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Dose-dependent enhancement of T-lymphocyte priming and CTL lysis following ionizing radiation in an engineered model of oral cancer

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Abstract

Objectives—Determine if direct tumor cell cytotoxicity, antigen release, and susceptibility to T-lymphocyte killing following radiation treatment is dose-dependent.

Materials and Methods—Mouse oral cancer cells were engineered to express full-length ovalbumin as a model antigen. Tumor antigen release with uptake and cross presentation of antigen by antigen presenting cells with subsequent priming and expansion of antigen-specific T-lymphocytes following radiation was modeled *in vitro* and *in vivo*. T-lymphocyte mediated killing was measured following radiation treatment using a novel impedance-based cytotoxicity assay.

Results—Radiation treatment induced dose-dependent induction of executioner caspase activity and apoptosis in MOC1 cells. *In vitro* modeling of antigen release and T-lymphocyte priming demonstrated enhanced proliferation of OT-1 T-lymphocytes with 8 Gy treatment of MOC1ova cells compared to 2 Gy. This was validated *in vivo* following treatment of established MOC1ova tumors and adoptive transfer of antigen-specific T-lymphocytes. Using a novel impedance – based cytotoxicity assay, 8 Gy enhanced tumor cell susceptibility to T-lymphocyte killing to a greater degree than 2 Gy.

Conclusion—In the context of using clinically-relevant doses of radiation treatment as an adjuvant for immunotherapy, 8 Gy is superior to 2 Gy for induction of antigen-specific immune

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responses and enhancing tumor cell susceptibility to T-lymphocyte killing. These findings have significant implications for the design of trials combining radiation and immunotherapy.

Keywords

Radiation; immunity; tumor microenvironment; T-lymphocyte priming; cytotoxic T-lymphocyte

Introduction

While many patients with head and neck squamous cell carcinoma (HNSCC) display a T-cell inflamed phenotype[1], only a small subset respond to programmed death (PD) pathway checkpoint inhibition[2]. Strategies to enhance response rates to checkpoint inhibition in HNSCC are needed. PD-based checkpoint blockade has the potential to unleash an existing anti-tumor immune response being blocked by the expression of PD-1/PD-L1, but cannot induce a *de novo* anti-tumor immunity[3]. The addition of PD-based checkpoint blockade to other anti-cancer treatments that have the potential to induce adaptive anti-tumor immune responses may be additive or synergistic due to reversal of adaptive immune resistance[3, 4]. Ionizing radiation (IR) is a mainstay of treatment for HNSCC and can induce anti-tumor immune responses via a number of defined mechanisms[5–7].

To provide a rationale for combining IR with immune-activating treatments, we hypothesized that IR could induce tumor cell death, causing release of tumor antigen for uptake and cross-presentation by antigen presenting cells (APC) with subsequent activation of antigen-specific T-lymphocytes. To accomplish this, we engineered mouse oral cancer (MOC) cells to express full-length ovalbumin as a well-defined model antigen and treated cells or tumors with clinically relevant doses of 2 Gy or 8 Gy IR. We demonstrated dose-dependent antigen release, processing and antigen-specific T-lymphocyte activation both *in vitro* and *in vivo* following IR, to a greater degree with 8 Gy than 2 Gy. Similarly, IR also significantly enhanced antigen-specific cytotoxic T-lymphocyte (CTL) killing of target cells in a novel, impedance based cytotoxicity assay to a greater degree with 8 Gy than 2 Gy. Given that standard-of-care treatment for HNSCC involves the use of fractionated low dose (2 Gy) IR, these results suggest that careful consideration should be given to experimental design in the setting of IR being used as an adjuvant treatment with immune-activating therapies such as checkpoint inhibition.

Materials and Methods

Cell culture and tumor growth

Syngeneic mouse oral cancer 1 (MOC1) cells were generated as described[8], cultured as described[9] and harvested with TrypLE Select to avoid cell surface epitope loss. To generate tumors, 5×10^6 cells were injected subcutaneously into the right leg of wild-type C57BL/6 (B6) mice in 30% matrigel (Corning). All studies involving tumor implantation and irradiation of mice received National Institutes of Health Animal Care and Use Committee approval (ASP#1364-14).

Generation of MOC1ova

A pBABE vector backbone containing full length ovalbumin and resistance genes (ampicillin and puromycin) was kindly provided by Dr. Gavin Dunn (Washington University in St. Louis). This plasmid and the retroviral envelop plasmid VSV-G were transformed into MAX efficiency DH5α cells on ampicillin impregnated LB plates for expansion. Isolation of plasmids was performed using an EndoFree Plasmid Maxi Kit (Qiagen). The ovalbumin and VSV-G plasmids were transfected into 293gp packaging cells in OptiMEM using Lipofectamine 2000. Viral-containing supernatants were collected at 48 hours. To prepare for transduction, MOC1 cells were plated on retronectin (TaKaRa) coated plates pre-seeded with retrovirus via centrifugation of viral supernatant. Following an overnight infection, transduced MOC1 cells were trypsinized and cultured in puromycin at a concentration pre-determined to be lethal to MOC1 cells (6 μg/mL). Transduction of ovalbumin containing plasmid was verified by puromycin resistance, flow cytometry for SIINFEKL presentation on H2-K^b, and cytotoxicity upon exposure to *ex vivo* generated OT-1 CTLs.

Radiation

Cells were harvested while in log growth phase and irradiated (2 or 8Gy) using a ^{137}Cs source (Gammacell-1000) at a dose rate of 0.74 Gy/min. Irradiated cells were washed three times before being plated for experiments. Mice bearing tumors were secured into custom lead-shielded jigs that expose the leg alone to radiation, and irradiated (2 or 8Gy) using a Pentak XRAD320 X-ray irradiator (Precision X-ray, Inc.) at a dose of 2.8 Gy/min.

