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Virus-like particle-drug conjugates induce protective, long-lasting adaptive anti-tumor immunity in the absence of specifically targeted tumor antigens

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Abstract

This study examined the ability of a papillomavirus-like particle drug-conjugate, belzupacap sarotalocan (AU-011) to eradicate subcutaneous tumors after intravenous injection and to subsequently elicit long-term anti-tumor immunity in the TC-1 syngeneic murine tumor model. Upon *in vitro* activation with near-infrared light (NIR), AU-011-mediated cell killing was pro-immunogenic in nature, resulting in the release of damage-associated molecular patterns such as DNA, ATP, and HMGB-1, activation of caspase-1, and surface re-localization of calreticulin and HSP70 on killed tumor cells. A single *in vivo* administration of AU-011 followed by NIR caused rapid cell death, leading to long-term tumor regression in ~50% of all animals. Within hours of treatment, calreticulin surface expression, caspase-1 activation, and depletion of immunosuppressive leukocytes were observed in tumors. Combination of AU-011 with immune checkpoint inhibitor antibodies, anti-CTLA-4 or anti-PD-1, improved therapeutic efficacy, resulting in 70–100% complete response rate that was durable 100 days post-treatment, with 50–80% of those animals displaying protection from secondary tumor re-challenge. Depletion of CD4⁺ or CD8⁺ T cells, either at the time of AU-011 treatment or secondary tumor re-challenge of tumor-free mice, indicated that both cell populations are vital to AU-011's ability to eradicate primary tumors and induce long-lasting anti-tumor protection. Tumor-specific CD8⁺ T-cell responses could be observed in circulating PBMCs within three weeks of AU-011 treatment. These data, taken together, support the conclusion that AU-011 has a direct cytotoxic effect on tumor cells and induces long-term anti-tumor immunity, and this activity is enhanced when combined with checkpoint inhibitor antibodies.

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Conflict of Interest:

RCK – employee of Aura Biosciences; patent

CDT – no conflict of interest

SS – no conflict of interest

ZL – employee of Aura Biosciences

EP – employee of Aura Biosciences; patent

SM – employee of Aura Biosciences

JTS – patent

Keywords

HPV VLP; photodynamic therapy; neoantigens; checkpoint inhibitors; tumor immunity

Introduction

We previously described the targeted cancer therapy, belzupacap sarotalocan (AU-011), a novel virus-like particle-drug conjugate (VDC) comprised of a modified human papillomavirus 16 (HPV16) virus-like particle (VLP) conjugated with ~200 molecules of the photoactivatable drug, IRDye-700DX (IR700) (1). The unique cytotoxic nature of IR700 was first reported by Kobayashi and colleagues (2), in the context of an anti-EGFR antibody-drug conjugate (ADC) and is currently being tested in a clinical trial for head and neck cancer ([ClinicalTrials.gov #NCT02422979](https://clinicaltrials.gov/ct2/show/study/NCT02422979)). Based on our promising preliminary findings, AU-011 is currently being assessed in two phase II clinical trials as a first-line treatment for choroidal melanoma ([ClinicalTrial.gov #NCT03052127](https://clinicaltrials.gov/ct2/show/study/NCT03052127), [#NCT04417530](https://clinicaltrials.gov/ct2/show/study/NCT04417530)). HPV VLPs display a natural tropism specifically targeting modified heparan sulfate proteoglycans (HSPGs) typically restricted to basement membranes in normal, intact tissues but also displayed on the surfaces of many tumors, making their use a novel approach for treating a broad range of tumor types (3,4). HSPGs in healthy, intact tissues remain inaccessible to VLP binding, thereby reducing off-target tissue association. When administered locally or systemically, AU-011 binds to the surface of tumor cells, and upon activation with near-infrared light (NIR), it induces rapid necrotic cell death. The requirement for directed NIR activation further provides the specificity of AU-011 treatment. Within minutes of activation with NIR light, VLP- or antibody-conjugated IR700 closely associated with the cell surface lead to localized generation of singlet oxygen and cell membrane disruption resulting in a rapid increase in cell size, indicating an influx of water, followed by release of intracellular components and ultimately necrotic cell death (1,5–8). This rapid and necrotic cell death may be pro-inflammatory and immunogenic in nature. Several hallmark features of immunogenic cell death (ICD), namely damage-associated molecular patterns (DAMPs), can be measured at the cellular level and within the tumor milieu and may successfully promote anti-tumor immune responses in the context of tumor therapy (9).

As cells undergo tumorigenesis, they acquire genetic alterations that favor immune evasion and survival. These changes can lead to the generation of mutated or otherwise altered self-proteins that could serve as tumor-restricted antigens (10). Researchers have demonstrated the utility of these “neoantigens” in cancer immunotherapy (11–14), and their identification and targeting has been at the forefront of current tumor immunotherapeutic discovery. Limitations do exist, the most glaring being that, unless consensus neoantigens can be established across an array of tumors types and patients, treatment modalities will remain patient-specific, leading to time consuming and costly therapy. As the field advances, it is important to consider therapeutics, such as AU-011, that can target a broad spectrum of cancer types, while simultaneously maintaining tumor-specific cytotoxicity.

The use of photodynamic therapy (PDT), applying a photosensitizing drug combined with light activation, to treat tumors, has been in practice for many years (15). Additionally, the ability of PDT to generate localized pro-inflammatory states within tumors, typically due to direct cell killing, reactive oxygen species (ROS) damage, and recruitment of immunocytes is well established (16–18). The immunogenic nature of PDT-like treatments makes them amenable to enhancement with the use of checkpoint inhibitors (19,20). Checkpoint blockades are effective tools for potentiating immunological responses within the tumor microenvironment (TME) and provide synergy when combined with pro-immunogenic therapies. Antibodies against CTLA-4 and PD-1 block the inhibitory signals being sent to T cells that lead them to become impaired or tolerogenic within the TME (21). There is evidence supporting checkpoint blockade treatment in which patients with tumors containing high mutational burdens are more responsive, implying that this blockade could facilitate the induction of T-cell responses to neoantigens within tumors previously shielded from the immune system and, when combined with a pro-immunogenic therapy, could augment anti-tumor immunity (22–25). Checkpoint inhibitors are currently being combined with a variety of other therapeutic modalities such as radiation (26), chemotherapy (27), oncolytic viruses (28), peptide and protein subunit vaccines (29,30), tumor-infiltrating lymphocyte (TIL) therapy (31), CAR-T therapy (32), as well as in combination with other antibody therapies (33,34), all with the estimation that the treatment regimens will synergize in the induction of effective anti-tumor immunity.

In this study, we examined the potential of AU-011 to induce the hallmarks of pro-immunogenic cell death both *in vitro* and *in vivo*, as evidenced by the rapid induction of DAMPs. Using an immunocompetent murine tumor model, we explored AU-011's capability to treat primary tumors, alter the TME, and induce long-term anti-tumor immunity, as evidenced by CD8⁺ and CD4⁺ T-cell-dependent protection from tumor re-challenge. We also examined the ability of AU-011 to synergize with the checkpoint inhibitors anti-CTLA-4 and anti-PD-1 in enhancing the overall anti-tumor response.

