Host Immunity Following Near-Infrared Photoimmunotherapy Is Enhanced with PD-1 Checkpoint Blockade to Eradicate Established Antigenic Tumors

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Abstract

Near-infrared photoimmunotherapy (NIR-PIT) induces immunogenic cell death but has mostly failed to induce durable antitumor responses in syngeneic tumor mouse models. We hypothesized that adaptive immune resistance could be limiting durable responses after treatment with NIR-PIT. We investigated the effects of combining NIR-PIT targeting cell-surface CD44 and PD-1 blockade in multiple syngeneic tumor models. In two of three models, NIR-PIT monotherapy halted tumor growth, enhanced dendritic cell tumor infiltration, and induced de novo tumor antigen-specific T-cell responses absent at baseline. The addition of PD-1 blockade reversed adaptive immune resistance, resulting in both enhanced preexisting tumor antigen-specific T-cell responses and enhanced de novo T-cell responses induced by NIR-PIT. Enhanced immune responses correlated with shared tumor antigen expression, suggesting that antigenicity is a major determinant of response to combination NIR-PIT and PD-1 blockade. Combination treatment induced complete rejection of MC38 tumors treated with NIR-PIT, as well as untreated, distant tumors. Accordingly, tumor antigen–specific T-cell responses were measured in both treated and untreated tumors, validating the development of systemic antitumor immunity. Mice that cleared tumors resisted subsequent tumor challenge, indicating the presence of systemic immune memory. Cumulatively, these results demonstrate reversal of adaptive immune resistance following induction of innate and adaptive immunity by NIR-PIT, resulting in high rates of tumor rejection and/or significant tumor growth control in antigenic syngeneic models of cancer.

Introduction

Near-infrared photoimmunotherapy (NIR-PIT) is a newly developed cancer treatment that uses a targeted monoclonal antibody–photoabsorber conjugate (1). Following antibody localization of the antibody–photoabsorber conjugate to a tumor cell-surface antigen, NIR light is used to induce selective cytolysis. NIR-PIT targeting EGFR with a cetuximab–antibody–photoabsorber conjugate has shown promising results in phase II clinical evaluation for the treatment of recurrent head and neck squamous cell carcinoma (NCT02422979) and has received breakthrough therapy designation by the Food and Drug Administration. Extensive preclinical evidence demonstrates that NIR-PIT is effective at inducing tumor cell lysis using a number of different antibody–antibody–photoabsorber conjugates (2–6).

NIR-PIT induces rapid, necrotic cell death that yields innate immune ligands that activate dendritic cells (DCs; ref. 7), consistent with immunogenic cell death (ICD; ref. 8). Yet NIR-PIT treatment of syngeneic tumors in wild-type mice has mostly failed to induce durable regression of established tumors (2), suggesting the presence of one or more mechanisms of resistance to formation of meaningful antitumor immunity. Expression of programmed cell death ligand-1 (PD-L1), in response to local immune activation and interferon (IFN) production, may bind the programmed cell death receptor (PD-1) on T lymphocytes and inhibit T-cell receptor signaling, proliferation, and mobility in a process known as adaptive immune resistance (9–11). Immune-checkpoint blockade (ICB) with anti–PD-1/anti–PD-L1 induces objective and sometimes durable antitumor immune responses, but only in a subset (15%–20%) of patients with recurrent/metastatic cancer (12, 13). Given that PD-1 inhibition reactivates T cells being inhibited by PD-1 signaling and that response to PD-1 blockade correlates with the presence of inducible adaptive immune responses (10, 14), anticancer treatments that enhance baseline adaptive immunity may enhance responses to PD-1 ICB.

In this study, we hypothesized that NIR-PIT could induce antitumor immunity being restricted by the PD-1/PD-L1 signaling axis and that PD-1 ICB could reverse innate immune resistance to induce durable, effective antitumor immune
responses. Using a CD44-targeting antibody–photobioabsorber conjugate, we demonstrated the ability of PD-1 ICB to significantly enhance antigen-specific antitumor immunity induced by NIR-PIT in multiple models of cancer. Combination treatment resulted in significant growth control or rejection of established colon (MC38) and lung (LLC) tumors. Assessment of antigen-specific tumor-infiltrating lymphocyte (TIL) responses against shared tumor-associated antigens revealed enhancement of preexisting and NIR-PIT–induced antigen-specific responses in tumors. Efficacy of the combination treatment between different syngeneic models correlated with NIR-PIT surface target antigen expression, baseline tumor-associated antigen (TAA) expression, and induction of DC tumor infiltration. Taken together, this data set provides insight into mechanisms of resistance following NIR-PIT and provides a rationale for the clinical combination of NIR-PIT and ICB.

Materials and Methods

Reagents

Water-soluble, silica-phthalocyanine derivative IRDye 700DX NHS ester (IR700) was obtained from LI-COR Biosciences. Anti-mouse/human CD44-specific mAb (clone IM7) and an anti-mouse PD-1 (CD279)–anti-mouse PD-1 (CD279) (mAb) (clone RMP1-14) were purchased from Bio X Cell. All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and Fisher Scientific (Megginson, IL), except as noted.

Synthesis of IR700-conjugated anti-CD44

Anti-CD44 (1.0 mg, 6.7 nmol/L) was incubated with IR700 NHS ester (65.1 μg, 33.3 nmol/L) in 0.1 M Na2HPO4 (pH 8.6) at room temperature for 1 hour and purified with a Sephadex G25 column (PD-10; GE Healthcare). Protein concentration was determined with Coomassie Plus protein assay kit (Thermo Fisher Scientific Inc.) by measuring the absorption at 595 nm with UV-Vis (8453 Value System; Agilent Technologies). IR700 concentration was measured by absorption at 689 nm to confirm the number of fluorophore molecules per monoclonal antibody (mAb). CD44–IR700 conjugate synthesis was controlled so that an average of two IR700 molecules were bound to each CD44 antibody. Fluorescence at 700 nm/L and the molecular weight of CD44–IR700 conjugates (~150 kD) were verified using sodium dodecyl sulfate-polyacrylamide (4%–20% gradient) gel electrophoresis (SDS-PAGE).