Caspase 3/7 and annexin V assay

MOC1 cells were irradiated and cultured for 12 hours before addition of CellEvent Caspase-3/7 Green Detection Reagent (ThermoFisher) per manufacturer protocol. Images were acquired on an Evos Cell Imaging System (ThermoFisher) and % positive cells was calculated manually from 10 high power fields (HPFs) per treatment condition. MOC1 cells were cultured for 24 hours before detection of apoptosis using the flow cytometry-based PE Annexin V Apoptosis Detection Kit I (BD Biosciences) per manufacturer protocol.

Flow cytometry

All analyses were performed on fresh cells or prepared tissue with exclusion of dead cells via 7AAD staining. Anti-SIINFEKL:H2-K^b (clone 25-D1.16), CD45.2 (104), CD11b (M1/70), CD11c (N418), CD19 (6D5), Vα2 (B20.1), ICAM (YN1/1.7.4), CD80 (16-10A1), and Fas (SA367H8) antibodies were from Biolegend and anti-calreticulin antibody (ab92516) was from Abcam. Isotype control antibodies and a “fluorescence minus one” method of antibody combination were used for specific staining validation. Data was acquired on a FACSCanto using FACSDiva software (BD Biosciences) and analyzed on FlowJo software vX10.0.7r2.

Colony formation assay

Clonogenic assays were performed on control or irradiated MOC1ova cells as described[10].

Cytotoxic T-lymphocyte (CTL) killing assay

To generate SIINFEKL-specific CTLs, splenocytes from OT-1 mice were cultured in the presence of SIINFEKL (2 μg/ml) with daily 2:1 splitting. After 72 hours in culture, >80% of remaining cells are CD8+Vα2+ cells (data not shown). Following the addition of OT-1 CTL effectors to 1×10^4 target cells plated in a 96-well E-Plate (ACEA Biosciences), alteration of impedance was acquired using the xCELLigence Real-Time Cell Analysis (RTCA) platform per manufacturer recommendations. Triton X-100 (0.2%) was added to some wells to verify complete loss of cell index with total cell lysis, and CTLs alone were plated up to 1×10^6/well to verify that they do not contribute to gain of impedance (data not shown). Percent loss of cell index for a given time point was calculated as: 1-(experimental cell index/control cell index).

In vitro cross-presentation and T-lymphocyte priming assay

MOC1ova cells (3×10^5) were irradiated and immediately placed in co-culture with unsorted wild-type B6 splenocytes at a 1:1 ratio with or without recombinant murine IFNβ at 500 U/mL. After 48 hours of co-culture, cells were harvested and either analyzed for SIINFEKL epitope cross-presentation of H2-K^b via flow cytometry using cell lineage markers or used as stimulators for naïve, sorted, CFSE-labelled OT-1 T-lymphocytes. Sorting of T-lymphocytes from whole spleen was performed using negative magnetic separation strategies (AutoMACS, Miltenyi) and the Pan T-cell Isolation Kit II (Miltenyi). T-lymphocytes were labelled with 5 μM CFSE (Sigma) for 3 minutes before quenching with 1×PBS/5% heat-inactivated FBS. Proliferation of CFSE-labelled T-lymphocytes was analyzed via flow cytometry and the division index was calculated via FlowJo software as described[11].

In vivo T-lymphocyte priming assay

Mice bearing MOC1ova tumors were irradiated and immediately tail-vein injected with $5–8\times10^6$ CFSE-labelled OT-1 T-lymphocytes. After 72 hours, spleens, tumor-draining lymph nodes and tumors were harvested and prepared as described[12]. Single cell suspensions were stained with primary conjugated antibodies and analyzed for CFSE-distribution of $7AAD^-CD45.2^+Vα2^+$ OT-1 T-lymphocytes by flow cytometry with calculation of division index as described above.

Statistical analysis

Tests of significance between pairs of data are reported as p-values, derived using a student's t-test with a two-tailed distribution and calculated at 95% confidence. Comparison of multiple sets of data was achieved with analysis of variance (ANOVA) with Tukey's multiple comparisons. All error bars indicate standard deviation. Statistical significance was set to $p<0.05$. All analysis was performed using GraphPad Prism v7.

Results

Higher doses of radiation induce greater executioner caspase activation and apoptosis

IR induces caspase-3-dependent apoptosis[13]. To determine if there was dose-dependent induction of caspase-3 activity, we exposed MOC1 cells to a single fraction of 0, 2 or 8 Gy and measured caspase-3/7 activity using a fluorescent DEVD peptide/DNA dye conjugate. Within 12 hours of exposure to IR, MOC1 cells treated with 8 Gy demonstrated significantly more caspase-3/7 activity than cells treated with 2 Gy (Figure 1A, TNF as a positive control). To determine if this increase correlated with increased apoptosis, cells were stained with annexin V and 7AAD 24 hours after exposure to IR. Treatment with 8 Gy induced both early and late apoptosis to a greater degree than 2 Gy (Figure 1B). Thus, 8 Gy IR induces executioner caspase activity and an apoptotic phenotype to a greater degree than 2 Gy in MOC1 cells.

