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Cisplatin and oxaliplatin induce similar immunogenic changes in preclinical models of head and neck cancer

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ABSTRACT

Objectives: Prior studies suggest that oxaliplatin is unique among platinum chemotherapy drugs in its ability to enhance anti-tumor immunity, but the immune mechanisms of different platinum chemotherapy drugs have not been previously compared in preclinical models of head and neck squamous cell carcinoma (HNSCC).

Materials and methods: Human HNSCC cell lines were treated with cisplatin or oxaliplatin, then assessed for markers associated with immunogenic cell death (ICD) and antigen processing. A syngeneic mouse model of oral cancer was then used to compare the effects of cisplatin vs. oxaliplatin, alone or in combination with anti-PD-1 immunotherapy, on tumor growth and survival. A subset of spleens and tumors were analyzed for ICD markers and immune cell infiltrates by flow cytometry.

Results: Cisplatin and oxaliplatin both increased cell surface levels of calreticulin, HSP70, MHC class I and PD-L1 in multiple cell lines. Inoculation of immunocompetent mice with cells killed *in vitro* by either drug resulted in failure of subsequently-injected live tumor cells to establish and grow in a small proportion of animals. Systemic cisplatin and oxaliplatin induced similar tumor growth delay when combined with anti-PD-1 therapy.

Conclusions: Treatment of HNSCC cells with platinum chemotherapy appears to induce some features of anti-tumor immunity, which may be enhanced by anti-PD-1 therapy. Cisplatin, the standard drug for HNSCC, appears to affect anti-tumor immunity in a similar fashion to oxaliplatin in these preclinical models.

Introduction

Head and neck squamous cell carcinoma (HNSCC) is the sixth most common cancer worldwide [1] and is caused by exposure to carcinogens (tobacco and alcohol) and/or human papillomavirus (HPV). Platinum chemotherapy drugs are the most commonly used systemic agents for HNSCC. Recent studies suggest that these drugs may enhance anti-tumor immunity [2–4].

Immunogenic cell death (ICD; Fig. 1) occurs when cells are exposed to select stressors, causing alterations in the cell membrane and immune signaling in the tumor microenvironment. During ICD, calreticulin (CRT) is exposed on the tumor cell surface, high-mobility group box 1 protein (HMGB1) and adenosine-5′-triphosphate (ATP) are released, and type 1 interferon (IFN) is produced by the tumor and/or immune cells. Exposure of heat-shock proteins (HSPs) 70/90 and release of CXCL10 can also occur [5,6]. These damage-associated molecular patterns (DAMPs) can stimulate the activation of $CD8^+$ dendritic cells (DCs) [7,8], which can then activate the adaptive immune system against tumor antigens [5,6,9].

Certain cytotoxic chemotherapy drugs have been found to induce

Abbreviations: ATP, Adenosine-5′-triphosphate; CDDP, Cisplatin; CRT, Calreticulin; CXCL10, C-X-C motif chemokine 10; DAMP, Damage-associated molecular pattern; DC, Dendritic cell; HSP, Heat shock protein; HMGB1, High mobility group box 1; HNSCC, Head and neck squamous cell carcinoma; ICD, Immunogenic cell death; IFN, Interferon; MDSC, Myeloid-derived suppressor cell; MFI, Median fluorescence intensity; MHC, Major histocompatibility complex; PD-L1, Programmed death-ligand 1; TCR, T-cell receptor

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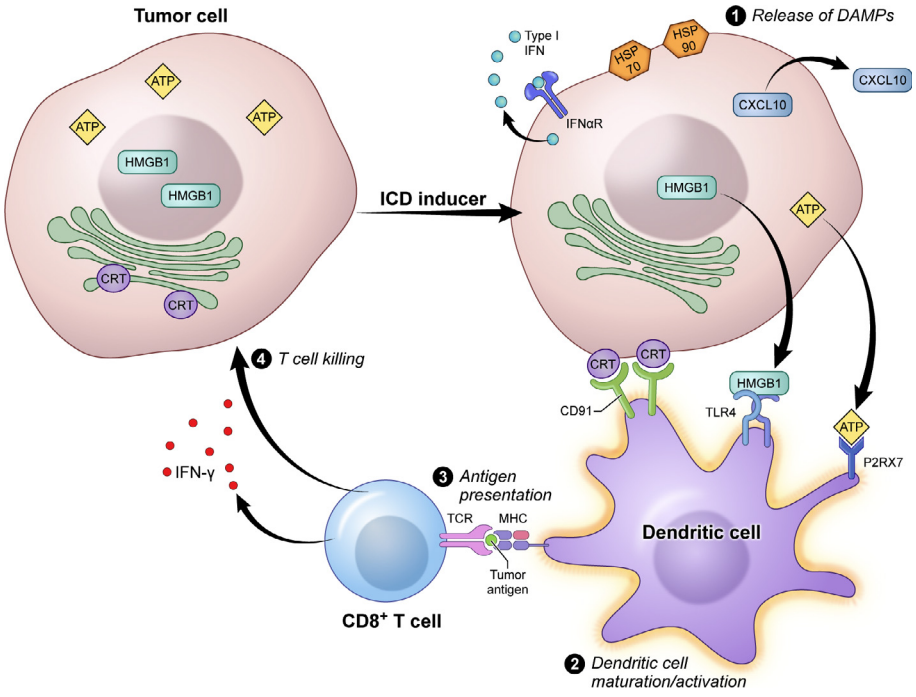

Fig. 1. Schema of immunogenic cell death. Immunogenic cell death (ICD) is the process by which stressed, dying cells induce translocation of damage-associated molecular patterns (DAMPs). The cell surface expression and the release of such molecular patterns can stimulate tumor antigen presentation, thereby activating adaptive immunity. CRT, calreticulin; HSP70/90, heat shock protein 70/90; ATP, adenosine triphosphate; HMGB1, high mobility group box 1; IFN, interferon; CXCL10, C-X-C motif chemokine 10; TCR, T-cell receptor; MHC, major histocompatibility complex.