Cell lines and culture

MC38 (colon cancer, kind gift from Claudia Palena, NCI) cells stably expressing luciferase (MC38-luc, generated via stable transduction with RedFect Red-Flu lentivirus from PerkinElmer per manufacturer recommendations), Lewis lung carcinoma (LLC; kind gift of James Hodge, NCI) cells, and MOC1 (murine oral carcinoma, kind gift from Ravindra Upshall, Washington University in St. Louis) cells were maintained in culture as previously described (15–17). Briefly, cells were cultured in IMDM/F12 (2:1) with 5% fetal calf serum, penicillin (50 U/mL) and streptomycin (50 μg/mL), 1% amphotericin, and EGF (5 ng/mL; Millipore). MOC1 cells were used from original stocks of exome-sequenced cells; otherwise, cell line authentication was performed via in vitro growth characteristics. Cells were maintained in culture for no more than 30 passages and routinely tested negative for Mycoplasma and murine-associated pathogens.

In vitro NIR-PIT

MC38-luc, LLC, or MOC1 cells (2 × 10^6) were seeded into 12-well plates, incubated for 24 hours, and then exposed to media containing CD44–IR700 (10 μg/mL) for 6 hours at 37°C. Cells were irradiated with a red light-emitting diode (LED, 690 ± 20 nm wavelength, L690-66-60; Manubeni America Co.) at a power density of 50 mW/cm². Cells were harvested with a cell scraper, stained with propidium iodide (PI, 2 μg/mL) at room temperature for 30 minutes, and then assessed for PI positivity on a BD FACScan (Beckton Dickinson Biosciences) using CellQuest software.

Animal and tumor models

All in vivo procedures were approved by the local Animal Care and Use Committee. Six- to 8-week-old female wild-type C57BL/6 mice (strain #000664) were purchased from The Jackson Laboratory. Tumors were established via subcutaneous injection of 6 × 10^6 cells in the caudal flank for each model. In some experiments, multiple subcutaneous MC38 tumors were established (contralateral caudal flank and bilateral cranial flanks). Established tumors were treated at volumes of approximately 50 mm^3 (4 to 5 mm in diameter; day 4 for MC38-luc and LLC tumors; day 18 for MOC1 tumors). Mice were shielded at sites of subcutaneous tumor transplantation prior to NIR light irradiation.

For NIR-PIT treatments and fluorescence/bioluminescence imaging (BLI), mice were anesthetized with inhaled 3% to 5% isoflurane and/or via intraperitoneal injection of 1 mg of sodium pentobarbital (Nembutal Sodium Solution, Ovation Pharmaceuticals Inc.). CD44–IR700 was administered via IV (tail-vein) injection, and NIR light was administered at 50 J/cm² on day 4 and 100 J/cm² on day 5. Previous results from our laboratory have demonstrated that two NIR light doses kill up to 80% of target-expressing cells (1, 2). For mice bearing multiple tumors, tumors not exposed to NIR were shielded from NIR light exposure with aluminum foil. Anti–PD-1 (clone RMP1-14, Bio X Cell, 100–200 μg/injection as indicated in figures) or isotype control (clone 2A3, Bio X Cell) was administered via intraperitoneal injection.

In some MC38 experiments, mice cleared of tumors after combination NIR-PIT and anti–PD-1 treatment were challenged via subcutaneous injection of MC38 (6 × 10^6) cells in the contralateral flank. Tumor volume (tumor volume = length × width^2 × 0.5) and animal weight were measured three times a week for MC38-luc and LLC tumors and two times a week for MOC1 tumors until the tumor volume reached 2,000 mm^3, whereupon the mice were euthanized with inhalation of carbon dioxide gas. For all immune correlate experiments, mice were euthanized via awake cervical dislocation.

Fluorescence imaging

In vivo, MC38-luc, LLC, or MOC1 cells (1 × 10^6) were seeded on cover-glass-bottomed dishes, incubated for 24 hours, and then exposed to CD44–IR700 (clone IM7, 10 μg/mL) for 6 hours at 37°C. Cells were then analyzed via fluorescence microscopy (BX61; Olympus America, Inc.) using a 590- to 650-nm excitation filter and a 665- to 740-nm band pass emission filter. Transmitted light differential interference contrast (DIC) images were also acquired.

In vivo, IR700 fluorescence and white light images were obtained using a Pearl Imager (700-nm fluorescence channel) and analyzed using Pearl Cam Software (LI-COR Biosciences). Regions of interest (ROI) within the tumor (n = 10) were
compared with adjacent nontumor regions as background (left dorsal). Average fluorescence intensity of each ROI was calculated.

**Bioluminescence imaging (BLI)**

*In vitro.* MC38-luc cells were seeded into 12-well plates (2 × 10^5 cells/well) or a 10-cm dish (2 × 10^5 cells), incubated for 24 hours, and then exposed to CD44-IR700 (10 μg/mL) for 6 hours at 37°C. Cells were treated with LED or NIR laser light (690 ± 5 nm, BWF5-690-8-600-0.37; B&W TEK Inc.) in phenol red-free culture medium. For luciferase activity, cells were exposed to D-luciferin (150 μg/mL; Gold Biotechnology) 1 hour after NIR-PIT treatment, and luciferase activity (photons/min) was obtained on a BLI system (Photon Imager; Biospace Lab) using M3 Vision Software (Biospace Lab). *In vivo,* D-luciferin (15 mg/mL, 200 μL) was injected intraperitoneally, and the mice were analyzed on a BLI system (Photon Imager) for luciferase activity (photons/min/cm²). ROIs were set to include the entire tumor with the adjacent nontumor region as background.

