






Exploiting the immunogenic potential of standard of care radiation or cisplatin therapy in preclinical models of HPV-associated malignancies

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ABSTRACT

Background While radiation and chemotherapy are primarily purposed for their cytotoxic effects, a growing body of preclinical and clinical evidence demonstrates an immunogenic potential for these standard therapies. Accordingly, we sought to characterize the immunogenic potential of radiation and cisplatin in human tumor models of HPV-associated malignancies. These studies may inform rational combination immuno-oncology (IO) strategies to be employed in the clinic on the backbone of standard of care, and in so doing exploit the immunogenic potential of standard of care to improve durable responses in HPV-associated malignancies.

Methods Retroviral transduction with HPV16 E7 established a novel HPV-associated sinonasal squamous cell carcinoma (SNSCC) cell line. Three established HPV16-positive cell lines were also studied (cervical carcinoma and head and neck squamous cell carcinoma). Following determination of sensitivities to standard therapies using MTT assays, flow cytometry was used to characterize induction of immunogenic cell stress following sublethal exposure to radiation or cisplatin, and the functional consequence of this induction was determined using impedance-based real time cell analysis cytotoxicity assays employing HPV16 E7-specific cytotoxic lymphocytes (CTLs) with or without N803 (IL-15/IL-15-R α superagonist) or exogenous death receptor ligands. In vitro observations were translated using an in vivo xenograft NSG mouse model of human cervical carcinoma evaluating cisplatin in combination with CTL adoptive cell transfer.

Results We showed that subpopulations surviving clinically relevant doses of radiation or cisplatin therapy were more susceptible to CTL-mediated lysis in four of four tumor models of HPV-associated malignancies, serving as a model for HPV therapeutic vaccine or T-cell receptor adoptive cell transfer. This increased killing was further amplified by IL-15 agonism employing N803. We further characterized that radiation or cisplatin induced immunogenic cell stress in three of three cell lines, and consequently demonstrated that upregulated surface expression of Fas and TRAIL-R2 death receptors at least in part mediated enhanced CTL-mediated lysis. In vivo, cisplatin-induced immunogenic cell stress synergistically potentiated CTL-mediated tumor control in a human model of HPV-associated malignancy.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Radiotherapy and select chemotherapies induce immunogenic cell stress, resulting in immunogenic cell death and immunogenic modulation in many cancer types, presenting an avenue to synthesize conventional therapies with immunotherapies. Whereas conventional therapies in combination with HPV therapeutic vaccines have been studied in murine models of HPV-associated malignancies, immunogenic cell stress resulting from radiation or cisplatin exposure has not been demonstrated in human models of HPV-associated malignancies.

WHAT THIS STUDY ADDS

⇒ Herein, we characterize induction of immunogenic cell stress in human models of HPV-associated malignancies with radiation or cisplatin exposure and consequently demonstrate enhanced cytotoxic lymphocyte-mediated tumor control both in vitro and in vivo employing adoptive cell transfer in combination with conventional therapies. We also introduce the first utilization of a transgenic HPV-associated human model of sinonasal squamous cell carcinoma.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ This study provides evidence and rationale to employ immuno-oncology strategies at the front line of cancer management in combination with conventional therapies to exploit the immunogenic potential of standard of care radiation or cisplatin therapy in HPV-associated malignancies.

Conclusion Standard of care radiation or cisplatin therapy induced immunogenic cell stress in preclinical models of HPV-associated malignancies, presenting an opportunity poised for exploitation by employing IO strategies in combination with standard of care.

INTRODUCTION

Globally, high-risk human papillomavirus (HPV) is responsible for nearly 1 in every

20 cases of cancer. Cervical carcinoma is the most common HPV-associated malignancy worldwide, with nearly all cases attributable to HPV, followed by HPV-associated anogenital malignancies and head and neck squamous cell carcinomas (HNSCC).¹ Incidence rates of HPV-associated oropharyngeal squamous cell carcinoma (OPSCC), representing the vast majority of HPV-associated HNSCC, are on the rise in the USA, causing more than two-thirds of cases of OPSCC.² While HPV-positive cases of OPSCC are more responsive to standard of care than HPV-negative cases, HPV-associated HNSCC maintains poor outcomes in the recurrent and metastatic settings.³ Locoregionally advanced cases of cervical carcinoma have a 5-year survival rate of about 70%, whereas in the recurrent or metastatic settings, outcomes are similarly poor as in HNSCC.⁴ While the clinical management of these diseases is complex and in many instances requires multimodal intervention, radiation and cisplatin play prominent roles in the first-line treatment of locoregionally advanced cervical carcinoma and HNSCC, as well as in first-line salvage attempts at treating recurrent and metastatic disease.^{2,4} Additionally, immune checkpoint inhibitors have gained approval in recurrent and metastatic cases of cervical carcinoma (pembrolizumab) and HNSCC (pembrolizumab and nivolumab), with broader ongoing investigation looking to move immune checkpoint inhibitors into the definitive treatment space at earlier stages of disease, notably with varying degrees of success to date.^{5,6}

Sinonasal squamous cell carcinoma (SNSCC) is a rare subtype of HNSCC of the nasal cavities and paranasal sinuses.⁷ When feasible, the typical first-line treatment is surgical resection. However, due to the close anatomic proximity to the skull base, orbits, and critical neurovascular structures, achieving negative surgical margins can be challenging. Indeed, positive margins have been reported in nearly 30% of the cases and are associated with decreased survival rates.⁸ Accordingly, primary management often includes adjuvant radiation with or without chemotherapy targeted at eradicating potential microscopic residual disease.^{8,9} Nonetheless, outcomes in SNSCC remain dismal, with a 5-year survival rate of about 50% owing to advanced stage of disease at diagnosis and high rates of recurrence ranging from 40% to 80%.^{7,9} Interestingly, it has recently been demonstrated that HPV is present in about one in three cases of SNSCC, raising the question whether the problem of residual disease and disease recurrence can be addressed with HPV-targeting immuno-oncology (IO) strategies.⁷

Whereas radiation and chemotherapy are primarily purposed as cytotoxic therapies in their clinical use, the last two decades have characterized an immunogenic potential for these standard therapies through the induction of immunogenic cell stress, resulting in phenotypic changes ranging from immunogenic modulation to immunogenic cell death. This body of investigation, though, has sparsely included representation of HPV-associated malignancies. We hypothesized that these

malignancies may uniquely benefit from this application considering the observation that outcomes in cervical carcinoma and HPV-associated HNSCC correlate with the level of immune infiltration, and that the viral etiology of these cancers contributes to a more inflamed tumor phenotype at baseline.^{2,10,11} Accordingly, we sought to investigate the immunogenic potential of radiation or cisplatin therapy in order to inform IO strategies that may be combined with standard of care with the express goal to exploit the immunogenic potential of standard of care in first-line treatments or first-line salvage treatments of HPV-associated malignancies where radiation and cisplatin are commonly used. Additionally, we employed an interleukin (IL)-15/IL-15-R α superagonist (N803) with a robust preclinical and clinical record of safety and efficacy to serve as an example of combination IO strategies that may be layered onto standard of care to further exploit this immunogenic potential.^{12,13} Notably, IL-15 agonism serves as a compelling IO strategy given its functional capacity to activate and expand the CD8⁺ T-cell and natural killer (NK)-cell compartments, while sparing similar effects on the regulatory T-cell compartment—a notable limitation of IL-2. Compared with IL-2, IL-15 agonism also spares induction of capillary-leak syndrome.^{12,14,15} Accordingly, N803 has demonstrated safety and activity in phase I trials in hematologic and solid malignancies, and more recently in healthy volunteers.^{13,16,17}

In the present study, we demonstrated that sublethal exposure to radiation or cisplatin significantly enhanced cytotoxic lymphocyte (CTL)-mediated target cell lysis in HPV-associated tumor models through induction of immunogenic cell stress, which was further amplified by way of IL-15 agonism. Mechanistically, we showed upregulation of immunomodulatory markers, including death receptors, which functionally mediated this effect following radiation or cisplatin exposure. To our knowledge, for the first time in the literature (1) we expressly characterized that radiation or cisplatin induced immunogenic cell stress in human tumor models of HPV-associated malignancies; (2) we mechanistically demonstrated that upregulation of TRAIL-R2 in part mediated enhanced CTL-mediated target cell lysis following radiation or cisplatin exposure; (3) we demonstrated that cisplatin synergized with CTL adoptive cell transfer (ACT) *in vivo* employing a xenograft model of human HPV-associated malignancy in NSG mice; and (4) we introduced the first use of a transgenic HPV-associated tumor model of SNSCC.

MATERIALS AND METHODS

Cell lines

The CaSki (HPV-associated cervical carcinoma) cell line was obtained from the American Type Culture Collection (ATCC) and was cultured according to ATCC guidelines. UPCI-SCC-90 and UPCI-SCC-152 (HPV-associated HNSCC pre-radiation and post-radiation therapy, respectively) were cultured as previously described.¹⁸

SCCNC5 (HPV-negative SNSCC) was cultured as previously described.¹⁹ E7-SCCNC5 was established for the first time as an HPV-associated SNSCC tumor model by retroviral transduction of SCCNC5 with HPV16 E7, as previously described.²⁰ Following transduction, HPV16 E7 expression was verified by flow cytometry (low-affinity nerve growth factor receptor (NGFR) tag in shared open reading frame) (APC-CD271 (NGFR), clone ME20.4, BioLegend) and sorted by fluorescent-activated cell sorting to achieve a purity >99%. All cell lines were serially tested for mycoplasma with negative results.

E7-specific CTL was established by T-cell receptor (TCR) retroviral transduction, as previously described.²⁰ Briefly, high avidity HLA-A*02-restricted HPV16 E7₁₁₋₁₉-specific TCR was transduced into healthy donor peripheral blood mononuclear cells, as described in Jin *et al.*²¹ This primary stock was then either used directly for initial CTL characterization assays (thawed, washed, rested overnight in media without IL-2) or rapidly expanded a second time to establish new stock as follows. Cells were thawed, washed, and stimulated with ImmunoCult Human CD2/CD3/CD28 T-cell activator (STEMCELL Technologies) in media supplemented with 3000 IU/mL of IL-2 (PeproTech) at a density of 2.5×10^5 to 5×10^5 cells/mL. After 5 days, cells were split every other day to maintain a density of 5×10^5 to 1×10^6 cells/mL with fresh media supplemented with IL-2, as above. On day 14, cells were harvested and cryopreserved. For use for cytotoxicity assays in combination with radiation or cisplatin, cells were thawed, washed, and restimulated using T-cell activator, as above, in media without IL-2 or with N803 (50 ng/mL) for 72 hours before effector addition to cytotoxicity assays. For *in vivo* use, primary stock was rapidly expanded a second time for 14 days, as above, without subsequent cryopreservation; on day 14, cells were harvested and injected into mice.

