotherapy aims to employ these characteristics of the immune system to attack and eradicate tumors.

A promising approach of cancer immunotherapy is therapeutic vaccination using synthetic long peptides (SLP) covering T cell epitopes of tumor antigens (1-4). Besides widely shared tumor antigens such as those expressed by virally induced tumors, this approach can also be applied to individual patient-specific neo-antigens (5, 6). Clinical studies using therapeutic SLP vaccination against cancer are ongoing based on encouraging results in preclinical tumor models (7-9). For instance, clinical Phase I/II studies using a set of overlapping peptides covering the E6 and E7 oncoproteins of Human Papillomavirus 16 (HPV16) have been successful in patients with HPV16-induced premalignant disease (10). This peptide vaccine formulation induced HPV16-specific T cell responses in all 20 patients and resulted in clinical responses in about 80% of patients and nearly 50% complete remissions correlating with robust effector T cell immunity. However, thus far this vaccine was not clinically effective against established HVP16+ cancer despite detectable vaccine-induced T cell responses (11, 12). This is one of the examples illustrating that successful treatment of advanced cancer requires combination protocols, as single-treatment modalities are insufficiently effective. Therapies causing immunogenic cell death are of particular interest for combination with immunotherapy, as the reduction of tumor burden and the immunogenic effects can enhance the efficacy of immunotherapy. Combinations of immunotherapy with conventional cancer therapies like chemotherapy or radiotherapy are already under investigation. In this study, we examine the use of Photodynamic Therapy (PDT), a tumor ablation method that is widely clinically applied for various premalignant and malignant lesions.

In PDT, an inactive light-sensitive molecule called photosensitizer is administered and subsequently activated by irradiation of the target area with visible light of a specific wavelength. The activated photosensitizer reacts with oxygen to form reactive oxygen species (ROS), which induce tumor cell death and vascular shutdown (13, 14). Besides direct cytotoxic effects on tumor cells, PDT has been shown to

cause the release of antigen and immunogenic factors such as damage-associated molecular patterns (DAMPs) from dying tumor cells (15-25). These immunological effects make PDT an attractive option for combinations with immunotherapy in the treatment of advanced tumors. Here, we use Bremachlorin, also known as Radachlorin, a novel photosensitizer that benefits from improved pharmacokinetics and high-wavelength irradiation reaching deeper tissue. Bremachlorin is currently being tested in clinical trials for basal cell carcinoma (BCC) and non-small-cell lung carcinoma (NSCLC) (26-31).

In this study, we investigated the combination of Bremachlorin-based PDT with therapeutic peptide vaccination in two mouse models of highly aggressive subcutaneous tumors. The tumor line TC-1 expresses the E6 and E7 oncoproteins of Human Papillomavirus 16 (HPV16) as a model for human HPV16-induced tumors, and has previously been shown to be sensitive for Bremachlorin-PDT (32, 33). RMA is an aggressive T cell lymphoma cell line induced by Rauscher murine leukemia virus (34). We show that PDT strongly ablated established fast-growing tumors, leading to a significantly longer survival and specific CD8+ T cell responses against the tumor. Combining PDT with therapeutic peptide vaccination efficiently eradicated established tumors, which was dependent on the presence of CD8 T cells. Importantly, combination treatment of primary tumors led to subsequent eradication of distant established secondary tumors and provided protection against repeated tumor challenge. Therefore, this successful combination of PDT and therapeutic vaccination, resulting in robust anti-tumor response and immunological memory, suggests a novel therapeutic combination strategy for advanced cancer.

Materials and Methods

Mice and cell lines

Wildtype C57BL/6 mice were obtained from Charles River Laboratories (France). Albino B6 mice (tyrosinase-deficient immunocompetent C57BL/6 mice) were bred in the animal breeding facility of the Leiden University Medical Center, the Netherlands. All experiments were approved by the animal experimental committee of the University of Leiden. The TC-1 mouse tumor cell line (a gift from T.C. Wu, John Hopkins University, Baltimore, MD) expressing HPV16 E6 and E7 oncoproteins was generated as previously described (32). RMA is a mutagenized derivative of RBL-5, a Rauscher Murine Leukemia Virus (MuLV)-induced T cell lymphoma line of C57BL/6 origin (34). Cell lines were assured to be free of rodent viruses and *Mycoplasma* by

regular PCR analysis. Authentication of the cell lines was done by antigen-specific T-cell recognition and the use of low passage number cells for all experiments. TC-1 cells were cultured as previously described (35). RMA cells were cultured in IMDM (Lonza) containing 8% Fetal Calf Serum (FCS, Greiner), 100 IU/mL penicillin/streptomycin (Gibco), 2 mM glutamin (Gibco) and 25 µM 2-mercaptoethanol. For tumor inoculation, 100,000 TC-1 or 1000 RMA tumor cells in 100 µL PBS were injected subcutaneously in the right flank of the mice. For tumor rechallenge, the identical injection was given in the left flank to distinguish possible outgrowth from regrowth of the original tumor. For double-tumor experiments, an identical TC-1 inoculation was given in the left flank 3 days after primary tumor inoculation. Tumors were measured 3 times per week with a caliper and the volume was calculated by multiplying the tumor diameters in three dimensions. Survival curves are based on the moment of sacrificing the mice upon reaching the maximally allowed tumor volume of 2000 mm^3.

Photosensitizer uptake and *in vitro* irradiation

In vitro Bremachlorin uptake by tumor cells was analyzed by incubating TC-1 tumor cells with Bremachlorin at the dose and time as indicated, washing the cells in PBS, and measuring the Bremachlorin fluorescence compared to control cells by flow cytometry (BD Calibur, emission channel FL4). In vivo Bremachlorin uptake by tumors was visualized using a Pearl Impulse imager (Li-cor). For photodynamic treatment *in vitro*, TC-1 tumor cells were incubated with 1 µg/mL Bremachlorin for 3 hours in 24 wells plates, then the cells were washed with PBS to remove all free photosensitizer, and fresh medium was added. Irradiation of the whole well followed immediately for 2 minutes at 116 mW/cm^2 (14 J/cm^2) using a 662 nm Milon Lakhta laser.

