



Original Articles

Olaparib enhances radiation-induced systemic anti-tumor effects via activating STING-chemokine signaling in hepatocellular carcinoma

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ABSTRACT

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Although Poly (ADP-ribose) polymerase (PARP) inhibitors have been clinically approved for cancers with BRCA mutations and are known to augment radiotherapy responses, their roles in promoting the abscopal effect and mediating immunotherapy in BRCA-proficient hepatocellular carcinoma (HCC) remain underexplored. Our study elucidates that olaparib enhances the radio-sensitivity of HCC cells. Coadministration of olaparib and irradiation induces significant DNA damage by generating double-strand breaks (DSBs), as revealed both in vitro and in immune-deficient mice. These DSBs activate the cGAS-STING pathway, initiating immunogenic cell death in abscopal tumors. STING activation reprograms the immune microenvironment in the abscopal tumors, triggering the release of type I interferon and chemokines, including CXCL9, CXCL10, CXCL11, and CCL5. This in turn amplifies T cell priming against tumor neoantigens, leading to an influx of activated, neoantigen-specific CD8⁺ T-cells within the abscopal tumors. Furthermore, olaparib attenuated the immune exhaustion induced by radiation and enhances the responsiveness of HCC to immune checkpoint inhibitors. Collectively, our data advocate that a synergistic regimen of PARP inhibitors and radiotherapy can strategically reinforce both local (primary) and systemic (abscopal) tumor control, bolstering HCC susceptibility to immunotherapy.

1. Introduction

Hepatocellular carcinoma (HCC), the third leading cause of cancer-related deaths, has a poor five-year survival rate [1]. While surgical tumor resection offers a potential cure for early-stage HCC patients, those with advanced HCC have limited therapeutic choices, predominantly radiotherapy and transarterial chemoembolization (TACE) [2]. Although chemotherapy has been widely used in solid tumors, the chemotherapeutic drugs used in HCC are limited. Sorafenib, a targeted therapy for advanced HCC, provides only modest benefits [3]. Thus,

revealing new therapies and exploring combination therapies for HCC is expected to change the landscape of HCC management.

Poly (ADP-ribose) polymerase (PARP) is a promising therapeutic target for cancers with both germline and somatic BRCA1 and BRCA2 mutations [4–6]. BRCA1 is essential for the repair of double-strand breaks (DSBs) via homologous recombination (HR), while PARP1 is predominantly involved in the repair of single-strand breaks (SSBs) [7]. PARP inhibitors, such as olaparib, niraparib, rucaparib, veliparib, and talazoparib, inhibit PARP-mediated alternative end joining (Alt-EJ) repair, allowing SSBs to escalate to DSBs during DNA replication [8]. In

Abbreviations: HCC, Hepatocellular Carcinoma; TACE, Transarterial Chemoembolization; PARP, Poly (ADP-ribose) Polymerase; DSB, Double-Strand Break; HR, Homologous Recombination; NHEJ, Nonhomologous End-Joining; IC50, half-maximal drug inhibitory concentration; SSB, Single-Strand Break; Alt-EJ, Alternative End Joining; NHEJ, Nonhomologous End-Joining; RT, Radiation Therapy; SBRT, Stereotactic Body Radiation Therapy; IR, Ionizing Irradiation; dsDNA, Double-Stranded DNA; cGAS, Cyclic GMPAMP Synthase; IFN, Interferon; STING, Stimulator of Interferon Genes; TBK1, TANK-Binding Kinase 1; IRF3, IFN Regulatory Factor 3; ICD, Immunogenic Cell Death; ICB, Immune Checkpoint Blockade; ICI, Immune Checkpoint Inhibition; CTG, Cell Titer GLO; HSA, highest single agent; DAMPs, Damage-associated molecular patterns; ATP, Adenosine triphosphate; HMGB1, High mobility group box 1; KO, Knock Out; WT, Wild Type; MDSCs, Myeloid-Derived Suppressor Cells; NK, Natural Killer; CXCL, CXC motif chemokine ligand; CCL, CC motif chemokine ligand; GZMB, Granzyme B; dMMR, DNA Mismatch Repair.

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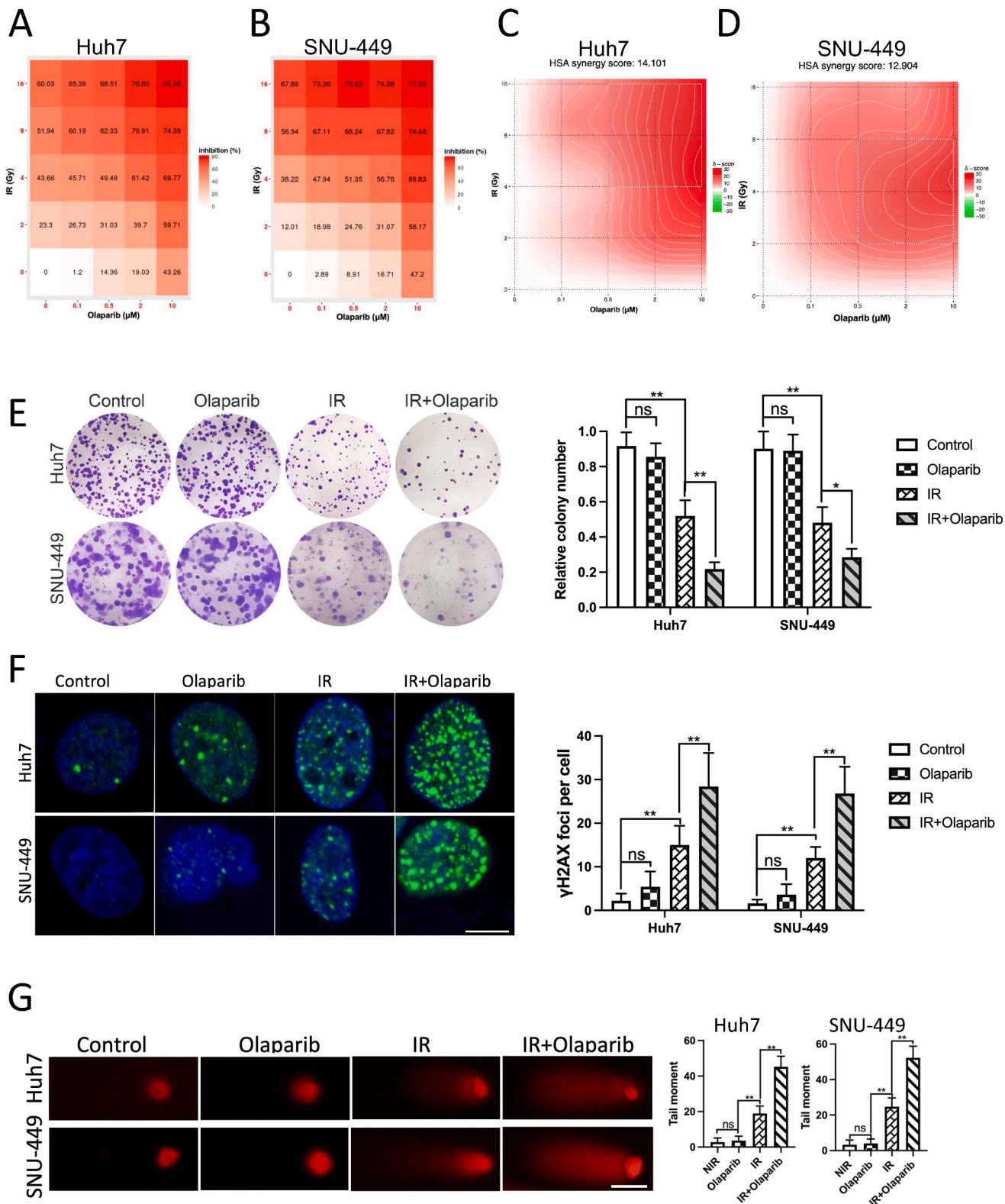