Experimental reagents

A Gammacell 1000 Elite with a cesium-137 source (Best Theratronics) was used for all irradiations. Cells were irradiated while in single cell suspension in the media. Cells were washed before replating in adhesion culture and rested for specified length of time (ie, treated for 72 hours implies a single fraction of radiation at time zero followed by 72 hours of rest). Cisplatin (Thymoorgan Pharmazie) was provided through the NIH pharmacy. IL-15/IL-15-R α superagonist (N803) was provided by ImmunityBio (Culver City, California, USA) through a Cooperative Research and Development Agreement with the National Cancer Institute, NIH.

Viability assays

Cells were plated in a 96-well plate at a density ranging from 5000 to 15,000 cells/well. Following treatment with a range of radiation or cisplatin concentrations for 72 hours, viability was determined using MTT assays (Abcam) following the manufacturer's protocol. Per cent viability was quantified as absorbance of treated group/

absorbance of untreated group after subtracting out background absorbance. A non-linear regression model ((inhibitor) vs normalized responses—variable slope) using GraphPad Prism V.9 (GraphPad Prism Software) was applied to establish a line of best fit. MTT assays were performed in technical triplicates.

Cytotoxicity assays

Cytotoxicity assays were performed using the impedance-based xCELLigence real-time cell analysis (RTCA) platform (Agilent) according to the manufacturer's recommendations. Cells were plated in a 96-well E-plate at a density ranging from 10,000 to 40,000 cells/well and allowed to adhere overnight before effector addition at 24 hours. Radiation and cisplatin groups were treated in tissue culture flasks for 48 and 24 hours, respectively, prior to plating on the RTCA platform with continued treatment for 24 hours before effector addition (ie, total 72-hour and 48-hour treatments, respectively, before effector addition). This format was adopted in order to ensure consistent effector to target (E:T) ratios between untreated and treated groups, as plating for a more extended time frame on the RTCA platform before effector addition would allow the untreated groups to expand while the treated groups would be inhibited, yielding disparate E:T ratios at the time of effector addition. Per cent lysis was determined by comparing normalized cell indices within each group (ie, untreated group, radiation treated group, cisplatin treated group) with effector addition versus without effector addition, quantified as $1 - (\text{normalized cell index with effector addition} / \text{normalized cell index without effector addition})$, providing internal control for any confounding effect of radiation or cisplatin on cell lysis. Effectors included HPV16 E7-specific CTL (described above), α -Fas activating antibody (clone CH11) (EMD Millipore), or KillerTRAIL activating protein (Enzo Life Sciences). Cytotoxicity assays were performed in technical triplicates and replicated in at least two independent experiments with similar results.

Flow cytometry

Following radiation or cisplatin treatment for 72 or 48 hours, respectively, cells were harvested, washed, and stained with LIVE/DEAD fixable blue dead cell stain (Thermo Fisher), followed by Fc block (TruStain FcX, BioLegend), cell surface staining with fluorophore-conjugated antibodies, and fixation (BD Cytofix, BD Biosciences). Fluorophore-conjugated antibodies included BV421-CD54 (ICAM-1) (clone HA58), BV605-CD274 (PD-L1) (clone 29E.2A3), BV785-CD95 (Fas) (clone DX2), and PE-CD262 (TRAIL-R2) (clone DJR2-4) from BioLegend; APC-HLA-A2 (clone BB7.2) from BD Biosciences; and AF488-calreticulin (clone 681233) from R&D Systems with appropriately matched isotype controls. Flow cytometry was performed using BD LSRFortessa (BD Biosciences), and analyses were performed using FlowJo V.10 (FlowJo). Viable populations were defined

by negative LIVE/DEAD staining of single cell gated populations. Net mean fluorescent intensity (net MFI) (geometric mean of isotype subtracted from geometric mean of marker) was used to correct for subtle shifts in the geometric mean of isotypes caused particularly by radiation treatment. Marked upregulation was defined by 10% increase in per cent positive cells or 1.5-fold increase in net MFI of total viable populations.

In vivo studies

All animal studies were approved and conducted in accordance with an Institutional Animal Care and Use Committee approved animal protocol (#LTIB-38 and #LTIB-57) and using ARRIVE1 reporting guidelines.²² Female NSG mice, 10–16 weeks old, (originally obtained from Jackson Laboratory; bred in-house at NCI, Bethesda, Maryland, USA) were inoculated subcutaneously with a suspension of CaSki at 1×10^6 cells per mouse in 50 μ L phosphate buffered saline (PBS) mixed with 50 μ L matrigel. Mice were randomized on day 11 to one of four groups, 8–10 mice per group: untreated control (PBS, intraperitoneal (ip)), cisplatin (5 mg/kg, ip), E7-specific CTL ACT (1×10^6 , ip), and combination. One injection (cycle) of cisplatin was administered on day 12 followed by E7-specific CTL ACT on day 14. Tumor growth was monitored, and animal studies were terminated 2 weeks following the last treatment.

Statistical analyses

Student's t-test was used to compare two groups. One-way or two-way analysis of variance with Tukey's post hoc multiple comparisons test was used to compare more than two groups. These were performed using GraphPad Prism V.9 (GraphPad Prism Software). Kolmogorov-Smirnov test was used to compare two populations in flow cytometric analyses using FlowJo V.10 (FlowJo). P values <0.05 were considered significant. Error bars in figures represent mean \pm SEM.

RESULTS

Characteristics of included HPV-associated HLA-A2⁺ tumor models

While previous studies have explored the combination of standard therapies with cancer vaccines in the context of HPV-associated malignancies, these studies employed the transgenic murine TC-1 tumor model, but such studies were not extended to include human tumor models of HPV-associated malignancies.^{23–25} Other studies have explored the induction of immunogenic cell stress across a broad range of human tumor models with standard therapies, but HPV-associated malignancies have been sparsely represented across these studies. Therefore, we selected representative human HLA-A2⁺ tumor models of HPV-associated cervical carcinoma, HNSCC, and SNSCC for study (table 1). CaSki is an established metastatic cervical carcinoma cell line. UPCI-SCC-90 and UPCI-SCC-152 are established HNSCC cell lines derived from

Table 1 Characteristics of included HPV-associated HLA-A2⁺ tumor models

Cell line	Model	HPV16 status (copy no.)
CaSki	Cervical carcinoma	Positive (60–600)*
UPCI-SCC-90	HNSCC (radiation naive)	Positive (739 per β -globin copy)**
UPCI-SCC-152	HNSCC (radiation refractory)	Positive (210 per β -globin copy)**
SCCNC5	Sinonasal squamous cell carcinoma	Negative
E7-SCCNC5	Sinonasal squamous cell carcinoma	E7 transduced

CaSki was an established metastatic cervical carcinoma cell line. UPCI-SCC-90 and UPCI-SCC-152 were established cell lines from the same base of tongue tumor; UPCI-SCC-152 represents a hypopharyngeal recurrence of UPCI-SCC-90 after radiation therapy. SCCNC5 was an established HPV-negative sinonasal squamous cell carcinoma cell line that was transduced with HPV16 E7 to serve as an HPV-positive sinonasal squamous cell carcinoma tumor model (E7-SCCNC5). HPV16 copy number reported from literature. *,²⁶ **,¹⁸ HNSCC, head and neck squamous cell carcinomas; HPV, human papillomavirus.

the same patient; UPCI-SCC-90 was established from a base of tongue tumor prior to radiation therapy, while UPCI-SCC-152 represents a hypopharyngeal recurrence in the same patient following radiation therapy.¹⁸ CaSki, UPCI-SCC-90, and UPCI-SCC-152 are HPV16-positive malignancies bearing high viral loads.^{18,26} SCCNC5 is an established HPV-negative SNSCC cell line.¹⁹ We established for the first time E7-SCCNC5 as a transgenic model of HPV-associated SNSCC by retroviral transduction of SCCNC5 with HPV16 E7, analogous to the transgenic expression of HPV16 E6/E7 in the well-established murine TC-1 tumor model.²⁷

Viability of HPV-associated tumor models after exposure to standard of care radiation or cisplatin

Radiation and cisplatin are standard cytotoxic therapies in HPV-associated malignancies, but balanced against their toxicity profiles affecting healthy tissues, tumor cell subpopulations may survive at clinically relevant concentrations of radiation or cisplatin exposure. We treated HPV-associated tumor models with a broad range of radiation or cisplatin concentrations for 72 hours and assessed viability relative to untreated controls via MTT assays (figure 1). At 10 Gy radiation—representing the summation of five fractions of 2 Gy radiation, or about 1 week of radiation therapy in the clinic—four of four tumor models maintained viability relative to untreated controls greater than 50% (figure 1A). Interestingly, UPCI-SCC-152, representing a radiation refractory tumor model, was more viable at 10 Gy radiation than UPCI-SCC-90 by a factor of about 50% (figure 1A). At 1 μ g/mL cisplatin—representing a clinically relevant plasma