Materials and Methods

Production of virus-like particle-drug conjugates (AU-011)

AU-011 production and purification is previously described (1). VLPs were generated by Paragon Bioservices (Baltimore, MD). Briefly, the HPV VLPs were generated in Freestyle 293F (ThermoFisher) cells by transfection with a plasmid co-expressing a modified HPV16 L1 gene and the wild-type L2 gene. VLPs self-assembled and were purified using a combination of sulfate- (EMD Millipore), cation- (SPXL; GE Healthcare Life Sciences), and anion- (QXL; GE Healthcare Life Sciences) based chromatography followed by covalent linkage to IRDye 700DX (IR700; LI-COR Biosciences) using N-hydroxysuccinimide reactive groups. Free dye was removed using tangential flow filtration, and AU-011 was then quantified using BCA analysis for protein content and the absorbance at 689nm was recorded for the purpose of dye quantification.

Cells and cell culture

Freestyle 293F (ThermoFisher) cells were obtained in 2014, grown in Freestyle growth media supplemented with 2 mM L-glutamine (ThermoFisher), determined to be mycoplasma-free, and authenticated prior to being banked at an early passage. TC-1 cells (kindly provided by Dr. T.C. Wu in 2007 (Johns Hopkins University, Baltimore, MD)(35)) and MB49luc (kindly provided by Dr. Denise Nardelli-Haeffliger in 2015 (CHUV, Lausanne, Switzerland)(36,37) were cultured in DMEM (Corning) supplemented with 10% FBS (Corning), and TC-1 cells were maintained in G418 (0.4 mg/mL; Invivogen). TC-1 cells were created by retrovirally transducing murine primary lung cells with HPV16 E6 and E7 followed by transduction with a plasmid expressing activated human c-Ha-ras. MB49luc cells were generated using 7, 12-dimethylbenz[a]anthracene induced urothelial carcinoma cells retrovirally transduced with firefly luciferase. MC38 cells were generated from murine colon cells after mice were injected with 1,2-dimethylhydrazine were obtained from Dr. James Hodges in 2017 (NCI, Bethesda, MD)(38) and cultured in DMEM supplemented with 10% FBS, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.1 mM non-essential amino acids, 10 mM HEPES, and 50 µg/mL gentamycin (Invitrogen). Upon receipt, cells were determined to be mycoplasma free, banked within three passages as required for expansion, and used for experiments within two passages after being thawed.

Animals

Eight-week-old female albino C57Bl/6 mice (Charles Rivers; Strain #562) were used for all studies unless otherwise noted. Animal studies described herein were approved by the Institutional Animal Care and Use Committees of the NCI (Bethesda, MD).

Immunizations

Mice were immunized subcutaneously three times at two-week intervals with either 10 µg each of HPV16 E6 and HPV16 E7 proteins (LSBio) or 15 µg each of H-2Db MHC class I peptides derived from their sequences: E6^{48–57}-EVYDFAFRDL (Selleckchem) and E7^{49–57}-RAHYNIVTF (Iba Lifesciences). Antigens were diluted into PBS with 1:10 ImjectAlum (Thermo) and 50 µg HMW-Poly I:C (Invivogen). One week after the third immunization, blood was obtained by retro-orbital collection using non-heparinized capillary tubes into EDTA coated tubes (BD Biosciences). Sample processing is described below.

Tumor implantation and treatment

TC-1 cells were detached using 10 mM EDTA and counted. A total of 5×10^5 cells (>98% viability) were implanted subcutaneously in the right hind flank of mice. When tumors reached a size of 40–80 mm³, they were randomized into treatment groups, such that group averages approximated 50 mm³ at the time of AU-011 treatment. AU-011 was delivered intravenously by tail vein injection, followed 10–14 hours later (unless otherwise noted) by exposure to 690 nm light (near-IR, NIR) using an MLL-III-690 laser (Opto Engine) connected to a fiber optic cable fitted with a collimator (25.4 mm BK7, CeramOptec). Doses of AU-011 used in the optimization and ICD studies were 25 µg, 35 µg, 50 µg, 75 µg, 100 µg, or 200 µg. All infiltrate and survival studies used 100 µg. NIR doses (J/cm²) used for optimizations studies were 6.25, 12.5, 25, 50, or 100. All subsequent studies used 50

J/cm². For studies using a short-term tumor viability readout, tumors were excised ~36 hours after NIR treatment (unless otherwise noted) and processed into single-cell suspensions as described in Kines et al. (1). Briefly, the tumors were cut into 1–2 mm³ size pieces, placed in a 4 mL solution of 1x PBS supplemented with DNase I (0.1 mg/mL; Roche) and collagenase A (0.5 mg/mL; Roche). Tumors were digested for 20 minutes in a 37°C shaker, transferred to a 70 µm cell strainer (Corning), and gently pressed thru using the pressing end of a 1 cc syringe. Cells were washed with 1x PBS supplemented with 2% FBS and used for downstream assays as described below. For studies assessing survival, tumors were measured twice weekly with study endpoints of tumor volumes reaching 1500 mm³ or 100 days. Studies involving tumor re-challenge consisted of subcutaneously implanting 5 × 10⁴ (>98% viability) TC-1 tumor cells in the opposite flank (left hind) 100 days after treatment with similar endpoints for survival. Anti-CTLA-4, anti-PD-1, or their matched isotype control were delivered in 200 µL intraperitoneally at a dose of 100 µg on days –3, 0, and +3, with AU-011 treatment occurring on day 0. Depleting antibodies, anti-CD4, anti-CD8, or matched isotype controls, were delivered on days –3 (200 µg), –1 (100 µg), +1 (100 µg), and +10 (100 µg) for depletion at the time of treatment. A similar schedule was employed for the tumor re-challenge studies with day 0 being the time of tumor implantation with an additional injection of depleting antibodies on day +17 (100 µg) to maintain depletion. All *in vivo* administered antibodies were diluted in InVivo Pure diluent (BioXCell).

Assessment of AU-011 binding and potency *in vitro*

The binding and potency protocol has been described previously (1); briefly, TC-1 cells in suspension were incubated with AU-011 (25 µg/mL, 5 µg/mL, 1 µg/mL, and 0.2 µg/mL), and half of the cells were irradiated with 25 J/cm² of 690 nm NIR light (Modulight ML6700-PDT with MLA Kit). The remaining cells served as untreated controls (0 J/cm²). Supernatants were sampled 15–30 minutes after *in vitro* AU-011+/-NIR treatment for downstream ICD analysis (described below), and cells were allowed 1–2-hour recovery at 37°C in DMEM supplemented with 10% FBS, unless otherwise noted, and were then stained for 20–30 minutes using LIVE/DEAD Yellow fixable viability dye following the manufacturer's instructions (Thermo Fisher), followed by 10-minute fixation in 4% paraformaldehyde (EMS). Cells were acquired using a BD FACS Canto II flow cytometer outfitted with an HTS (BD Biosciences). Data were analyzed using FlowJo v10 and plotted using GraphPad Prism v8.