Fig. 1. Olaparib sensitized HCC cells to irradiation. (A–B) The combined inhibitory effects of various combinations of IR (0, 2, 4, 8, and 16 Gy) and olaparib (0, 0.1, 0.5, 2, and 10 μ M) were assessed in Huh7 (A) and SNU-449 (B) cells using the CTG assay. The dose-response matrix was generated using SynergyFinder. (C–D) The HSA synergy scores and synergy maps were visualized using the SynergyFinder. (E) Representative images of the colony formation assay in Huh7 and SNU-449 cells treated with 100 nM olaparib, 4 Gy irradiation and the combination. Quantification of the colony number is indicated on the right. (F) Representative γ H2AX-positive foci in Huh7 and SNU-449 cells treated with 100 nM olaparib, 2 Gy irradiation, and the combination (scale bar, 10 μ m). Quantification of the γ H2AX foci is indicated on the right. (G) Representative comet assay images of Huh7 and SNU-449 cells treated with 100 nM olaparib, 4 Gy irradiation, and the combination (scale bar, 20 μ m). Analysis of comet tail length is indicated on the right. (* $P < .05$; ** $P < .01$; ns, no significance).

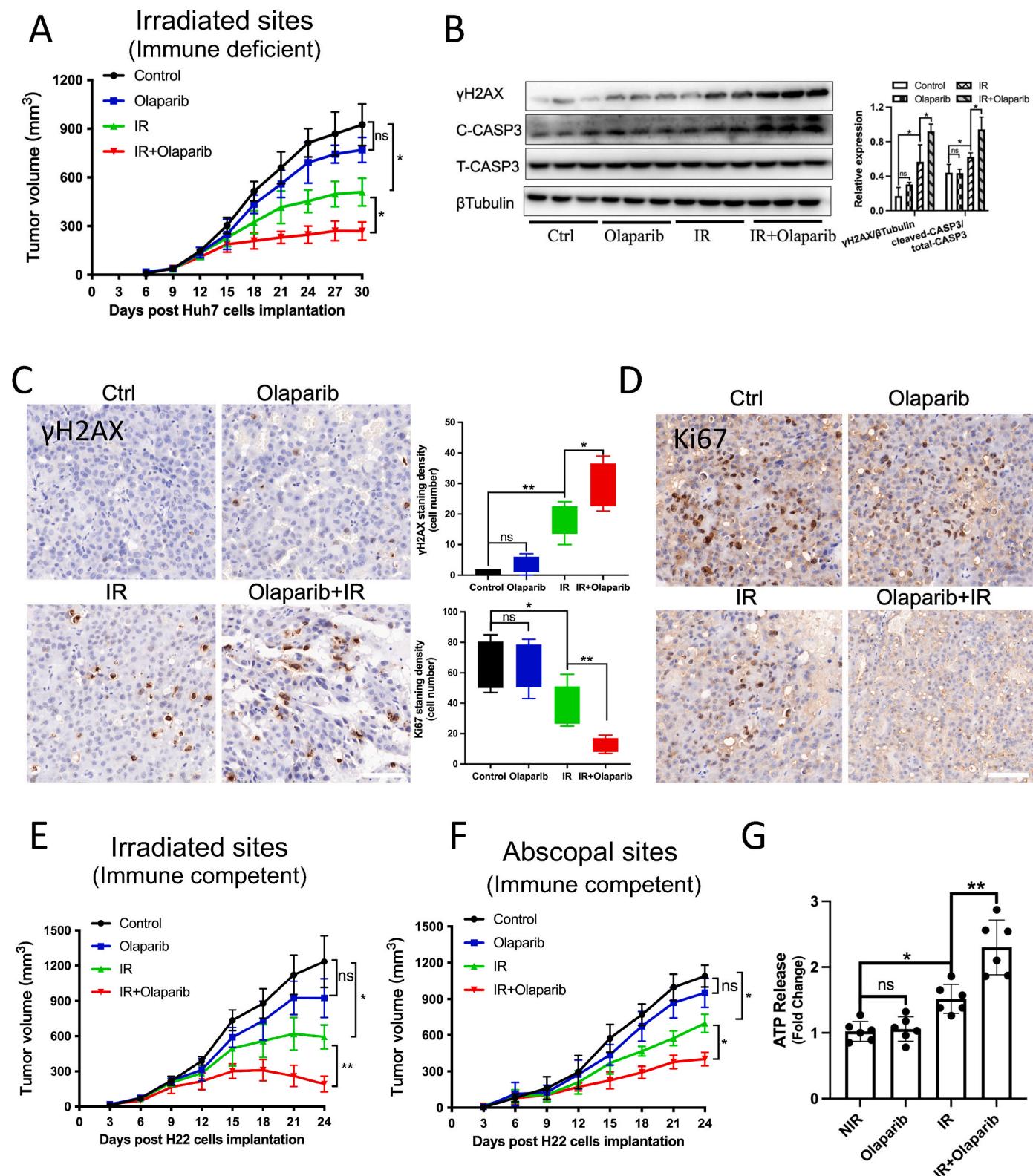


Fig. 2. Olaparib augments irradiation-induced DNA damage *in vivo*. (A) Growth curves from Huh7 tumors in immune deficient mice treated with olaparib, IR and the combination. (B) Western blot of γ H2AX, caspase 3 and cleaved caspase 3 in Huh7 tumor tissues in mice treated with olaparib, IR and the combination. (C) Representative immunohistochemistry images and quantification of γ H2AX staining in Huh7 tumor tissues in mice treated with olaparib, IR and the combination. (D) Representative immunohistochemistry images and quantification of Ki67 staining in Huh7 tumor tissues in mice treated with olaparib, IR and the combination (scale bar: 100 μ m). (E–F) Growth curves from primary (E) and abscopal (F) H22 tumors in immune competent mice treated with olaparib, IR and the combination. (G) Fold increase of ATP in mice receiving olaparib, IR and the combination. (* $P < .05$; ** $P < .01$; ns, no significance).

the absence of HR, cells often resort to nonhomologous end-joining (NHEJ) for repair, explaining the resistance of many cancers to PARP inhibitors without BRCA mutation [8]. This makes patients with wild-type BRCA1, in which HR is functional, ineligible for the benefits of PARP blockade therapy [9]. HCC remains insensitive to PARP inhibitors owing to low BRCA mutation rates [10]. A phase II study focusing on patients with sorafenib-resistant advanced HCC found that a temozolamide and veliparib combination therapy failed due to a poor overall response rate [11]. Yet, HCC patients harboring BRCA2 germline mutations showed an objective response [10].

Radiation therapy (RT), especially stereotactic body radiation therapy (SBRT), is a promising therapy to achieve local control in advanced HCC patients. A previous study indicated that SBRT provides better local control and a similar overall survival rate compared to TACE [12]. Ionizing irradiation (IR) impedes tumor progression by inducing DNA breaks, including DSBs and SSBs, which can lead to genomic instability in tumor cells and even cell death. Concurrently, DSBs and SSBs can be repaired by various pathways, including two major pathways, HR and NHEJ [13]. Sensitivity to radiation therapy differs among cells due to variations in their ability to process DNA damage. Recent studies have revealed that IR induces the production of DSBs and SSBs, potentially activating the PARP-mediated NHEJ pathway, thus facilitates the coadministration of IR and olaparib [14]. Consequently, PARP inhibitors are considered as radiosensitizers that suppress SSB repair and subsequently promote DSB generation. Several proof-of-concept experiments have shown that PARP inhibitors amplify radiotherapy-induced DNA damage in HCC [15]. However, the underlying mechanisms remain to be fully elucidated.