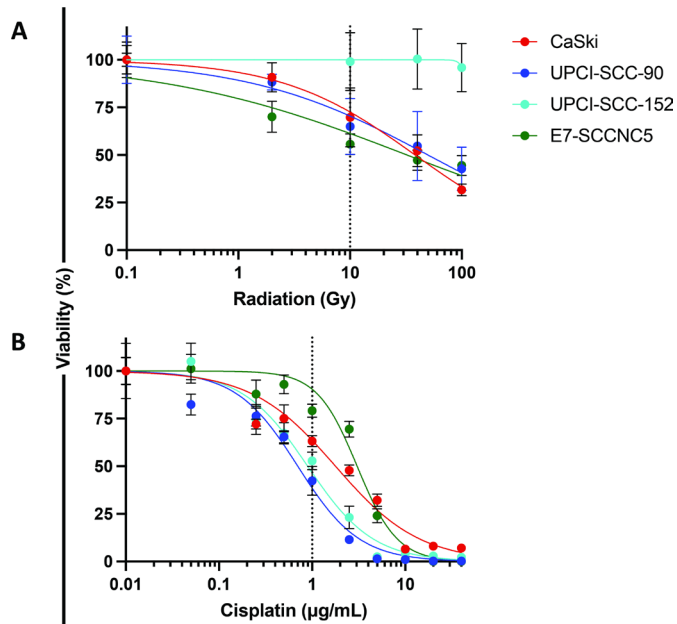


Figure 1 Viability of HPV-associated tumor models after exposure to standard of care radiation or cisplatin. HPV-associated tumor models were treated with increasing concentrations of (A) radiation or (B) cisplatin, and viability was determined by MTT assays at 72 hours as a percentage relative to untreated control. Dotted lines represent sublethal clinically relevant concentrations of radiation (10 Gy) or cisplatin (1 $\mu\text{g}/\text{mL}$) used for subsequent studies. HPV, human papillomavirus.

concentration according to pharmacokinetic studies²⁸—viability relative to untreated controls ranged from about 40% in UPCI-SCC-90 to nearly 80% in E7-SCCNC5 (figure 1B). These clinically relevant concentrations of radiation (10 Gy) or cisplatin (1 $\mu\text{g}/\text{mL}$) were adopted for all subsequent studies. Taken together, these data represent the phenomenon that tumor cell subpopulations may survive at clinically relevant concentrations of radiation or cisplatin exposure, and it was hypothesized that IO strategies could then be employed to eradicate these surviving tumor cell subpopulations.

HPV16 E7-specific CTL effector function was significantly enhanced by IL-15 agonism

Serving as a model for HPV therapeutic vaccine or TCR ACT, a previously described E7-specific CTL was employed for our study.^{20,21} Using an impedance-based real-time cell analysis cytotoxicity assay, we demonstrated specificity of the CTL model for HPV16 E7 by comparing per cent lysis of the E7-negative SCCNC5 parent cell line to per cent lysis of the E7-positive E7-SCCNC5 daughter cell line, representing HPV-negative and HPV-positive SNSCC, respectively. At a range of E:T ratios spanning one log, E7-SCCNC5 lysis was significantly greater than SCCNC5 lysis by as much as 8.6-fold ($p < 0.0001$), demonstrating specificity of the CTL model for HPV16 E7 (figure 2A). These data also functionally validate E7-SCCNC5 as a transgenic model of HPV-associated SNSCC.

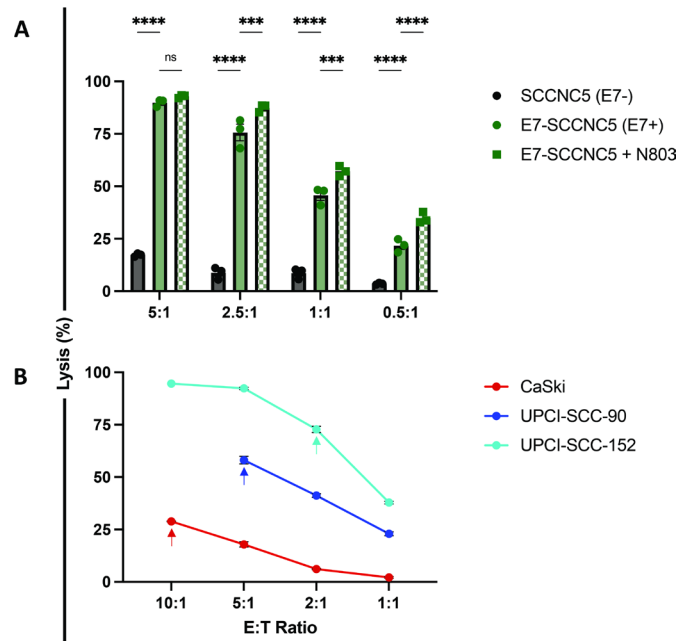


Figure 2 HPV16 E7-specific cytotoxic lymphocyte effector function was significantly enhanced by IL-15 agonism. (A) SCCNC5 (parent, E7 negative) and E7-SCCNC5 (daughter, E7 transduced) were co-cultured with HLA-A2 restricted HPV16 E7-specific cytotoxic lymphocyte (CTL) at increasing effector to target (E:T) ratios with and without effector pretreatment with IL-15 superagonist (N803, 50 ng/mL) for 24 hours. Lysis was determined at 4 hours as a percentage relative to control without effector addition. (B) Three endogenous HPV16-positive tumor models were co-cultured with HPV16 E7-specific CTL, and lysis was determined at 4 hours, as above. Arrows represent E:T ratios used for subsequent studies (CaSki, 10:1; UPCI-SCC-90, 5:1; UPCI-SCC-152, 2:1; E7-SCCNC5, 2:1, not shown). ns $p > 0.05$, *** $p < 0.001$, **** $p < 0.0001$. HPV, human papillomavirus; IL, interleukin.

N803 is a compelling IO agent that functions as an IL-15/IL-15-R α superagonist to activate and expand the CD8⁺ T-cell and NK-cell compartments while sparing similar effects on the regulatory T-cell compartment.¹²⁻¹⁴ To determine whether the CTL model was sensitive to IL-15 agonism, CTLs were pretreated with N803 before effector addition to the above cytotoxicity assay (figure 2A). At an E:T ratio of 2.5:1, E7-SCCNC5 lysis was significantly enhanced by a factor of more than 15% with N803 pretreatment versus without N803 pretreatment ($p = 0.0002$). Taken together, these data demonstrate that the CTL model is specific for HPV16 E7 and sensitive to IL-15 agonism.

After characterizing the HPV16 E7-specific CTL model using the E7-SCCNC5 transgenic model of HPV-associated SNSCC, we sought to further investigate effector function against endogenous HPV16-positive tumor models (figure 2B). In three of three endogenous HPV16-positive tumor models, we demonstrated increasing target cell lysis with increasing E:T ratios, and for all subsequent studies, the following E:T ratios were adopted with opportunity for improvement through employing IO strategies:

CaSki at 10:1, UPCI-SCC-90 at 5:1, UPCI-SCC-152 at 2:1, and E7-SCCNC5 at 2:1.

Radiation or cisplatin exposure significantly enhanced CTL-mediated HPV-associated target cell lysis, which was further enhanced by IL-15 agonism

Standard therapies, including radiation and cisplatin, have been shown to induce immunogenic cell stress and, in turn, enhance immune-mediated killing across many cancer types; HPV-associated malignancies, however, have been sparsely represented across this body of literature. Accordingly, we sought to investigate whether radiation or cisplatin exposure would enhance CTL-mediated lysis in tumor models of HPV-associated malignancies. At 72

hours following 10 Gy radiation exposure, CTL-mediated lysis of CaSki significantly increased by a factor of 21.1% ($p < 0.0001$), of UPCI-SCC-90 by 2.6-fold ($p < 0.0001$), of UPCI-SCC-152 by 2.1-fold ($p < 0.0001$), and of E7-SCCNC5 by 2.3-fold ($p < 0.0001$) (figure 3A). Furthermore, CTL pretreatment with N803 further enhanced CTL-mediated lysis across four of four cell lines following radiation exposure. With N803 pretreatment and following radiation exposure, CTL-mediated lysis of CaSki significantly increased by an additional factor of 11.0% ($p = 0.0018$), of UPCI-SCC-90 by 7.1% ($p = 0.0005$), of UPCI-SCC-152 by 16.2% ($p = 0.0030$), and of E7-SCCNC5 by 6.9% ($p = 0.0004$) (figure 3A). Following 48 hours of 1 $\mu\text{g}/\text{mL}$