**Histologic analysis**

Tumors (day 10 for MC38-luc and LLC tumors, day 24 for MOC1 tumors) were excised, formalin-fixed and paraffin-embedded, and sectioned at 10 μm. Following standard H&E staining, brightlight photomicrographs were obtained on an Olympus BX61 microscope.

**Immunofluorescence**

Formalin-fixed paraffin-embedded sections were stained as described (18). Briefly, sections were deparaffinized in an ethanol gradient, then blocked in separate incubations with bloxall (Vector Laboratories), 2.5% normal goat serum (Vector Laboratories), and Renaissance antibody diluent (Biocare Medical). A primary antibody targeting CD4 (Invitrogen, clone 4SM95, 1:75 dilution) in Renaissance antibody diluent was added for 45 minutes on an orbital shaker. Slides were washed five times, then stained with an anti-rat secondary antibody (Vector Laboratories). Following four more washes, slides were stained with TSA-conjugated Opal650 (PerkinElmer, 1:150 dilution) in Amplification Plus buffer (PerkinElmer). Slides were washed four times with 1× TBST. Slides were washed, exposed to antigen stripping buffer (0.1 M glycine pH10 + 0.5% tween 20), and reblocked as above. A primary antibody targeting CD8 (Invitrogen, clone 4SM15, 1:75 dilution) in Reinsaissance antibody diluent was added for 45 minutes. Anti-rat secondary antibody (Vector Laboratories) was added as above. Following four more washes, slides were stained with TSA-conjugated Opal520 (PerkinElmer, 1:150 dilution) in Amplification Plus buffer (PerkinElmer). Nuclei counterstaining was achieved with Spectral DAPI (PerkinElmer, 1:500). Slides were rinsed once with ddH₂O, over-slipped with Vectashield hard mount (Vector Laboratories), and sealed with clear nail polish (Revlon).

**Flow cytometry**

*In vitro.* MC38-luc, LLC, or MOC1 cells (2 × 10^5) were seeded into 12-well plates and incubated for 24 hours. The cells were then exposed to media containing CD44-IR700 (10 μg/mL) for 6 hours at 37°C. Cells were harvested and analyzed on a BD FACSCalibur (BD Biosciences) using CellQuest software. To validate specific binding of CD44-IR700, cells were incubated with excess unconjugated CD44 antibody (clone IM7, Bio X Cell, 100 μg) prior to incubation with CD44-IR700.

*In vivo,* tumors were harvested (day 10 for MC38-luc and LLC tumors, day 24 for MOC1 tumors) and immediately digested as previously described (19). Briefly, minced tumors were digested using the mouse tumor dissociation kit (Miltenyi) and the gentleMACS dissociator (Miltenyi). Digests were filtered and suspended in cold 1× PBS in 1% BSA for staining. Following FcγR (CD16/32) blocking (clone 93, 1 μg/mL, 10 minutes on ice), single-cell suspensions were stained with fluorophore-conjugated primary antibodies: anti-mouse CD45.2 (clone 104), CD3 (clone 145-2C11), CD8 (clone 53-6.7), CD4 (clone GK1.5), PD-1 (clone 29F.1A12), CD11c (clone N418), F4/80 (clone BM8), CD11b (clone M1/70), Ly-6C (clone HK1.4), Ly-6G (clone 1A8), I-A/I-E (clone M5/114.15.2), PD-L1 (clone 10F.9G2), CD25 (clone PC61.5.3), CTLA-4 (clone UC10-4B9), CD31 (clone 390), PDGFR (clone APA5), and CD44 (clone IM7; all from BioLegend). Cells were incubated with primary antibodies for 1 hour in a 1% BSA/1× PBS buffer. Suspensions were washed, stained with a viability marker (7AAD or zombie aqua; BioLegend), and analyzed on a BD Canto using BD FACSDiva software. Isotype controls and “fluorescence minus one” controls were used to validate staining specificity. FoxP3+ regulatory CD4+ T lymphocytes (Tregs) were stained using the Mouse Regulatory T-Cell Staining Kit #1 (eBioscience) and associated fix and permeabilization buffers per the manufacturer’s protocol. Post-acquisition analysis was performed with FlowJo vX10.0.7r2.

**Antigen-specific TIL reactivity**

Minced fragments of fresh MC38, LLC, or MOC1 tumors were incubated in RPMI-1640 media supplemented with glutamine (2 mmol/L), HEPES (25 mmol/L), 1 mmol/L sodium pyruvate, 10 mmol/L β-mercaptoethanol, 5% FBS, and recombinant murine IL2 (100 U/mL; BioLegend) for 72 hours. Untouched TILs were enriched to >95% purity with negative magnetic sorting (Pan T-Cell Isolation Kit II, AutoMACSpro, Miltenyi Biotec). Antigen-presenting cells (APC; whole splenocytes from naïve, WT B6 mice from Taconic, irradiated with 50 Gy) were pulsed for 1 hour with peptides of interest: class I–restricted antigen p15E 504-611 (H-2Kb–restricted KSPWFITL; ref. 20), survivin/Birc5 57-64 (H-2Kb–restricted QCFCFKEEL; ref. 21), Twist315-323 (H-2Db–restricted KSPWFITL; ref. 20), and Trp53 232-240 (H-2Db–restricted SIINFEKL; ref. 21). Peptides were synthesized by Peptide2.0. Antigen-pulsed APCs and TILs were coincubated for 24 hours at a 3:1 APC:TIL ratio. Supernatants (50 μL) were analyzed for IFNγ production by ELISA (Mouse IFNγ Quantikine ELISA kit, R&D Systems) per the manufacturer’s recommendations. TILs alone, APCs alone, and peptide stimulations with ovalbumin 257-264 (H-2Kb–restricted SIINFEKL) and VSV-N52-59 (H-2Dβ–restricted GYYVQGL) were used as controls.