Recent research has suggested that radiotherapy induces the production of double-stranded DNA (dsDNA), which activates the cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS). Activation of cGAS stimulates stimulator of interferon (IFN) genes (STING), which binds to TANK-binding kinase 1 (TBK1) and then activates the transcription factor IFN regulatory factor 3 (IRF3). Subsequently, IRF3 initiates the transcription of type I IFN, which plays a role in host defense [16,17]. Previous studies have reported that STING-mediated innate immunity is important for radiation-induced antitumor response. STING signaling can enhance the recruitment and expansion of cytotoxic effector cells at tumor sites [18]. Additionally, STING signaling activation stimulates immunogenic cell death (ICD), inhibiting tumor growth and improving responses to ICB [19]. Preclinical models revealed variable degrees of efficacy of STING agonists and several clinical trials involving their efficacy, alone or in combination with other treatments, are currently underway [20]. However, the role and mechanism of STING signaling in non-irradiated abscopal tumors remain poorly understood.

Previously, the abscopal effect garnered only fringe attention due to its rarity. However, this has changed with the advent of systemic therapeutics, particularly immune checkpoint inhibition (ICI) therapy [21, 22]. The combination of RT with immunotherapy has significantly increased the occurrence of the abscopal effect. An increasing number of studies attribute the occurrence of this phenomenon to an antitumor immune response [23,24]. However, little is known about the role of PARP inhibitors in the abscopal effect. Herein, we investigated the role of PARP inhibition in combination with RT in HCC. On the one hand, we wanted to explore whether olaparib could act as a radiosensitizing agent by inducing DNA damage. On the other hand, we intend to evaluate the impact of olaparib in combination with RT on the abscopal effect by enhancing the antitumor response. Our findings may provide a rationale for the contribution of olaparib to DNA damage in primary tumors and the improvement of abscopal tumor control.

2. Materials and methods

For details regarding the materials and methods used, please refer to the supplemental materials.

3. Results

3.1. Olaparib sensitizes HCC cells to irradiation

To investigate whether olaparib sensitizes HCC cells to radiation, human HCC cell lines Huh7, HCCLM3, and SNU-449 cells were treated with 100 nM or 500 nM olaparib with or without 4 Gy of X-ray irradiation. As shown in [Supplemental Fig. 1A](#), incubation with 100 nM or 500 nM olaparib for 96 h did not significantly affect cell viability, as assessed by the Cell Titer GLO (CTG) luminescent cell viability assay. However, cell viability was significantly inhibited by the combination of olaparib and irradiation ([Supplemental Fig. 1A](#)). To quantify potential synergistic interactions for the irradiation and olaparib combination, we used SynergyFinder to obtain the dose-response matrix from Huh7 and SNU-449 cells treated with 0, 2, 4, 8, and 16 Gy irradiation with or without 0, 0.1, 0.5, 2, and 10 μ M olaparib [25]. We observed that IR inhibited cell viability in a dose-dependent manner, and this effect was enhanced in combination with a gradient dose of olaparib ([Fig. 1A](#) and B). Remarkably, the highest single agent (HSA) synergy scores for both Huh7 and SNU-449 cells were 14.1 and 12.9, respectively—values above 10, implying a likely synergistic interaction ([Fig. 1C](#) and D). Moreover, the colony formation assay also demonstrated a significant decrease in the clonogenic ability of the cells upon combination treatment ([Fig. 1E](#)). To validate these findings, we performed staining for γ H2AX histones, indicative of double-stranded DNA breaks [DSBs]. Few DSBs were observed without exposure to IR. However, the cotreatment with IR and olaparib led to a significant increase in the number of γ H2AX-positive foci in the nuclei, indicating enhanced levels of unrepaired DNA damage ([Fig. 1F](#)). The neutral comet assay further affirmed the extent of DNA damage, evidenced by pronounced tail elongation in the combination treatment group ([Fig. 1G](#)). These findings were further supported by the cotreatment with irradiation and veliparib, another clinically used PARP inhibitor, as revealed by the CTG assay ([Supplemental Fig. 1B](#)) and γ H2AX histones staining ([Supplemental Fig. 1C](#)).

To extrapolate the efficacy of this combination to other immunogenic tumors, we subjected the lung cancer cell line A549 and breast cancer cell line MDAMB231 to combined IR and olaparib treatment. Similarly, cotreatment significantly suppressed cell viability, whereas 100 nM olaparib alone did not exhibit marked cytotoxicity ([Supplemental Figs. 1D–E](#)). Since PARP inhibitors are known to induce cell death in those with homologous recombination deficiency (HRD), we sought to determine whether HRD status influenced this combination therapy. HCT116, recognized as an HRD cell line due to its mutated BRCA1 gene, was selected for investigation. Notably, we observed that the half-maximal drug inhibitory concentration (IC50) for olaparib in HCT116 cells was considerably lower than that in HCC cell lines ([Supplemental Fig. 1F](#)), yet a commensurate synergistic impact was discerned ([Supplemental Fig. 1G](#)). Taken together, these findings suggest that the concurrent administration of olaparib and irradiation synergistically exerts an antiproliferative effect by inducing DNA damage in HCC cell lines, transcending their HRD genetic status.

3.2. Olaparib augments irradiation-induced DNA damage *in vivo*

Based on the previous findings, we next investigated whether olaparib could sensitize tumors to radiotherapy *in vivo*. To achieve this, we established a subcutaneous tumor model using the human HCC cell line Huh7 with irradiation (8 Gy \times 3 fractions over three consecutive days) and with or without olaparib (10 mg/kg daily for two weeks, [Supplemental Fig. 2A](#)). Tumor growth was monitored for 30 days. Paralleling the response *in vitro*, olaparib alone exhibited limited antitumor effects, while irradiation alone showed some degree of antitumor activity ([Fig. 2A](#)). However, a remarkable antitumor effect was observed in the combination group, as evidenced by significant tumor regression ([Fig. 2A](#) and [Supplemental Fig. 2B](#)), without observed changes in body weight ([Supplemental Fig. 2C](#)).