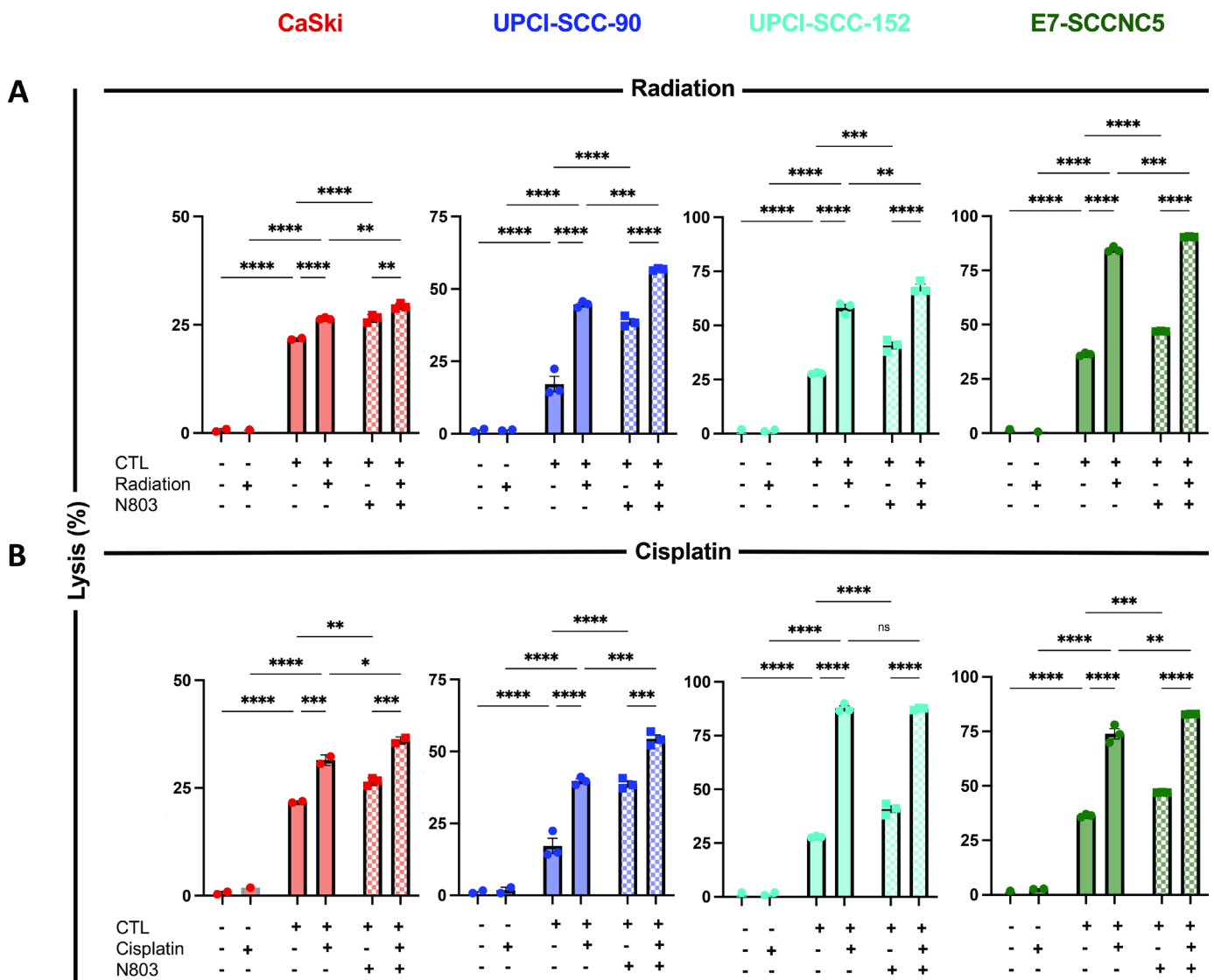


Figure 3 Radiation or cisplatin exposure significantly enhanced CTL-mediated HPV-associated target cell lysis, which was further enhanced by IL-15 agonism. HPV-associated tumor models were treated with (A) radiation (10 Gy, 72 hours) or (B) cisplatin (1 $\mu\text{g}/\text{mL}$, 48 hours) followed by co-culture with HPV16 E7-specific CTL at established effector to target ratios (see figure 2) with and without effector pretreatment with IL-15 superagonist (N803, 50 ng/mL) for 72 hours after effector stimulation with $\alpha\text{-CD2}/\text{CD3}/\text{CD28}$, as described in Materials and methods. Lysis was determined at 4 hours (CaSki, UPCI-SCC-90) or 18 hours (UCPI-SCC-152, E7-SCCNC5) as a percentage relative to control without effector addition in each respective group. Experiments were replicated at least twice with similar results. ns $p > 0.05$, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. CTL, cytotoxic lymphocyte; HPV, human papillomavirus; IL, interleukin.

cisplatin exposure, CTL-mediated lysis of CaSki significantly increased by a factor of 44.5% ($p=0.0002$), of UPCI-SCC-90 by 2.3-fold ($p<0.0001$), of UPCI-SCC-152 by 3.1-fold ($p<0.0001$), and of E7-SCCNC5 by 2.0-fold ($p<0.0001$) (figure 3B). Furthermore, CTL pretreatment with N803 further enhanced CTL-mediated lysis following cisplatin exposure. With N803 pretreatment and following cisplatin exposure, CTL-mediated lysis of CaSki significantly increased by an additional factor of 14.3% ($p=0.0107$), of UPCI-SCC-90 by 37.0% ($p=0.0003$), and of E7-SCCNC5 by 12.2% ($p=0.0013$) (figure 3B). At a time point of 18 hours, CTL-mediated lysis of UPCI-SCC-152 following cisplatin exposure did not significantly increase with N803 pretreatment ($p>0.9999$) owing to maximal effect at this time point (figure 3B); however, at an earlier time point of 4 hours when the dependent variable was not saturated, CTL-mediated lysis of UPCI-SCC-152 following cisplatin exposure significantly increased by an additional factor of 13.6% with N803 pretreatment ($p=0.0009$) (data not shown). Taken together, these data demonstrate that radiation or cisplatin exposure significantly enhances CTL-mediated lysis in four of four HPV-associated tumor models, which can further be enhanced with IL-15 agonism by employing N803.

Radiation or cisplatin exposure markedly upregulated immunomodulatory markers in representative HPV-associated tumor models

We next sought to interrogate markers of immunomodulation following radiation or cisplatin exposure in three

of four representative tumor models of HPV-associated malignancies. We included programmed death ligand-1 (PD-L1) in our panel of investigation given the established role of immune checkpoint inhibitors in cervical carcinoma and HNSCC. At 72 hours following 10 Gy radiation exposure or 48 hours of 1 $\mu\text{g}/\text{mL}$ cisplatin exposure, viable single cell populations were assessed by flow cytometry for HLA-A2 (antigen presentation machinery), calreticulin ('eat-me' signal for dendritic cell activation), ICAM-1 (adhesion molecule and costimulatory ligand), Fas and TRAIL-R2 (death receptors), and PD-L1 (ligand for T-cell exhaustion). We defined marked upregulation as 10% increase in per cent positive cells or 1.5-fold increase in net MFI relative to untreated controls (table 2).

HLA-A2 was markedly upregulated with radiation or cisplatin exposure in CaSki—which had the lowest HLA-A2 expression at baseline—and with radiation exposure in UPCI-SCC-90. While net MFI numerically increased with cisplatin exposure in UPCI-SCC-90 and with radiation or cisplatin exposure in E7-SCCNC5, these did not meet our defined threshold for marked upregulation. Calreticulin, ICAM-1, and PD-L1 were markedly upregulated with radiation or cisplatin exposure in three of three cell lines tested. Fas was markedly upregulated with radiation or cisplatin exposure in CaSki and E7-SCCNC5, and with radiation exposure in UPCI-SCC-90. While per cent positive cells and net MFI numerically increased with cisplatin exposure in UPCI-SCC-90, these did not meet our defined threshold for marked upregulation. Finally,

Table 2 Radiation or cisplatin exposure markedly upregulated immunomodulatory markers in representative HPV-associated tumor models

CaSki	Viability	HLA-A2	Expression (% positive, (net MFI))				
			Calreticulin	ICAM-1	Fas	TRAIL-R2	PD-L1
Control	87.9	23.7 (349)	6.69 (79)	43.5 (957)	98.5 (6158)	99.7 (6469)	86.0 (2118)
Radiation	83.8	70.7 (1494)	27.3 (205)	51.3 (2533)	99.3 (22,153)	99.6 (17,999)	91.9 (5674)
Cisplatin	84.8	61.4 (948)	25.2 (174)	51.1 (2107)	98.2 (19,602)	99.5 (12,401)	92.9 (4191)
UPCI-SCC-90							
Control	89.3	91.5 (1539)	1.53 (20)	7.19 (87)	5.75 (268)	35.5 (78)	1.43 (20)
Radiation	87.1	91.1 (2357)	2.84 (69)	15.6 (220)	8.95 (481)	30.2 (163)	2.53 (52)
Cisplatin	89.7	96.0 (1996)	2.41 (39)	12.6 (146)	9.17 (340)	61.4 (195)	2.15 (45)
E7-SCCNC5							
Control	88.0	99.7 (34,159)	72.3 (30)	26.8 (49)	99.6 (9810)	99.6 (2034)	52.2 (228)
Radiation	86.2	98.6 (34,816)	56.5 (120)	17.8 (163)	99.5 (28,830)	98.6 (5510)	33.9 (652)
Cisplatin	90.4	99.8 (44,581)	79.5 (85)	26.8 (131)	99.8 (28,308)	99.6 (4641)	52.9 (479)

Representative HPV-associated tumor models for cervical carcinoma (CaSki), head and neck squamous cell carcinoma (UPCI-SCC-90), and sinonasal squamous cell carcinoma (E7-SCCNC5) were treated with radiation (10 Gy, 72 hours) or cisplatin (1 $\mu\text{g}/\text{mL}$, 48 hours) followed by flow cytometric interrogation of immunomodulatory markers representing antigen presentation machinery (HLA-A2), dendritic cell 'eat me' signal and activator (calreticulin), adhesion molecule and costimulatory ligand (ICAM-1), death receptors (Fas, TRAIL-R2), and T-cell exhaustion ligand (PD-L1). Viability represents per cent of single cell gated population with negative LIVE/DEAD staining. Percentages indicate per cent positive cells of the total viable population. Numbers in parentheses indicate net MFI (geometric mean of isotype subtracted from geometric mean of marker) of total viable population. Numbers in bold represent marked upregulation relative to control, as defined by 10% increase in per cent positive cells or 1.5-fold increase in net MFI. Experiment was replicated twice with similar results.

HPV, human papillomavirus; MFI, mean fluorescent intensity.

TRAIL-R2 was markedly upregulated with radiation or cisplatin exposure in three of three cell lines tested (table 2). Taken together, these data demonstrate that HPV-associated tumor models are phenotypically altered with sublethal radiation or cisplatin exposure to upregulate markers of immunomodulation, including PD-L1.

Radiation or cisplatin exposure significantly upregulated death receptors Fas and TRAIL-R2, which functionally mediated enhanced HPV-associated target cell lysis

Following the observation that Fas and TRAIL-R2 were markedly upregulated with sublethal radiation or cisplatin exposure (table 2), in conjunction with the observation that sublethal radiation or cisplatin exposure significantly enhanced CTL-mediated HPV-associated target cell lysis (figure 3), we sought to investigate the functional significance of Fas and TRAIL-R2 death receptor upregulation

in three of four representative tumor models of HPV-associated malignancies. At 72 hours following 10 Gy radiation exposure or 48 hours of 1 $\mu\text{g}/\text{mL}$ cisplatin exposure, Fas net MFI significantly increased in CaSki by 3.0-fold or 2.1-fold, respectively ($p < 0.001$); in UPCI-SCC-90 by a factor of 51.3% or 8.5%, respectively ($p < 0.001$); and in E7-SCCNC5 by 3.9-fold or 3.2-fold, respectively ($p < 0.001$) (figure 4A,B). When treated with exogenous Fas ligand in cytotoxicity assays following sublethal radiation or cisplatin exposure, these significant increases in Fas net MFI translated to significant increases in target cell lysis in CaSki by 2.8-fold or 3.1-fold, respectively ($p < 0.0001$); in UPCI-SCC-90 from 0.1% to 6.1% or 8.9%, respectively ($p = 0.0038, 0.0011$); and in E7-SCCNC5 from 0.0% to 37.5% or 44.7%, respectively (untreated control rounded to 0.0%, unable to calculate fold increase; $p = 0.0009$,