Photodynamic Therapy

Tumors were treated 9 days (TC-1) or 14 days (RMA) after inoculation, both at an average tumor diameter of 5 mm. First, 20 mg/kg Bremachlorin photosensitizer (RadaPharma International) was injected intravenously in the tail vein, followed by irradiation of the tumor 6 hours later using a 662 nm Milon Lakhta laser. A continuous irradiation protocol of 1000 seconds at 116 mW/cm^2 (116 J/cm^2) was used based on optimization experiments (data not shown). For irradiation, the skin in the tumor area was shaved and the mice were anaesthetized by inhalation of isoflurane and positioned horizontally on a heat mat. Precision irradiation of the tumor was ensured by using a fiber fixed vertically above the mouse, and the exposed area was precisely adjusted using a diaphragm.

Serum analysis for HMGB1

Serum was obtained from blood samples taken 1 hour after PDT treatment, or at the same time for untreated controls. The HMGB1 serum level was determined by a sandwich ELISA kit (IBL International) following the manufacturers protocol.

Ex vivo lymph node analysis

TC-1 tumor-bearing animals received the standard PDT treatment as described above, and were sacrificed after 6 days and the tumor-draining inguinal lymph node was obtained, together with the contralateral inguinal lymph node. The lymph nodes were incubated with 2.5 mg/mL Liberase TL (Roche) for 20 minutes at 37°C and single-cell suspensions were made using 70 μm cell strainers (BD Biosciences). The cells were then stained with fluorescently labeled antibodies against CD3ε, CD8α, CD11c and with 7-AAD and APC-labeled tetramer for flow cytometry analysis.

Flow cytometry

All flow cytometry analyses were performed by suspending cells in FACS buffer (PBS with 0.5% BSA and 0.02% sodium azide) and analysis on a BD FACS Calibur. Antibodies against CD3, CD8 or CD11c and the dyes Annexin V and 7-AAD were purchased from BD, eBioscience and BioLegend. The APC-labelled H-2Db RAHYNIVTF tetramer was own production.

Synthetic long peptide vaccination

The SLP vaccine for TC-1 (sequence GQAEPDRAHYNIVTFCCKCDSTLRLCVQSTHVDIR), including both a CD4 and a CD8 epitope from the HPV16 E7 oncoprotein, was given on day 7 and 21 after tumor inoculation by injecting 150 μg peptide subcutaneously in the left flank of the mouse (35). The peptide was dissolved in a 100 μL PBS and mixed 1:1 with Incomplete Freunds Adjuvant (IFA), which was then emulsified for 30 minutes on a vortex. The peptide vaccine for RMA tumors contains epitopes from Rauscher Murine Leukemia Virus (MuLV) and existed of a single vaccination on day 14 containing 20 nmole of the Env-encoded CD4 epitope EPLTSLTPRCNTAWNRLKL and 50 nmole of the Gag-encoded CD8 epitope CCLCLTVFL (36) complemented with 20 μg CpG (ODN 1826, Invivogen), in 100 μL PBS subcutaneously in the tail-base region.

Systemic blood analysis for specific CD8 T cell response

The systemic tumor-specific CD8 T cell response was determined by taking venous blood samples from the tail vein 8 days after peptide vaccination or on the same day for non-vaccinated animals. After erythrocyte lysis of the blood samples, the tumor-specific CD8 T cell response was determined by flow cytometry analysis

after staining of the cells with CD3ε, CD8β, and APC-conjugated tetramers for the relevant peptide-MHC complex on the CD8 T cell.

CD8+ T cell depletion

Hybridoma cells producing depleting CD8 mAb (clone 2.43) were cultured in Protein-Free Hybridoma Medium (Gibco), and mAbs were purified using a Protein G column. To deplete CD8 T cells, mice received an intraperitoneal (i.p.) injection of 150 µg anti-CD8 antibodies on day 8 after tumor inoculation, followed by periodical depletion of 50 µg antibodies every 5 days until day 30 after tumor inoculation. All control mice received in parallel similar amounts of isotype control rat immunoglobulin G. The effective T-cell depletion was assured by flow cytometry analysis of blood lymphocytes stained for cell surface expression of CD8.

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5.0 software. Data are shown as the mean ± SEM for each group, and comparison of groups was performed by two-tailed Student's t-test, with the exception of survival curves which were compared using the LogRank Mantel-Cox test. Statistical differences were considered significant at $p < 0.05$.

Results

Efficient photosensitizer uptake allows strong tumor ablation

For effective PDT, sufficient photosensitizer uptake by tumor cells is required to ensure irradiation-induced cell death. Both TC-1 and RMA tumor cells showed a dose-dependent uptake after incubation with Bremachlorin (**Supplementary Figure S1a**). Irradiation of Bremachlorin-treated TC-1 cells using visible light resulted in >98% cell death based on Annexin V and 7-AAD analysis, which was completely dependent on the presence of both the photosensitizer and the irradiation (**Supplementary Figure S1b**). Photosensitizer uptake in established tumors was shown by intravenously injecting mice bearing subcutaneous TC-1 or RMA tumors with Bremachlorin, which after 6 hours accumulated in the tumor area (**Supplementary Figure S2**). To analyze whether this photosensitizer accumulation is sufficient for photodynamic ablation, growing TC-1 tumors with a diameter of 5 mm were irradiated with a focused laser beam 6 hours after injection of Bremachlorin. After a clear inflammatory reaction in the treated area in the first days after PDT, a strongly flattened tumor lesion remained with a necrotic appearance. This resulted in a significant delay in tumor

growth of at least 7 days, after which tumor outgrowth resumed with a growth rate similar to untreated tumors (**Figure 1a**).