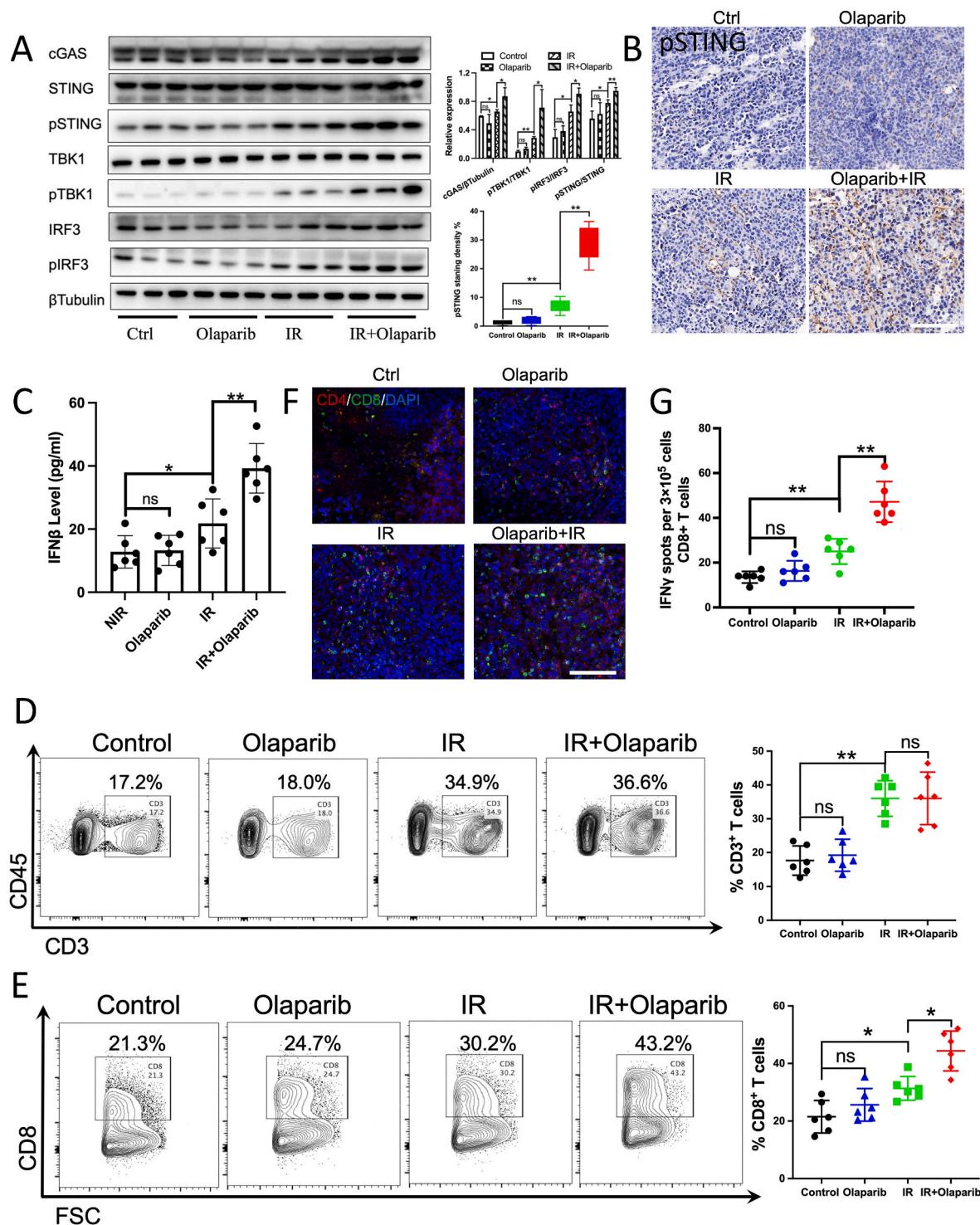


Fig. 3. STING activation reprograms the abscopal tumor immune microenvironment. (A) Representative immunoblots and quantification of cGAS-STING-IRF3 signaling in primary H22 tumor tissues in mice treated with olaparib, IR and the combination. (B) Representative immunohistochemistry images and quantification of phosphor-STING staining in primary H22 tumor tissues in mice treated with olaparib, IR and the combination (scale bar: 100 μ m). (C) IFN β levels in mice treated with olaparib, IR and the combination. (D-E) FACS analysis of infiltrating CD45+CD3 lymphocytes (D) and CD8 $^{+}$ cytotoxic T cells (E) after treatment with olaparib, IR and the combination. (F) Representative multiple color staining of CD4 $^{+}$ and CD8 $^{+}$ T cells in abscopal tumor tissues in mice treated with olaparib, IR and the combination (scale bar: 100 μ m). (G) IFN γ -producing CD8 $^{+}$ T cells were enumerated by ELISPOT assay. (* $P < .05$; ** $P < .01$; ns, no significance).

To assess DNA damage in the tumor tissues, we performed Western blot for γ H2AX. This revealed no significant difference in DSBs between the control group and the olaparib-alone group. However, a notable increase in DSBs was observed in the combination group compared to the irradiated group (Fig. 2B). Pathological studies were also conducted

to further evaluate DNA damage in the tumor tissues. Consistently, an increased number of DSBs were observed in the combination group, as indicated by the quantification of γ H2AX positive cells (Fig. 2C). The accumulation of IR-induced DSBs, facilitated by olaparib, impaired the repair of DSBs and resulted in cell apoptosis. Ki67 staining demonstrated

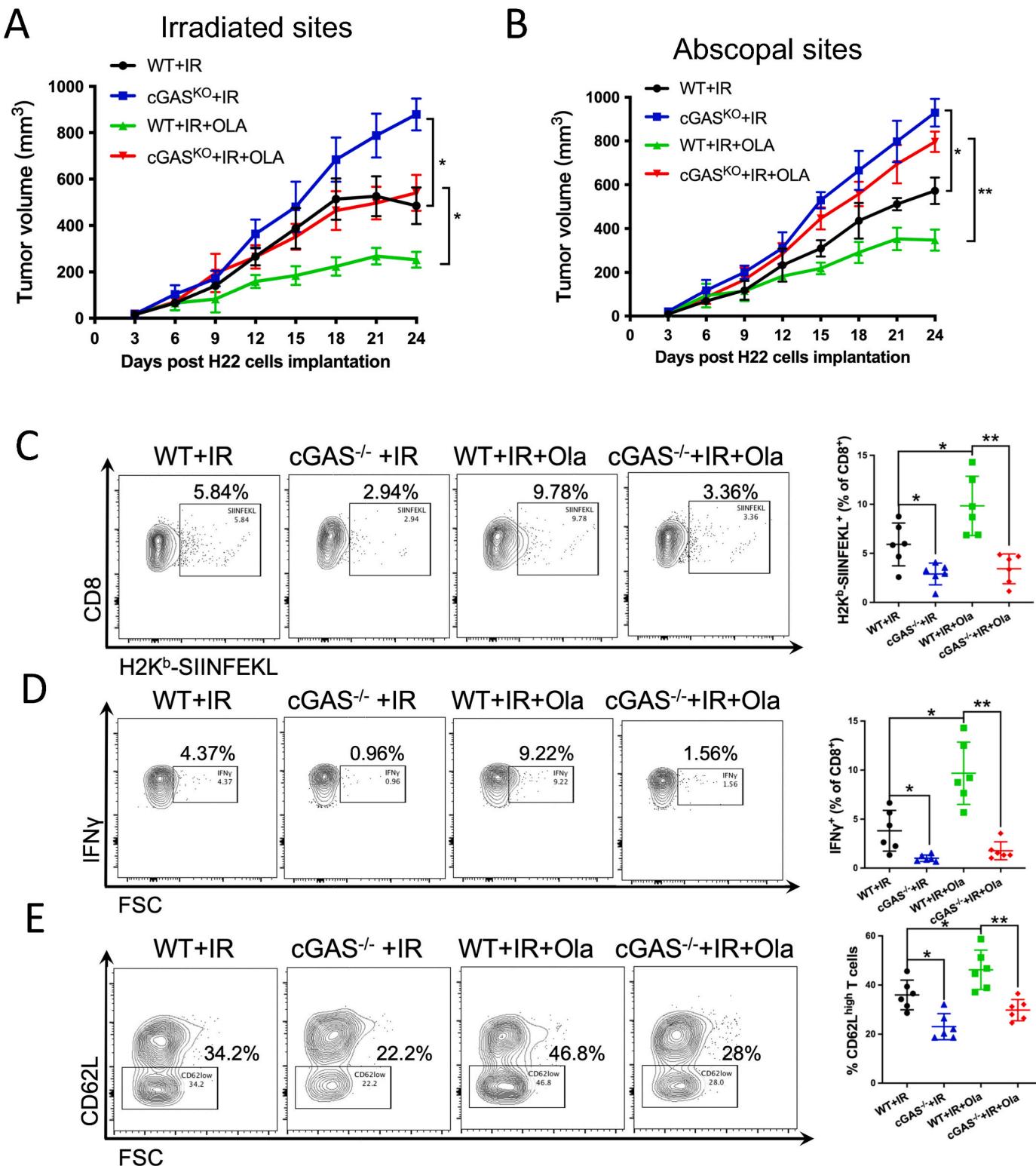


Fig. 4. cGAS-STING signaling is required for radiotherapy-driven and olaparib-enhanced systemic anti-tumor effects. (A–B) Growth curves of primary (A) and abscopal (B) H22 tumors in immunocompetent and cGAS KO mice treated with olaparib, IR, and the combination. (C) FACS analysis of infiltrating CD8⁺ T lymphocytes primed with OVA-tetramers from abscopal H22 tumors after treatment with olaparib, IR and the combination. (D) FACS analysis of infiltrating IFN γ + CD8⁺ T cells from abscopal H22 tumors after treatment with olaparib, IR and the combination. (E) FACS analysis of infiltrating CD44^{medium}CD62L^{low} CD8 memory/effector T cells from abscopal H22 tumors after treatment with olaparib, IR and the combination. (*P < .05; **P < .01).

no significant difference in cell viability between the control group and the olaparib alone group. However, there was a significant inhibition of cell viability in the combination group compared to the irradiation group (Fig. 2D). The expression of cleaved caspase 3 supported the synergistic effect between IR and olaparib (Fig. 2B). Taken together, these findings supported the idea that olaparib contributes to IR-induced DNA damage.