Generation of MOC1 cells expressing a well-defined model antigen

To study antigen release from dead or dying tumor cells, we engineered MOC1 cells to express a well-defined antigen. MOC1 cells were stably transduced with a plasmid encoding full length ovalbumin as a model antigen. To measure cell surface presentation of the known ovalbumin MHC class I epitope SIINFEKL, we stained cells with an antibody that specifically binds SIINFEKL presented on MHC class I H2-K^b. Flow cytometric analysis reveals IFNγ-inducible presentation of SIINFEKL on the surface of MOC1ova cells (Figure 2A). Relatively low SIINFEKL presentation is achieved even in the presence of IFNγ, possibly due to competition within the cell for peptide processing and MHC class I loading. To validate that SINFEKL presented on the surface of MOC1ova cells serves as a CTL antigen, we performed CTL assays using *ex vivo* generated OT-1 CTLs as effectors in an impedance-based cytotoxicity assay. OT-1 CTLs induced E:T ratio-dependent loss of MOC1ova (Figure 2B, **top panel**) but not parental MOC1 cell index (Figure 2B, **bottom panel**). As a measure of cytotoxicity, a 100:1 E:T ratio induces complete loss of MOC1ova cell index, whereas 1:1 E:T ratio induces no loss of cell index. These data validate the expression and processing of full length ovalbumin into the MHC class restricted peptide SIINFEKL that serves as a CTL antigen on the surface of MOC1ova cells.

Higher doses of radiation induce antigen release, cross presentation and priming of antigen-specific T-lymphocytes *in vitro*

To model whether IR can induce antigen release and subsequent priming of T-lymphocytes *in vitro*, we first assessed whether splenocytes serving as antigen presenting cells (APCs) could take up ovalbumin released by irradiated cells and cross-present SIINFEKL on H2-K^b. Figure 3A demonstrates representative histograms and quantification of SIINFEKL presentation via H2-K^b on the surface of $CD11c^+$ dendritic cells (DCs), other $CD11b^+$ myeloid cells and $CD19^+$ B-lymphocytes. APCs exposed to irradiated MOC1ova cells cross-presented SIINFEKL, to a greater degree with 8 Gy than 2 Gy in myeloid cells and B-lymphocytes and to a similar degree in DCs. Cross-presentation of SIINFEKL on APC appeared to be dependent upon the presence of IFNβ (lower panels), consistent with the work of Fuertes et al. and Diamond et al. [14, 15]. Presentation of SIINFEKL via H2-K^b on the surface of the tumor cells did not appear to be significantly enhanced with IR. To

evaluate the ability of SIINFEKL presenting APCs to prime antigen-specific T-lymphocytes, APCs exposed to irradiated MOC1ova cells were co-cultured with naïve, CFSE-labelled OT-1 T-lymphocytes (Figure 3B). OT-1 T-lymphocytes exposed to APCs co-cultured with MOC1ova cells irradiated to 8 Gy proliferated to a significantly greater degree. Taken together, these data indicate that 8 but not 2 Gy IR induces antigen release to a degree that it becomes available to APCs for IFNβ-dependent cross-presentation and activation of antigen-specific T-lymphocytes.

Higher doses of radiation induce priming of antigen-specific T-lymphocytes *in vivo*

To validate these *in vitro* findings *in vivo*, we established MOC1ova tumors in wild-type B6 mice. Following irradiation of tumors with a single dose of 0, 2 or 8 Gy, we assessed the proliferation of adoptively transferred naïve, CFSE labelled OT-1 T-lymphocytes harvested from the spleen, tumor-draining lymph node and tumor. Using mice bearing parental MOC1 tumors without ovalbumin as a negative control, analysis indicated that *in vivo* baseline OT-1 T-lymphocyte proliferation was enhanced to a greater degree with 8 Gy treatment compared to 2 Gy in all tissue compartments analyzed (Figure 4, **representative histograms and dot plots on left and quantification of division index on right**). Together, these data indicate enhanced *in vivo* antigen-specific T-lymphocyte priming and expansion in both the periphery and tumor microenvironment following 8 Gy IR compared to 2 Gy.

Radiation enhances tumor cell susceptibility to CTL-mediated lysis in a dose-dependent fashion

While IR did not appear to enhance antigen presentation (SIINFEKL:H2-K^b) on the surface MOC1ova cells, we hypothesized that increasing doses of IR would nevertheless sensitize tumor cells to CTL-mediated cytotoxicity. Exposure of MOC1ova cells to 8 Gy IR slowed proliferation compared to 2 Gy treated cells, but did not inhibit outgrowth of cells that survived IR (Figure 5A). To validate these findings and for comparison purposes, colony formation assays revealed that exposure of MOC1ova cells to 8 Gy IR significantly limited MOC1ova survival fraction compared to 2 Gy treated cells (Figure 5B). While OT-1 CTLs at a 1:1 E:T ratio had no measureable effects on non-irradiated MOC1ova cells, IR reversed resistance to CTL-mediated lysis with measureable loss of cell index in MOC1ova cells treated with 2 Gy and near-complete loss of cell index in cells treated with 8 Gy within 12 hours (Figure 5C **panels, quantified in** 5D). Expression of cell surface calreticulin, ICAM, CD80 and Fas, involved in CTL:target cell interaction and sensitivity to CTL-mediated lysis, was increased in a dose-dependent fashion following IR (Figure 5E). These data support that IR sensitizes MOC1ova cells to CTL-mediated lysis to a greater degree following 8 Gy than 2 Gy, potentially through enhanced CTL:target cell interaction and/or enhanced Fas expression.

Discussion

With the FDA-approval of immune checkpoint inhibitors, immunotherapy has emerged as an exciting new treatment option for patients with recurrent/metastatic HNSCC. Combining checkpoint inhibition with standard of care therapies, such as IR, may enhance response rates through a number of mechanims. Appropriately executed pre-clinical research is

critical to inform the design of clinical trials aiming to demonstrate such enhanced responses. IR is attractive as a potential adjuvant to immunotherapy given its widespread use in the definitive management of both early and advanced stage HNSCC[16] as well as its demonstrated ability to enhance responses to many types of immunotherapy under specific experimental conditions[7, 17–21].