PDT induces an anti-tumor immune response

As we aimed to use Bremachlorin-based PDT in combination with immunotherapy, we analyzed the immunological effects of PDT in our model. It has previously been shown that PDT can contribute to anti-tumor immune responses through the release of DAMPs such as HMGB1 (17, 18). Serum analysis of TC-1 tumor-bearing mice 1 hour after PDT showed a significant increase in HMGB1 compared to untreated mice (**Figure 1b).**

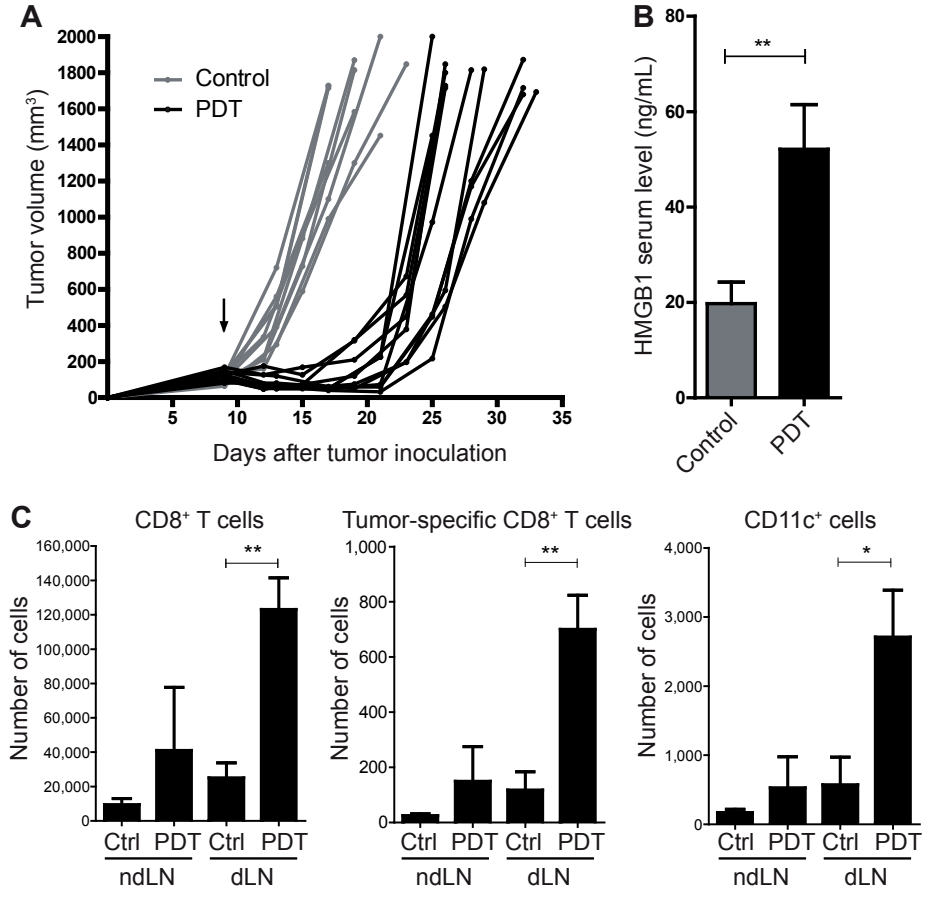

Figure 1. PDT strongly delays tumor outgrowth and induces an immune response against the tumor. (A) Tumor outgrowth curves of subcutaneous TC-1 tumors in BL/6 mice treated with PDT on day 9 (arrow) after tumor inoculation, compared to untreated control tumors. Pooled data of 2 independent experiments, n=10-12 mice. **(B)** ELISA serum analysis for HMGB1 in 9 mice at 1 hour after PDT versus untreated control mice. Pooled data of 2 independent experiments, n=9 mice. **(C)** Flow cytometry analysis of TC-1 tumor-draining lymph nodes (dLN) or contralateral non-draining lymph nodes (ndLN) of 4 mice at 6 days after PDT in comparison to untreated control mice (Ctrl). Single-cell suspensions from lymph nodes were stained for CD3ε, CD8α, CD11c and the Db-RAHYNIVTF Tetramer (Tm) for the tumor antigen-specific T cell receptor. Y-axes show absolute numbers of total CD8 T cells (CD3+ CD8+), tumor-antigen specific CD8 T cells (CD3+ CD8+ Tm+) or CD11c+ cells. Statistical analysis by Student's T test, significance is indicated by asterisks: * $p<0.05$, ** $p<0.01$.