3.3. Olaparib triggers abscopal tumor cell apoptosis and immunogenic cell death

Mounting evidence has revealed that radiotherapy has profound immunostimulatory effects and can lead to abscopal responses. Therefore, we aimed to investigate the antitumor response to olaparib and IR treatment in an immune proficient mouse model. We utilized murine HCC H22 cells as a model. C57 mice bearing bilateral subcutaneous tumors received irradiation of the right tumors, and the tumor growth of the irradiated (primary) and nonirradiated (abscopal) tumors was monitored. Similarly, olaparib treatment showed no significant difference in tumor growth in immunocompetent mice (Fig. 2E). However, we observed a more pronounced synergistic effect at the primary tumor sites in immunocompetent mice compared to nude mice (Fig. 2E).

In the abscopal sites, we observed tumor shrinkage in the nonirradiated sites as well (Fig. 2F). A similar synergistic effect in controlling the growth of the abscopal tumor was observed at the abscopal sites. Notably, the abscopal effect was abolished in immune deficient mouse, highlighting the crucial role of the immune system in systemic tumor control (Supplemental Fig. 3A). Tumor cell proliferation was significantly inhibited in the abscopal tumor following treatment with both RT and olaparib (Supplemental Fig. 3B). Noteworthy is the observation that immune cellular constituents remained largely unperturbed by this dual treatment, as evinced by CD45 and cleaved caspase 3 co-staining (Supplemental Fig. 3C). Immunogenic cell death (ICD), an inflammatory form of cell death, manifests as various types like necroptosis and pyroptosis. During ICD, dying cells trigger the release of hallmark immunostimulatory damage-associated molecular patterns (DAMPs), such as adenosine triphosphate (ATP), high mobility group box 1 (HMGB1) and translocation of calreticulin [26]. We next confirmed whether ICD contributes to the abscopal effect. Our group and others have found that irradiated cells promote the release of HMGB1 and can induce the abscopal effect [27]. Therefore, we examined the expression of ATP and HMGB1, common and widely accepted DAMPs associated with ICD. We found that RT triggers the release of ATP, which can be significantly enhanced by olaparib (Fig. 2G). Similarly, the combination of olaparib and RT significantly stimulated the secretion of HMGB1 compared to RT alone (Supplemental Fig. 3D). These findings suggest that ICD may play a key role in the DNA damage induced abscopal effect.

Our group, along with others, has found that irradiated cells promote the release of DAMPs and micronuclear formation and subsequently activate the cGAS-STING-mediated Type I IFN response. Consequently, our examinations spanning the structural and morphological spectra of nuclei within Huh7 and SNU-449 cells, in response to IR and olaparib, manifested elevated mitotic catastrophes upon olaparib co-treatment (Supplemental Fig. 3E). Type I IFNs have been demonstrated to contribute to systemic antitumor adaptive immunity following radiotherapy (6, 7). We then investigate how cGAS-STING signaling contributes to the abscopal effect. As expected, we confirmed the activation of cGAS-STING-IRF3 signaling through immunoblotting of cGAS and the phosphorylation levels of STING, TBK1, and the transcription factor IRF3 after irradiation, especially within the group treated with both IR and olaparib (Fig. 3A). Additionally, the potentiating effect of olaparib on cGAS-STING activation was validated by assessing phosphorylated STING intensity via immunohistochemistry staining (Fig. 3B). Considering that the antitumor response in the abscopal sites appears to be dependent on IFN β production, via the promotion of cross-presentation by antigen presenting cells, we observed enhanced IFN β production in

the combination group, while olaparib as a single agent had little effect (Fig. 3C).

3.4. STING activation reprograms the immune microenvironment in the abscopal tumor

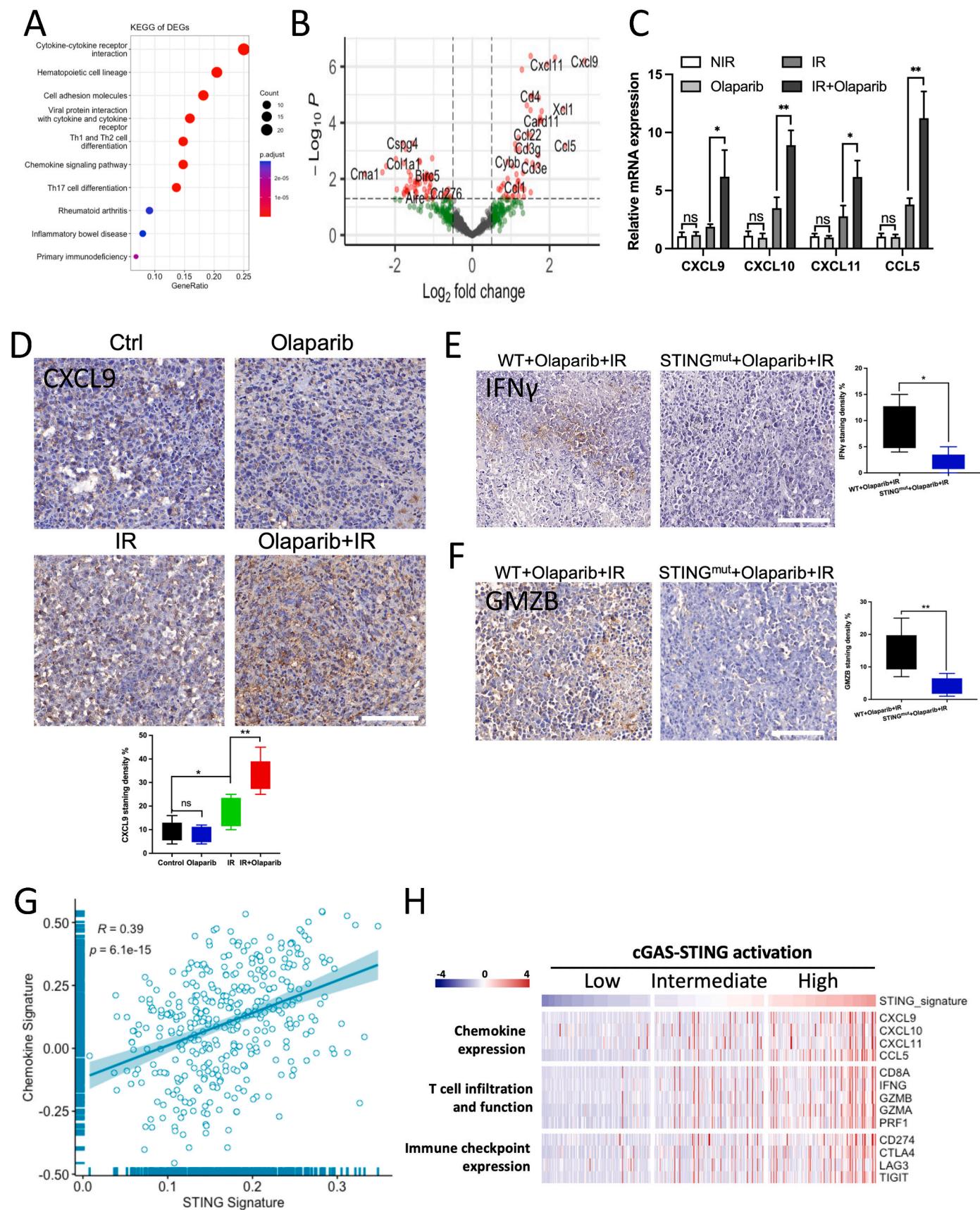
The absence of the abscopal effect in immune deficient mice indicates that the growth delay of abscopal tumors depends on a competent immune system. Consequently, we detected alterations in the tumor immune microenvironment at the abscopal sites. Following irradiation, we observed a significant increase in lymphocytes within the abscopal tumors (Fig. 3D). However, when comparing IR treatment alone to IR combined with olaparib, no significant difference in this increase was noted (Fig. 3D). Notably, the population of CD4 $^+$ T helper cells did not show significant changes (Supplemental Fig. 4). Conversely, CD8 $^+$ cytotoxic T cells were obviously increased in the combination group compared to the irradiation alone group (Fig. 3E). This upregulation of CD8 $^+$ cytotoxic T cells in the tumor microenvironment was confirmed through multiple-color staining (Fig. 3F). These findings suggest that the local immune microenvironment is responsible for immune surveillance of abscopal tumors.