**Quantitative RT-PCR**

RNA from whole MC38, LLC, or MOC1 tumor lysates generated via Tissue Lyser II mechanical dissociation was purified using the RNeasy Mini Kit (Qiagen) per the manufacturer’s protocol. RNA purity and quantity were assessed on a Nanodrop Spectrophotometer based on absorbance at 260/280 and 260/230. CDNA was synthesized utilizing a high-capacity cDNA reverse transcription kit with an RNase inhibitor (Applied Biosystems) beginning with 2 μg or RNA template. A TaqMan Universal PCR master mix was used to assess the relative expression (ΔΔCT) of target genes...
compared with Gapdh on a Viia7 qPCR analyzer (Applied Biosystems) in technical triplicates. Custom primers were designed to flank nucleotide regions encoding the MHC class I–restricted epitopes for each TAA (Supplementary Table S1).

Statistical analysis

Data are expressed as means ± SEM from a minimum of five experiments, unless otherwise indicated. Statistical analyses were carried out using GraphPad Prism version 7 (GraphPad Software). Student t test was used to compare the treatment effects with that of control in vitro. To compare tumor growth in a reinoculated tumor model of MC38-luc, the Mann–Whitney test was used. For multiple comparisons, a one-way analysis of variance (ANOVA) followed by the Tukey test was used. The cumulative probability of survival based on volume (2,000 mm³) was estimated in each group with a Kaplan–Meier survival curve analysis, and the results were compared using the log-rank test. A P value of <0.05 was considered statistically significant.

Results

Confirmation of CD44 expression as a target for NIR-PIT

Using gel electrophoresis, CD44–IR700 conjugates demonstrated no appreciable aggregates, having a similar molecular weight to CD44 mAb alone. Conjugates demonstrated strong fluorescent intensity (Fig. 1A) and peak absorbance around 690 nm (Fig. 1B) as expected for IR700. Flow-cytometric and fluorescent microscopic analysis revealed a high fluorescent signal in MC38-luc (Fig. 1C and D), LLC (Supplementary Fig. S1A and S1B), and MOC1 (Supplementary Fig. S2A and S2B) cells after exposure to CD44–IR700. This signal was completely reversed in the presence of excess unconjugated CD44 mAb, verifying binding specificity. NIR light exposure of tumor cells exposed to CD44–IR700 induced immediate cellular swelling, bleb formation, and rupture of vesicles indicative of necrotic cell death in all three cell lines (Supplementary Videos S1–S3). These morphologic changes were observed within 15 minutes of NIR exposure (Fig. 1D; Supplementary Videos S1B and S2B). Bioluminescence imaging demonstrated decreased luciferase activity in a light-dose–dependent manner (Fig. 1E and F) in MC38-luc cells. Based on incorporation of propidium iodine, NIR induced cell death in a light-dose–dependent manner in MC38-luc (Fig. 1G), LLC (Supplementary Fig. S1C), and MOC1 (Supplementary Fig. S2C) cells exposed to CD44–IR700. NIR nor CD44–IR700 alone did not induce significant alterations in cell viability. These data validated that NIR–PIT targeting CD44 induced specific cell death in MC38-luc, LLC, and MOC1 cells in vitro.

CD44 expression within MOC1, LLC, and MC38-luc tumor compartments

To verify target expression of CD44 in vivo, size-matched MOC1 (day 24), LLC (day 10), and MC38 (day 10) tumors were assessed for CD44 expression within different tumor compartments via flow cytometry (Supplementary Fig. S3A). Significant heterogeneity in tumor and stromal cell–specific CD44 expression was observed, with LLC and MC38-luc tumor cells expressing significantly greater CD44 compared with MOC1. Expression of CD44 on immune cell subsets was more homogeneous between MOC1, LLC, and MC38-luc tumors and was greater than CD44 expression on tumor and stromal cells on a cell-by-cell basis as measured by mean fluorescence intensity (MFI). Whole tumor accumulation of CD44–IR700 1 day after injection, which is dependent upon multiple factors including target antigen expression and vascularity, was significantly greater in MC38-luc tumors (P < 0.001) compared with LLC or MOC1 tumors (Supplementary Fig. S3B and S3C).

In vivo effect of NIR-PIT and PD-1 mAb in mice bearing unilateral tumors

We previously demonstrated the antitumor effects of NIR-PIT targeting CD44 in syngeneic murine models of oral cancer (2) and that NIR-PIT treatment, in general, can lead to ICD and promote antitumor inflammatory responses (7). Thus, we hypothesized that the addition of systemic PD-1 ICB to local CD44-directed NIR-PIT would reverse adaptive immune resistance (10) and enhance antitumor responses. The NIR-PIT and PD-1 mAb treatment regimen and imaging protocol (Fig. 2A) for treatment of unilateral MC38-luc tumors (Fig. 2B) are depicted. Compared with control or PD-1 mAb alone groups, NIR-PIT resulted in a near-immediate decrease in tumor fluorescence signal, likely due to dispersion of IR700 from dying cells (Fig. 2C). Combination NIR-PIT and PD-1 mAb treatment resulted in dramatically decreased bioluminescence compared with control or single treatment groups (Fig. 2D and E). Histologic (H&E) analysis of treated tumors revealed extensive tumor necrosis and microhemorrhage in tumors treated with NIR-PIT, whereas groups treated with PD-1 mAb demonstrated greater leukocyte infiltration (Fig. 2F). Although primary tumor growth was inhibited following NIR-PIT or PD-1 mAb alone compared with control (Fig. 2G), combination treatment resulted in significant tumor control and complete rejection of established MC38-luc tumors in 9 of 13 (70%) mice and resulted in significantly prolonged survival (Fig. 2H). Neither skin necrosis nor systemic toxicity was observed within any treatment group.