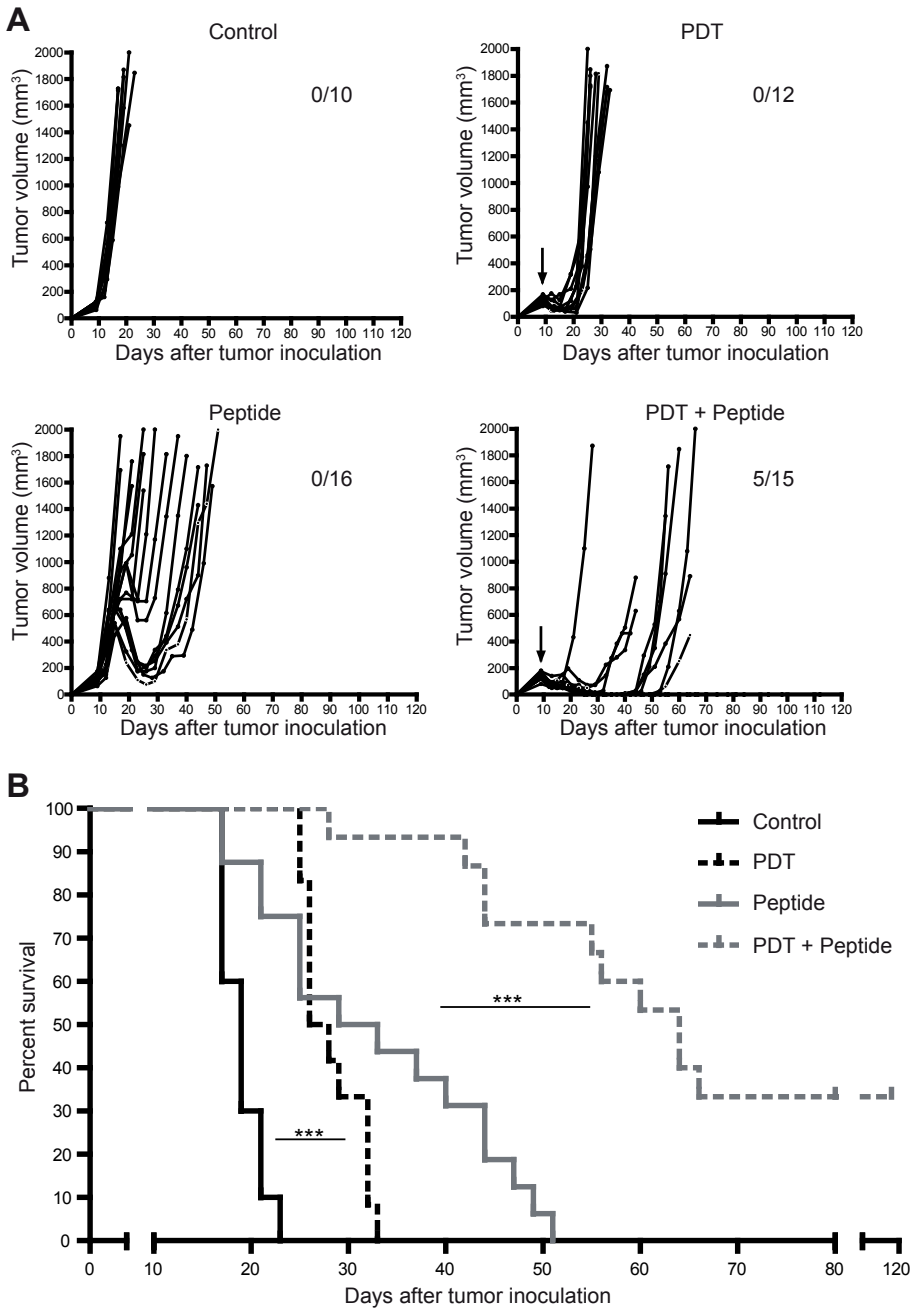

Figure 2. Curative combination treatment of established TC-1 tumors by PDT and synthetic long peptide vaccination. (A) Tumor outgrowth curves and **(B)** survival curves of TC-1 tumor-bearing mice treated with PDT, peptide vaccination or combined treatment, compared to untreated control tumors. PDT was done on day 9 after tumor inoculation (arrows), peptide was administered subcutaneously in IFA in the contralateral flank on day 7 and 21. Pooled data of 2 independent experiments, 10-16 mice. The fractions of mice that cleared the tumor are indicated. Survival curve statistics by LogRank X^2 test. Statistical significance is indicated by asterisks: *** $p<0.001$.

To investigate the immunological consequences of the massive tumor cell death induced by PDT, we analyzed the tumor-draining lymph nodes 6 days after PDT treatment of TC-1 tumors and compared them to contralateral lymph nodes not draining the irradiated tumor area. PDT induced a strong tumor antigen-specific CD8 T cell response in the tumor-draining lymph nodes, accompanied by a significant increase in the total number of CD8 T cells which was not increased in the non-draining nodes of the same animals (**Figure 1c).** Untreated tumor-bearing mice mounted only a minimal CD8 T cell response against the tumor, quantitatively similar to non-draining lymph nodes of PDT-treated mice. Strikingly, also the numbers of CD11c+ dendritic cells (DC) were strongly increased in the draining nodes of the PDT-treated tumor, suggesting that the DC facilitate cross-presentation of tumor-associated antigen to T cells in local lymphoid organs to stimulate anti-tumor responses.

Combination of PDT and therapeutic vaccination eradicates established tumors
Altogether, the strong tumor ablation and beneficial immunological effects of Bremachlorin-PDT make it an attractive candidate for combination with immunotherapy. As we have previously shown that the TC-1 tumor model is susceptible to therapeutic synthetic long peptide (SLP) vaccination (7), we combined Bremachlorin-PDT with SLP vaccination following the experimental setup depicted in **Supplementary Figure S3**. Single treatments of PDT or peptide vaccination of established TC-1 tumors each resulted in a significant delay in tumor outgrowth and increased survival, but neither treatment was curative**.** However, when PDT was combined with SLP vaccination, overall survival was strongly increased and over one third of mice were cured **(Figure 2)**.

Combination treatment protects against tumor rechallenge and eradicates established secondary tumors
All mice cured from their TC-1 tumor after combination therapy of PDT and SLP vaccination subsequently rejected TC-1 tumor cells injected at a distant location two to three months after primary curative treatment, indicating the induction of protective systemic immunity **(Supplementary Figure S4a)**. To investigate whether combination therapy can also eradicate existing established distant tumors, mice were inoculated with TC-1 tumors in both flanks followed by combination therapy where PDT was only applied on the primary tumor in the right flank, as depicted in **Supplementary Figure S4b.** The outgrowth of secondary tumors was not delayed by PDT of the contralateral primary tumor **(Figure 3a).** Mice treated by peptide vaccination showed an initial regression of both primary and secondary tumors, but none of the mice were cured from both tumors and all were eventually sacrificed due to tumor outgrowth. In contrast, combination treatment of PDT and peptide

vaccination caused definite cure from both primary and secondary tumors in almost 40% of mice, similar to the experimental model with a single TC-1 tumor. This can be appreciated when comparing the long-term survival between peptide vaccination and combination treatment from day 50 onwards **(Figure 3b).**