Antibodies for flow cytometry and microscopy

Calreticulin antibodies (APC and 594 conjugates; 1G6A7) and HSP70 antibodies (AF488 and 594 conjugates; NBP1–77455) were purchased from Novus Biologicals and used at the manufacturer's recommended dilutions for flow cytometry and microscopy. Anti-Fc receptor (CD16/CD32) was used for blocking in flow cytometry experiments (BioXCell 2.4G2; 1µg/10⁵ cells). Antibodies used for flow cytometry were purchased from BioLegend unless noted and used at the manufacturer's recommended dilution: anti-CD45 (30-F11), anti-CD3 (17A2), anti-CD4 (RM4–5), anti-CD8 (53–6.7), anti-CD8α (Invitrogen #MCD0830), anti-FoxP3 (MF-14), anti-CD25 (3C7), anti-NKp46 (29A1.4), anti-IFNγ (XMG1.2), anti-TNFα (MP6-XT22), anti-IL2 (JES6–5H4), anti-CD11b (M1/70), anti-F4/80 (BM8), anti-Gr-1 (R36–8C5), anti-Ly-6C (HK1.4), anti-Ly-6G (1A8), and anti-CD19 (6D5). H-2Db/HPV16

E7^{49–57}-(RAHYNIVTF) MHC Class I tetramer was purchased from MBL International and used at the recommended dilution. The following antibodies used for *in vivo* work were purchased from BioXCell: anti-CTLA-4 (9D9) and matched isotype control (MPC-11); anti-PD-1 (RMP1-14) and matched Isotype control (2A3); anti-CD4 (GK1.5) and matched Isotype control (LTF-2); anti-CD8 α (53–6.7) and matched Isotype control (2A3).

Flow Cytometry for viability, calreticulin, and HSP70

Cells or implanted tumors were treated with AU-011+/-NIR as described and, after 1 hour (*in vitro*; TC-1 cells) or 36 hours (*in vivo*; TC-1, MB49luc, or MC38 cells), processed into single-cell suspensions as described above (1). All staining and washing steps were performed using as diluent 1x PBS supplemented with 2% FBS unless otherwise noted. Cells were blocked by incubation at 4°C with anti-Fc receptor for 30 minutes. Antibodies for surface detection of calreticulin, HSP70, and CD45 (*in vivo* only in order to discriminate tumor cells from infiltrating immune cells) were added and incubated for an additional 30 minutes at 4°C. Cells were washed, stained for viability using LIVE/DEAD Yellow fixable viability dye for 20 minutes at room temperature, washed, and fixed for 10 minutes at room temperature in 4% paraformaldehyde (PFA). Cells were acquired using a BD FACS Canto II flow cytometer outfitted with an HTS (BD Biosciences). Data were analyzed using FlowJo v10 by first selecting the cell population within FSC-A/SSC-A gate and then gating CD45⁻ (tumor) and CD45⁺ (immune cells) for *in vivo* studies. Each population was then assessed for AU-011 (APC-Cy7 channel), viability (Pacific Orange channel), HSP70 (FITC channel), and calreticulin (APC channel) expression. The percentage of viable cells and geometric mean fluorescence (GMFI) of HSP70 and calreticulin were plotted using GraphPad Prism v8.

Cell-free DNA

Cell-free DNA was measured in supernatants sampled 15–30 minutes after *in vitro* AU-011+/-NIR treatment (described above). Samples were tested using the protocol described in Goldshtein et al. (39) using SYBR-Gold Nucleic acid stain (ThermoFisher). Briefly, the stock solution was diluted 1:1000 in DMSO followed by 1:8 dilution into PBS. Cell supernatants were centrifuged for 1 minute at 2000 RPM prior to sampling. Ten microliters of test sample were combined with 40 μ L of the SYBR-Gold solution (final 1:10000) in black 96-well microtiter plates (Corning), and fluorescent data was acquired immediately using 485nm ex/520nm em filters (BMG CellSTAR). Data are reported as fluorescence intensity with background subtracted.

ATP release

ATP release was measured in supernatant sampled 15–30 minutes after *in vitro* AU-011+/-NIR treatment and centrifugation (described above). Fifty microliters of supernatant were combined with 50 μ L of Cell-titer Glo (Promega) in a white 96-well microtiter plate (PerkinElmer). The plate was placed on an orbital shaker for 2 minutes at room temperature, followed by 10 minutes on the bench top. Luminescence was measured using the BMG CellSTAR (BMG), and data are reported as relative light units (RLU) with background subtracted.

HMGB-1 ELISA

Secreted HMGB-1 was measured in supernatants sampled 15–30 minutes after *in vitro* AU-011+/-NIR treatment and centrifugation (described above). HMGB-1 was measured using an ELISA (Chondrex), following the manufacturers protocol. Absorbance at 450nm/630nm was measured using the BMG CellSTAR (BMG), data are reported as ng/mL of HMGB-1.

Caspase-1 assay

Caspase-1 activity was measured 1 hour after *in vitro* AU-011+/-NIR treatment (described above) using the FAM-FLICA Caspase-1 reagent (ImmunoChemistry Technologies). For TC-1 tumors treated *in vivo*, tumors were removed ~36 hours after treatment and processed into single-cell suspensions as described above (1), and the cells were then treated following the same protocol for *in vitro* experiments. The caspase-1 reagent was added to the cells and incubated 1 hour at 37°C as per the manufacturer's protocol. Cells were then centrifuged and washed, followed by anti-CD45 staining, LIVE/DEAD Yellow viability staining, and 4% PFA fixation as described above. Cells were acquired using a BD FACS Canto II flow cytometer outfitted with an HTS (BD Biosciences). Data were analyzed using FlowJo v10 by first selecting the cell population within the FSC-A/SSC-A gate and then subdivided into CD45⁻ (tumor) and CD45⁺ (immune cells) populations. Each population was then assessed for viability using the Pacific Orange and caspase-1 activity using the FITC channel. The percentage of viable cells and geometric mean fluorescence (GMFI) of caspase-1 were plotted using GraphPad Prism v8.