The release of DAMPs can promote antigen cross-presentation. Moreover, ICD can lead to adaptive immune responses mediated by cytotoxic T lymphocytes, indicating the vital role of ICD in systemic immunosurveillance. To determine whether the infiltration of neoantigen-specific CD8 $^+$ T cells into abscopal tumors resulted from boosted spontaneous CD8 $^+$ T cell priming, we generated mice bearing bilateral subcutaneous H22-overexpressing ovalbumin (H22-OVA) tumors as a model. We observed a higher number of IFN γ spot-forming cells in the combination group compared to olaparib alone in abscopal tumors, indicating that olaparib potentiates T cell priming against tumor neoantigens (Fig. 3G). Collectively, these results suggest that ICD contributes to the restriction of abscopal tumors by inducing antitumor adaptive immune responses.

3.5. cGAS-STING signaling is required for radiotherapy-driven and olaparib-enhanced systemic anti-tumor effects

We next determined whether the cGAS-STING pathway mediates olaparib-induced radiosensitivity. To address this question, we utilized cGAS knockout mice deficient in the innate pathway to explore the impact of systemic innate immunity on radiotherapy-driven and olaparib-enhanced primary and abscopal responses. Wild type (WT) and cGAS knockout (KO) mice were subjected to the previously described treatment, which involved IR with or without olaparib. In this model, both the primary and abscopal tumors in cGAS KO mice showed accelerated growth (Fig. 4A and B). Although the synergistic effect of IR and olaparib was still evident in the primary tumors, this effect was greatly diminished in innate immunity-deficient mice (Fig. 4B). IFN β staining in the abscopal tumors revealed that the type I IFN was severely blunted in cGAS KO host mice (Supplemental Fig. 5). These results highlight the crucial role of the cGAS-STING-IFN β axis in abscopal tumor control.

Initiating the abscopal effect crucially depends on the generation of neoantigens [23]. Emerging evidence suggests that activation of cGAS-STING signaling is crucial to enhance antigen presentation and contributes to the priming and activation of T cells [28]. Thus, we determined neoantigen-specific CD8 $^+$ T cells generated in abscopal H22-OVA tumors. Higher levels of CD8 $^+$ T cells expressing the SIINFEKL MHC-I tetramer were detected in the IR and olaparib combination group compared to the IR alone group (Fig. 4C). In cGAS KO mice, the upregulation of tumor-infiltrating antigen-specific CD8 $^+$ T-cells in response to IR, with or without olaparib treatment, was abrogated. Consequently, only a minimal number of IFN γ + CD8 $^+$ T cells were detected in cGAS KO host mice (Fig. 4D). Furthermore, the substantial increase in CD44 $^{\text{medium}}$ CD62L $^{\text{low}}$ CD8 memory/effector T cells observed in the combination group within the abscopal tumors were also abolished by cGAS



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Fig. 5. The STING-dependent CXCL9/CXCL10/CXCL11/CCL5 chemokine axis drives immune surveillance of abscopal tumors. (A) KEGG analysis identified marked enhancement of pathways in a mouse model treated with combined STING agonist and PARP inhibitor therapy. (B) Volcano plot showing dysregulated genes in a mouse model treated with the combination of a STING agonist and PARP inhibitor. (C) mRNA levels of CXCL9, CXCL10, CXCL11, and CCL5 in abscopal H22 tumor tissues in mice treated with olaparib, IR and the combination. (D) Representative immunohistochemistry images and quantification of CXCL9 staining in abscopal H22 tumor tissues in wild type and STING mutated mice treated with olaparib, IR and the combination (scale bar: 100 μ m). (E) Representative immunohistochemistry images and quantification of IFN γ (E) and GMZB (F) staining in abscopal H22 tumor tissues in wild type and STING mutated mice treated with combination of olaparib and IR (scale bar: 100 μ m). (G) Correlation of STING signature (including cGAS, STING, TBK1, and IRF3) and Chemokine signature (CXCL9, CXCL10, CXCL11, and CCL5) in hepatocellular carcinoma tissues from TCGA dataset ($R = 0.39$, $P = 6.1^{-15}$). (H) Heat-map depicting the correlations of STING signature and chemokine expression, CD8 $^+$ T cell infiltration and function, and immune checkpoint expression. (* $P < .05$; ** $P < .01$; ns, no significance).

depletion (Fig. 4E). These results indicate that the activation of cGAS-STING signaling and type I IFN production potentiate T-cell priming and elicit immunological memory against tumor neoantigens.

3.6. Immune surveillance of abscopal tumors is driven by the STING-dependent CXCL9/CXCL10/CXCL11/CCL5 chemokine axis

To investigate how cGAS-STING signaling contributes to immune surveillance, we analyzed immune gene expression and immune-related pathways in a mouse model treated with combined STING agonist and PARP inhibitor therapy (GSE204858). NanoString immune gene expression analysis revealed that 46 genes were upregulated ($p < .05$ and fold change >2) out of 750 immune genes, while 53 genes were downregulated ($p < .05$ and fold change <0.5 , *Supplemental Fig. 6A*). KEGG analysis identified significant enhancement of several pathways, such as cytokine-cytokine receptor interaction, cell adhesion molecules, and chemokine signaling pathway, associated with CD8 $^+$ T cell adhesion in the combination of olaparib and STING agonist treatment compared to olaparib alone (Fig. 5A).

We observed that CXCL9, CXCL10, CXCL11, and CCL5 were the most significantly upregulated chemokines (Fig. 5B). Considering that CXCL9, CXCL10, CXCL11, and CCL5 are crucial chemokines for inducing antitumor immunity in response to type I IFN [29–31], we assessed their expression when RT and olaparib were coadministered in our mouse model. As expected, we observed increased mRNA levels of CXCL9, CXCL10, CXCL11, and CCL5 in the combination treatment group (Fig. 5C). Moreover, the enhanced secretion of CXCL9 was confirmed through immunohistochemistry staining (Fig. 5D). The activation of CD8 $^+$ T cells was also observed based on CD69 staining (*Supplemental Fig. 6B*). In the STING-mutated mouse model, however, there was almost no upregulation of IFN γ levels (Fig. 5E). Thus, the GMZB expression was significantly downregulated in the tumors of STING-deficiency host mice, indicating STING signaling activation enhanced granzyme release (Fig. 5F). To further dissect the influence of cGAS-STING signaling on CXCL9, CXCL10, CXCL11, and CCL5 expression, we treated human Huh7 cells with olaparib and 8 Gy irradiation. Consistent with these findings, the combined treatment increased mRNA levels of CXCL9, CXCL10, CXCL11, and CCL5 (*Supplemental Fig. 6C*). In contrast, the STING inhibitor H151 blocked the upregulation of CXCL9, CXCL10, CXCL11, and CCL5 induced by olaparib and irradiation (*Supplemental Fig. 6C*).