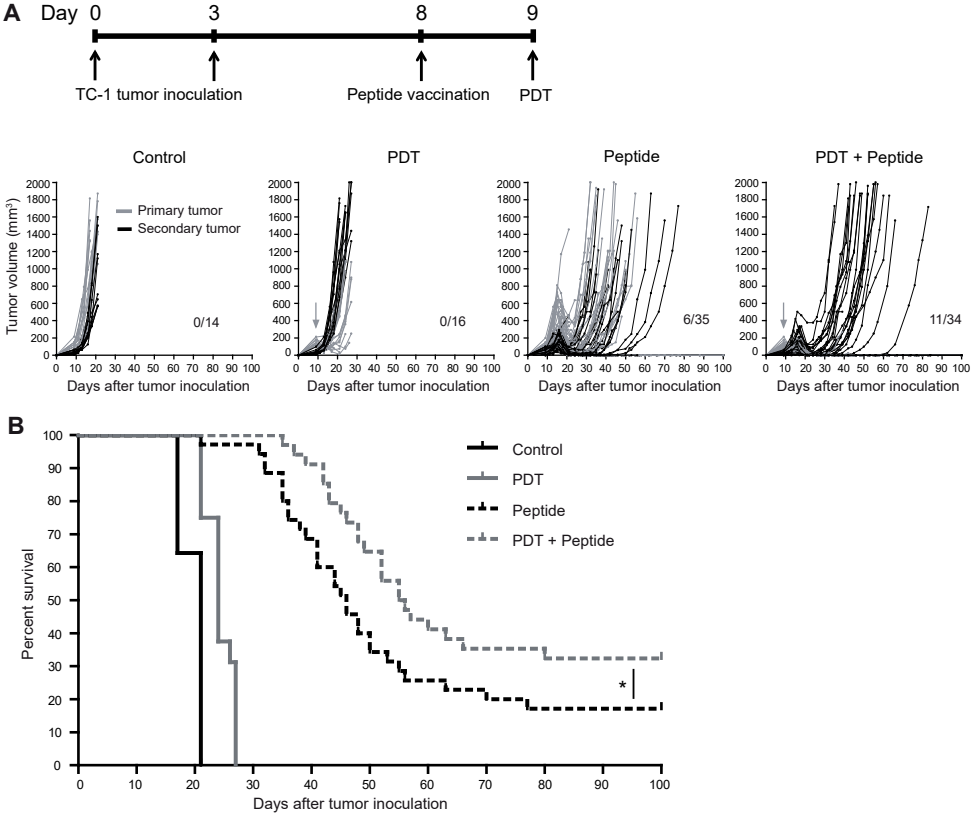

Figure 3. Combination treatment of primary tumors leads to durable eradication of distant tumors. (A) Tumor outgrowth curves of mice bearing established subcutaneous TC-1 tumors in both flanks, treated with systemic peptide vaccination on day 8 followed by PDT of only the primary tumor in the right flank on day 9 (arrows). Primary tumors (grey lines) were inoculated on day 0 in the right flank, secondary tumors (black lines) on day 3 in the left flank. The fractions of mice that cleared both tumors are indicated. **(B)** Corresponding survival curves, statistical analysis by LogRank X2 test. Statistical significance is indicated by asterisks: * $p<0.05$.

Treatment-induced anti-tumor CD8 T cells are essential for therapeutic efficacy

As we found that PDT induces a local immune response in lymph nodes and that combination therapy using local PDT is also able to cure distant secondary tumors, we analyzed the systemic CD8 T cell response against the tumor. Using specific MHC tetramer staining to identify tumor antigen-specific CD8 T cells, we could show that SLP vaccination raised the levels of CD8+ T cells specific for the HVP16 E7 epitope used for vaccination in circulating blood as we have reported previously (**Figure 4a)** (7)**.** Importantly, also PDT significantly increased percentage of tumor antigen-specific CD8+ T cells circulating in blood, supporting the immunogenic effects of

PDT described earlier. Moreover, PDT even further increased the SLP-induced CD8 T cell response, reflecting the efficacy of combination treatment in tumor control. To analyze whether these tumor-specific CD8 T cells are responsible for the observed tumor control, TC-1 tumor-bearing mice treated with PDT and SLP vaccination were depleted of all CD8+ cells using an anti-CD8 antibody. Periodical screening of systemic venous blood confirmed a persisting reduction in the number of CD8 T cells of over 98% during the experiment (data not shown). In the absence of CD8 T cells, the curative effect of PDT and SLP combination treatment was abrogated, suggesting a crucial role of CD8 T cells in this combination treatment protocol **(Figure 4b)**.

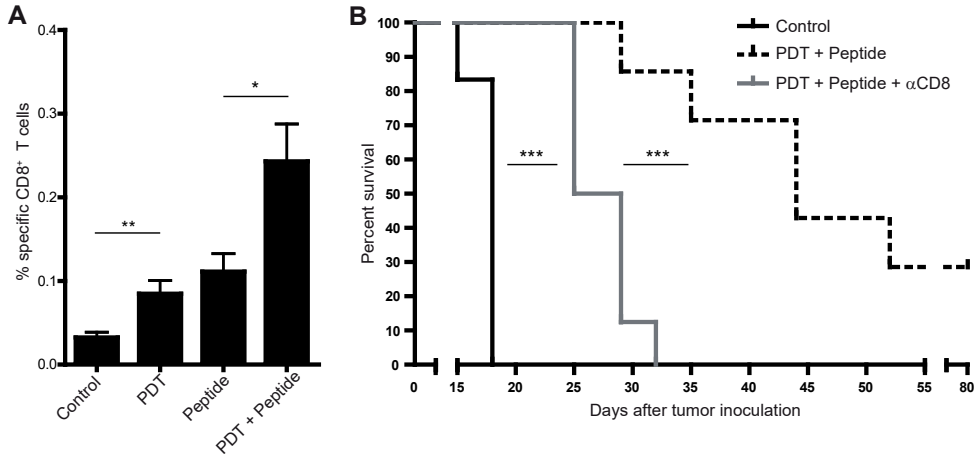

Figure 4. The strong effect of combination treatment is dependent on a treatment-induced systemic CD8 T cell response against the tumor. (A) Tetramer staining showing the percentage of CD8 T cells in tail vein blood that is specific for the HPV16 E7 epitope expressed by TC-1 tumor cells, 8 days after treatment. **(B)** Survival curves of TC-1 tumor-bearing mice treated with PDT and peptide vaccination during antibody-mediated depletion of CD8 T cells. PDT was performed on day 9 after tumor inoculation, peptide was administered subcutaneously in IFA in the contralateral flank on day 7 and 21. Depleting antibody was administered i.p. regularly from day 8 to day 45, resulting in >98% depletion of CD8+ cells in the blood within 24h after injection. Pooled data of individual experiments with in total 16-24 mice per group. Statistical analysis of Figure A by Student's T test and of Figure B by LogRank X^2 test. Statistical significance is indicated by asterisks: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Efficient PDT-vaccination combination in virally-induced lymphoma tumors