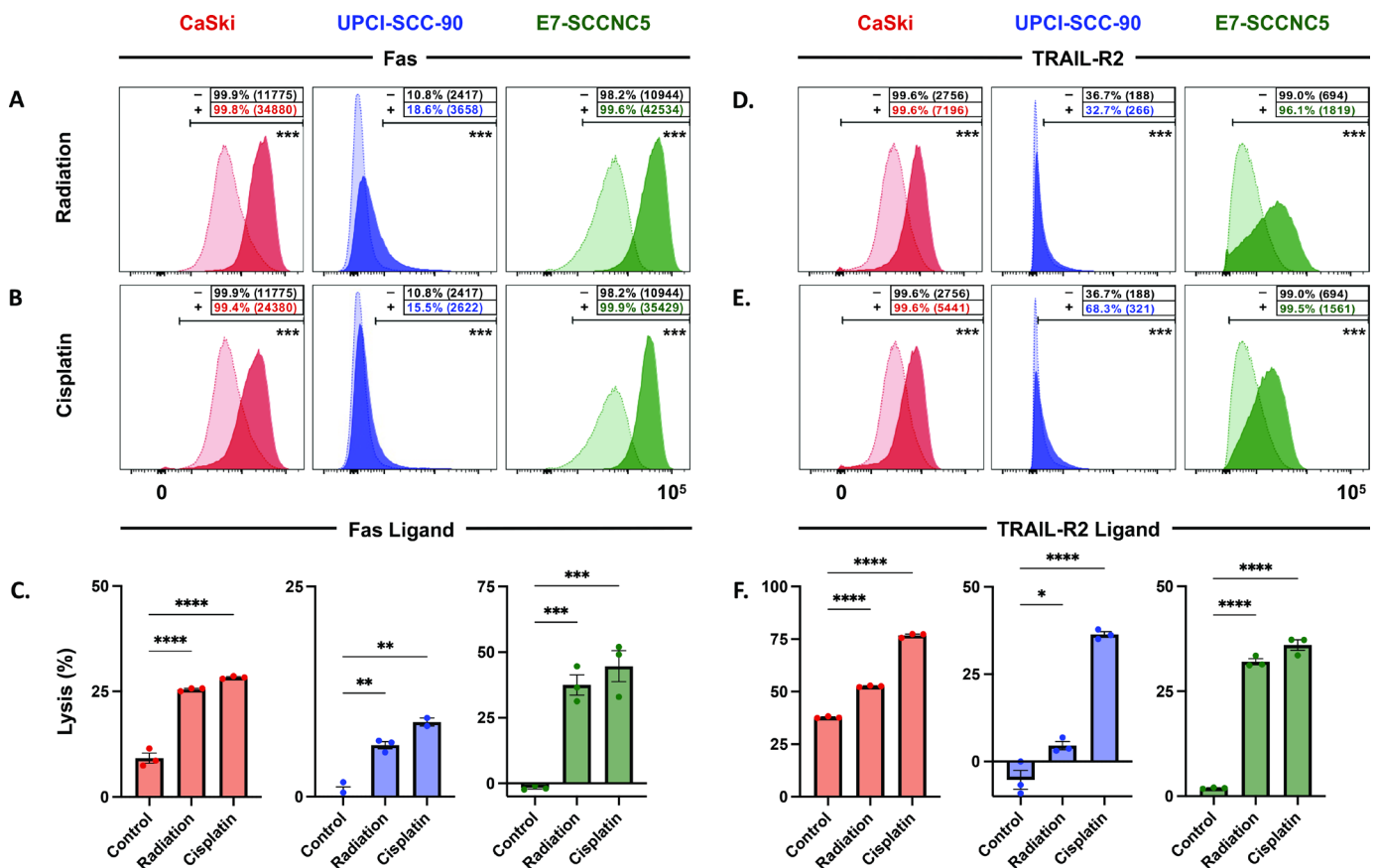


Figure 4 Radiation or cisplatin exposure significantly upregulated death receptors Fas and TRAIL-R2, which functionally mediated enhanced HPV-associated target cell lysis. Representative HPV-associated tumor models for cervical carcinoma (CaSki), head and neck squamous cell carcinoma (UPCI-SCC-90), and sinonasal squamous cell carcinoma (E7-SCCNC5) were treated with (A, D) radiation (10 Gy, 72 hours) or (B, E) cisplatin (1 $\mu\text{g}/\text{mL}$, 48 hours) followed by flow cytometric interrogation of death receptors (A, B) Fas and (D, E) TRAIL-R2. Light shades and black, top boxes (-) depict untreated control groups. Dark shades and colored, bottom boxes (+) depict treatment groups. Gates represent per cent positive cells of the total viable population. Percentages indicate per cent positive cells of the total viable population. Numbers in parentheses indicate net mean fluorescent intensity (geometric mean of isotype subtracted from geometric mean of marker) of total viable population. Statistical significance determined by Kolmogorov-Smirnov test. (Bottom panel) Following radiation or cisplatin exposure, tumor models were co-incubated with (C) exogenous Fas ligand (α -Fas antibody, CH11 clone; 1 $\mu\text{g}/\text{mL}$, 10 $\mu\text{g}/\text{mL}$, 1 $\mu\text{g}/\text{mL}$, respectively) or (F) exogenous TRAIL-R2 ligand (KillerTRAIL; 20 ng/mL, 500 ng/mL, 500 ng/mL, respectively), and lysis was determined at 4 hours (KillerTRAIL, E7-SCCNC5) or 18 hours (others) as a percentage relative to control without effector addition in each respective group. Experiments were replicated twice with similar results. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. HPV, human papillomavirus.

0.0004) (figure 4C). Following sublethal radiation or cisplatin exposure, TRAIL-R2 net MFI significantly increased in CaSki by 2.6-fold or 2.0-fold, respectively ($p < 0.001$); in UPCI-SCC-90 by 1.4-fold or 1.7-fold, respectively ($p < 0.001$); and in E7-SCCNC5 by 2.6-fold or 2.3-fold, respectively ($p < 0.001$) (figure 4D,E). When treated with exogenous TRAIL-R2 ligand in cytotoxicity assays following sublethal radiation or cisplatin exposure, these significant increases in TRAIL-R2 net MFI translated to significant increases in target cell lysis in CaSki by a factor of 39.2% or 2.0-fold, respectively ($p < 0.0001$); in UPCI-SCC-90 from 0.0% to 4.5% or 36.4%, respectively (untreated control rounded to 0.0%, unable to calculate fold increase; $p = 0.0138$, $p < 0.0001$), and in E7-SCCNC5 by 16.8-fold or 18.9-fold, respectively ($p < 0.0001$) (figure 4F). Taken together, these data demonstrate a functional mechanism through which sublethal radiation or cisplatin exposure enhances CTL-mediated target cell lysis in tumor models of HPV-associated malignancies by way of Fas and TRAIL-R2 death receptor upregulation, which in turn mediates enhanced target cell lysis through functional engagement of these death receptors.

Cisplatin significantly potentiated CTL-mediated tumor control following in vivo ACT

Having characterized that radiation or cisplatin induces immunogenic cell stress in tumor models of HPV-associated malignancies, which in turn mediates enhanced CTL-mediated target cell lysis, we sought to investigate this effect in vivo (figure 5). For this study, we selected CaSki as a representative human tumor model of HPV-associated malignancy, which, notably, was least potentiated to enhanced CTL-mediated target cell lysis in vitro (figure 3). NSG mice bearing CaSki tumors were

treated with a single cycle of cisplatin (5 mg/kg, ip) followed 2 days later by ACT of 1×10^6 E7-specific CTLs (figure 5A). Whereas cisplatin and ACT had no effect on tumor growth alone ($p = 0.52$ and $p = 0.82$, respectively), the combination demonstrated significant tumor control compared with no treatment ($p = 0.0004$), as well as compared with cisplatin monotherapy ($p = 0.0168$) and ACT monotherapy ($p < 0.0001$), demonstrating synergy (figure 5B). Taken together, these data suggest that antigen specific antitumor immunity is significantly enhanced by exploiting the immunogenic potential of standard of care in an in vivo human tumor model of HPV-associated malignancy.

DISCUSSION

In the last two decades, the observation that radiation and chemotherapy may have immunogenic potential in addition to their cytotoxic properties is one that has been well documented but underwhelmingly exploited with standard of care in the clinic. Additionally, characterization of this immunogenic potential, resulting from immunogenic cell stress, across many cancer types has sparsely included representation of HPV-associated malignancies. This area of investigation is of particular interest as, for example, HPV-positive HNSCC at baseline has a favorable immune-infiltrated tumor microenvironment owing to its viral etiology as compared with HPV-negative HNSCC that sets a compelling foundation on which to employ IO strategies.¹¹ Moreover, viral antigens, including E6 and E7, that are uniformly and constitutively expressed by HPV-transformed malignant cells make ideal tumor-associated antigens in principle to target with therapeutic

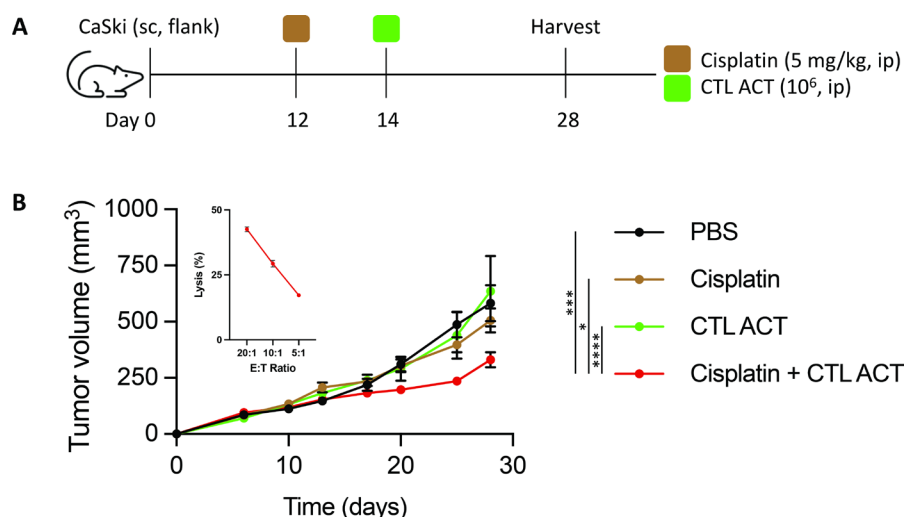


Figure 5 Cisplatin significantly potentiated cytotoxic lymphocyte-mediated tumor control following in vivo adoptive cell transfer. (A) Pictogram representing experimental design. (B) NSG mice bearing CaSki tumors were treated with one cycle of cisplatin (5 mg/kg, ip) on day 12 and/or E7-specific CTL ACT (1×10^6 , ip) on day 14 post-tumor inoculation (sc). Inset demonstrating in vitro CTL lytic activity against CaSki at time of ACT at E:T ratios of 5:1, 10:1, and 20:1. Tumor volume (mm^3) was monitored for 2 weeks following last treatment. Two-way analysis of variance with Tukey's post hoc multiple comparisons test was used to compare tumor growth curves. ACT, adoptive cell transfer; CTL, cytotoxic lymphocyte; E:T, effector to target; ip, intraperitoneal; PBS, phosphate buffered saline; sc, subcutaneous. * $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$.