ICD and/or upregulate MHC class I expression [2,3]. Our study focused on platinum chemotherapy drugs. Cisplatin is a standard treatment for head and neck squamous cell carcinoma (HNSCC). Carboplatin is a cisplatin analog with less toxicity that is administered to HNSCC patients who cannot cisplatin [10]. Oxaliplatin is a third-generation platinum compound used for colorectal cancer [11]. Preclinical studies involving non-HNSCC cancer types suggest that oxaliplatin is a bona fide ICD inducer, but cisplatin is not [6,9]. The widely-held idea that oxaliplatin is more immunogenic than cisplatin is based on a few studies suggesting that cisplatin fails to induce calreticulin exposure on the tumor cell surface, which can be restored by enhancing ER stress [6,9,12,13]. We aimed to investigate whether oxaliplatin might be a better inducer of ICD and MHC class I/PD-L1 expression in HNSCC models, which would provide a rationale for using oxaliplatin, instead of cisplatin, in future clinical paradigms combining platinum chemotherapy with immunotherapies [14].

We used human HNSCC cell lines to compare the effects of cisplatin versus oxaliplatin on the release and/or expression of calreticulin (CRT), heat shock proteins (HSPs), HMGB1, ATP, MHC class 1 and PD-L1 *in vitro*. A syngeneic mouse model of oral cancer was used to assess the ability of tumor cells killed by these agents to promote ICD *in vivo*, and to compare the effects of these chemotherapy drugs with or without PD-1 blockade on tumor growth.

Materials and methods

Cell lines

Human UMSCC-46 and UMSCC-74A cell lines (HPV-negative) were obtained from Dr. T. Carey at the University of Michigan and were authenticated and maintained as described [15]. UPCI SCC90 cells (HPV-positive) were obtained from Dr. R. Ferris and Dr. S. Gollin at the University of Pittsburgh and were validated as described [16,17]. Human cell lines were maintained in MEM or DMEM with 1% penicillin/streptomycin, 1% glutamine and 10% FBS. Mouse oral cancer 1 (MOC1) cells were obtained from Dr. R. Uppaluri in 2014 and were authenticated and maintained as previously described [18,19]. Cell lines were regularly tested for Mycoplasma and cultured for no more than 6 months or 20 passages before use.

Antibodies and reagents

Recombinant human interferon-gamma (IFN-γ) and antibodies to human MHC class I and PD-L1 were obtained from Biolegend. Antibodies to calreticulin (CRT), HSP70 and HSP90 were obtained from Abcam. The anti-PD-1 antibody for *in vivo* mouse treatments (clone RMP1-14) was from BioXCell. Fluorescent-conjugated flow cytometry antibodies for mouse tumor experiments were obtained from eBioscience (CD137/41BB) or Biolegend (CD8, CD45, CD80, CD11b, CD11c, CD107a, Ly6G, Ly6C, H-2Kb/H-2Db). Pharmaceutical grade chemotherapy was obtained from the veterinary pharmacy at NIH.

In vitro drug treatments and cell death assays

Cells were plated at 50,000–100,000 cells per well in 6-well plates and allowed to adhere overnight prior to drug treatments. Cell lines were treated with a range of platinum chemotherapy doses for 72 h, then assessed for cell death by annexin/7AAD flow cytometry assays (BD Biosciences) according to manufacturer instructions. Cell death curves were generated using GraphPad Prism software and used to estimate the dose required to kill 40% of the cells in 72 h (LD_{40}).

Flow cytometry

Cell lines were harvested with TrypLE Select. For intracellular staining, cells were fixed in 2% PFA and permeabilized with 100% methanol overnight at −20 °C. Cytometry samples were acquired with a BD FACS Canto cytometer, then analyzed using FlowJo software. Cell lines treated for 48 h and stained for cell surface markers were not stained with 7AAD or gated for live cells, as preliminary 48-hour experiments in all cell lines showed that cell death was negligible. Viability of cells from mouse tumors was verified using 7AAD staining. Median fluorescence intensity values for isotype controls were subtracted for each condition, and "fluorescence minus one" controls were tested for each multicolor panel.

Immunofluorescent microscopy

Cell lines were plated at 60,000–80,000 cells per well in Lab-Tek®II

8 well-chamber slides, allowed to adhere overnight, then drug treated for 48 h, fixed with 4% paraformaldehyde, incubated in acetone at −20 °C for 10 min, and stained with antibodies according to manufacturer specifications before examination with a Zeiss LSM 780 confocal microscope. Identical laser and software settings were used across samples.

ELISA assays

Fresh supernatants from cells cultured with chemotherapy were collected. Samples were assessed using ELISA kits for HMGB1 (IBL International) according to the manufacturer's instructions. Plates were analyzed with a Biotek uQuant microplate reader.

ATP and WST-1 cell proliferation assays

Cell lines were plated at 1,000–50,000 cells per well in 96 well plates in complete media, then drug treated for 48 h in serum-free media. After treatment, Luminescent ATP Detection Assay Kit (Abcam) or WST-1 Cell Proliferation Reagent (Sigma) were used according to the manufacturer's instructions. Plates were analyzed with a Biotek microplate reader.

In vivo mouse studies

Wildtype, female C57BL/6 mice (aged 6–8 weeks) were obtained from Charles River. For ICD vaccination studies, MOC1 cells were treated *in vitro* with the IC_{90} of cisplatin, oxaliplatin, or mitoxantrone (determined by MTT assay) for 72 h. Additional cells were treated with 100 Gy radiation. Dead/dying cells were rinsed in PBS, then injected into the left flank (5×10^6 chemotherapy-treated cells or 2×10^6 radiation-treated cells). One week later, mice were injected in the right flank with live MOC1 cells (3×10^6 cells, in Matrigel).

For systemic therapy experiments, mice were injected in the right flank with MOC1 cells (5×10^6 cells, in Matrigel) and allowed to grow for 14 days, then randomized into treatment groups. Mice were then treated for 5 weeks with cisplatin (5 mg/kg/week), oxaliplatin (6.5 mg/kg/week) and/or an antibody to PD-1 (200 mcg, twice/week, concurrently with chemotherapy) or vehicle (saline/PBS) by intraperitoneal injection. Chemotherapy doses were based on preliminary experiments to determine the maximum tolerated dose that did not induce excess weight loss or toxicity. Chemotherapy-treated mice received saline supplementation as described [4]. In a subset of mice, tumors were harvested during the second week of treatment, digested into single cell suspensions as previously described [19], and analyzed by flow cytometry. All animal experiments were approved by the Animal Care and Use Committee at NIDCD.