Here we have demonstrated that a single dose of 8 Gy is superior to 2 Gy in terms of both tumor cell antigen release with subsequent priming of antigen specific T–lymphocytes by cross-presenting APCs and direct enhanced tumor cell sensitivity to CTL killing. Our use of well-established oral cavity tumor cells (MOC1) engineered to express full length ovalbumin allowed us to model antigen release, uptake and cross presentation by APCs and stimulation of antigen-specific T-lymphocytes both *in vitro* and *in vivo*. Dose-dependent induction of MOC1 apoptosis following IR is significant given evidence that apoptotic cells serve as efficient antigen sources[22]. Our demonstration of the absolute requirement of type I interferon for effective APC antigen cross-presentation supports the work of Diamond et al. [15] and Fuertes et al.[14] demonstrating abrogation of cross presentation and activation of anti-tumor immunity with loss of type I IFN receptor expression. With our MOC1ova model system, it is unclear if the antigen available for uptake by APCs at baseline or following IR is in the form of cellular (tumor cell debris) or free antigen. Others have demonstrated enhanced priming of antigen-specific T-lymphocytes in melanoma (B16) tumor draining lymph nodes following IR using similar techniques[23, 24], but have utilized higher single doses (15–20 Gy) that may not be feasible clinically in patients with HNSCC. This work is the first to demonstrate dose-dependent priming of adoptively transferred antigen-specific T-lymphocytes *in vivo* following IR in the peripheral compartment, TDLN and tumor microenvironment following IR treatment at doses clinically relevant to HNSCC.

Though cross presentation of cellular antigen is typically a function attributed to CD11c+ DCs, our findings of antigen cross-presentation *in vitro* by CD11c+ DCs, other CD11c− myeloid cells and B-lymphocytes is supported by the findings of others[25, 26]. We do not know which professional APC cell type is ultimately responsible for cross presentation of antigen *in vivo* in our model system, but this could be achieved with selective antibody-based elimination or genetic alteration of specific APC types within IR treated mice bearing MOC1ova tumors.

Enhanced CTL lysis of irradiated tumor cells has been demonstrated in a number of cancer models[5, 6, 27]. Traditional CTL assays utilize release of isotopes that allow quantification of target cell lysis at one specific time point, typically 4 hours. Use of the impedance-based CTL assay, which continuously measures the adherence of target cells following the addition of effector CTLs, allows real-time analysis of target cell cytotoxicity kinetics for an extended period with high sensitivity. Here we were able to demonstrate complete loss of cell impedance within about 24 hours of adding effector CTLs at a low E:T ratio (1:1) in cells irradiated with 8 Gy. While irradiation did not appear to significantly enhance SIINFEKL antigen presentation on MHC class I in this model, other potential mechanisms include increased tumor cell immunogenicity and susceptibility to Fas ligand-mediated killing.

Our comparison of single doses of 2 Gy and 8 Gy has clinical relevance for patients with HNSCC. Conventional fractionated IR for HNSCC utilizes 1.8–2.0 Gy doses. While we did not evaluate the effects of multiple treatments with 2 Gy on T-lymphocyte priming or tumor cell sensitivity to CTL killing, mounting evidence suggests that fractionated IR is immunosuppressive[23, 24, 28], potentially due to serial exposure of highly radiosensitive lymphocytes to IR. A comparison dose of 8 Gy was chosen based upon demonstrated safety of stereotactic body radiation therapy in patients with HNSCC with minimal toxicity in the reirradiation setting[29–31]. Our demonstration of enhanced antigen-specific T-lymphocyte priming with higher individual doses of IR will be instrumental for the design of clinical trials utilizing single fraction of hypofractionated IR as an adjuvant for immunotherapy.

Clearly low-dose fractionated IR is the standard of care approach for the upfront treatment of both early and late stage HNSCC. However, this IR regimen is based upon principles of sub-lethal repair of normal cells, redistribution of tumor cells into more radiosensitive portions of the cell cycle, and re-oxygenation of tumor cells and is administered with the intent of inducing direct tumor cell toxicity[28]. In the setting of IR as an adjuvant for immunotherapy, how fractionation and individual fraction doses of IR alter cell death, immunogenicity, antigen release and the function of infiltrating immune cells must be considered. Much work is needed, including determining the effects of single dose versus fractionated radiotherapy on the function of immune cells within the tumor microenvironment and what volume of the tumor needs to be irradiated to have adequate tumor antigen release to induce immunity. Our use of a single cancer model limits the generalizability of these findings, and these results should be validated in other carcinoma models. However, our data demonstrating superior tumor cell antigen release, antigen cross-presentation by APCs, priming of antigen-specific T-lymphocytes and tumor cell sensitivity to CTL killing with a single dose of 8 Gy compared to 2 Gy provides some of the pre-clinical information critical for the appropriate, data-driven design of clinical trials combining IR and checkpoint inhibition.