Flow cytometry for tumor infiltrates

TC-1 tumor bearing mice were treated *in vivo* with diluent buffer alone or 100 µg of AU-011 +/- anti-CTLA-4 or matching isotype. 50 J/cm² of NIR light was applied to the tumor 12 hours after AU-011 or buffer injection as previously described. A subset of animals did not receive NIR and were used to measure baseline levels of infiltrating cells 24 hours after AU-011 injection. Tumors from buffer-treated mice were simultaneously stained for all immune markers (described below) and AU-011 (25 µg/mL) to measure AU-011 binding to each subset of immune cells. NIR-treated tumors were removed 1 hour, 2 hours, 6 hours, or 12 hours after NIR treatment. All tumors were processed into single-cell suspensions as described above. Animals receiving NIR alone serve as the negative controls for the kinetics study (time = 0 hour). All staining and washing steps were performed using as diluent 1x PBS supplemented with 2% FBS unless otherwise noted. Cells were blocked by incubation at 4°C with anti-Fc receptor for 30 minutes. Antibodies for detection of surface markers (see list above; used at manufacturer's recommended dilution) were added and incubated for an additional 30 minutes at 4°C. Cells were washed, stained for viability using LIVE/DEAD Yellow fixable viability dye for 20 minutes at room temperature, washed, and fixed for 10 minutes at room temperature in 4% PFA. For those cells being stained for FoxP3, cells were not fixed after the viability staining step, rather they were subjected to FoxP3 staining using the True-Nuclear Transcription factor buffer system (BioLegend) as per the manufacturer's recommended dilutions and protocol. Cells were acquired using a BD FACS Canto II flow cytometer outfitted with an HTS

(BD Biosciences). Cells were first gated on the singlet population (FSC-H/FSC-A). The CD45⁺ population was then selected for downstream analysis. Markers used to distinguish the infiltrating populations were: CD19⁺ B cells, NKp46⁺ NK cells, CD8⁺ T cells, CD4⁺ T cells, CD4⁺CD25⁺FoxP3⁺ T-regulatory cells (Tregs), F4/80⁺CD11b⁺Gr-1⁻ tumor-associated macrophage (TAMs), CD11b⁺Ly6C⁺ monocytic myeloid-derived suppressor cells (M-MDSCs), and CD11b⁺Ly6G⁺ polymorphonuclear myeloid-derived suppressor cells (PMN-MDSCs). Viability was then measured for each subset. Data were analyzed using FlowJo v10 and the viability and overall percentage of each population was plotted using GraphPad Prism v8.

***In vitro* re-stimulation of PBMCs**

TC-1 and MC38 tumor cells were cultured in recombinant murine IFN γ (1 μ g/mL; Prospec) for 24 hours, followed by irradiation with 120 Gy (Mark I gamma irradiator (JL Shepherd); cells kindly provided by Nicolas Çuburu). Blood was retro-orbitally collected from immunized and TC-1 tumor-bearing mice (AU-011 + NIR treated and untreated) into EDTA coated tubes (BD Biosciences), and red blood cells were lysed for 5 minutes using ACK buffer (Invitrogen). Cells were washed in 1x PBS supplemented with 2% FBS and the residual cell pellet (hereafter referred to as PBMCs) was resuspended in culture media (RPMI (Corning) supplemented with 10%FBS, 50 μ M β -mercaptoethanol, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 U/mL penicillin/ 50 μ g/mL streptomycin (Invitrogen)). Half of the cells were then blocked using anti-Fc receptor as described above, followed by surface staining with H-2Db/HPV16 E7^{49–57}-(RAHYNIVTF) MHC Class I tetramer, anti-CD3, anti-CD8, anti-CD4, and anti-CD45 for 30 minutes at 4°C followed by staining with LIVE/DEAD Yellow viability dye and 4% PFA fixation as described above. The remaining cells were plated with irradiated tumor cells at a ratio of 3:1, or with the E6 and E7 peptides (described above; 1 μ g/well) at for 8 hours in culture media at 37°C, 5% CO₂. Golgi-stop and Golgi-plug (BD) were added for the last 6 hours using the manufacturer's recommended dilutions. Cells were blocked with anti-Fc receptor and stained for lymphoid surface markers and viability using LIVE/DEAD Yellow fixable dye as described above. Intracellular cytokine staining (anti-TNF α , anti-IFN γ , and anti-IL2) was then performed using the Cytofix/Cytoperm reagents following the manufacturer's protocol (BD). Cells were acquired using a BD FACS Canto II flow cytometer outfitted with an HTS (BD Biosciences). For tetramer analysis, lymphocytes were first gated (FSC-A/SSC-A) and only viable cells were further analyzed. The CD3⁺CD45⁺ population was then selected and from these cells, CD8⁺ T cells were analyzed for tetramer positivity. Intracellular cytokine analysis of cells relied first on a gate of singlet cells (FSC-H/FSC-A). Viable cells were determined from this population followed by a lymphocyte gate (FSC-A/SSC-A). CD3⁺CD8⁺ T cells were then analyzed for cytokine production (TNF α , IFN γ , and IL2). Data were analyzed using FlowJo v10 and the overall percentage of each population was plotted using GraphPad Prism v8.

Statistical Analysis

Graph Pad Prism v8 was used to calculate all p-values. Unless otherwise noted, data were analyzed using two-tailed unpaired T-test. All survival statistics were calculated using Log-Rank analysis from Kaplan-Meier survival plots.

Results

AU-011 induces the hallmarks of immunogenic cell death *in vitro*

AU-011-mediated cytotoxicity *in vitro* occurs within minutes of NIR exposure, and treated tumors appear visibly necrotic within hours of treatment (1). In pilot studies performed using immunocompetent mice, we found AU-011 treated animals whose tumors regressed were often protected from tumor challenge months after the initial treatment (Supplemental Fig. S1A–B), indicating the potential induction of an adaptive anti-tumor immune response. We therefore performed *in vitro* studies to determine if AU-011-mediated cell death could generate potent immune stimulatory conditions. Murine TC-1 tumor cells were treated with buffer alone or escalating doses of AU-011 (0.2 µg/mL, 1 µg/mL, 5 µg/mL or 25 µg/mL) and either kept in the dark or exposed to 25 J/cm² NIR light and the ICD markers were examined (surface re-localization of calreticulin and HSP70 and release of DNA, ATP and HMGB-1 into the supernatant). Caspase-1 activity was also measured, as it can be a key player in generating a pro-immunogenic environment after necrotic cell death (40). Each of these markers could be observed within 15–60 minutes, after NIR treatment (Fig. 1A–F, Supplemental Fig. S2A). A dose-dependent response was observed, and upregulation of the markers was conditional on exposure to NIR light. Because the membrane disruption that occurs at the time of AU-011 treatment might allow the detecting antibodies to enter the cell, we confirmed by microscopy that calreticulin and HSP70 were expressed on the surface of the cells (Supplemental Fig. S2B). Taken together, these *in vitro* data support the conclusion that an immunogenic milieu is generated by AU-011 mediated tumor cytotoxicity.