vaccine or ACT.²⁹ Accordingly, we sought to characterize the immunogenic potential of radiation and cisplatin in tumor models of HPV-associated malignancies in order to inform rational combination IO strategies that might be employed in the clinic on the backbone of standard of care, and in so doing exploit the immunogenic potential of standard of care to improve durable responses in HPV-associated malignancies. Such rational combination IO strategies should aim to support four phases of an effective antitumor immune response: to engage, to expand, to enable, and to evolve a tumor antigen specific immune response, which we will apply as a framework for discussion.³⁰

To serve as a model for HPV therapeutic vaccine or ACT, we employed a high avidity HLA-A*02-restricted HPV16 E7₁₁₋₁₉-specific CTL, which has been characterized preclinically, and in a phase I clinical trial has demonstrated safety and clinical activity in the form of ACT.^{21 31} This model for HPV therapeutic vaccine or ACT illustrates the first phase of an effective antitumor immune response: to engage a tumor antigen specific T-cell response. The CTL model was demonstrated as specific for HPV16 E7 and could mediate lysis of target cells infected with HPV16 (figure 2, table 1). We further demonstrated on the cognate side of the target cells marked upregulation of antigen presentation machinery, represented by HLA-A2, in two of three HPV-associated tumor models with radiation or cisplatin exposure, which may function to enhance the first phase of engaging a tumor antigen specific T-cell response by upregulation of major histocompatibility complex (MHC) class I (table 2).

The second phase of an effective antitumor immune response is expansion, which includes proliferation and immune infiltration into the tumor microenvironment.³⁰ While our in vitro CTL cytotoxicity assays were controlled for E:T ratios between untreated and treated groups, N803—an IL-15/IL-15-R α superagonist—has demonstrated robust preclinical and clinical activity in activating and expanding the CD8⁺ T-cell and NK-cell compartments.^{12 13} In our study, we demonstrated upregulation of ICAM-1, a co-stimulatory ligand and adhesion molecule, in three of three HPV-associated tumor models with radiation or cisplatin exposure, which may function as a co-stimulatory ligand to activate and expand a tumor antigen specific T-cell response, while additionally functioning as an adhesion molecule to facilitate immune infiltration (table 2).

The third phase of an effective antitumor immune response is to enable a strong effector response.³⁰ We demonstrated that sublethal exposure to radiation or cisplatin at clinically relevant concentrations significantly enhanced CTL-mediated target cell lysis in four of four HPV-associated tumor models, including in one radiation refractory tumor model (figures 1 and 3, table 1). Additionally, we demonstrated that N803 further enhanced this effect through CTL activation, improving CTL-mediated target cell lysis in four of four HPV-associated tumor models (figures 2 and 3). In vivo, a single cycle

of cisplatin or E7-specific CTL ACT failed to mediate significant tumor control as monotherapies, whereas in combination they demonstrated synergy, mediating significant tumor control (figure 5)—while employing the least remarkable HPV-associated tumor model from the in vitro phase of study (figure 3). These results build on a previous study characterizing the E7-specific TCR employed here, which demonstrated tumor control following ACT of 1×10^7 CTLs, whereas 1×10^6 CTLs had no effect.²¹ Here, we observed that ACT of 1×10^6 E7-specific CTLs had no effect alone but was potentiated to mediate significant tumor control when combined with a single, subtherapeutic cycle of cisplatin (figure 5). Interestingly, one group demonstrated that oxaliplatin (another immunogenic platinum-based chemotherapy similar to cisplatin) facilitated increased tumor infiltration of adoptively transferred chimeric antigen receptor (CAR)-T cells in a murine model of lung carcinoma, suggesting an additional mechanism through which subtherapeutic doses of cisplatin and E7-specific CTL ACT may have synergized in our study.³²

We went on to show that upregulation of death receptors Fas and TRAIL-R2 functionally mediated enhanced target cell lysis with radiation or cisplatin exposure in three of three HPV-associated tumor models (table 2, figure 4). Fas serves as a canonical death receptor mediating immune-mediated target cell lysis, while dysfunction of Fas-mediated apoptosis has been characterized as a tumor mechanism of immune evasion.³³ Accordingly, parallel upregulation of the distinct death receptor TRAIL-R2 may function as a fail-safe in case of Fas dysfunction. We have previously demonstrated upregulation of TRAIL-R2 as a mechanism for enhanced immune-mediated target cell lysis in the context of immunogenic cell stress induced with small molecule inhibitors, but this is the first time we demonstrated this mechanism with radiation or chemotherapy.^{34 35} Our group and others have previously and extensively characterized the role of Fas upregulation in studies of immunogenic cell stress involving radiation or chemotherapy, including cisplatin.^{36 37} Our data confirm and extend these observations, wherein radiation or cisplatin exposure upregulates death receptors Fas and TRAIL-R2 in tumor models of HPV-associated malignancies, establishing a condition poised for immune-mediated killing.

We also demonstrated upregulation of PD-L1 with radiation or cisplatin exposure in three of three HPV-associated tumor models (table 2), lending rationale to combine standard of care with immune checkpoint inhibitors.³⁸ Consistent with this observation, others have also demonstrated upregulation of PD-L1 through induction of cGAS/STING signaling following radiation or cisplatin exposure.³⁸⁻⁴⁰ One group elegantly described upregulation of PD-L1 as an immune escape mechanism in a clinical study of radiation and ipilimumab (α -cytotoxic T-lymphocyte-associated antigen 4), which was subsequently relieved by α -PD-L1 checkpoint inhibition.⁴¹ Additionally, we have previously demonstrated that N803

synergizes with immune checkpoint inhibitors in preclinical studies via N803-mediated upregulation of PD-L1 on immune cell subsets through induction of interferon- γ signaling, consistent with an activated phenotype, which in turn is relieved by immune checkpoint inhibition.⁴² Immune checkpoint inhibitors are approved in the metastatic and recurrent settings of cervical carcinoma (pembrolizumab) and HNSCC (pembrolizumab and nivolumab), and there are ongoing efforts to investigate these at earlier stages of disease in combination with standard of care with curative intent—although with variable degrees of success to date, which will further be discussed below.^{5 6} Immune checkpoint inhibitors illustrate the third phase of an effective antitumor immune response by enabling a strong effector response through relief of exhausted programmed cell death protein-1 (PD-1)/PD-L1 signaling in activated tumor antigen specific T cells.

The fourth phase of an effective antitumor immune response is the evolution of the tumor antigen specific T-cell response.³⁰ Here, we demonstrated upregulation of calreticulin surface expression with radiation or cisplatin exposure in three of three HPV-associated tumor models (table 2). Calreticulin is an endoplasmic reticulum resident chaperone protein that traffics to the tumor cell surface under conditions of immunogenic cell stress, serving as a potent immunogenic signal to dendritic cells to facilitate tumor cell phagocytosis and tumor antigen cross presentation, additionally serving directly to enhance CTL-mediated target cell lysis.^{43 44} Furthermore, we have previously demonstrated that radiation not only upregulates antigen processing and presentation machinery, as we have demonstrated in the present study through upregulation of HLA-A*02 (table 2), but also increases the peptide repertoire that is presented through MHC class I by increased degradation of intracellular proteins as well as increased translation of nascent proteins.⁴⁵ These features collectively serve to mediate antigen cascade and, in turn, evolution of the tumor antigen specific T-cell response, which our group has previously characterized in both the preclinical and clinical settings.^{46 47}

Taken together, our data demonstrate that radiation or cisplatin is capable of inducing immunogenic cell stress in HPV-associated tumor models, which in principle may facilitate tumor antigen specific T-cell engagement (phase 1) through upregulation of HLA-A*02; expansion (phase 2) through upregulation of ICAM-1, as well as through IL-15 agonism (ie, N803); enabling (phase 3) through upregulation of death receptors Fas and TRAIL-R2, as well as through immune checkpoint inhibition of upregulated PD-L1; and evolution (phase 4) through upregulation of calreticulin surface expression, which may facilitate antigen cascade. Other groups have demonstrated synergy with radiation or cisplatin in combination with HPV16 therapeutic vaccines in TC-1 murine models of HPV-associated malignancies, but we present the first characterization of immunogenic cell stress in human

tumor models of HPV-associated malignancies, which may functionally mediate the synergy observed by other groups in TC-1 murine models. Indeed, few of those studies characterized upregulation of MHC class I and Fas with radiation or cisplatin treatment in TC-1 murine models of HPV-associated malignancies.^{23 25} Interestingly, one study demonstrated significantly greater synergy with HPV16 therapeutic vaccine followed by radiation as compared with the reverse schedule,²⁴ as has been demonstrated by others,⁴⁸ which has implications for the scheduling of combination IO strategies with standard of care in the clinic. This would follow the ideal paradigm that an active antitumor immune response is present at the time of standard of care in order to best exploit the immunogenic potential of standard of care. For example, applying this paradigm, while additionally borrowing from principles learned from clinical trials in HNSCC,⁵ the data presented here would support a rational clinical trial design in SNSCC—where the aim is to eradicate residual disease following surgery⁸—as follows: HPV16 therapeutic vaccine, N803, and immune checkpoint inhibition employed in the neoadjuvant setting would engage and expand a tumor antigen specific immune response, while additionally exploiting the tumor as an in situ vaccine, followed by surgical resection and adjuvant radiation with or without chemotherapy, which is standard of care. In this way, an active antitumor immune response would be present at the time of radiation with or without chemotherapy in order to exploit the immunogenic potential of standard of care to eradicate residual disease in the adjuvant setting. There is an ongoing phase II clinical trial in newly diagnosed HPV-associated HNSCC that follows this paradigm investigating HPV therapeutic vaccine followed after 1 week by pembrolizumab followed again after 1 week by cisplatin-based chemoradiotherapy (NCT04369937). Notably, moving IO strategies to the front line of cancer care—in addition to exploiting the immunogenic cell stress induced by standard of care, as we have demonstrated—benefits from a less immunoevaded tumor microenvironment, whereas at later stages of disease in heavily pretreated populations, immunoevading fortifies a more recalcitrant tumor microenvironment to IO strategies.⁴⁹ These concepts, in addition to the data presented herein, give rationale to investigate IO strategies in HPV-associated malignancies with standard of care at the time of diagnosis or at the time of disease progression or recurrence in combination with first-line therapies and first-line salvage therapies.