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Research highlights

- Ionizing radiation (IR) induces apoptosis in a dose-dependent fashion
- 8 Gy IR induces release of tumor antigen to a greater degree than 2 Gy
- 8 Gy IR induces T-cell priming *in vitro* to a greater degree than 2 Gy
- 8 Gy IR induces T-cell priming in peripheral and tumor compartments *in vivo*
- Higher single doses of IR should be considered when combining IR and immunotherapy

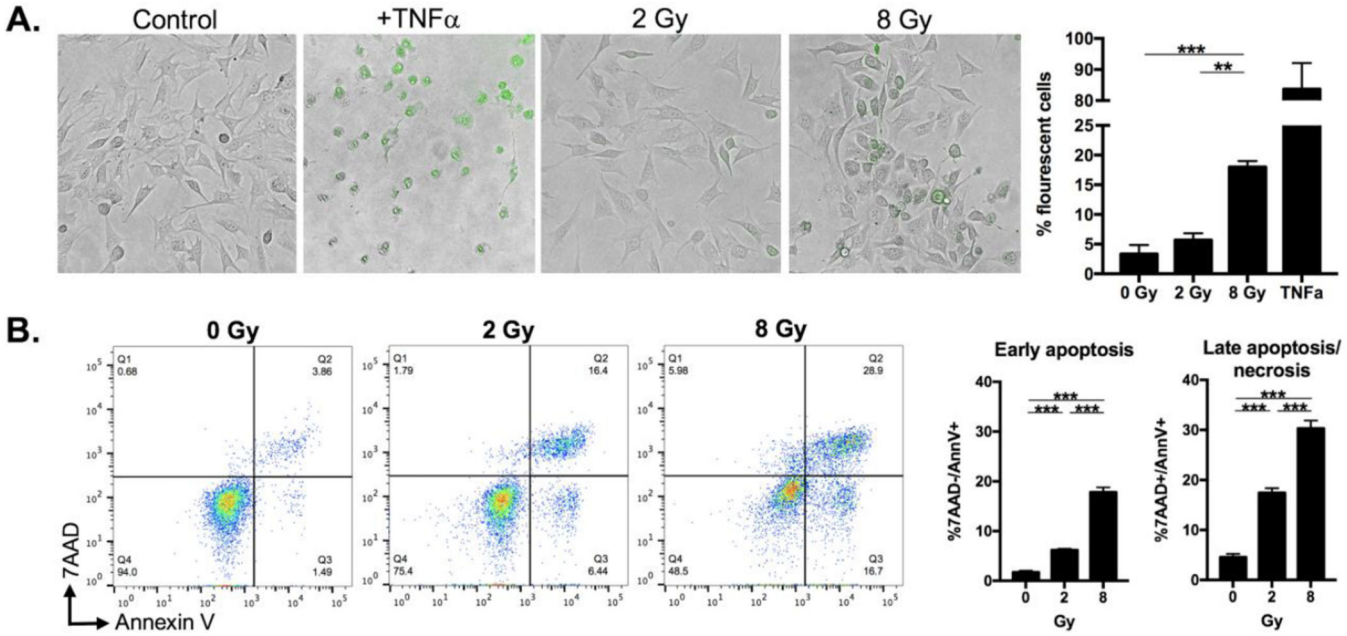

Figure 1. Ionizing radiation dose-dependent induction of caspase 3/7 activity and apoptosis

A, induction of caspase 3/7 activity after a single dose of ionizing radiation. MOC1 cells were irradiated and cultured for 12 hours before caspase 3/7 detection reagent added. Representative photomicrographs at 20× magnification. TNFα (100 ng/mL) used as positive control. Quantification from 10 HPFs per condition. **B**, induction of apoptosis and necrosis after a single dose of ionizing radiation. MOC1 cells were irradiated, cultured for 24 hours, harvested, and immediately stained and analyzed via flow cytometry. Late apoptosis/ necrosis defined as annexin V and 7AAD double positive; early apoptosis as annexin V positive but 7AAD negative. Representative results from one of at least three independent experiments are shown. **, p,0.01; ***, p<0.001.