Optimization of the *in vivo* treatment protocol

We designed several short-term *in vivo* cytotoxicity studies, in order to optimize treatment conditions in the TC-1 model. We tested a dose range of AU-011 (25 µg–100 µg; Fig. 2A), time to NIR post-intravenous AU-011 injection (30 minutes–72 hours; Fig. 2B), and laser fluence (6.25 J/cm²–100 J/cm²; Fig. 2C). As previously described with the ADC (6,8), *in vitro* treatment of tumor cells with AU-011+NIR similarly induced ROS, which likely plays a role in tumor cytotoxicity (Supplemental Fig. S3A). Antibody-IR700-mediated cytotoxicity leads to enhanced vascularity and rapid accumulation of blood within the tumor, but a reduced flow rate leading to accumulation of drug within the tumor (8,41). Reperfusion, and thus re-oxygenation of the TME as reported with the ADC, could potentiate AU-011 activity with repeated low fluence NIR treatments delivered in short bursts. We therefore compared a single administration of 50 J/cm² to a cumulative dose consisting of fractionated administration of 12.5 J/cm² four times at five-minute intervals to allow for continued tumor damage and reperfusion (both NIR treatments were initiated 12 hours after AU-011 injection). Using suboptimal doses of AU-011 (25 µg, 35 µg and 50 µg), we observed a subtle, albeit distinct, difference between the single (S) and fractionated (F) NIR treatments in both the short-term cytotoxicity assay and survival studies, implying that fractionation of NIR treatment could enhance AU-011 efficacy (Fig. 2D, Supplemental Fig. S3B). Taking all data into consideration, we established a standard treatment protocol involving delivery of 100 µg of AU-011 followed by 50 J/cm² of fractionated NIR light within 12+/-2 hours post-injection in order to ensure accumulation of AU-011 and

maximum treatment effect. Of note, similar *in vivo* dose responses were also observed using two additional murine tumor models, MC38 and MB49luc (Supplemental Figs S3C–D).

AU-011 induces markers of immunogenic cell death *in vivo*

We next examined calreticulin surface expression and caspase-1 activity *in vivo*. Animals received buffer or one of two doses of AU-011 (50 µg or 100 µg), all followed by NIR treatment. Tumors were harvested ~36 hours post-NIR and were processed into single-cell suspensions. Calreticulin and CD45 surface expression, along with caspase-1 activity and cell viability, were measured. Both caspase-1 activity and calreticulin surface expression were increased in tumor cells treated with AU-011+NIR compared to buffer+NIR, and the intensity of both markers was directly correlated with the percent of non-viable cells (Fig. 3A–C; Supplemental Fig. S4). A similar effect was observed on the CD45⁺ cell population, posing the question of AU-011's impact on the tumor-infiltrating immune cell population. These data suggest that AU-011-mediated tumor cytotoxicity generates a pro-immunogenic microenvironment that, with the plausible concomitant release of tumor neoantigens, could potentiate anti-tumor immunity.

Impact of AU-011 on the tumor-infiltrating immunocyte population

In vitro, HPV VLPs bind to macrophages, dendritic cells, B cells, and neutrophils (42–44), leading us to question the impact of AU-011 on the tumor-resident immune infiltrates, including suppressive populations such as tumor-associated macrophages (TAMs) and myeloid-derived suppressor cells (MDSCs). We first determined the baseline frequency of eight subsets of cells (CD19⁺ B cells, NKp46⁺ NK cells, CD8⁺ T cells, CD4⁺ T cells, CD4⁺CD25⁺FoxP3⁺ T-regulatory cells [Tregs], F4/80⁺CD11b⁺Gr-1⁺ TAMs, CD11b⁺Ly6C⁺ M-MDSCs, and CD11b⁺Ly6G⁺ PMN-MDSCs) within the CD45⁺ tumor-infiltrating population in animals 24 hours after receiving an intravenous injection of buffer, AU-011, or AU-011+anti-CTLA-4 in the absence of NIR exposure (Fig. 4A, Supplemental Fig. S5). We noted a small, albeit significant decrease in B cells, Tregs, and PMN-MDSCs with the addition of anti-CTLA-4, and an increase in TAMs. To determine if AU-011 was binding any of these cell subsets, it was added *ex vivo* to naïve tumor cell suspensions at the time of immune marker staining, and we observed binding to B cells, TAMs, M-MDSCs, and to a lesser extent, PMN-MDSCs, but not to NK cells or any of the T-cell subsets (Fig. 4B).

We next assessed the impact of AU-011+NIR treatment on the infiltrating immunocyte population. Due to the rapid nature of tumor death and regression, tumor infiltrates were examined 30 minutes, 2 hours, 6 hours, and 12 hours post-NIR treatment in mice receiving AU-011 or buffer, and changes in the overall cell populations and viability were noted (Fig. 4C–D). Tumors treated with buffer+NIR were also harvested at these time points, and data were pooled and reported as time “0” to provide a reference for the cell population in the absence of AU-011; no difference across time points was observed for these tumors. We noted an increase of the innate immune cell populations, MDSC subsets, and TAMs immediately following NIR treatment, whereas the adaptive T-cell and B-cell populations decreased (Fig 4C–D). This rapid influx of innate cells, primarily the PMN-MDSCs which may also contain pro-inflammatory cells such as neutrophils, may contribute to the inflamed TME, leading to the rapid and sustained loss of T cells and B cells. By 12 hours post-NIR,

TAMs and PMN-MDSCs returned to baseline frequencies, whereas all other cell populations remained low. An increase in non-viable cells over time was noted for each of the cell populations, although there was an immediate impact on B-cell viability, perhaps due to direct binding and cytotoxic effects of AU-011 (Fig. 4C–D).

Combination of checkpoint inhibitors and AU-011 enhances efficacy

In preliminary TC-1 survival studies, we typically observed a 40–60% complete response rate (CRR) with AU-011, and of the mice exhibiting complete responses, approximately 60–80% were protected from subsequent tumor re-challenge, implying that adaptive anti-tumor immunity was generated (Supplemental Fig. S1A–B). Efficacy was also noted in the MC38 and MB49luc models (Supplemental Fig. S6A–D). Considering these observations in conjunction with the aforementioned data supporting ICD, we evaluated the activity of AU-011 combined with immune checkpoint inhibitors, anti-PD-1 or anti-CTLA-4, to determine if they could enhance both AU-011's efficacy and induction of anti-tumor immunity. Animals received checkpoint inhibitors or an isotype matched control antibody in conjunction with AU-011 treatment (Fig. 5). Animals were then followed for 100 days, at which time all tumor-free survivors (complete responders, CR) were re-challenged with TC-1 tumor cells and followed for an additional 100 days. Durable complete responses (100 days) were observed in approximately 50% of animals that received AU-011 or anti-CTLA-4 as a single agent (44.4% Isotype+AU-011 and 52.6% anti-CTLA-4; Fig. 5A and 5C) whereas anti-PD-1 treatment resulted in 28.6% CRs. However, 100% of animals receiving combination therapy with AU-011+anti-CTLA-4 had durable CRs, demonstrating a significant additive effect in two pooled experiments. Combining AU-011 with anti-PD-1 resulted in an increased number of tumor-free animals (71.4%) but was inferior to the combination of AU-011+anti-CTLA-4. Upon tumor re-challenge with TC-1 cells, 50% and 66.6% of animals from the AU-011 and AU-011+anti-CTLA-4 groups were protected from tumor growth, respectively, and all animals were protected from challenge in the anti-CTLA-4 alone, anti-PD-1 alone, and AU-011+anti-PD-1 groups (Fig. 5B). In an additional murine tumor model, MB49luc, we noted a similar enhancement of AU-011 efficacy when combined with anti-PD-1, yet protection from tumor re-challenge was unchanged between groups (Supplemental Fig. S6D). Overall, the data support improved CRR after the combination treatments, demonstrating beneficial enhancement of single-agent therapies. Although combination treatment with anti-PD-1 did not result in CRR in all animals, it did result in complete protection from tumor re-challenge, whereas treatment with AU-011+anti-CTLA-4 provided no added benefit for long-term protection.