Similar approaches were taken in mice bearing established unilateral LLC or MOC1 tumors using similar treatment regimens and imaging protocols (Supplementary Figs. S4A and S5A). Similar to MC38-luc tumors, treatment of LLC or MOC1 tumors with NIR-PIT resulted in near-immediate loss of IR700 fluorescent signal (Supplementary Figs. S4B and S5B) indicating on-target effects. Treatment of LLC tumor–bearing mice with combination NIR-PIT and PD-1 mAb significantly enhanced primary tumor control (Supplementary Fig. S4C) and survival (Supplementary Fig. S4D) over control or either treatment alone, and resulted in rejection of 1 of 12 (8%) established tumors. Treatment of MOC1 tumor–bearing mice with combination NIR-PIT and PD-1 mAb induced rejection of 1 of 13 (8%) established tumors and resulted in statistically enhanced survival compared with control, but cumulative primary tumor growth following combination treatment was not enhanced over either treatment alone (Supplementary Fig. S5C and S5D). Taken together, these results demonstrated CD44 on-target effects of NIR-PIT in MC38-luc, LLC, and MOC1 tumor–bearing mice, with significant enhancement of primary tumor control and survival with the addition of PD-1 ICB in the MC38-luc and LLC models.

Antigen-specific immunity is induced with NIR-PIT and enhanced by PD-1 ICB

Following completion of treatment, some MC38-luc tumors were processed into single-cell suspensions and assessed for infiltration of immune cells with flow cytometry. Tumors treated with NIR-PIT demonstrated significantly enhanced infiltration...
Figure 1.
Confirmation of CD44 expression as a target for NIR-PIT in MC38-luc cells and evaluation of in vitro NIR-PIT. A, Validation of CD44-IR700 by SDS-PAGE (left: Colloidal Blue staining; right: IR700 fluorescence). Diluted anti-CD44 was used as a control. B, Absorbance curve of CD44–IR700. C, Expression of cell-surface CD44 in MC38-luc cells was examined with flow cytometry. CD44-blocking antibody was added to some wells to validate specific staining. Representative histograms were shown. D, Differential interference contrast (DIC) and fluorescence microscopy images of MC38-luc cells. Change in MC38-luc cellular architecture following 15 minutes of NIR light exposure shown. Scale bars, 20 μm. E, Bioluminescence imaging (BLI) demonstrating luciferase activity in MC38-luc cells following NIR light. F, Quantification of MC38-luc luciferase activity after labeling with CD44-IR700 and treatment with NIR light (n = 5; **, P < 0.01 vs. untreated control, Student t test). G, Membrane permeability of MC38-luc cells, as measured by PI staining, after labeling with CD44-IR700 and treatment with NIR light (n = 5; *, P < 0.05 vs. untreated control; **, P < 0.01 vs. untreated control, Student t test). Each value represents mean ± SEM of five independent experiments.
by CD8+ and CD4+ lymphocytes (Fig. 3A) that expressed greater PD-1. Mice treated with systemic PD-1 mAb demonstrated PD-1 target saturation, as very low levels of PD-1 were detectable on the surface of TILs from these tumors by flow cytometry after staining with the same antibody clone (RMP1-14). This enhanced CD8+ and CD4+ TIL infiltration was verified by multiplex immuno-fluorescence (IF). In control- or PD-1 mAb–treated tumors, few CD8+ TILs nested along the tumor–stromal interface but did not infiltrate the tumor (Fig. 3B, left). Following NIR-PIT, more CD8+ TILs infiltrated throughout the tumor, but many TILs were still arrested at the tumor–stromal interface. Infiltration into the tumor was significantly enhanced with the addition of PD-1 mAb (Fig. 3B, right). In additional experiments, TILs were extracted from control- or treated MC38-luc tumors via IL2, and assessed for antigen-specific IFNγ responses to multiple known H-2Kb– or H-2Kd–restricted TAAs (Fig. 3C). TILs from control tumors demonstrated measurable responses to H-2Kb–restricted survivin/Birc5 (QCFFCFREI) and H-2Dd–restricted Trp53 232–240 (KYMNSSCM) and enhanced baseline responses to p15E6 604–611. Treatment with PD-1 mAb enhanced these NIR-PIT–induced or enhanced antigen-specific responses.

NIR-PIT also enhanced tumor infiltration of MHC class II+ DCs and F4/80+ macrophages polarized to express greater MHC class II (Fig. 3D). Immunosuppressive neutrophilic-myeloid (PMN-myeloid) and regulatory CD4+ T lymphocytes (Tregs) were variably altered by combination treatment (Fig. 3E). MC38-luc tumor cell–specific PD-L1 expression was verified but did not change with treatment, whereas infiltrating immune cell PD-L1 was significantly greater than tumor cell expression and increased with combination treatment (Fig. 3F).