Statistical analyses

Data were analyzed by one-way ANOVA with *post hoc* Tukey's multiple comparisons tests. Tumor growth curves were compared by repeated measures two-way ANOVA with *post hoc* Tukey's multiple comparisons tests. Animal survival curves were made using the Kaplan-Meier method with comparison by log-rank (Mantel-Cox) testing. GraphPad Prism software was used for statistical testing. $P < 0.05$ was used to determine statistical significance.

Results

Cisplatin and oxaliplatin induced similar immune markers in HNSCC cell lines

Two HPV(−) HNSCC cell lines and one HPV(+) cell line were treated with a range of cisplatin and oxaliplatin doses, then analyzed by annexin/7AAD flow cytometry assays (Fig. 2A) to establish the doses required to kill 40% of the cells (LD_{40}) over 72 h (Table 1). Treatment of additional cells with these doses was then stopped prior to cell death (24–48 h) and analyzed for expression of CRT, HSP 70, HMGB1, MHC class I, and PD-L1 by flow cytometry (Fig. 2). Mitoxantrone (MTX) was used as a positive control for ICD [9], and interferon-gamma (IFN-γ) was used as a positive control for MHC class I and PD-L1 expression. Increases in surface calreticulin and HSP70 were noted at 24–48 h, whereas decreased intracellular HMGB1 was only noted after 48–72 h (data not shown). At 48 h, UMSCC-46 cells showed similarly modest alterations in ICD-related DAMPs with cisplatin and oxaliplatin, whereas UMSCC-74A demonstrated the most pronounced changes in these markers with cisplatin (Fig. 2C). In HPV(+) SCC90 cells, modest changes in these DAMPs did not reach statistical significance for either drug despite robust changes with MTX. The HPV-negative cells exhibited modest increases in MHC class I and PD-L1 after platinum chemotherapy, with no clear difference between drugs (Fig. 2D). We also assessed DAMPs by immunofluorescent microscopy of two cell lines grown in chamber slides (Fig. 3). By microscopy, cisplatin and oxaliplatin treatment increased levels of calreticulin and HSP70; however, this appeared to be cytoplasmic and did not clearly localize to the cell surface (Fig. 3). Calreticulin exposure on the cell surface is a critical component of ICD, but intracellular calreticulin also contributes to antigen processing within the endoplasmic reticulum [20,21]. We have previously shown that intracellular calreticulin increases in cisplatin-treated HNSCC cells [4], and the present study suggests that total calreticulin levels were similarly increased by cisplatin and oxaliplatin (Fig. 3). Taken together, these data suggest that HNSCC cells may exhibit some of the early hallmarks of ICD and enhanced antigen processing machinery in response to moderate doses of platinum chemotherapy. Oxaliplatin did not appear to be superior to cisplatin in its ability to induce these changes.

Cisplatin and oxaliplatin both fail to induce robust secretion of HMGB1 and ATP

In addition to intracellular flow cytometry as an indirect measure of HMGB1 release in platinum chemotherapy treated cells, we also directly assessed HMGB1 release by ELISA. Two HNSCC cell lines were treated with the LD_{40} of cisplatin and oxaliplatin for 48 h and the cell culture supernatants were collected and analyzed for extracellular HMGB1 (Fig. 4A, B). Cisplatin and oxaliplatin both failed to induce a significant increase in extracellular HMGB1 at 48 h.

ATP release, another important event in ICD, was also assessed (Fig. 4C–F). Multiple attempts to measure extracellular ATP in the supernatants showed no significant increase in extracellular ATP following treatment of cells with mitoxantrone or platinum chemotherapy (data not shown). In all trials the ATP was dramatically reduced compared to assay standards despite using techniques to enhance its preservation [22,23]. We therefore measured intracellular ATP and normalized it to the number of live cells, since cell number increased in control samples but remained static with platinum drugs and decreased with MTX. Total intracellular ATP decreased significantly only after treatment with mitoxantrone (Fig. 4C, E). However, when normalized to cell count, the ATP amounts within individual treated cells were comparable to untreated cells. These data showing lack of a clear increase in these later markers of ICD suggest that moderate, physiologic doses of platinum chemotherapy drugs do not induce complete ICD by 48 h.

Cisplatin- and oxaliplatin-killed cells modestly promote ICD in vivo

To further explore the possibility that oxaliplatin and/or cisplatin promote ICD in models of HNSCC, we used mouse vaccination experiments. Immunocompetent mice were inoculated with mouse oral cancer (MOC1) cells killed *in vitro* by cisplatin, oxaliplatin, radiation, or mitoxantrone (positive controls), followed by re-challenge with live