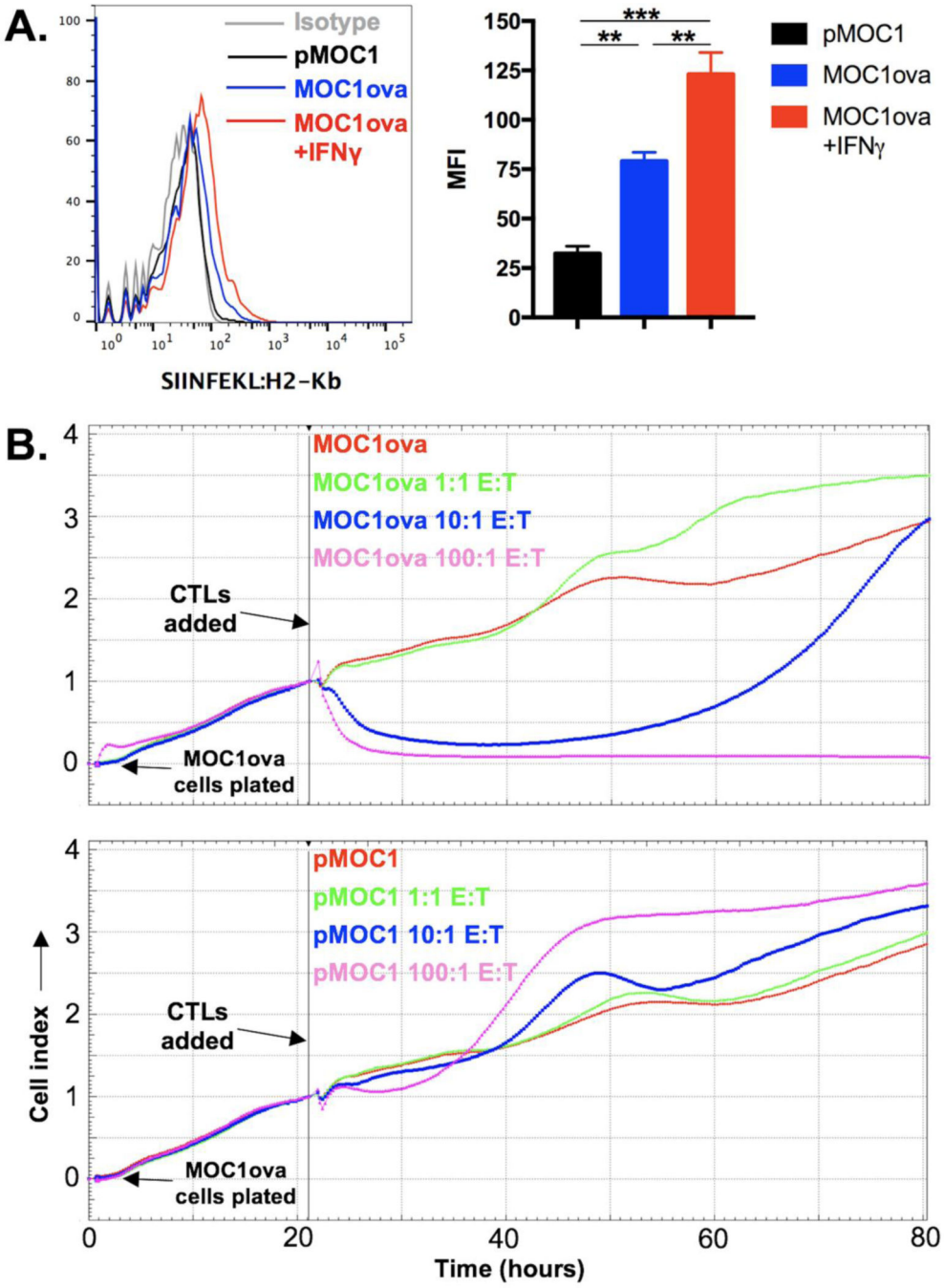

Figure 2. Validation of MOC1 cells engineered to express full-length ovalbumin as a model antigen

A, MOC1 cells stably transduced with full length ovalbumin were exposed to IFNγ (10 ng/mL) for 24 hours and assessed via flow cytometry for presentation of SIINFEKL on H2-K^b. Histograms displayed on the left, MFI quantification on the right. **B**, using changes in impedance as a measure of target cell killing, *ex vivo* generated OT-1 CTLs were applied to either MOC1ova (top panel) or parental MOC1 (bottom panel) cells at the indicated E:T ratios. Target cells were plated and impedance was allowed to rise for 22 hours before the

addition of effectors. Representative results from one of at least three independent experiments are shown. **, p,0.01; ***, p<0.001.

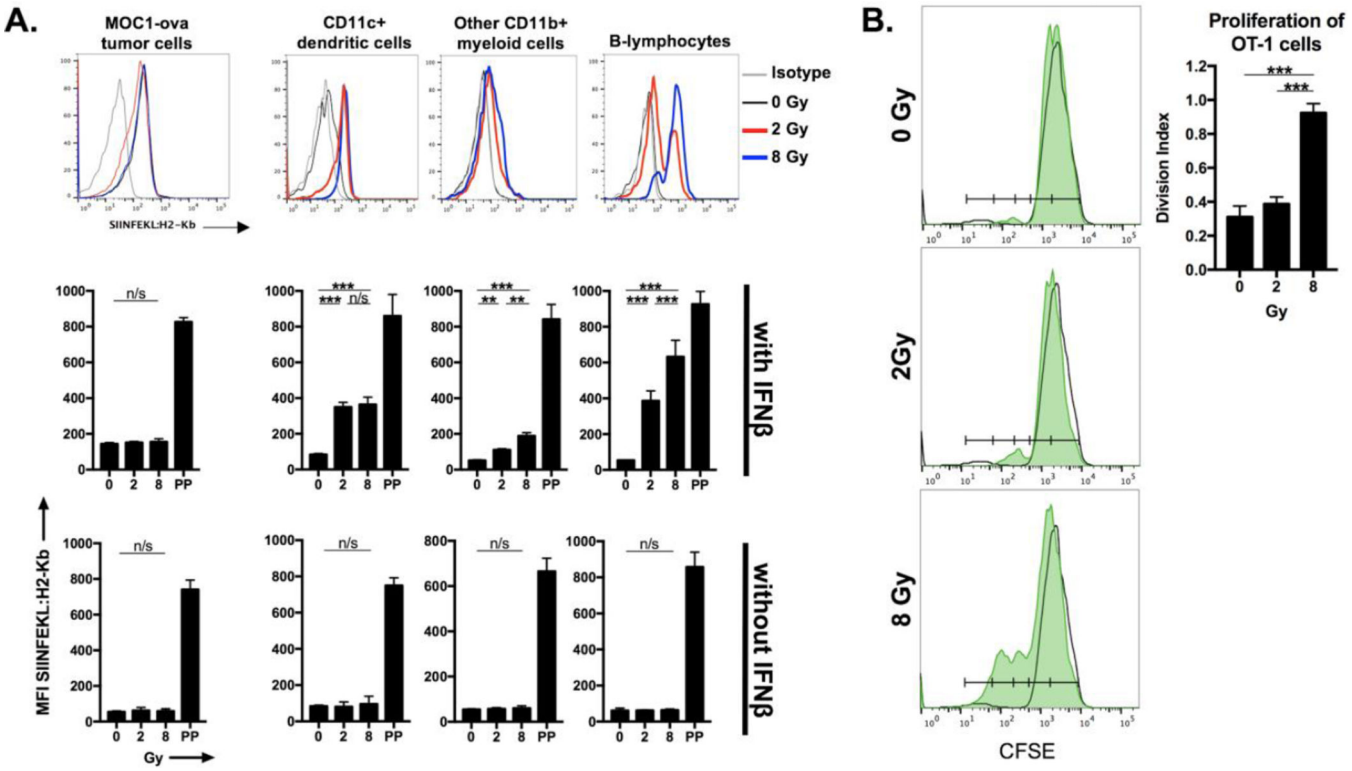

Figure 3. Antigen cross-presentation and T-lymphocyte priming *in vitro* following ionizing radiation