Similar immune correlate experiments were carried out in LLC and MOC1 tumors. LLC tumors treated with PD-1 mAb and NIR-PIT alone or in combination demonstrated enhanced TIL infiltration (Supplementary Fig. S6A). Antigen-specific LLC TILs demonstrated measurable baseline responses to p15E6 604–611 and H-2Dd–restricted Twist 125–133 (TQSLNEAFA) similar to...
MC38-luc tumors, NIR-PIT treatment induced responses to survivin/Birc5. Responses to Birc5 and Twist but not p15E were enhanced with PD-1 mAb treatment (Supplementary Fig. S6B). NIR-PIT treatment of LLC tumors enhanced infiltration of MHC class II+ DCs and MHC class II expression on macrophages (Supplementary Fig. S6C). PMN-myeloid cells and Tregs were variably altered following treatments (Supplementary Fig. S6D), and LLC tumor and immune cell–specific PD-L1 expression was enhanced with treatment (Supplementary Fig. S6E). In contrast to MC38-luc or LLC tumors, MOC1 tumors treated with NIR-PIT demonstrated few immune correlative alterations. CD8+ and CD4+ TIL infiltration was enhanced with PD-1 mAb but not NIR-PIT (Supplementary Fig. S7A). Baseline TIL antigen-specific responses to p15E604–611 were enhanced with systemic PD-1 mAb treatment, but responses to other shared tumor antigens were not induced with NIR-PIT treatment (Supplementary Fig. S7B).

To investigate possible explanations for the lack of TIL responses against TAAs in MOC1, we measured relative expression of each antigen within MC38-luc, LLC, and MOC1 cells. Using primers designed to flank the MHC class I–restricted
epitope coding region, PCR results indicated low expression of Birc5, Twist1, and Trp53 gene transcripts in MOC1 relative to MC38-luc and LLC (Supplementary Fig. S8). Greater antigen expression generally correlated with baseline TIL responses. Higher relative Trp53 expression in MC38-luc cells and Twist1 expression in LLC cells correlated to enhanced TIL responses against the class I–restricted epitopes from these genes after combination NIR-PIT and PD-1 mAb treatment. Although correlative, these data suggest that enhanced TIL responses after treatment may be dependent on baseline tumor antigen expression. Taken together, these results indicated that NIR-PIT can induce de novo, polyclonal antigen-specific TIL responses against MHC class I–restricted tumor antigens in MC38-luc and LLC tumor–bearing mice, and that these responses can be enhanced with systemic PD-1 ICB.

Combination NIR-PIT and PD-1 ICB induces an abscopal antitumor effect in mice

Given evidence of induction of tumor antigen–specific immunity following NIR-PIT in MC38-luc tumor–bearing mice, we next assessed whether local NIR-PIT combined with systemic PD-1 mAb could induce antitumor immunity in a separate, distant tumor not treated with NIR-PIT. Treatment and imaging regimens (Fig. 4A) were similar for mice bearing bilateral MC38-luc tumors as previously described, but only the right flank tumor was treated with NIR-PIT (Fig. 4B). NIR-PIT induced near-immediate loss of IR700 fluorescent signal in the treated tumor, whereas loss of IR700 signal intensity in the untreated tumor was delayed for several days (Fig. 4C). Conversely, bioluminescence of both right (treated with NIR-PIT) and left (untreated) MC38-luc tumors decreased concurrently after combination treatment (Fig. 4D and E). Histologic analysis of both right and left tumors revealed...
NIR-PIT Combined with PD-1 Blockade Eradicates Tumors

![Image](https://www.aacrjournals.org/cancerimmunolres/2019/7(3)/CIR.18-0546/Figure5A.png)

**Figure 5.** Immune correlative and functional effects of NIR-PIT and PD-1 mAb in mice bearing bilateral MC38-luc tumors. **A,** Bilateral MC38-luc tumors (day 10, n = 5/group) treated with PD-1 mAb with or without NIR-PIT and bilateral control tumors were harvested, digested into single-cell suspensions, and analyzed for TIL via flow cytometry. Presented as absolute number of infiltrating cells per 1.5 × 10⁶ live cells analyzed. PD-1 expression shown as inset (MFI). P < 0.05; **,** P < 0.001, t test with ANOVA. Representative data from one of two independent experiments shown. **B,** TILs were extracted from tumors treated as above (n = 5/group) via an IL2 gradient, enriched via negative magnetic selection, and stimulated with irradiated splenocytes pulsed with peptides representing known MHC class I-restricted epitopes from selected TAAs. IFNγ production with ANOVA. Representative data from one of two independent experiments shown. **C,** Flow-cytometric analysis of tumor-infiltrating DCs and macrophages, with quantification of macrophage polarization based on MHC class II expression. **,** P < 0.05; **,** P < 0.001, t test with ANOVA. **D,** Flow-cytometric analysis of tumor-infiltrating PMN-myeloid and Tregs. **,** P < 0.05; **,** P < 0.001, t test with ANOVA. **E,** Flow-cytometric analysis of PD-L1 expression on CD45.2 CD31 PDGFR+ tumor cells. N = 5/group.