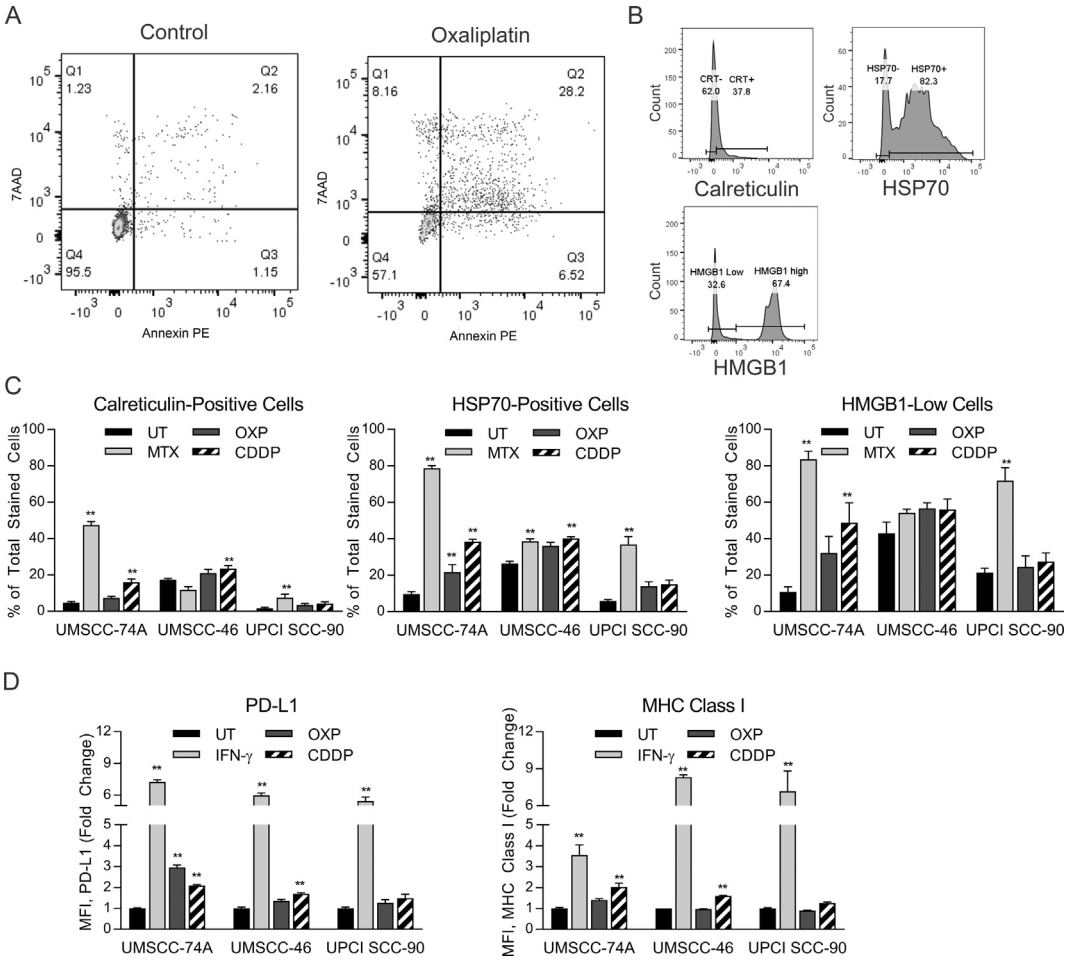

Fig. 2. Induction of immune markers as measured by flow cytometry is similar after treatment with cisplatin versus oxaliplatin in HNSCC cell lines. Three cell lines were untreated (UT) or treated with mitoxantrone (MTX, 1 μg/ml, positive control), cisplatin (CDDP), or oxaliplatin (OXP) and analyzed by flow cytometry. *A*, example annexin/7AAD plots of cells cultured in drug-free medium or platinum chemotherapy for 72 h. In *B-D*, cells were treated with drugs for only 48 h, then stained for surface levels of calreticulin (CRT), heat shock protein 70 (HSP70), MHC class I, PD-L1, or intracellular levels of HMGB1. *B*, representative gating strategies for calreticulin, HSP70 and HMGB1. *C*, percent of cells found to be positive for CRT/HSP70 or low in HMGB1. *D*, Median fluorescence intensity (MFI) of MHC class I and PD-L1. Data are mean + SEM, n = 9–12 from at least 3 independent experiments, **p < 0.01 vs. untreated.

Table 1
Doses of cisplatin and oxaliplatin needed to kill 40% of HNSCC cells at 72 h, as determined by annexin/7AAD flow cytometry assays in three HNSCC cell lines.

Cell line	UMSCC-46	UMSCC-74A	UPCI SCC90
LD_{40} of cisplatin (μg/ml)	0.23	2.45	0.64
LD_{40} of oxaliplatin (μg/ml)	0.96	24.90	2.63

MOC1 cells one week later (Fig. 5A). Vaccination with radiation-killed cells induced a robust immune response, as half of the mice failed to form tumors after re-challenge (Fig. 5B). Vaccination with cells killed by cisplatin and oxaliplatin only produced tumor rejection in a small proportion of animals. However, several of the mice inoculated with cisplatin-killed cells exhibited delayed tumor growth compared to control animals (Fig. 5B). These data suggest that high doses of both cisplatin and oxaliplatin have a modest ability to promote ICD, and cisplatin-killed cells may have a superior ability to induce tumor growth delay.

Mice treated with cisplatin or oxaliplatin plus anti-PD-1 therapy had similar delayed tumor growth and survival

We previously found additive anti-tumor activity using cisplatin and an anti-PD-1 antibody concurrently in the MOC1 model [4]. Since experiments thus far suggested that cisplatin and oxaliplatin may have similar effects on anti-tumor immunity, we next compared the efficacy of cisplatin versus oxaliplatin when combined with anti-PD-1 *in vivo*. MOC1 tumor-bearing mice were treated with cisplatin or oxaliplatin alone or with concurrent anti-PD-1 antibody for five weeks. Combination of anti-PD-1 with cisplatin or oxaliplatin induced a statistically significant tumor growth delay versus untreated animals ($p < 0.01$, Fig. 6A). Survival was not significantly increased for any of the treatment groups vs. control (median survival 63 days, Fig. 6B), though it approached statistical significance for oxaliplatin + anti-PD-1 (median survival 79.5 days, $p = 0.13$) and cisplatin + anti-PD-1 (median survival 80 days, $p = 0.06$). These results suggest similar efficacy of cisplatin and oxaliplatin in delaying tumor growth when combined with anti-PD-1 immunotherapy in this preclinical model of HNSCC.

Platinum chemotherapy drugs increased calreticulin, MHC class I, and PD-L1 surface expression in some tumors in vivo

To further analyze the immune effects of platinum chemotherapy at the cellular level *in vivo*, tumors were collected from a subset of mice 9 days after initiating treatment (2 days following the second dose of chemotherapy and third dose of anti-PD-1). Increased tumor cell surface expression of MHC class I, PD-L1, and calreticulin appeared to be most pronounced in tumors from animals treated with cisplatin and