AU-011 efficacy is dependent upon CD4⁺ and CD8⁺ T cells

The ability of animals to reject tumor re-challenge 100 days after treatment led us to investigate whether adaptive cellular immunity was required in the context of AU-011 as a single-agent, or in combination with anti-CTLA-4. We therefore independently depleted the two primary populations of T cells, CD4⁺ and CD8⁺ T cells, either at the time of treatment of the primary tumor (Table 1; Supplemental Fig. S7A–B) or at the time of secondary tumor re-challenge (Table 1; Supplemental Fig. S7C–D). A role for both CD4⁺ and CD8⁺ T cells was observed regardless of the treatment regimen, and both cell types were important at the time of primary treatment and at the time of secondary tumor re-challenge. These

results point to the importance of the induction of adaptive tumor-specific immunity as a key feature of AU-011 treatment.

Tumor-specific CD8⁺ T-cell responses in the absence of targeted antigens

Vaccines that induce strong T-cell responses against the HPV16 E6 and E7 oncoproteins are historically protective in the TC-1 tumor model (45). We examined these responses within the PBMCs of mice three weeks after AU-011 or AU-011+anti-CTLA-4 treatment. E6 and E7 protein and peptide vaccinated mice were used as controls. Using an MHC class I tetramer (H-2Db/HPV16 E7^{49–57} (RAHYNIVTF)) capable of detecting a known protective HPV16 E7 epitope after E7-targeted vaccination in the TC-1 model, we detected low and varied tetramer-specific T cells amongst all animals tested (Fig. 6A; Supplemental Fig. S8A, C). Although there was a noticeable increase in tetramer-positive cells after AU-011 treatment, alone or combined with anti-CTLA-4, it was not statistically significant. These data highlight the notion that AU-011 treatment may be inducing responses against other subdominant tumor antigens or neoepitopes. Although peptide-immunized mice generated detectable responses (Fig. 6A), animals immunized with whole E6 and E7 protein did not generate strong tetramer responses, further affirming that the tetramer-specific E7 epitope may not be immunodominant under native protein MHC-processing conditions.

In order to examine broader anti-tumor responses, we re-stimulated PBMCs with E6 and E7 peptides, irradiated TC-1 tumor cells, or irradiated MC38 tumor cells, which served as a non-specific tumor control, and measured cytokine production after 8hrs of exposure. Enhanced secretion of IFN γ and TNF α was noted in the PBMCs from AU-011 and combination treated animals after stimulation with TC-1 tumor cells compared to MC38, and this difference was significant in the combination group (IFN γ , n.s.; TNF α , $p=0.0375$; IFN γ /TNF α , $p=0.049$)(Fig. 6B, C, E; Supplemental Fig. S8B–C). IL2 responses alone were unchanged; however, cells secreting IL2 with either IFN γ or TNF α were increased in the AU-011 and combination treated animals, both compared to naïve animals, as well as between TC-1- and MC38-stimulated groups (Fig. 6D, F, G; Supplemental Fig. S8B). PBMCs from peptide-immunized mice did not generate strong responses when re-stimulated with the TC-1 tumor lysate, although they were significant compared to PBMCs from naïve animals (IFN γ , $p<0.0001$; IFN γ /TNF α , $p=0.024$), affirming that there are likely other protective immunodominant epitopes present in the tumor milieu. Taken together, these data demonstrate that AU-011 treatment is capable of inducing tumor-specific, multi-functional CD8⁺ T cells within three weeks of a single treatment.

Discussion

The data reported herein describe the ability of AU-011 followed by NIR treatment to induce potent and durable anti-tumor responses in the absence of conventionally targeted tumor-associated antigens. Treatment with AU-011 resulted in rapid cell death paired with the hallmarks of immunogenic cell death (i.e. DAMPs). Although the primary anti-tumor activity of AU-011 involves ablative physical damage to the cell membrane, the residual pro-inflammatory milieu that remains is capable of stimulating a protective, long-term anti-tumor immune response. Approximately half of the animals treated with a single treatment

of AU-011 alone displayed CRR, and of those, a majority demonstrated long-lasting anti-tumor immunity by rejecting subsequent tumor re-challenge. We sought to potentiate this anti-tumor response by combining AU-011 with anti-CTLA-4 or anti-PD-1 checkpoint blockade, which prevent tumor cells and resident antigen-presenting cells from sending tolerizing and inhibitory signals to T cells. The combination treatments resulted in a 70–100% complete response rate across three independent experiments.

We observed a small, but significant, reduction in tumor-resident B cells, Tregs and PMN-MDSC with the addition of anti-CTLA-4. There is precedence for T-reg depletion with anti-CTLA-4 therapy thought to be Fc-mediated clearance (46), and because B cells also express CTLA-4, it is plausible that they too are cleared by a similar mechanism. It is noteworthy that, in contrast to some tumor immunotherapy models, tumors never reappeared at the primary tumor site in CR mice after tumor re-challenge, even in animals where the secondary tumor grew progressively. This observation suggests that either the primary tumors were completely eradicated by the initial treatment or that the residual tumor cells were mainly controlled by tumor-resident T cells that could not be effectively recruited to a distant tumor site.

ICD can be identified by several hallmark features, namely the induction of DAMPs, such as release of ATP, DNA, and HMGB-1, and the surface re-localization of stress factors such as HSP70/90 and calreticulin (9). Another such indicator of ICD is the activation of caspase-1, a molecule known to cleave and activate pro-inflammatory cytokines IL1 β and IL18 (40,47,48). Each of these ICD components is thought to play a role in attracting and stimulating antigen-presenting cells in the localized, inflammatory TME. We demonstrated that AU-011-mediated tumor cell killing resulted in the generation of anti-tumor immunity in the absence of a specifically targeted tumor antigen, and we observed the consistent induction of DAMPs after AU-011 treatment of tumor cells, both *in vitro* and *in vivo*. We also noted upregulation of some ICD markers on the tumor-infiltrating immunocytes, suggesting that a component of AU-011 efficacy *in vivo* may be the localized cytotoxicity towards the resident immune cell population.