Induction of antigen-specific immunity in distant tumors not treated with NIR-PIT

Flow-cytometric analysis of single-cell suspensions from both right (treated with NIR-PIT) and left (untreated) tumors revealed similar enhancement of CD8⁺ and CD4⁺ TIL accumulation (Fig. 5A). Assessment of antigen-specific reactivity demonstrated that TILs from both treated and untreated tumors reacted to the same MHC class I-restricted antigens (Fig. 5B), indicating the presence of systemic antigen-specific immunity. TIL responses were similar in magnitude to p15E604 and survivin/Birc5/E61 and survivin/Birc5/E61 and survivin/Birc5/E61 and survivin/Birc5/E61 and survivin/Birc5/E61 and survivin/Birc5/E61 but responses to Tp53/232-240 were diminished in tumors not treated with NIR-PIT compared with treated. Increased MHC class II⁺ DCs and macrophages (Fig. 5C), increased PMN-myeloid cells, and decreased Tregs (Fig. 5D) were observed in treated but not untreated tumors, suggesting these changes are a direct result of NIR-PIT and not a result of systemic antitumor immunity. MC38-luc infiltrating immune cell PD-L1 expression (Fig. 5E) was enhanced in both right (treated) and left (untreated) tumors in mice receiving combination treatment, indicating that immune cell PD-L1 expression may be independent of NIR-PIT. These results together demonstrated that combination NIR-PIT and PD-1 ICB can lead to the development of systemic tumor antigen–specific immunity capable of eliminating an established untreated tumor, but that enhanced innate immunity and alterations in immunosuppressive cell subsets appear to occur locally as a more direct effect of NIR-PIT.

Combination NIR-PIT and PD-1 ICB leads to control of multiple distant tumors

We next investigated whether treatment of a single MC38-luc tumor could lead to rejection of multiple established distant tumors within an individual mouse. Similar treatments (Fig. 6A) were used to deliver NIR-PIT to one of four established MC38 tumors (Fig. 6B). NIR-PIT induced near-immediate loss of IR700 fluorescent signal in the single treated tumor, whereas resolution of IR700 signal intensity in the three untreated tumors was delayed for several days (Fig. 6C). Conversely, bioluminescence of both the single treated and three untreated MC38-luc tumors decreased concurrently after combination treatment (Fig. 6D and E). Histologic analysis revealed necrosis and increased leukocyte infiltration in all tumors from treated mice but not tumors from control mice (Fig. 6F). Systemic PD-1 mAb and treatment of a single MC38-luc tumor with NIR-PIT resulted in similar patterns of necrosis and microhemorrhage and increase leukocyte infiltration (Fig. 4F). Combination treatment resulted in significant primary tumor control and complete tumor rejection of both right and left tumors in 8 of 10 mice (80%; Fig. 4G), leading to enhanced survival compared with untreated mice (Fig. 4H).
in growth control of multiple MC38-luc tumors. Twelve of 15 (80%) treated mice (Fig. 6G) completely rejected all four tumors, resulting in enhanced survival compared with control (Fig. 6H). Thus, treatment of a single focus of tumor with local NIR-PIT plus systemic PD-1 ICB is sufficient to induce systemic immunity capable of eliminating multiple sites of distant disease not treated with NIR-PIT.

Development of immunologic memory after combination NIR-PIT and PD-1 ICB

To assess for the presence of immunologic memory, mice were treated with NIR-PIT and PD-1 mAb as before (Supplementary Fig. S9A). Mice that demonstrated a complete response to combination treatment were challenged 30 days later with injection of MC38-luc cells in the contralateral flank (Supplementary Fig. S9B). Whereas control mice were readily engrafted with MC38-luc tumors, mice that previously rejected established MC38-luc tumors resisted engraftment and did not grow tumors (Supplementary Fig. S9C, survival in Supplementary Fig. S9D), demonstrating the presence of immunologic memory.

As depicted in Fig. 7, this and previous work has demonstrated that NIR-PIT induces CD44-specific tumor cell death, leading to the release of multiple tumor antigens. NIR-PIT also promotes a proinflammatory tumor microenvironment, resulting in the cross-priming of multiple antigens and the development of a polyclonal antigen-specific T-cell response. This effector response is limited by PD-1/PD-L1 expression and adaptive immune resistance, which is effectively reversed with the addition of PD-1 ICB.

Discussion

This and other work has demonstrated on-target cytolytic effects of NIR-PIT monotherapy (1–6), as well as the ability of
NIR-PIT combined with PD-1 blockade eradicates tumors

NIR-PIT to induce ICD capable of inducing DC maturation (7). Batf3⁺ DCs are required for production of chemokine signals that drive trafficking of effector T cells into tumors (23), and type I interferon production by DCs is required for innate activation of adaptive T-cell immunity (24, 25). Here, we demonstrated that NIR-PIT treatment alone induced tumor cell death in multiple models, yet durable antitumor responses were not consistently achieved as established tumors failed to reject completely after monotherapy despite a wild-type immune background. In mice bearing MC38 colon adenocarcinomas or LLC lung carcinomas, NIR-PIT enhanced tumor accumulation of MHC class II⁺ DCs, indicating that DC priming had occurred. TILs within tumors treated with NIR-PIT expressed more PD-1, consistent with their activation, and both tumor and infiltrating immune cells expressed consistent or greater PD-L1 after NIR-PIT, suggesting that adaptive immune resistance could be limiting antitumor immunity. The addition of PD-1 ICB to CD44 NIR-PIT significantly enhanced tumor control in mice bearing MC38 and LLC tumors and led to the induction of robust antitumor immunity. PD-1 ICB reversed adaptive immune resistance, likely induced by local interferon production following NIR-PIT, resulting in systemic antitumor immunity sufficiently potent to induce complete rejection of multiple established tumors. This work serves as proof of concept that PD-1 ICB can be used to enhance antitumor immunity induced by NIR-PIT, which can be formulated to target any number of tumor cell-surface antigens.