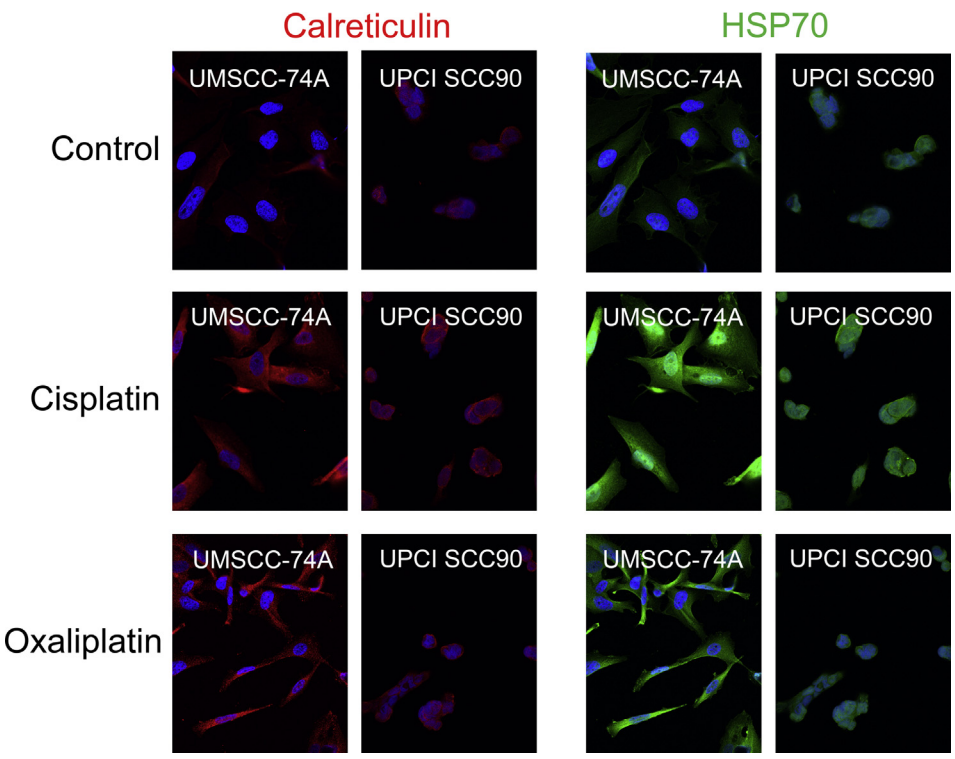

Fig. 3. Intracellular calreticulin and heat shock proteins as measured by immunofluorescent microscopy are similarly increased after treatment with cisplatin or oxaliplatin in HNSCC cell lines. Two cell lines were grown on chamber slides and treated with cisplatin or oxaliplatin for 48 h, stained with Hoechst to label nuclei, then fixed and stained with fluorescent antibodies to calreticulin or HSP70. Slides were then analyzed by confocal microscopy with identical laser settings across samples. Micrographs shown are representative of triplicate samples from two independent experiments.

anti-PD-1 (Fig. 6C). However, these differences did not reach statistical significance, though they were nearly significant for calreticulin ($p = 0.06$, one-way ANOVA).

Platinum chemotherapy drugs may differentially affect CD8+ T cells, but not myeloid cells, when combined with PD-1 blockade

Tumors from treated animals were also analyzed for immune cell subsets by flow cytometry. The number of CD8+ T cells was significantly higher in tumors from animals treated with anti-PD-1 + oxaliplatin versus cisplatin (Fig. 6D). However, the percentage of CD8+ T cells with CD137 (4-1BB costimulatory molecule) expression was notably higher in tumors from animals treated with anti-PD-1 antibody; an effect that was lost with the addition of oxaliplatin but not cisplatin. Expression of CD107a, a marker of activation on CD8+ T cells, was increased with anti-PD-1 but was reduced back to baseline levels when either chemotherapy drug was added. Levels of dendritic cells and myeloid-derived suppressor cells were not significantly different among the treatment groups (data not shown). These results suggest that cisplatin and oxaliplatin, when paired with PD-1 blockade, have no appreciable effects on myeloid cells but may affect CD8+ T cells. Oxaliplatin may be more likely to increase the overall number of CD8+ T cells in tumors, whereas cisplatin may be less likely to impair some aspects of T cell function, such as CD137 costimulation.

Discussion

Previous preclinical studies involving non-HNSCC cancer types suggest that oxaliplatin is a bona fide ICD inducer, but other platinum drugs are not [6,9,12,13]. In particular, cisplatin is thought to be a suboptimal ICD inducer due to its failure to induce calreticulin exposure on the cell surface [6,9,12,13]. It should be noted that the doses of cisplatin used in those studies were supraphysiologic, and time points selected were short. In our studies, we carefully titrated the doses to determine a moderate dose capable of killing 40% of cells over a 72 h period, and all of these doses were within the range of serum concentrations seen in patients treated with cisplatin for HNSCC [24]. At these doses, we noted increased calreticulin both in the cells and at the cell surface in multiple cell lines, with no appreciable differences between cisplatin and oxaliplatin. Our experiments failed to show all the classic markers for ICD, including release of HMGB1 and ATP into the supernatants, likely because we stopped the experiments at 48 h when cell death was negligible. However, even high doses (LD_{90}) failed to induce robust ICD in our mouse vaccination experiments. We posit that the doses of cisplatin or oxaliplatin monotherapy that would be required to induce classic ICD in experimental settings would be much higher than achievable serum concentrations in patients, and thus prior studies suggesting superior ICD with oxaliplatin should not be used to justify choosing this drug over cisplatin in trials of chemoimmunotherapy.

Mouse vaccination experiments, which are the gold standard for demonstration of ICD [21], suggested that cisplatin and oxaliplatin may both be relatively weak ICD inducers. In a syngeneic mouse model, cisplatin and oxaliplatin produced a virtually identical tumor growth delay, whether used alone or in combination with PD-1 blockade. Our *in vivo* mouse experiments also suggest that cisplatin and oxaliplatin may affect the number and function of CD8+ T cells in different ways: oxaliplatin significantly increased the number of CD8+ cells, whereas cisplatin was less likely to negate the anti-PD-1-induced increase in the proportion of CD137-positive cells. These differential effects of cisplatin and oxaliplatin are somewhat preliminary based on the small number of animals, but the results were statistically significant, and suggest intriguing avenues for further development.

Other studies in the literature have demonstrated HMGB1 release following treatment of various cell lines with cisplatin [13,25]. In our study, we determined HMGB1 release by direct (ELISA) and indirect (flow cytometry) means. The indirect measurement of HMGB1 release by flow cytometry allows cells to be analyzed individually for low HMGB1 content, and thus the experiments are controlled for the total cell number. We found that the indirect method was more sensitive for detecting HMGB1 changes among treatment groups, though it is possible that decreased transcription due to cellular stress may in part account for the decreased intracellular HMGB1.