A, MOC1ova cells were irradiated and cultured with naïve B6 splenocytes in the presence or absence of IFNβ (500 units/mL). Co-cultured cells were harvested and tumor cells ($CD45.2^{-}$), dendritic cells ($CD11b^{+/-}CD11c^{+}$), other myeloid cells ($CD11b^{+}CD11c^{-}$) and B-lymphocytes ($CD19^{+}$) were assessed for cell surface SIINFEKL:H2-K^{b} via flow cytometry. In the bar graph quantifying SIINFEKL presentation, also quantified is SIINFEKL presentation following SIINFEKL peptide pulse (PP; 2 μg/mL × 4 hours) as a representation of antibody specificity and for comparison purposes. **B**, following co-culture of irradiated MOC1ova cells and naive B6 splenocytes in the presence of IFNβ for 48 hours, splenocytes were co-cultured with sorted, CFSE-labelled naïve OT-1 T-lymphocytes for 72 hours. Proliferation of OT-1 T-lymphocytes was measured by CFSE spread on flow cytometry and quantified via calculation of the division index. Unfilled histograms represent histograms of unstimulated CFSE-labelled naïve OT-1 T-lymphocytes alone. Representative results from one of at least two independent experiments are shown. **, p,0.01; ***, p<0.001. n/s, non-significant.

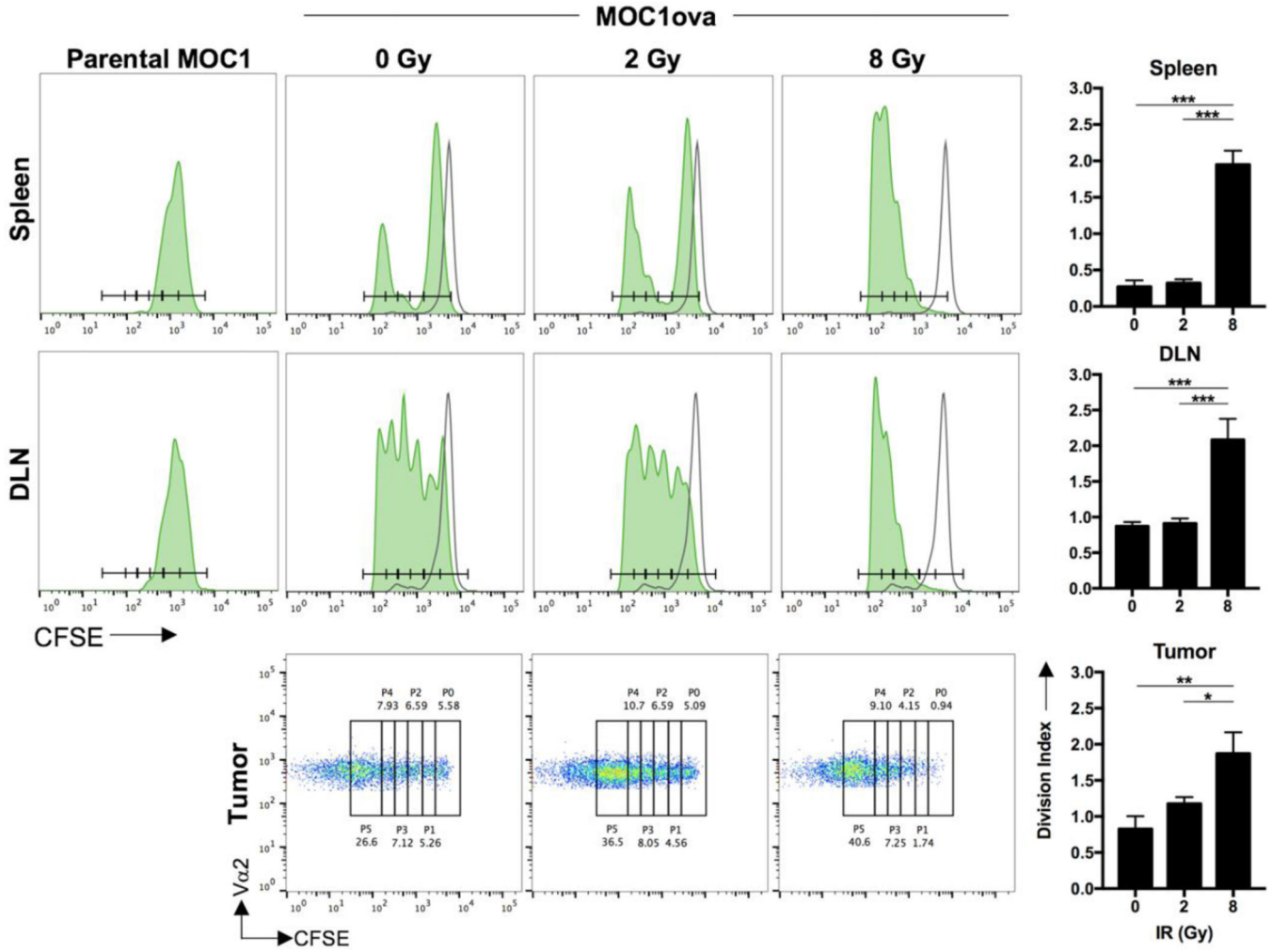

Figure 4. Antigen-specific T-lymphocyte priming *in vivo* following ionizing radiation

Established (>0.1 cm^3 volume) MOC1ova tumors in wild-type B6 mice were irradiated and CFSE-labelled OT-1 T-lymphocytes were adoptively transferred. CFSE spread of Vα2+ T-lymphocytes was assessed from the spleen, tumor-draining lymph node and tumor via flow cytometry (n=5 mice/group). Unfilled histograms represent CFSE-labelled OT-1 T-lymphocytes adoptively transferred into naive, non-tumor bearing mice. Representative CFSE histograms (spleen, lymph node) or dot plots (tumor) are shown with quantification of division index on the right. CFSE spread of adoptively transferred OT-1 T-lymphocytes into parental MOC1 tumor-bearing mice were used as a control for antigen-specific T-lymphocyte proliferation (left histograms). Representative results from one of at least two independent experiments are shown. *, $p<0.05$; **, p,0.01; ***, $p<0.001$.