Seminal work by Schreiber and colleagues demonstrated that PD-1 ICB enhances the activity of TILs targeting neoantigens derived from tumor-specific mutated but expressed genes (tumor-specific antigens, TSAs; ref. 26). We did not identify TSAs, but rather, our work assessed specific TIL responses against a panel of TAAs known or suspected to be shared among MC38, LLC, and MOC1 and with previously identified MHC class I-restricted epitopes (20–22). We demonstrated enhanced TIL responses following NIR-PIT against antigens not targeted at baseline. Not to be confused with the concept of “antigen spread,” which refers to a polyclonal T-cell response following induction of T-cell immunity against a specific antigen with approaches such as peptide vaccination (16, 27), our work demonstrated the ability of NIR-PIT to induce release of multiple TAAs that were processed...
by DCs leading to a polyclonal T-cell immune response. This work definitively demonstrates the development of polyclonal T-cell responses following tumor-targeting cytolytic therapy. Dovedi and colleagues demonstrated the ability of IR to increase accumulation of TILs derived from preexisting T-cell clones based upon sequencing of the TCR repertoire (28). Why NIR-PIT but not other cytotoxic treatment is able to induce a polyclonal T-cell response remains unclear, but may lie with the ability of NIR-PIT to concurrently induce DC maturation through induction of ICD.

Evidence of de novo antigen-specific T-cell responses against new antigens following PD-1 ICB is lacking (29). Our data demonstrated enhancement of preexisting TAA-specific TIL responses following treatment with PD-1 mAb alone, supporting that ICB enhances the antitumor activity of preexisting but dysfunctional antigen-specific TILs. However, PD-1 ICB enhanced de novo TAA-specific TIL responses induced following NIR-PIT as well. Assessment of distant, untreated tumors demonstrated infiltration and activation of TILs that were induced in the treated tumor with combination NIR-PIT and PD-1 ICB, definitively demonstrating the development of systemic antigen-specific antitumor immunity. This has clinical implications for the metastatic disease setting, where if feasible, treatment of one or more sites of disease with tumor-targeting NIR-PIT along with systemic PD-1 ICB could result in systemic antitumor immune responses in lesions not treated with NIR-PIT—analogous to the so-called abscopal effect infrequently observed with ionizing radiation (30, 31).

As a proof-of-concept study, we targeted CD44 to induce cytolyis, targeting the CD44 cell–surface antigen with NIR-PIT may have multiple benefits. The tumor lines used in this study all expressed high CD44 in vitro, but tumor cell CD44 expression in vivo was much more heterogeneous between models. CD44 is expressed on tumor cells to a degree in most tumors (32, 33) and can serve as a tumor cell stemness marker (34–36), suggesting that targeting these cells with NIR-PIT may eliminate the cells most resistant to other anticancer treatments. However, CD44 is also expressed on activated immune cells within the tumor treatment field, raising the concern that CD44-targeting NIR-PIT could eliminate desirable effector immune cells. Despite this, significant antitumor immunity was induced with combination CD44-targeting NIR-PIT and anti–PD-1. Although not captured in our immune analyses, at day 10 after the start of treatment, one possible explanation is that one to two NIR-PIT treatments targeting an antigen expressed on tumor-infiltrating immune cells ’reset’ the immunosuppressive immune microenvironment by eliminating immunosuppressive subsets, similar to induction chemotherapy before adoptive cell transfer immunotherapy (37). Tumor-associated macrophages, MDSCs, and Tregs expressed high CD44. IR700 conjugated to an EGFR-targeting antibody is currently in clinical development (RM-1929, NCT02422979). Although EGFR-targeting antibodies are FDA approved, providing a significant clinical development advantage, it remains to be seen whether NIR-PIT targeting tumor cells alone (with EGFR, for example) will have the same immunostimulatory effects as NIR-PIT targeting more broadly expressed surface antigens.

Several possibilities exist to explain why we did not observe a significant combinatorial effect in MOC1 tumors. First, CD44 tumor–specific expression was significantly lower on MOC1 cells in vivo compared with LLC or MC38, suggesting that antigen expression is important for the cytolytic effect of NIR-PIT. Next, baseline expression of survivin, Twist1, and Tp53 TAA was low within MOC1 tumors relative to LLC and MC38. Accordingly, TIL responses against these antigens were not induced following NIR-PIT treatment of MOC1 tumors as they were in LLC and MC38. There was no increase in tumor MHC class II+ DCs or lymphocyte infiltration following NIR-PIT treatment of MOC1 tumors as there was in LLC and MC38, indicating a lack of DC priming. These results suggest that assessment of basic tumor inflammatory responses after NIR-PIT, namely, tumor DC and lymphocyte infiltration, could serve as biomarkers of response and select patients most likely to respond to the addition of PD-1 ICB.

Limitations of this study include our inability to replicate the IR700 conjugate currently in clinical development that targets cell-surface EGFR (NCT02422979). Multiple attempts to generate an antibody with high specificity for mouse EGFR were unsuccessful. Given our previous work targeting CD44 with NIR-PIT, we used this cytolytic approach for proof-of-concept studies on antigen release and the development of polyclonal T-cell responses described here. It is possible that using PIT-NIR to target other surface antigens could variably alter T-cell priming, especially if NIR-PIT targeting of CD44 immunosuppressive immune cell subsets contributes to immune activation. This requires further study.

In conclusion, we have demonstrated the ability of tumor-targeting NIR-PIT to induce tumor cell death and innate priming of polyclonal, antigen-specific T-cell responses in models of colon and lung cancer. These polyclonal responses were enhanced via reversal of adaptive immune resistance with PD-1 ICB, leading to durable antitumor immunity, eradication of both treated and distant untreated tumors, and formation of immunologic memory. These results provide a strong preclinical rationale for the treatment of patients with locoregionally advanced or metastatic malignancies with combination NIR-PIT and PD-1 ICB in the trial setting.

Disclosure of Potential Conflicts of Interest
P.L. Choyke has ownership interest in a U.S. government patent on photodynamic immunotherapy. No potential conflicts of interest were disclosed by the other authors.

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References


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