Additional technical challenges were found in determining the ATP

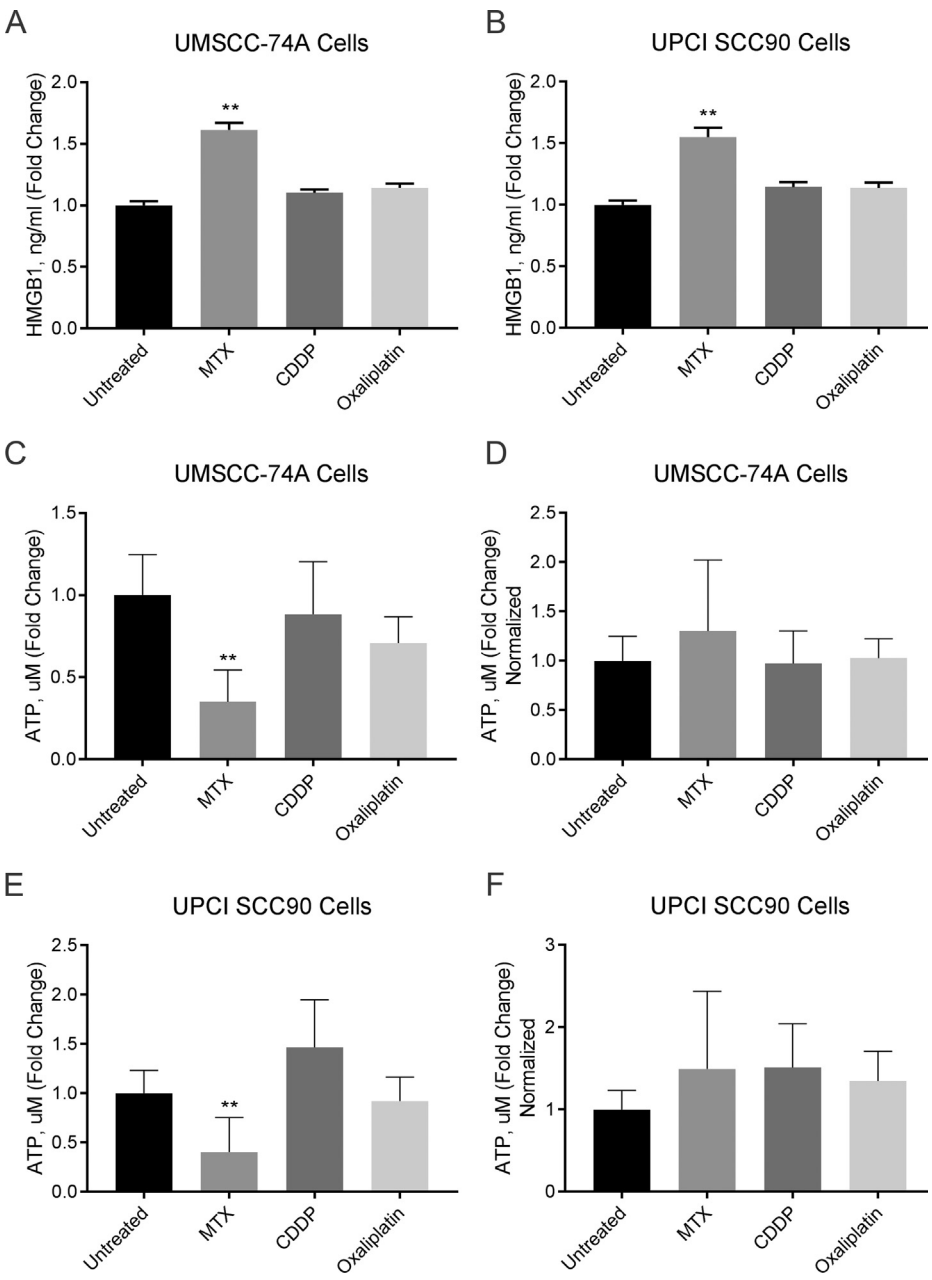

Fig. 4. Cisplatin and oxaliplatin induce minimal release of HMGB1 and ATP in HNSCC cell lines. Two HNSCC cell lines were treated with chemotherapy drugs (LD_{40}) or mitoxantrone (MTX, 1 μg/ml, positive control) for 48 h. After treatment, supernatants were collected and measured for HMGB1 by ELISA (*A-B*), and cells were collected and analyzed for intracellular ATP by bioluminescence assay (*C-F*). The total intracellular ATP concentration was analyzed (*C, E*) and then normalized to cell number (*D, F*) by WST-1 cell proliferation assay. Data are mean + SEM, n = 18 from 4 independent experiments for HMGB1 ELISA, n = 8 from two independent experiments for ATP, $^{**}p < 0.01$ vs. untreated. HMGB1, high mobility group box 1; ATP, adenosine-5′-triphosphate.

content in our human cell lines. Several luciferase assays performed in two independent labs showed consistently lower ATP in chemotherapy-treated cells compared with controls, even with mitoxantrone, a known inducer of ATP secretion [9]. Previous studies indicate that HNSCC tumor cells have overexpression of ectonucleoside triphosphate diphosphohydrolase 1 (CD39), a cell membrane protein that degrades extracellular ATP [9,26]. Other studies have successfully measured extracellular ATP using a luciferase assay, but most of these experiments again used supraphysiologic doses of chemotherapy drugs for short treatment periods and did not account for cell number [12,13,27-29]. We were unable to reproduce these results in our cell lines despite trying multiple treatment periods, identical doses of mitoxantrone, and inhibition of ecto-ATPDases with ARL 67156 [22,23].

Our study has important clinical implications. Cisplatin is the most commonly used systemic drug for HNSCC. A switch to carboplatin or oxaliplatin would be highly desirable, as these drugs do not cause the severe inner ear and renal toxicities seen with cisplatin [30]. Though oxaliplatin, similar to cisplatin, may function as a radiosensitizer, its use in HNSCC so far has been limited to studies of recurrent/metastatic disease or nasopharyngeal carcinoma [31–36]. As a result, it is unclear whether oxaliplatin would be equally effective to cisplatin when combined with radiation for previously untreated, locally advanced HNSCC of other anatomic subsites.