AU-011 treatment can be categorized as next-generation PDT, but, in contrast to small molecule-based PDT, the photosensitizing drug is specifically directed to the tumor cell surface by the HPV VLP. VDCs have several advantages over ADCs that are directed towards a specific tumor cell surface antigen. First, the multivalent binding assures a high avidity interaction with the tumor cells and delivers hundreds of cytotoxic dye molecules. Second, their size (50–60 nm diameter) allows them to passively exit the characteristically leaky vasculature of tumors. Third, the VLPs have a much broader spectrum of tumor type binding, and absence of binding to normal intact tissues, than currently available monoclonal antibodies that target tumor cell surface-associated antigens. Fourth, the VLP component of the VDC may serve as an additional immunogenic catalyst within the tumor. HPV VLPs bind to macrophages, B cells, dendritic cells, and neutrophils (42–44) and are capable of stimulating antigen-presenting cells through TLR-4 engagement and NF κ - β production (49), thus potentially serving as a localized adjuvant in the context of AU-011-induced cytotoxicity. Lastly, we demonstrate that AU-011 treatment is cytopathic towards resident suppressor cells such as TAMs, MDSCs, and CD4⁺ Tregs within hours of treatment. The

role of these cells is to maintain a suppressed and tolerizing state, thereby facilitating tumor immune escape or immune impairment. Thus, their removal from the TME could prove beneficial to the induction of anti-tumor immunity while the tumor is being cleared (50). Of note was the difference in the kinetics of the loss of the various immune populations. The tumor-associated B cells and T cells were quickly lost from the tumor within 2 hours of NIR treatment. It is possible that these cells were more susceptible to the localized ROS activity resulting from AU-011 treatment and the increasingly hostile and inflamed environment compared to the more resistant innate immune cells.

In the depletion studies, we found that not only were both CD4⁺ and CD8⁺ T cells required for complete regression of primary tumors, but they also played a role in long-term protection from re-challenge. Although CD8⁺ T cells have long been considered the major cytotoxic cells responsible for tumor control, the importance of CD4⁺ T cells has also proven important. TC-1 tumors do not express class II molecules (35), but functional CD4⁺ T cells have been found infiltrating other class II-negative tumors, where they can recruit macrophages and secrete cytokines responsible for upregulating co-stimulatory molecules on tumors and resident immunocytes. They also facilitate antigen cross-presentation and are involved in enhancing epitope/antigen spreading. Murine and human studies assessing predicted neoantigens have demonstrated a preponderance of CD4⁺ T cells with reactivity to neoepitopes and have also demonstrated loss of anti-tumor immunity in the absence of CD4⁺ T cells (11,51–55). High-avidity CD8⁺ T cells are apt to become exhausted, therefore generation and maintenance of lower-affinity CD4⁺ and CD8⁺ T cells against neoepitopes may be key to long-term anti-tumor immunity. The observed dependence upon both T-cell types in the long-term anti-tumor responses observed after AU-011 treatment demonstrates that it plays a role in inducing adaptive tumor-specific immunity and highlights it as a therapeutic modality that may potentiate the release of tumor neoantigens into the TME.

The TC-1 tumor model was initially chosen to study the pro-immunogenic potential of AU-011 due to its expression of HPV16 oncoprotein E7, for which a well-defined protective MHC class I epitope exists. However, we discovered early-on that the E7-specific T-cell response elicited by AU-011 treatment was variable. This finding raised the possibility that, due to the nature of AU-011-mediated cell cytotoxicity, unidentified neoantigens often dominated the T-cell responses generated after treatment. Consistent with this conjecture, Stevanovic et al. (53) reported analysis of TIL samples from HPV-positive cervical cancer patients and noted that the majority of T cells were not reactive to the E6 and E7 viral antigens, but rather were specific for tumor neoantigens. Similarly, we noted that multifunctional cytokine secretion from PBMCs stimulated with TC-1 tumor cells was higher than when stimulated with specific HPV MHC class I peptides. These responses were not observed when PBMCs were stimulated with the unrelated MC38 tumor cells, indicating the responses were TC-1-specific.

The rapid rise of genomics is allowing for a more specific identification of potential tumor neoantigens. However, the applications of these technologies to tumor immunotherapies are patient-specific, labor intensive, and are often restricted by host genetic factors (HLA), which ultimately limits their widespread application (13,14). AU-011 has the possibility to become an “off-the-shelf” cancer therapy that is tumor- antigen agnostic and is effective

as a single agent, or in combination with other immune-oncology treatments, leading to durable, complete responses and prevention of tumor recurrence. Tumor accessibility to NIR light treatment is a major limitation, although the latest developments of fiber optic systems minimizes this constraint by allowing direct visualization and access to tumors in previously inaccessible locations. Due to the broad tumor tropic nature of AU-011, we believe that this treatment modality has the potential to be applied to a wide range of tumor types that are poorly treated today and improve the outcomes for patients with cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Synopsis:

AU-011 is composed of an HPV VLP conjugated to photoactivatable IRDye-700DX. Their broad, tumor-tropic nature combined with light-induced cytotoxic activity induces tumor immunogenic cell death, boosts anti-tumor immunity, and elicits long-term anti-tumor immunity.

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Figure 1. AU-011 induces immunogenic cell death.

TC-1 cells were treated *in vitro* with buffer alone (0 µg) or 0.2 µg/mL, 1 µg/mL, 5 µg/mL, 25 µg/mL of AU-011. Cells were washed and either kept in the dark (open bars) or exposed to 25 J/cm² of NIR light (solid bars). Cells were then assessed for surface localization of A) calreticulin or B) HSP70 by flow cytometry 1 hour after NIR treatment, and the supernatant was used to measure C) DNA release, D) ATP release, and E) HMGB-1 release within 15–30 minutes of NIR treatment. F) Caspase-1 activity was measured 1 hour after NIR treatment. Data represent the mean values (+/- SEM) from four independent experiments.

All other data are the mean values ($\pm$ SEM) of 2 or 3 experiments performed in triplicate. p-values are calculated using unpaired T-test analysis.

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Figure 2. Optimization of TC-1 *in vivo* treatment conditions.

A) AU-011 dose. TC-1 tumor-bearing animals received increasing doses of AU-011 by intravenous injection (25 µg, 50 µg, 75 µg or 100 µg), followed 12 hours later by 50 J/cm² NIR treatment. Tumors were removed ~36 hours after NIR treatment, collagenase digested, and viability was measured. Data are reported as percent of dead cells within the tested population, n=5/group and is representative of a single experiment. B) Using a fixed dose of AU-011 (100 µg) and NIR treatment (50 J/cm²), a time course was performed in which animals received NIR treatment at 30 minutes, 2 hours, 6 hours, 12 hours, 24 hours, 48

hours, or 72 hours post-AU-011 injection. Tumors were processed as described; n=5/group and is representative of a single experiment. C) Using a fixed dose of AU-011 (100 µg) and NIR treatment at 12 hours post-injection, a titration of NIR fluence was performed in which animals received 6.25, 12.5, 25, 50 or 100 J/cm² of NIR light. Tumors were processed as described; n=5/group and is representative of a single experiment. D) TC-1 tumor-bearing animals received increasing sub-optimal doses of AU-011 by intravenous injection (25 µg, 35 µg or 50 µg), followed 12 hours later by 50 J/cm² NIR treatment delivered either as a single administration (**S**), or delivered as a cumulative dose fractionated over four treatments of 12.5 J/cm² at five-minute intervals (**F**). Tumors were processed as described. Data are reported as percent of dead cells within the tested population. Data pooled from three experiments, n = 5/group. All data are plotted as mean ± SEM.