It would be prudent to address the recently tempered enthusiasm in HNSCC for immune checkpoint inhibitors at the front line of treatment in combination with standard of care after the early termination of the JAVELIN Head and Neck 100 phase III clinical trial on the grounds of futility.^{5 50} Whereas immune checkpoint inhibitors have demonstrated benefit at earlier stages of disease in other cancer types—most notably in the PACIFIC phase III clinical trial in locally advanced non-small cell lung cancer⁵¹—the JAVELIN trial failed to show improvement

in progression-free survival with concurrent and adjuvant addition of avelumab (α -PD-L1) to standard cisplatin-based chemoradiotherapy in locally advanced HNSCC. While treatment groups were matched for HPV status, only one-third of the patients were HPV-positive. It is also interesting to note that exploratory analysis demonstrated a trend toward benefit in the experimental group among a small subset of patients with high PD-L1 expression (>25%). Notably, it is still yet to be determined whether addition of immune checkpoint inhibitors to standard of care will demonstrate benefit in locoregionally advanced cervical carcinoma, with two ongoing phase III clinical trials aiming to answer this question (NCT03830866, NCT04221945).⁶ Other considerations include when to schedule IO strategies relative to standard therapies, with increasing evidence suggesting enhanced benefit in the neoadjuvant setting versus the adjuvant setting.^{48 52 53} Compared with the JAVELIN trial, the ongoing phase II clinical trial discussed above is investigating HPV therapeutic vaccine and pembrolizumab (α -PD-1) in the neoadjuvant setting before initiation of cisplatin-based chemoradiotherapy (NCT04369937). Moreover, while the primary aim of our study was to demonstrate an immunogenic potential for standard therapies in HPV-associated malignancies, future studies will focus attention on optimizing specific IO strategies in combination with standard therapies to exploit the immunogenic potential of standard therapies described herein. Notably, two recent preclinical studies have illustrated the utility of multipronged combination IO strategies by demonstrating immune-mediated synergy through combining five IO agents in one study and five IO agents in combination with chemotherapy in a second study, wherein monotherapies and permutations of each combination had significantly decreased effect.^{54 55} Accordingly, it is suggested that the results of the JAVELIN trial, while intrinsically disappointing, present an opportunity to explore combination IO strategies beyond immune checkpoint inhibitors alone layered onto standard of care. Importantly, the JAVELIN trial at minimum, among other trials, has demonstrated the safety of immune checkpoint inhibitors in combination with standard therapies in the clinic. Thus, given the well-established safety profiles of cancer vaccines and N803,^{17 56} a triplet combination of HPV therapeutic vaccine, N803, and immune checkpoint inhibition in conjunction with cisplatin-based chemoradiotherapy offers a safe and rational path forward for future investigation following the evidence demonstrated herein that standard therapies have immunogenic potential in HPV-associated malignancies.

Finally, to further elaborate on clinical options, it is important to highlight that we have previously demonstrated across the many modalities of radiotherapy a similar capacity to induce immunogenic cell stress in tumor cell populations.³⁶ This includes brachytherapy, which can be employed in locoregionally advanced cervical carcinoma, as well as proton therapy and stereotactic radiotherapy,^{57 58} which can be employed in HNSCC

and SNSCC to discriminate between tumor and adjacent critical organs in this complex anatomical region.⁹ Second, there is a robust pipeline of clinical investigation involving HPV therapeutic vaccines, including some in phase III clinical trials, and two important takeaways are their strong safety profile and their ability to engage an antigen specific immune response.^{59 60} Accordingly, these offer a safe foundation on which to build combination IO strategies, as suggested above. ACT may also be considered in this space to engage an effective antigen specific immune response. Third, IL-15 agonism is a compelling IO strategy to layer in combination with HPV therapeutic vaccine in order to expand and enable an effective anti-tumor immune response,¹⁴ but there are many additional IO strategies than can be explored in this space.⁶⁰ One ongoing phase II clinical trial has demonstrated impressive early activity in patients with advanced relapsed or refractory HPV-associated malignancy on the backbone of HPV therapeutic vaccine in combination with bintrafusp alfa (a bifunctional α -PD-L1/transforming growth factor- β trap conjugate) and NHS-IL-12 (a tumor targeting antibody/IL-12 conjugate), demonstrating the power of multipronged combination IO strategies in the clinic (NCT04287868).⁶¹

One potential limitation of our study was an inherent inability to investigate the effect of immune checkpoint inhibition using the present E7-specific CTL model, as it was previously characterized that these CTLs have little to no expression of PD-1 and were therefore unlikely to respond to immune checkpoint inhibition.³¹ We confirmed and extended those results, demonstrating that pembrolizumab had no effect on the present E7-specific CTL model in vivo (online supplemental figure 1). In addition, our in vivo study used an experimental design wherein a single dose of N803 was administered on day 14 at the time of CTL ACT, which had no effect on outcome by day 28 considering that the half-life of N803 in mice and in humans is about 1 day (online supplemental figure 1).^{17 62} While our in vivo study benefited from interrogating a xenograft model of human HPV-associated malignancy, future studies will address these limitations using the TC-1 syngeneic mouse model, which will allow interrogation of the addition of checkpoint inhibition and N803 to cisplatin or radiation therapy. Finally, it should be noted that our study design used for the first time a transgenic HPV-associated SNSCC model that may or may not recapitulate the true oncogenic profile of naturally occurring HPV-associated SNSCC, as a cell line with the latter profile has not yet been established.

In conclusion, we provide evidence that radiation or cisplatin induces immunogenic cell stress, which in turn functionally enhances immune-mediated killing in tumor models of HPV-associated malignancies. We support the rationale that multipronged IO strategies should be brought into combination with standard of care to exploit the immunogenic potential of standard of care to improve outcomes in HPV-associated malignancies where

radiation and cisplatin are employed as first-line therapies or first-line salvage therapies.

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REFERENCES

- de Martel C, Plummer M, Vignat J, *et al*. Worldwide burden of cancer attributable to HPV by site, country and HPV type. *Int J Cancer* 2017;141:664–70.
- Johnson DE, Burtneis B, Leemans CR, *et al*. Head and neck squamous cell carcinoma. *Nat Rev Dis Primers* 2020;6:92.
- Fakhry C, Zhang Q, Nguyen-Tan PF, *et al*. Human papillomavirus and overall survival after progression of oropharyngeal squamous cell carcinoma. *J Clin Oncol* 2014;32:3365–73.
- Cohen PA, Jhingran A, Oaknin A, *et al*. Cervical cancer. *Lancet* 2019;393:169–82.
- Nenclares P, Rullan A, Tam K, *et al*. Introducing checkpoint inhibitors into the curative setting of head and neck cancers: lessons learned, future considerations. *Am Soc Clin Oncol Educ Book* 2022;42:511–26.
- Sherer MV, Kotha NV, Williamson C, *et al*. Advances in immunotherapy for cervical cancer: recent developments and future directions. *Int J Gynecol Cancer* 2022;32:281–7.
- Lopez DC, Hoke ATK, Rooper LM, *et al*. Human papillomavirus-related carcinomas of the sinonasal tract. *Curr Otorhinolaryngol Rep* 2022;10:291–302.
- Torabi SJ, Spock T, Cardoso B, *et al*. Margins in sinonasal squamous cell carcinoma: predictors, outcomes, and the endoscopic approach. *Laryngoscope* 2020;130:E388–96.
- Ferrari M, Taboni S, Carobbio ALC, *et al*. Sinonasal squamous cell carcinoma, a narrative reappraisal of the current evidence. *Cancers* 2021;13. doi:10.3390/cancers13112835. [Epub ahead of print: 07 06 2021].
- Kang Y, Huang J, Liu Y, *et al*. Integrated analysis of immune infiltration features for cervical carcinoma and their associated immunotherapeutic responses. *Front Cell Dev Biol* 2021;9:573497.
- Welters MJP, Ma W, Santegoets SJAM, *et al*. Intratumoral HPV16-Specific T cells constitute a type I-Oriented tumor microenvironment to improve survival in HPV16-Driven oropharyngeal cancer. *Clin Cancer Res* 2018;24:634–47.
- Kim PS, Kwilas AR, Xu W, *et al*. IL-15 superagonist/IL-15R α /Sushi-Fc fusion complex (IL-15SA/IL-15R α Su-Fc; ALT-803) markedly enhances specific subpopulations of NK and memory CD8⁺ T cells, and mediates potent anti-tumor activity against murine breast and colon carcinomas. *Oncotarget* 2016;7:16130–45.
- Romee R, Cooley S, Berrien-Elliott MM, *et al*. First-in-human phase 1 clinical study of the IL-15 superagonist complex ALT-803 to treat relapse after transplantation. *Blood* 2018;131:2515–27.
- Knudson KM, Hodge JW, Schlom J, *et al*. Rationale for IL-15 superagonists in cancer immunotherapy. *Expert Opin Biol Ther* 2020;20:705–9.
- Waldmann TA. The shared and contrasting roles of IL2 and IL15 in the life and death of normal and neoplastic lymphocytes: implications for cancer therapy. *Cancer Immunol Res* 2015;3:219–27.
- Margolin K, Morishima C, Velcheti V, *et al*. Phase I trial of ALT-803, a novel recombinant IL15 complex, in patients with advanced solid tumors. *Clin Cancer Res* 2018;24:5552–61.
- Rubinstein MP, Williams C, Mart C, *et al*. Phase I trial characterizing the pharmacokinetic profile of N-803, a chimeric IL-15 superagonist, in healthy volunteers. *J Immunol* 2022;208:1362–70.
- Olthoff NC, Huebbers CU, Kolligs J, *et al*. Viral load, gene expression and mapping of viral integration sites in HPV16-associated HNSCC cell lines. *Int J Cancer* 2015;136:E207–18.
- García-Inclán C, López-Hernández A, Alonso-Guervós M, *et al*. Establishment and genetic characterization of six unique tumor cell lines as preclinical models for sinonasal squamous cell carcinoma. *Sci Rep* 2014;4:4925.
- Lee MY, Robbins Y, Sievers C, *et al*. Chimeric antigen receptor engineered NK cellular immunotherapy overcomes the selection of T-cell escape variant cancer cells. *J Immunother Cancer* 2021;9:e002128.
- Jin BY, Campbell TE, Draper LM, *et al*. Engineered T cells targeting E7 mediate regression of human papillomavirus cancers in a murine model. *JCI Insight* 2018;3. doi:10.1172/jci.insight.99488. [Epub ahead of print: 19 04 2018].
- Kilkenny C, Browne WJ, Cuthill IC, *et al*. Improving bioscience research reporting: the ARRIVE guidelines for reporting animal research. *PLoS Biol* 2010;8:e1000412.
- Salomon N, Selmi A, Grunwitz C, *et al*. Local radiotherapy and E7 RNA-LPX vaccination show enhanced therapeutic efficacy in preclinical models of HPV16⁺ cancer. *Cancer Immunol Immunother* 2022;71:1975–1988.
- Zhang F, Zheng Z, Barman AK, *et al*. Optimal combination treatment regimens of vaccine and radiotherapy augment tumor-bearing host immunity. *Commun Biol* 2021;4:78.
- Ye GW, Park J-B, Park Y-J, *et al*. Increased sensitivity of irradiated murine cervical cancer tumors to E7 subunit vaccine-driven CTL-mediated killing induces synergistic anti-tumor activity. *Mol Ther* 2007;15:1564–70.
- Meissner JD. Nucleotide sequences and further characterization of human papillomavirus DNA present in the CaSKI, SiHa and HeLa cervical carcinoma cell lines. *J Gen Virol* 1999;80 (Pt 7):1725–33.