Importantly, platinum chemotherapy drugs often induce severe nausea and vomiting that requires administration of corticosteroids, which was not simulated in our experiments and could have an impact on the efficacy of these drugs when combined with immunotherapy. Carboplatin, another platinum drug used for HNSCC, is often combined with taxanes and usually does not require the co-administration of steroids due to its lower incidence of severe nausea [37,38]. Carboplatin is thought to be less effective than cisplatin when combined with radiation for HNSCC, though large comparative studies are lacking [10,37,39]. In preliminary experiments, the increases in DAMPs, MHC class I and PD-L1 were slightly less compelling with carboplatin versus cisplatin and oxaliplatin (data not shown), therefore we did not include a carboplatin group in our murine experiments. However, based on the

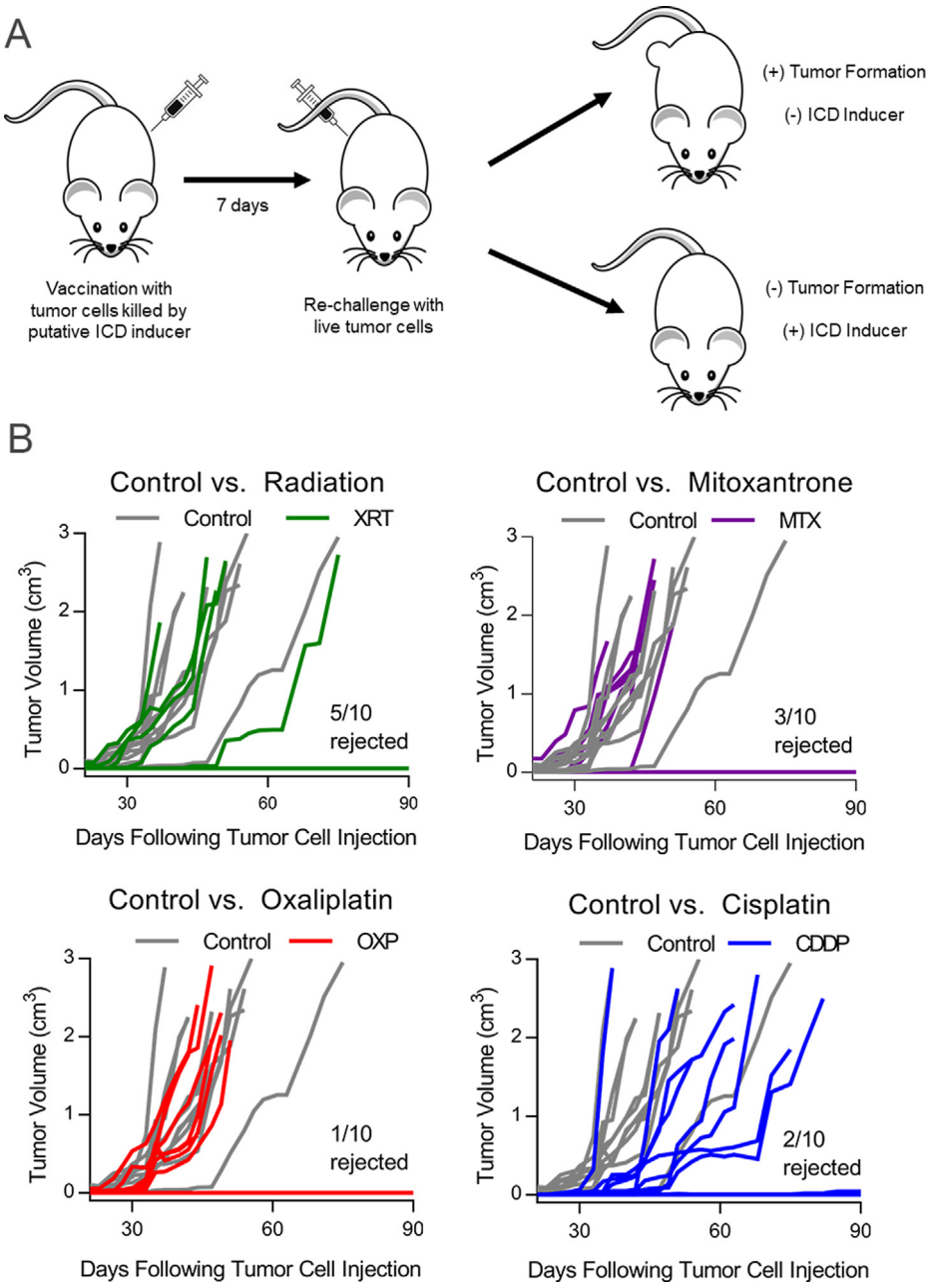

Fig. 5. Platinum chemotherapy drugs modestly promote ICD *in vivo*. Mice were inoculated with mouse oral cancer (MOC1) cells killed *in vitro* by radiation (XRT, 100 Gy, positive control), mitoxantrone (MTX, 1 μg/ml × 72 h, positive control) oxaliplatin (OXP, 6 μg/ml × 72 h, LD_{90}), or cisplatin (CDDP, 2 μg/ml × 72 h, LD_{90}), followed by re-challenge with live MOC1 cells one week later. LD_{90} doses were determined by MTT assay. *A*, treatment schema, adapted from Kepp et al. [21]. *B*, tumor growth of individual animals after vaccination with cells killed *in vitro*, compared with sham (saline) injection (grey lines).

improved toxicity profile of carboplatin versus cisplatin and the lack of need for corticosteroids [10,24,37,39], the combination of carboplatin, taxanes and immunotherapy may be an attractive strategy for HNSCC. A clinical trial of induction carboplatin, nab-paclitaxel and nivolumab (anti-PD-1) for patients with HPV-related HNSCC (OPTIMA-II; NCT03107182) is currently recruiting and will provide valuable insight on the efficacy of this therapeutic combination.