Figure 3. *In vivo* markers for AU-011-induced immunogenic cell death.

TC-1 tumor bearing mice received an intravenous injection of buffer alone, 50 µg AU-011, or 100 µg AU-011 and all animals were treated with NIR light after 12 hours. Tumor cell suspensions were generated ~36 hours after NIR treatment. A) Viability was measured. Cells were blocked and stained for B) calreticulin and CD45, and C) caspase-1 activity was measured for 1 hour after the suspension was generated. n=5 mice/group from a single representative experiment. All data are plotted as mean $\pm$ SEM. p-values calculated using a two-tailed unpaired T-test.

Figure 4. Impact of AU-011 treatment on tumor-infiltrating immunocyte populations.

A) TC-1 tumor-bearing mice received an intravenous injection of dilution buffer or 100 µg AU-011+isotype or AU-011+anti-CTLA-4 (antibodies were delivered as described on days -3 and 0, with day 0 being AU-011 administration). Tumors were removed 24 hours after AU-011 injection and processed into single-cell suspensions and stained for the listed cell subsets. B) During the surface staining, tumor suspensions from the buffer-treated mice were also incubated with 25 µg/mL of AU-011; n=7 mice/group, *p<0.0001. A series of animals bearing TC-1 tumors were treated *in vivo* with 100 µg AU-011, followed 12 hours later with

NIR as described. Tumors were excised and analyzed by flow cytometry at various time points post-NIR for the impact of treatment on the frequency and viability of C) TAMs, M-MDSCs, and PMN-MDSCs, D) as well as B- and T-cell populations. Time “0” mice are pooled data from all time points from mice that received buffer+NIR and provide baseline populations and viability; n=3–27 animals/group pooled from six experiments. All data are plotted as mean $\pm$ SEM.

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Figure 5. Anti-CTLA-4 enhances AU-011 efficacy.

A) and C) TC-1 tumor-bearing animals received buffer alone (dotted black line), isotype control (MCP-11)+100 µg of AU-011 (solid dark gray line), anti-CTLA-4 (dashed black line), anti-CTLA-4+100 µg AU-011 (black solid line), anti-PD-1 (dashed light gray line), or anti-PD-1+100 µg AU-011 (light gray solid line). All groups received 50 J/cm² NIR treatment delivered fractionated over four treatments of 12.5 J/cm² at 5 minute intervals 12 hours after AU-011 or buffer injection. Tumors were measured twice weekly and when tumors reached ~1500 mm³ in size, animals were euthanized; n=18–19 mice/group;

n=7 mice/group for anti-PD-1 and anti-PD-1+AU-011. *p=0.0001, Isotype+AU-011 vs. anti-CTLA-4+AU-011; **p=0.0006, anti-CTLA-4 vs. anti-CTLA-4+AU-011. B) All remaining animals that were tumor-free at day 100 were challenged with TC-1 tumor cells. Naïve age-matched controls were included, and similar endpoints were applied; n=9–18/group; n=2–5/group for anti-PD-1 and anti-PD-1+AU-011. *p=0.0123; anti-CTLA-4 vs. Isotype+AU-011; **p=0.0476; anti-CTLA-4 vs. anti-CTLA-4+AU-011. All survival statistics were calculated using Log-Rank analysis from Kaplan-Meier survival plots. Anti-CTLA-4 data are pooled from two independent experiments and anti-PD-1 data are from one experiment.

Figure 6. Anti-tumor immune responses measured using PBMCs of treated mice. Positive control mice were immunized subcutaneously with either E6 and E7 proteins or peptides with poly I:C, and responses were measured one week after the third immunization. Tumor-bearing animals were treated with buffer alone, 100 ug AU-011, or 100 ug AU-011+anti-CTLA-4 as described. A) Three weeks after treatment, E7-specific tetramer responses were measured in the PBMCs. (B-G) PBMCs were re-stimulated *in vitro* with irradiated TC-1 (white bars), irradiated MC38 (black bars), or E6 and E7 peptides (gray bars) for 8 hours. B) IFN γ , C) TNF α , D) IL2, E) IFN γ and TNF α , F)

IFN γ and IL2, and G) TNF α and IL2 production in CD8⁺ T cells. n=3–5/group from a single representative experiment; data reported are mean $\pm$ SEM. p-values were calculated using two-tailed, unpaired T-tests: **IFN γ - E6/E7 peptide re-stimulation**, naïve vs. peptide immunized p=0.0127, naïve vs. protein immunized p=0.0107, naïve vs. AU-011+anti-CTLA-4 p=0.029; **TC-1 re-stimulation**, naïve vs. peptide immunized p<0.0001, naïve vs. protein immunized p=0.044, naïve vs. AU-011+anti-CTLA-4 p=0.0125; **Protein immunized, re-stimulation with E6/E7 peptides vs. MC38** p=0.046; **TNF α - TC-1 re-stimulation**, naïve vs. AU-011+anti-CTLA-4 p=0.0279; **AU-011 treatment**, re-stimulation with MC38 vs. peptides p=0.003; **AU-011+anti-CTLA-4 treatment**, re-stimulation with TC-1 vs. MC38 p=0.0375, re-stimulation with TC-1 vs. peptides p=0.0409; **IL2 - AU-011 treatment**, re-stimulation with TC-1 vs. peptides p=0.0489; **IFN γ /TNF α - E6/E7 peptide re-stimulation**, naïve vs. peptide immunized p=0.0157, **TC-1 re-stimulation**, naïve vs. peptide immunized p=0.024, naïve vs. AU-011+anti-CTLA-4 p=0.0498; **AU-011+anti-CTLA-4 treatment**, re-stimulation with TC-1 vs. MC38 p=0.049; **TNF α /IL2 - AU-011 treatment**, re-stimulation with peptides vs. MC38 p=0.0195.

Table 1.

Tumor-free animals after CD4⁺ T-cell or CD8⁺ T-cell depletion at the time of treatment or at the time of tumor re-challenge

		Tumor-free animals (TF)					
		Time of treatment			Time of Challenge		
	Depletion	# TF animals/# total	% tumor-free	p-value (vs. Isotype)	# TF animals/# total	% tumor-free	p-value (vs. Isotype)
Naïve (challenge)	--	--	--	--	0/9	0%	--
	Isotype	4/10	40%	--	8/10	80%	--
	CD4	0/9	0%	0.0227	1/9	11.10%	0.0022
	CD8	0/8	0%	0.0029	3/10	30%	0.0163
anti-CTLA-4 + AU-011 + NIR	Isotype	8/10	80%	--	5/8	62.50%	--
	CD4	2/9	22%	0.0057	2/10	20%	0.1209
	CD8	0/9	0%	<0.0001	3/9	33%	0.3816
	Isotype	0/10	0%	--	--	--	--
No treatment	CD4	2/8	25%	--	--	--	--
	CD8	0/8	0%	--	--	--	--

p-values obtained using Log-rank analysis of Kaplan-Meier survival plots (Supplemental Fig. S7)