- 27 Lin KY, Guarnieri FG, Staveley-O'Carroll KF, et al. Treatment of established tumors with a novel vaccine that enhances major histocompatibility class II presentation of tumor antigen. *Cancer Res* 1996;56:21–6.
- 28 Urien S, Lokiec F. Population pharmacokinetics of total and unbound plasma cisplatin in adult patients. *Br J Clin Pharmacol* 2004;57:756–63.
- 29 Hinrichs CS, Restifo NP. Reassessing target antigens for adoptive T-cell therapy. *Nat Biotechnol* 2013;31:999–1008.
- 30 Wolfson B, Hodge JW. Next Generation Therapeutic Strateg-Es: Evolving cancer immunotherapy through agents that Engage, Expand and Enable the anti-tumor immune response. *Immunomedicine* 2021;1.
- 31 Nagarsheth NB, Norberg SM, Sinkoe AL, et al. TCR-engineered T cells targeting E7 for patients with metastatic HPV-associated epithelial cancers. *Nat Med* 2021;27:419–25.
- 32 Srivastava S, Furlan SN, Jaeger-Ruckstuhl CA, et al. Immunogenic chemotherapy enhances recruitment of CAR-T cells to lung tumors and improves antitumor efficacy when combined with checkpoint blockade. *Cancer Cell* 2021;39:193–208.
- 33 French LE, Tschopp J. Defective death receptor signaling as a cause of tumor immune escape. *Semin Cancer Biol* 2002;12:51–5.
- 34 Kwilas AR, Ardiani A, Gameiro SR, et al. Androgen deprivation therapy sensitizes triple negative breast cancer cells to immune-mediated lysis through androgen receptor independent modulation of osteoprotegerin. *Oncotarget* 2016;7:23498–511.
- 35 Fenerty KE, Padget M, Wolfson B, et al. Immunotherapy utilizing the combination of natural killer- and antibody dependent cellular cytotoxicity (ADCC)-mediating agents with poly (ADP-ribose) polymerase (PARP) inhibition. *J Immunother Cancer* 2018;6:133.
- 36 Hodge JW, Ardiani A, Farsaci B, et al. The tipping point for combination therapy: cancer vaccines with radiation, chemotherapy, or targeted small molecule inhibitors. *Semin Oncol* 2012;39:323–39.
- 37 Merritt RE, Mahtabifard A, Yamada RE, et al. Cisplatin augments cytotoxic T-lymphocyte-mediated antitumor immunity in poorly immunogenic murine lung cancer. *J Thorac Cardiovasc Surg* 2003;126:1609–17.
- 38 Sato H, Okonogi N, Nakano T. Rationale of combination of anti-PD-1/PD-L1 antibody therapy and radiotherapy for cancer treatment. *Int J Clin Oncol* 2020;25:801–9.
- 39 Grabosch S, Bulatovic M, Zeng F, et al. Cisplatin-induced immune modulation in ovarian cancer mouse models with distinct inflammation profiles. *Oncogene* 2019;38:2380–93.
- 40 Deng L, Liang H, Xu M, et al. Sting-Dependent cytosolic DNA sensing promotes radiation-induced type I interferon-dependent antitumor immunity in immunogenic tumors. *Immunity* 2014;41:843–52.
- 41 Twyman-Saint Victor C, Rech AJ, Maity A, et al. Radiation and dual checkpoint blockade activate non-redundant immune mechanisms in cancer. *Nature* 2015;520:373–7.
- 42 Knudson KM, Hicks KC, Alter S, et al. Mechanisms involved in IL-15 superagonist enhancement of anti-PD-L1 therapy. *J Immunother Cancer* 2019;7:82.
- 43 Obeid M, Tesniere A, Ghiringhelli F, et al. Calreticulin exposure dictates the immunogenicity of cancer cell death. *Nat Med* 2007;13:54–61.
- 44 Gameiro SR, Jammeh ML, Wattenberg MM, et al. Radiation-induced immunogenic modulation of tumor enhances antigen processing and calreticulin exposure, resulting in enhanced T-cell killing. *Oncotarget* 2014;5:403–16.
- 45 Reits EA, Hodge JW, Herberts CA, et al. Radiation modulates the peptide repertoire, enhances MHC class I expression, and induces successful antitumor immunotherapy. *J Exp Med* 2006;203:1259–71.
- 46 Hodge JW, Sharp HJ, Gameiro SR. Abscopal regression of antigen disparate tumors by antigen cascade after systemic tumor vaccination in combination with local tumor radiation. *Cancer Biother Radiopharm* 2012;27:12–22.
- 47 Gulley JL, Arlen PM, Bastian A, et al. Combining a recombinant cancer vaccine with standard definitive radiotherapy in patients with localized prostate cancer. *Clin Cancer Res* 2005;11:3353–62.
- 48 Liu J, Blake SJ, Yong MCR, et al. Improved efficacy of neoadjuvant compared to adjuvant immunotherapy to eradicate metastatic disease. *Cancer Discov* 2016;6:1382–99.
- 49 O'Donnell JS, Teng MWL, Smyth MJ. Cancer immunoeediting and resistance to T cell-based immunotherapy. *Nat Rev Clin Oncol* 2019;16:151–67.
- 50 Lee NY, Ferris RL, Psyrrri A, et al. Avelumab plus standard-of-care chemoradiotherapy versus chemoradiotherapy alone in patients with locally advanced squamous cell carcinoma of the head and neck: a randomised, double-blind, placebo-controlled, multicentre, phase 3 trial. *Lancet Oncol* 2021;22:450–62.
- 51 Antonia SJ, Villegas A, Daniel D, et al. Overall survival with Durvalumab after chemoradiotherapy in stage III NSCLC. *N Engl J Med* 2018;379:2342–50.
- 52 Tarhini AA, Eads JR, Moore KN, et al. Neoadjuvant immunotherapy of locoregionally advanced solid tumors. *J Immunother Cancer* 2022;10:e005036.
- 53 Bilusic M. What are the advantages of neoadjuvant immunotherapy over adjuvant immunotherapy? *Expert Rev Anticancer Ther* 2022;22:561–3.
- 54 Fabian KP, Malamas AS, Padget MR, et al. Therapy of established tumors with rationally designed multiple agents targeting diverse Immune-Tumor interactions: engage, expand, enable. *Cancer Immunol Res* 2021;9:239–52.
- 55 Fabian KP, Padget MR, Fujii R, et al. Differential combination immunotherapy requirements for inflamed (warm) tumors versus T cell excluded (cool) tumors: engage, expand, enable, and evolve. *J Immunother Cancer* 2021;9:e001691.
- 56 Gatti-Mays ME, Redman JM, Collins JM, et al. Cancer vaccines: enhanced immunogenic modulation through therapeutic combinations. *Hum Vaccin Immunother* 2017;13:2561–74.
- 57 Gameiro SR, Malamas AS, Bernstein MB, et al. Tumor cells surviving exposure to proton or photon radiation share a common immunogenic modulation signature, rendering them more sensitive to T cell-mediated killing. *Int J Radiat Oncol Biol Phys* 2016;95:120–30.
- 58 Bernstein MB, Krishnan S, Hodge JW, et al. Immunotherapy and stereotactic ablative radiotherapy (ISABR): a curative approach? *Nat Rev Clin Oncol* 2016;13:516–24.
- 59 Smalley Rumfield C, Roller N, Pellom ST, et al. Therapeutic vaccines for HPV-associated malignancies. *Immunotargets Ther* 2020;9:167–200.
- 60 Wolfson B, Franks SE, Hodge JW. Stay on target: Reengaging cancer vaccines in combination immunotherapy. *Vaccines* 2021;9. doi:10.3390/vaccines9050509. [Epub ahead of print: 15 05 2021].
- 61 Strauss J, Floudas CS, Sater HA. Phase II evaluation of the triple combination of PDS0101, M9241, and bintrafusp alfa in patients with HPV 16 positive malignancies (abstr; 2021 ASCO annual meeting I, virtual). *J Clin Oncol* 2021;39:2501–2501.
- 62 Han K-ping, Zhu X, Liu B, et al. IL-15:IL-15 receptor alpha superagonist complex: high-level co-expression in recombinant mammalian cells, purification and characterization. *Cytokine* 2011;56:804–10.