Given that cGAS-STING signaling activates CXCL9, CXCL10, CXCL11, and CCL5 chemokine signaling, which contributes to the abscopal effect, we examined the STING signature (cGAS, STING, TBK1, IRF3) and the Chemokine signature (CXCL9, CXCL10, CXCL11, CCL5) in TCGA-LIHC cohort. The STING signature showed a strong positive correlation with the expression of CXCL9, CXCL10, CXCL11, and CCL5 (Fig. 5G and H). In addition, the STING signature also exhibited a positive correlation with CD8 $^+$ T cell infiltration and function markers (CD8A, IFNG, GZMB, GMZA, PRF1) (Fig. 5H). These data indicate that the combination treatment of RT and Olaparib induces STING activation, enhancing CD8 $^+$ T cell reactivity toward abscopal tumors.

3.7. Olaparib impacts immune exhaustion following radioimmunotherapy

Recently, immunotherapy has revealed a new era for cancer therapy

in a variety of cancer types. However, few studies have revealed a positive response to immune checkpoint blockade in HCC [32]. Interestingly, we also observed that STING activation correlated with the expression of immune checkpoint markers (CD274, CTLA4, LAG3, TIGIT) (Fig. 5H). As olaparib contributes to the radiotherapy-induced immune response [33], we hypothesize that it may also improve the antitumor effect of radiotherapy combined with ICIs. H22 tumor-bearing mice were treated with olaparib daily before radiation, and anti-CTLA4 was administered twice a week beginning on the last day of radiation (Fig. 6A). To remove the impact of DNA damage induced by radiation in the tumor control, we monitored tumor growth and analyzed immune cell infiltration in the abscopal tumors. The addition of anti-CTLA4 significantly inhibited tumor growth, and most tumors disappeared at the end of the experiment. To analyze immune cell infiltration in the tumor microenvironment, tumors were resected one week after anti-CTLA4 administration. Previous results indicate that olaparib and IR cotreatment increased activated CD8 $^+$ T-cell infiltration. Next, we wanted to explore whether myeloid-derived suppressor cells (MDSCs) suppress immune cell infiltration. FACS analysis revealed that olaparib and IR alone had no significant impact on MDSCs (Fig. 6C). However, the combination of olaparib and IR significantly reduced the population of MDSCs. Notably, the triple therapy further enhanced the downregulation of MDSCs compared to IR with olaparib. MDSCs are known to suppress the functions of natural killer (NK) and T cells [34]. Therefore, we examined NK cell infiltration and found that NK cells were significantly upregulated in the triple therapy group (Fig. 6D). Furthermore, we examined exhausted T cells (CD45 $+$ CD3 $^+$ CD8 $+$ TIM3 $+$), which are associated with impaired anti-tumor immunity. Olaparib and IR alone did not have a significant impact on exhausted T cells. However, the combination of olaparib and IR decreased the levels of exhausted T cells, and this effect was further enhanced by anti-CTLA4 coadministration (Fig. 6E). Our results demonstrate that olaparib enhances immune cell infiltration and attenuates immune exhaustion following radioimmunotherapy (Fig. 7).

4. Discussion

Radiotherapy has achieved efficacious tumor control in locally advanced HCC patients, and SBRT has been evaluated as a safe and efficient approach for treating HCC [35]. However, administering curative doses might lead to liver injury, thus limiting the application of radiotherapy [27]. Thus, to reduce radiation-induced side effects and improve safety, various novel therapeutic agents are being explored in combination with radiotherapy to achieve better control of primary and abscopal tumors.

PARP inhibitors have been approved for the treatment of several cancers, such as ovarian, breast, pancreatic, and prostate cancers, with BRCA mutations. Patients with BRCA mutations exhibit replication stress and genomic instability [36], and they have shown significant benefits from PARP inhibitors [37]. To enhance the sensitivity of PARP inhibitors and overcome resistance, different combination strategies have been explored and have demonstrated favorable synergistic effects [38]. Combining with ionizing radiation has been shown to significantly increase DNA damage and induce cell death [39]. Mechanistically, olaparib traps the PARP complex at sites with irradiation-produced SSBs. This impairs DNA repair, leading ultimately to the generation of

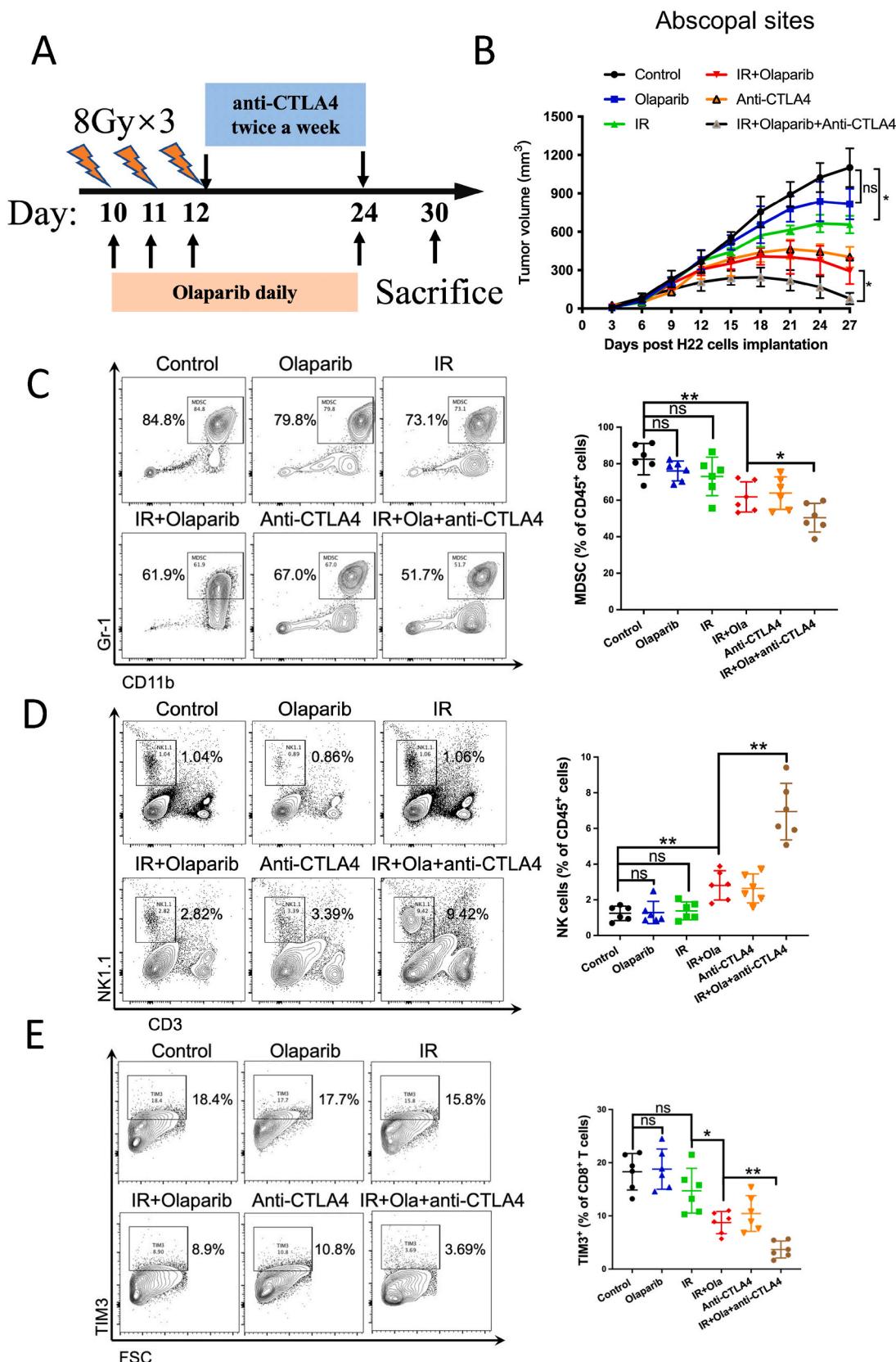


Fig. 6. Olaparib impacts immune exhaustion following radioimmunotherapy. (A) Schematic of olaparib, IR and anti-CTLA4 treatment in immunocompetent mice. (B) Growth curves from abscopal H22 tumors in immune competent mice treated with olaparib, IR, anti-CTLA4, and the combination. (C–E) FACS analysis of infiltrating MDSCs (C), NK cells (D), and exhausted T cells (E) from abscopal H22 tumors after treatment with olaparib, IR, anti-CTLA4, and the combination. (* $P < .05$; ** $P < .01$; ns, no significance).