Conclusions

Cisplatin and oxaliplatin chemotherapy appear to induce similar immunogenic changes when used at clinically relevant doses in pre-clinical models of HNSCC. Furthermore, additive activity is seen with cisplatin or oxaliplatin and anti-PD-1 therapy in a syngeneic mouse model of HNSCC. These results suggest that among platinum chemotherapy drugs, cisplatin is an appropriate choice for use in clinical paradigms combining chemotherapy with PD-1 blockade and perhaps other immunotherapies. The use of oxaliplatin or carboplatin with immunotherapy for HNSCC deserves further study, as these drugs may have less toxicity.

Declaration of Competing Interest

CVW and NCS have received research funding from Astex Pharmaceuticals, which have not been applied to this project or related projects.

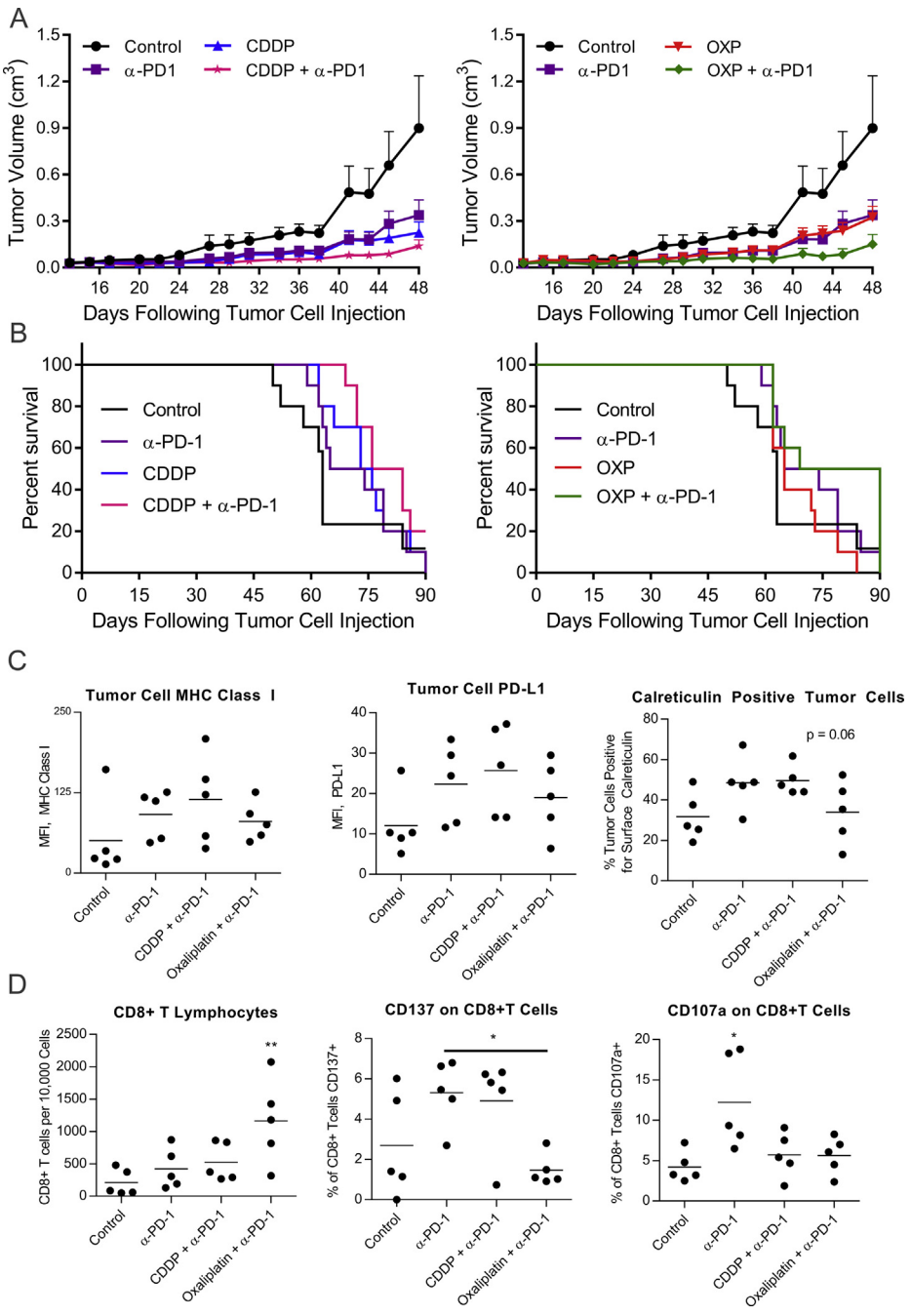

Fig. 6. MOC1 tumor-bearing mice treated with cisplatin or oxaliplatin +/− anti-PD-1 therapy had similar tumor growth delay and survival. MOC1-bearing mice were treated with cisplatin, oxaliplatin, and anti-PD-1 antibody alone or in combination. Tumor cells were harvested 9 days after the first dose of chemotherapy and anti-PD-1 treatment, digested by mechanical and enzymatic methods, then analyzed by flow cytometry. *A,* tumor growth shown as mean + SEM for each group, separated into two graphs for clarity. Combination of anti-PD-1 with cisplatin or oxaliplatin induced statistically significant growth delay vs. control ($p < 0.01$). N = 9–10 mice per group. *B,* Kaplan-Meier curves showing percent survival. *C,* Tumor cell surface expression of MHC class I, PD-L1, and calreticulin were increased by cisplatin in a subset of tumors. Gated on CD45-negative, live cells from the entire tumor. Data from individual animals and mean are shown. One-way ANOVA approached but did not reach statistical significance for calreticulin ($p = 0.06$). MFI, median fluorescence intensity. *D,* Cisplatin and oxaliplatin appear to differentially affect the number and function of intratumoral CD8+ T lymphocytes when combined with anti-PD-1. Immune cells were gated by CD45-positive, live cells from the entire tumor. Data from individual animals and mean are shown. * $p < 0.05$, ** $p < 0.01$.

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Authors' contributions

SP and WY collected, analyzed and interpreted data, then drafted and revised the manuscript. RX and MP helped collect and analyze data. JWH and CVW interpreted data, offered feedback on the project and revised the manuscript. NCS designed the project, collected, analyzed, and interpreted data, drafted and revised the manuscript, and supervised the project. All authors read and approved the final manuscript.

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