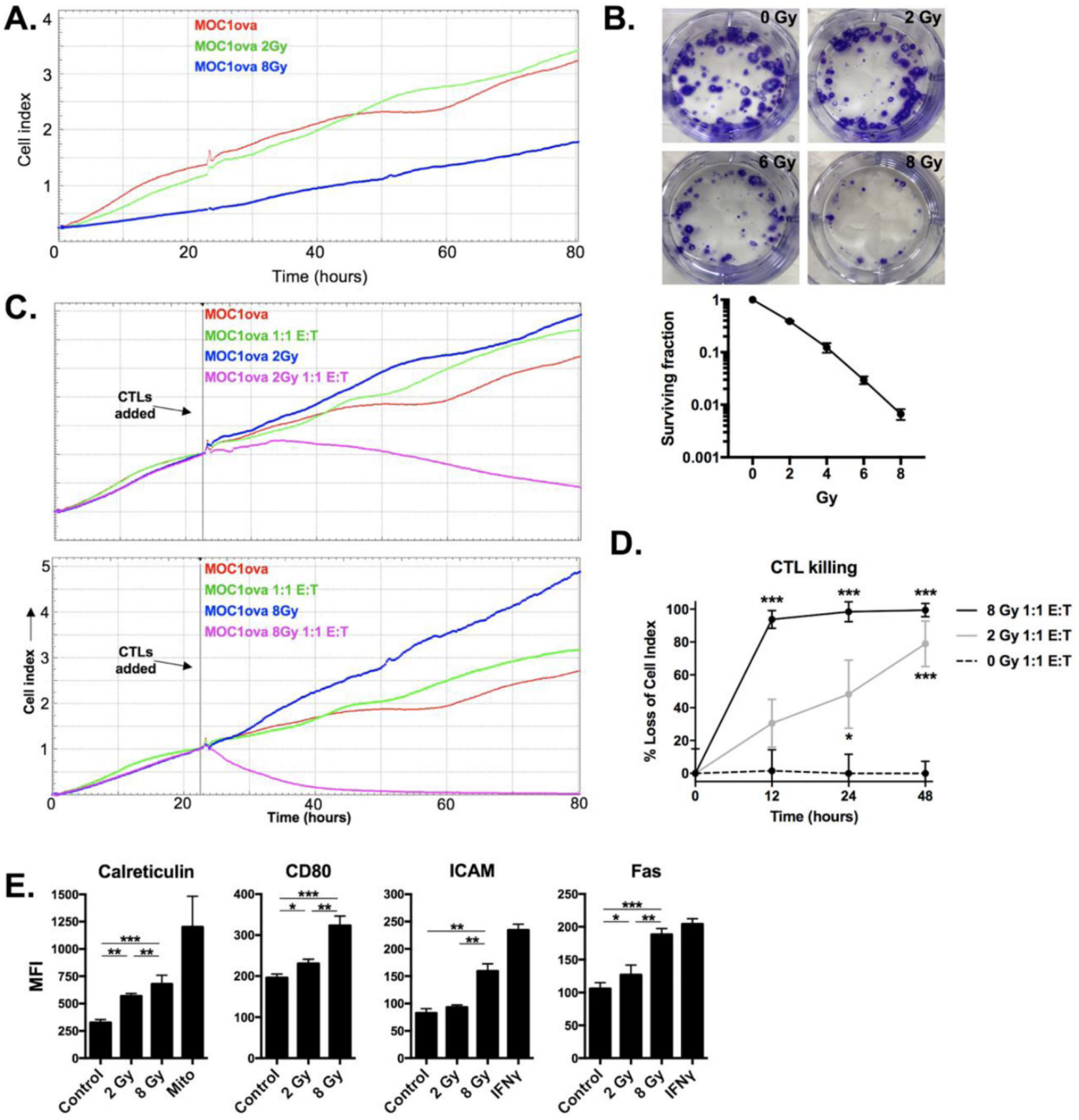

Figure 5. Enhanced sensitivity to CTL-mediated killing following ionizing radiation

A, MOC1ova cells were irradiated, cultured, and growth was measured via change in impedance. Data is not normalized to any time point. **B**, cells were irradiated with either 2, 4, 6 or 8 Gy, and standard clonogenic assays were performed. Representative photos of wells demonstrating colony formation for each condition are shown on top panels, with survival fraction quantified below. Two independent experiments were performed with similar results. **C**, irradiated MOC1ova cells were co-cultured with *ex vivo* generated OT-1 CTLs at a 1:1 E:T ratio. Comparative impedance curves for each experimental condition (2 vs 8 Gy)

were normalized to the time at which effectors were added (vertical black line). **C**, percent loss of cell index quantified at 12, 24 and 48 hours after the addition of effector CTLs. Asterisks indicate significant differences compared to 0 Gy. **D**, MFI quantification of cell surface expression of immunogenicity markers on MOC1 cells 24 hours following IR or the addition of positive control (mitoxantrone 1 μM; IFNγ 10 ng/mL). Representative results from one of three independent experiments are shown. *, $p<0.01$; **, $p<0.01$; ***, $p<0.001$.