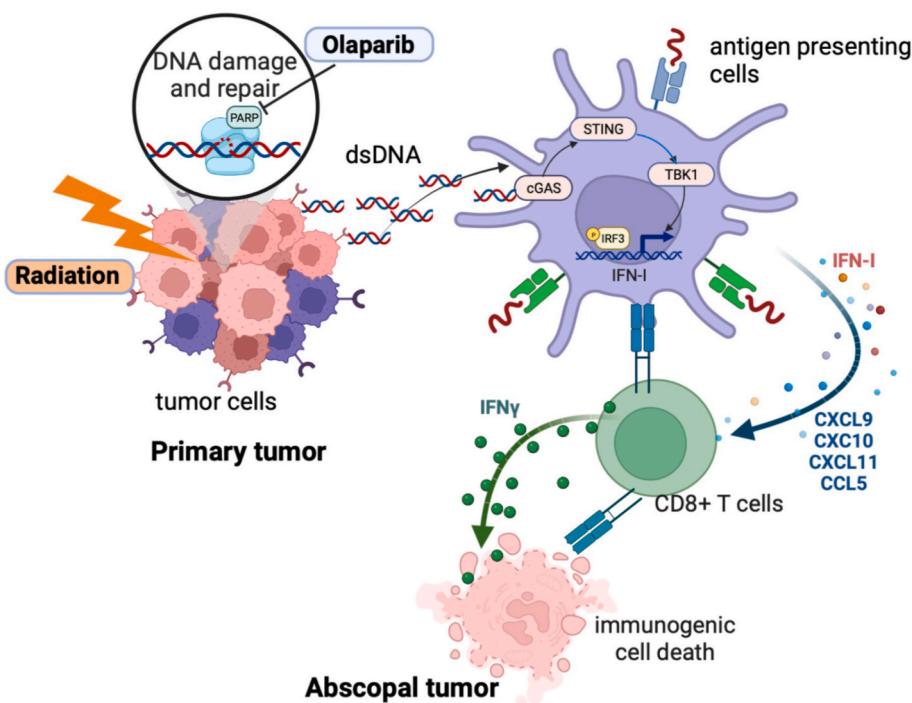


Fig. 7. Schematic of proposed mechanism of how cGAS-STING-dependent innate immune response contributes to radiation-induced and olaparib-enhanced systemic anti-tumor effects.

DSBs. Following radiation-caused DNA damage, BRCA1 is exported from the nucleus to the cytoplasm [40], making HR-proficient tumor cells susceptible to PARP inhibitors [41]. In vitro studies also found that low-dose irradiation activates PARP activity, enhancing the efficacy of PARP1 inhibitors [42]. In a preclinical model, PARP inhibitor resistance due to BRCA1-independent HR restoration can be reversed by radiotherapy [43]. In our study, we observed that a low concentration of olaparib resulted in minimal damage to DNA but sensitized HCC cells with germline BRCA1 competence to irradiation, both *in vitro* and *in vivo*. Our results indicate that olaparib sensitizes HCC cells to irradiation by impeding the DNA repair pathway.

The abscopal effect, characterized by an antitumor immune response occurring outside the irradiated site, is dependent on the activation of the immune system [24]. Previous studies have demonstrated that the antitumor response observed in abscopal tumors is absent in immunodeficient mice [44]. The cGAS-STING pathway plays a crucial role in antitumor immunity by triggering the production of type I IFN and facilitating T cell priming. Our recent study has identified that irradiation activates the cGAS-STING innate immune pathway by inducing dsDNA production. We confirmed the activation of the cGAS-STING-IRF3 signaling pathway within irradiated tumors [45]. Moreover, it has been found that the activation of cGAS-STING induced by PARP inhibitors is dependent on the trapping of PARP1 [46]. The STING pathway has been reported to remodel the tumor immune microenvironment and stimulate immunogenic cell death [19]. Consequently, we observed more pronounced tumor regression in immune proficient mice compared to immune deficient mice in irradiated tumors. Emerging evidence suggests that the production of type I IFN attracts cytotoxic T cells to the tumor site, triggering an adaptive immune response [47]. In BRCA-deficient ovarian and triple-negative breast cancers, treatment with PARP inhibitors increases intratumoral CD8⁺ T cells due to activation of the cGAS-STING pathway and is more pronounced in HR-deficient cells [48,49]. Consistently, we found that IFN β expression was upregulated in abscopal tumors. Immune cell infiltration analysis revealed higher levels of CD8⁺ T cells, especially for the populations of IFN γ +, neoantigen specific, and memory/effectector T

cells, in the group receiving the combination of IR and olaparib. However, the antitumor effect was abrogated with cGAS depletion. Notably, our study also revealed that the combination of olaparib and IR significantly reduced the number of immunosuppressive cells and improved the efficacy of anti-CTLA4 treatment in preventing immune escape. These data provide fundamental mechanistic insights into the role of the cGAS-STING pathway in mediating systemic anti-tumor effects.

Chemokines, known for their thermotactic properties, are crucial for antitumor immunity [50]. Specifically, C-X-C motif chemokine ligand 10 (CXCL10) and C-C motif chemokine ligand (CCL5) have been shown to recruit CD8⁺ T cells to tumors and stimulate the production of Granzyme B production, thereby enhancing their antitumor activities [51]. CXCL9, CXCL10 and CXCL11 are well-known ligands of CXCR3 in directing the migratory properties of CD8⁺ T cells and NK cells in the context of antitumor immunity and inflammatory autoimmunity [52]. The genetic deletion of CCL5 has been identified as a potential approach to attenuate T cell inflammation and has been served as a target for autoimmune diseases such as Multiple Sclerosis, Rheumatoid Arthritis, and Inflammatory Bowel Disease [53]. In DNA mismatch repair (dMMR) colorectal cancers, the selective recruitment of systemic CD8⁺ T cells responding to the abundant neoantigens strictly depends on the upregulation of cGAS-STING-mediated CCL5 and CXCL10 [53]. In addition, CXCL9, CXCL10, and CXCL11 are required for antitumor immune responses following immune checkpoint blockade [54]. Depletion of CXCL9, CXCL10, and CXCL11 ablated CD8⁺ T-cell infiltration and the antitumor response to dual immune checkpoint blockade. Therefore, the activation of the cGAS-STING-CXCL9/10/11 axis enhances the therapeutic efficacy of anti-CTLA4 treatment.

In summary, the combined biological effects of PARP inhibition and RT not only focus on DNA damage repair but also activate the cGAS-STING innate immune pathway. This leads to the recruitment of cytotoxic T cell and helps prevent immune exhaustion. Our results suggest that PARP inhibition may be a promising strategy to improve the tumor response to RT, particularly in abscopal tumors. This provides a rationale for further investigating the combination of PARP inhibition and radioimmunotherapy in advanced HCC.

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Data availability statement

All data are available upon request.

CRediT authorship contribution statement

Genwen Chen: Writing – original draft, Funding acquisition, Formal analysis, Data curation. **Danxue Zheng:** Software, Investigation, Data curation. **Yimin Zhou:** Formal analysis, Investigation, Validation, Writing – review & editing. **Shisuo Du:** Supervision, Project administration, Methodology, Conceptualization. **Zhaochong Zeng:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2023.216507>.

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