Next, we applied PDT and peptide vaccination in another aggressive tumor system, the RMA lymphoma model for which we have previously described efficient prophylactic peptide vaccination, which prevented tumor outgrowth through the effects of both CD4 and CD8 T cells (36). Previous attempts in our group to treat established RMA tumors by therapeutic peptide vaccination have never been successful. Here, we show that combination of Bremachlorin-PDT and therapeutic peptide vaccination in mice bearing subcutaneous RMA tumors resulted in significantly prolonged survival compared to either single treatment alone, similar

to our observations in the TC-1 model **(Figure 5)**. All mice cured of their primary tumor were able to reject RMA tumor cells upon rechallenge at a distant location over two months after treatment (data not shown), suggesting that also in this model PDT and peptide vaccination induced systemic immunity against the tumor.

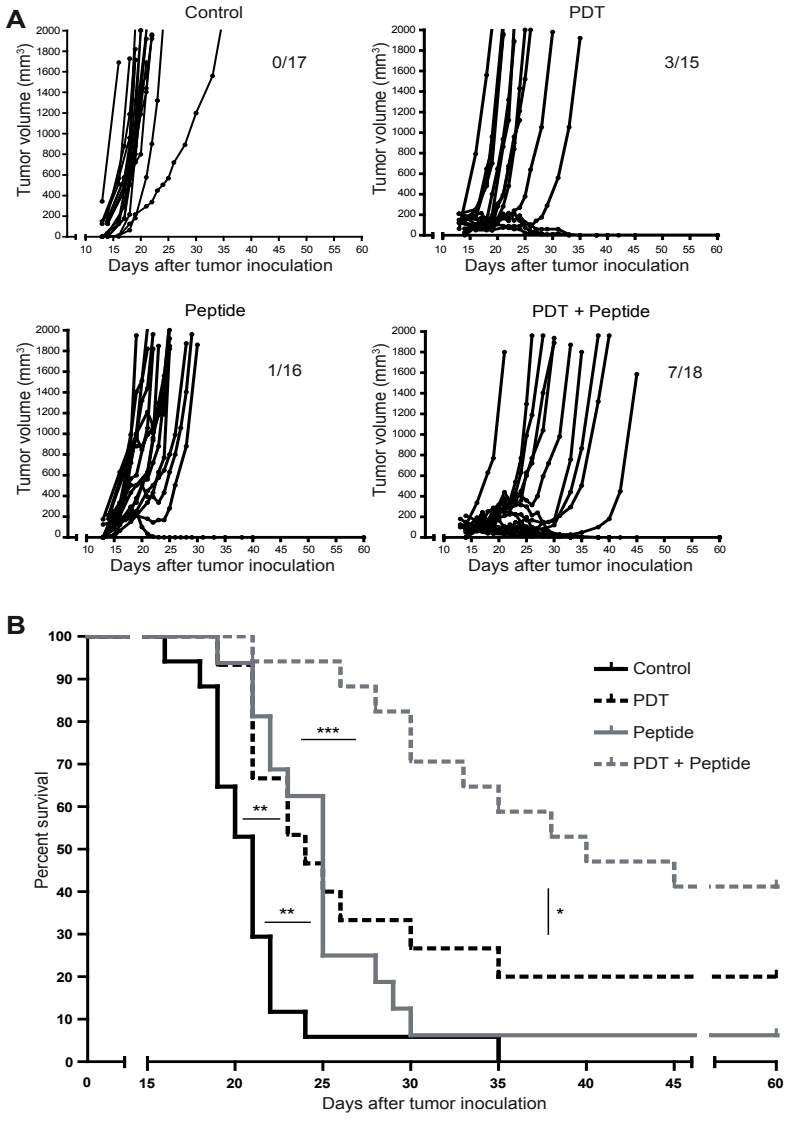

Figure 5. Therapeutic treatment of murine leukemia virus-induced lymphoma by PDT and tumor-specific peptide vaccination. (A) Tumor outgrowth curves and **(B)** survival curves of RMA tumor-bearing mice treated with PDT, peptide vaccination, or combined therapy, compared to untreated control tumors. PDT was given on day 14 after tumor inoculation, the peptide vaccine was mixed with CpG and administered subcutaneously in the tail-base in PBS on day 12. The fractions of mice that cleared the tumor are indicated. Survival curve statistics by LogRank X2 test. Statistical significance is indicated by asterisks: * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Discussion

In this study we suggest a novel therapeutic combination strategy for advanced metastatic cancer, consisting of PDT-mediated tumor ablation and tumor-specific peptide vaccination. In two independent aggressive tumor models we show that ablation of established tumors using Bremachlorin-PDT strongly reduces tumor burden and at the same time induces anti-tumor T cell responses, which was significantly enhanced when combined with therapeutic long peptide vaccination. Importantly, the systemic anti-tumor CD8 T cell response induced by combination treatment was essential for the therapeutic effect, and likely provided long-term protection since all cured mice did not develop a tumor after renewed injection of tumor cells at a different body site. The relevance of the systemic immune response was emphasized by the eradication of distant secondary tumors after combination therapy of primary tumors. Our combination protocol therefore meets the requirements of an efficient treatment strategy for advanced cancer that we discussed earlier: a strong anti-tumor effect to eradicate known tumors, and a lasting systemic immune response to identify possible metastatic tumor sites.

PDT, like other tumor ablation therapies, aims to strongly affect tumor cells while minimizing damage to healthy tissue. The non-toxic nature of the two individual components of PDT, the photosensitizer and the irradiation with visible light, allows precise restriction of the photodynamic effect to the target region. Pharmacokinetic optimization of photosensitizers has been aimed at a better accumulation in tumors and a faster clearance from other tissues. This has led to new generations of photosensitizers, which moreover are optimized for the use of higher wavelength irradiation light. This increases the effect range of PDT, as a higher wavelength of light penetrates deeper through tissue. The use of flexible interstitial optical fibers allows both precision irradiation of complexly localized tumors and the treatment of bulky tumor masses (13).

Several animal tumor models expressing known tumor antigens are available to study the immunological effects of PDT on an antigen-specific level; however, many concern artificially introduced model antigens such as chicken ovalbumin which have no clinical relevance (19). To overcome this limitation, a recent study used a murine mastocytoma tumor expressing P1A, the mouse homolog of human MAGE cancer/testis antigens, and showed antigen-specific immune responses against this clinically relevant tumor antigen and corresponding effects on tumor growth (37). In this study, we used two mouse tumor models expressing known epitopes from oncogenic viruses as a model for HPV- or Leukemia Virus-induced cancer in humans, and showed successful combination treatment of these tumors by PDT and peptide

vaccination. These tumor models expressing viral epitopes are of clinically relevance to a large group of cancer patients as around 15% to 20% of human cancer is estimated to be virally induced (38, 39). Importantly, the application of combination therapy using PDT and SLP vaccination may theoretically be extended to virtually any type of cancer, as was illustrated by recent studies identifying neo-epitopes in mouse tumors and subsequent successful vaccination with long peptides containing these tumor-specific neo-epitopes (5, 6).

Our findings in the TC-1 mouse model for HPV16-induced human tumors are of particular interest as PDT is currently already clinically studied in the treatment of HPV16-induced gynecological lesions (40-42). These studies used topical administration of the second-generation photosensitizer 5-ALA, and reported inefficient photosensitizer distribution through the target tissue leading to incomplete responses. The use of novel photosensitizers such as Bremachlorin may help to resolve this issue. Combination treatments of PDT and immunotherapy to improve the therapeutic effect are being investigated preclinically and clinically using non-specific immunostimulatory agents (43-45). However, tumor-specific immunotherapy such as therapeutic peptide vaccination with HPV antigens may be preferred to ensure a stronger and target-specific effect. Alternatively, to overcome the tumor-mediated immune suppression, T cell checkpoint blocking antibodies form an attractive therapeutic option for combination with PDT in order to boost the anti-tumor T cell response and relieve the immune system from suppression (46, 47). Taken together, this successful combination of systemic immunotherapy and local tumor ablation, which are independently already clinically applied, proposes an attractive clinical treatment strategy for advanced cancer.

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Supplementary Information

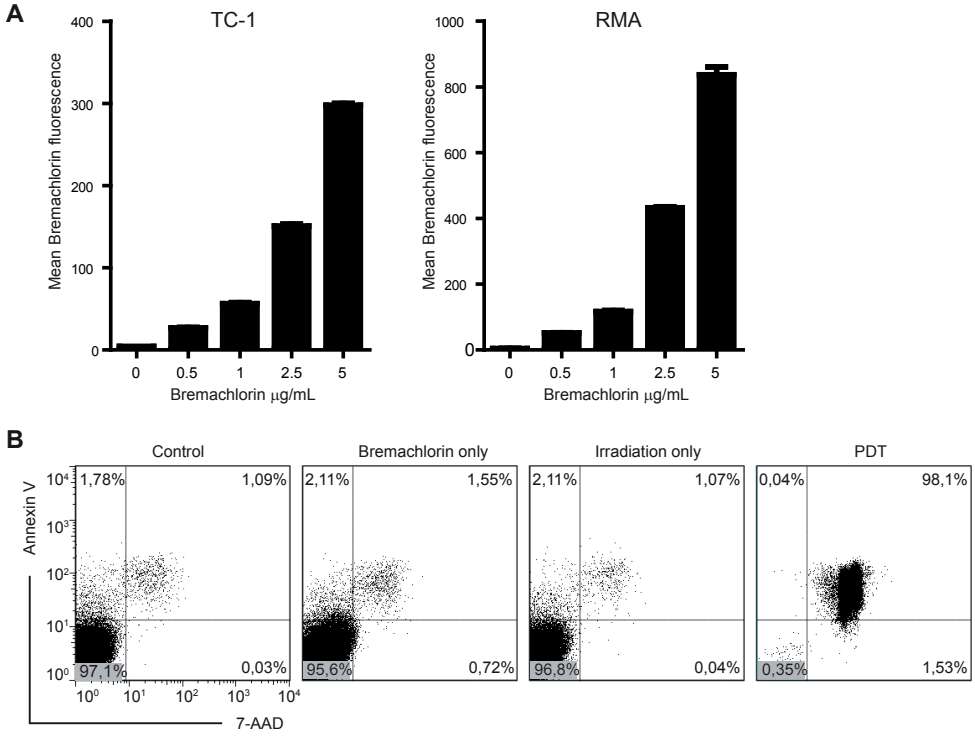

Figure S1. Efficient photosensitizer uptake enables specific killing following laser irradiation. (A) Flow cytometry of Bremachlorin fluorescence on TC-1 tumor cells incubated with different concentrations of Bremachlorin for 3 hours at 37°C. **(B)** Flow cytometry plots showing Annexin V and 7-AAD staining of TC-1 tumor cells 16 hours after in vitro PDT treatment. Cells were incubated with 1 μg/mL Bremachlorin for 3 hours, which had shown to give high cellular uptake, and irradiated for 2 minutes. Control samples were untreated cells, photosensitizer-only or irradiation-only cells. Representative plots of 3 experiments.

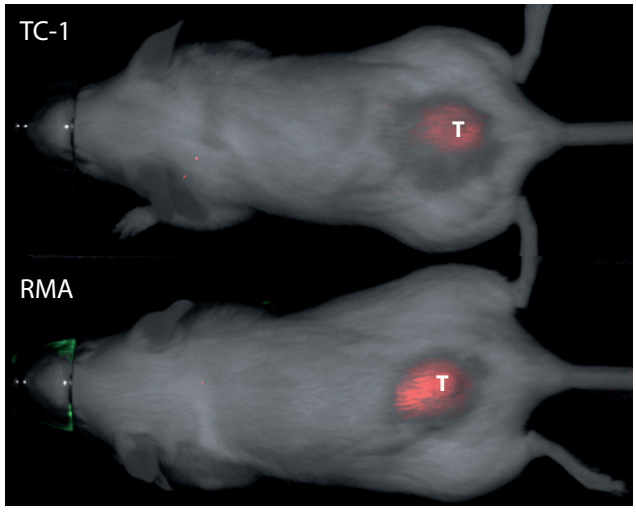

Figure S2. Bremachlorin photosensitizer accumulates in tumors. In vivo imaging of Bremachlorin fluorescence 6 hours after injection of 20 mg/kg Bremachlorin in Albino B6 mice bearing subcutaneous TC-1 or RMA tumors. Bremachlorin fluorescence in the 700 nm emission filter is represented by the red color, the tumor is indicated by a white 'T'. Representative pictures from 4 mice per tumor model.

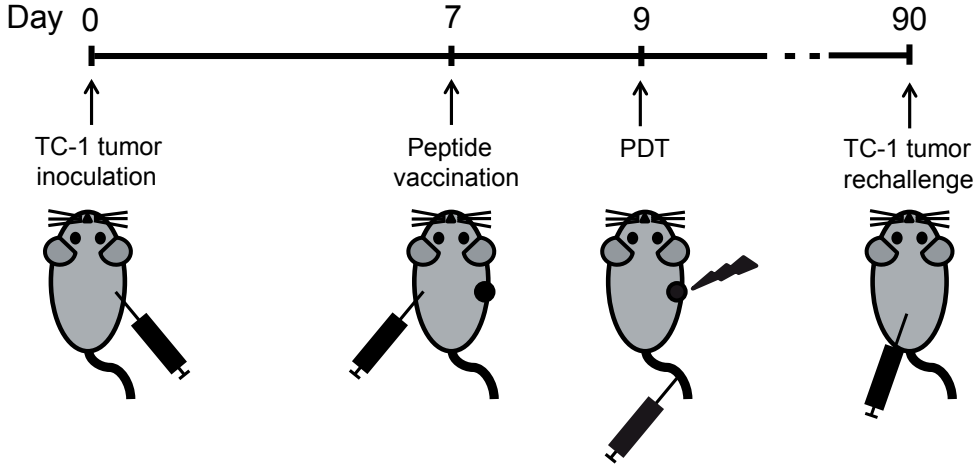

Figure S3. Graphical overview of the experimental setup in the TC-1 tumor model. The setup of the experiments using the TC-1 tumor model (single-tumor setting) is shown on a timescale starting with tumor inoculation at day 0.

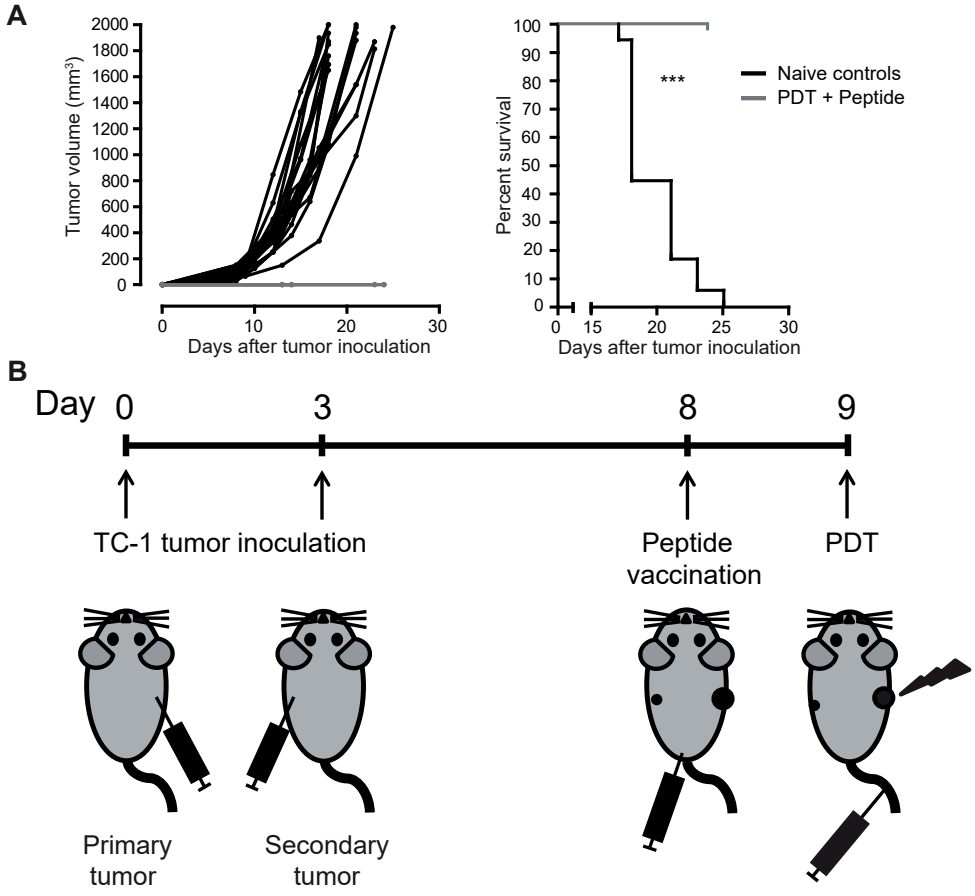

Figure S4. TC-1 tumor rechallenge and metastasis model. (A) Tumor outgrowth and survival curves after TC-1 tumor challenge in mice cured from their TC-1 tumor by combination therapy of PDT and peptide vaccination, compared to naïve control mice. Pooled data from 3 independent experiments, 13-18 mice per group. Survival curve statistics by LogRank X^2 test, statistical significance *** = $p<0.001$. **(B)** The experimental setup of the TC-1 metastasis model is shown on a timescale starting with inoculation of the primary tumor at day 0. Mice were inoculated with TC-1 tumor cells in both flanks followed by systemic peptide vaccination and PDT of only the primary tumor.