



Original Articles

Talazoparib engages innate immune activation via PARP trapping–dependent cGAS/STING activation in Ewing Sarcoma

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ARTICLE INFO

Keywords:

Ewing sarcoma
Talazoparib
cGAS/STING pathway
PARP inhibitors resistance
Immune response

ABSTRACT

Ewing sarcoma (EwS) shows a limited clinical response to poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi), despite promising preclinical data. In this study, we compared five PARPi with different PARP-trapping capacities in PDX-derived cell lines and mouse models. Talazoparib, the strongest PARP-trapping agent, showed markedly greater efficacy than olaparib or veliparib. It triggered extensive DNA damage, micronuclei formation, and activation of the cyclic GMP-AMP synthase (cGAS)/stimulator of interferon genes (STING) pathway, leading to robust type I interferon and pro-inflammatory cytokine release, an effect not seen in osteosarcoma. *In vivo*, talazoparib also reshaped the tumor microenvironment, increasing macrophage infiltration and reducing tumor growth. *In vitro*, conditioned media from treated EwS cells promoted M0-like macrophage polarization towards an inflammatory M1-like status. These immunostimulatory effects were initiated by tumor-derived interferons and were absent in talazoparib-resistant and olaparib-treated EwS cells, underscoring the importance of the PARP trapping activity of PARPi rather than catalytic inhibition. Combination of talazoparib with exogenous 2'-3'-cyclic GMP-AMP (cGAMP) does not further increase phagocytosis of EwS cells when co-cultured with macrophages, and no additive effects were observed under the tested conditions. Thus, talazoparib is a potent cytotoxic agent with innate immune activation/macrophage-mediated effects, prompting further clinical evaluation in this tumor type.

1. Introduction

Ewing sarcoma (EwS) is an aggressive pediatric cancer of the bone and soft tissue, that is driven predominantly by the chromosomal translocation t (11; 22) (q24; q12), which generates the oncogenic fusion protein EWSR1::FLI1 (also named EWS::FLI1) [1]. EWS::FLI1 reshapes the epigenetic landscape of EwS, functioning as a master regulator of chromatin states that can either activate or repress enhancer activity [2]. This dual activity contributes to tumor aggressiveness and a

high level of heterogeneity in the phenotype, differentiation, and metastatic potential. EWS::FLI1 also affects DNA repair and regulates the chemosensitivity of EwS cells. It promotes the accumulation of R-loops, three-stranded nucleic acid structures that threaten genome stability and disrupts homologous recombination (HR) DNA repair by sequestering the breast cancer gene (BRCA)1 [3]. Thus, despite robust BRCA1 expression and absence of mutations [4–6], EwS is considered a “BRCA-deficient-like” tumor because BRCA1 is trapped in transcription complexes and unable to respond to DNA damage. This functional

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<https://doi.org/10.1016/j.canlet.2026.218704>

Received 21 January 2026; Received in revised form 26 June 2026; Accepted 1 July 2026

Available online 2 July 2026

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impairment of BRCA1 underlies the marked sensitivity of EwS cells to DNA-damaging agents such as etoposide and to poly (ADP-ribose) polymerase (PARP) inhibitors (PARPi) [3], which are established therapies for BRCA1/2-mutated cancers with homologous recombination deficiency (HRD), and for tumors with “BRCAness”, molecular alterations that mimic BRCA loss [7]. Preclinical studies have consistently demonstrated strong sensitivity of EwS to PARPi, particularly olaparib [8,9]. However, clinical trials have demonstrated only minimal benefits, largely attributable to the rapid development of resistance [10–14]. This gap highlights the need for a deeper understanding of the mechanisms underlying PARPi activity and resistance in this tumor context.

PARP1 is the most extensively studied member of the PARP family and plays a central role in DNA repair. Upon binding to DNA breaks, its catalytic activity is triggered, leading to chromatin relaxation and recruitment of PAR-binding proteins that coordinate the downstream repair processes. Inhibition of PARP1 with small-molecule compounds, most of which act by competing with NAD⁺ at the catalytic site [15], results in impairment of DNA repair and accumulation of DNA lesions. Depending on the compound, this inhibition can also promote the trapping of PARP–DNA complexes at damage sites, further exacerbating genomic instability. In addition, PARP1 and its inhibitors are increasingly being recognized for their roles beyond DNA repair, including transcriptional regulation, ribosome biogenesis, programmed cell death, and tumor immunity [15,16]. In particular, the relationship between PARP inhibition and tumor immunity remains complex and poorly understood. R-loops and DNA damage can generate micronuclei with fragile nuclear envelopes prone to rupture, releasing DNA into the cytoplasm and triggering the activation of the cyclic GMP-AMP synthase (cGAS)/stimulator of interferon genes (STING) signaling pathway [17, 18] as well as phosphorylation and activation of the signal transducer and activator of transcription (STAT)1 [19]. This signaling cascade induces the production of type I interferons (IFNs) and pro-inflammatory cytokines, which in turn promote the recruitment of natural killer (NK) cells, cytotoxic T cells, and macrophages [20–22]. However, tumor cells rarely produce type I IFNs spontaneously [23], suggesting that STING-mediated interferon signaling may be suppressed by tumor cells to facilitate their evasion from immunosurveillance. EwS is typically a “cold” tumor with low mutational burden, poor T-cell infiltration [24, 25], and enrichment of myeloid-cell infiltration, including immunosuppressive M0- and M2-like tumor-associated macrophages (TAMs) [26–28]. A higher prevalence of M0- and M2-like macrophages in EwS has been associated with a poorer prognosis [26,29], supporting strategies aimed at reprogramming TAMs toward an inflammatory M1-like phenotype. In this study, we dissected the role of PARPi in connecting DNA damage and innate immune activation in EwS. We investigated whether PARPi with varying trapping activities can induce DNA damage sufficient to activate the cGAS/STING pathway. We showed that talazoparib, but not other PARPi, robustly activated cGAS/STING, driving type I IFNs signaling and efficiently reprogrammed intratumoral macrophages from an immunosuppressive M0/M2-like state toward an anti-tumor M1-like phenotype. Our findings show that talazoparib not only targets DNA repair but also shapes the EwS tumor microenvironment (TME), underscoring that impaired cGAS/STING signaling may cause resistance to talazoparib.

2. Material and methods

2.1. Sex as a biological variable

Sex was not considered a biological variable in our study. Our study exclusively examined female mice because the results of PARPi efficacy were not sex dependent.

2.2. EwS cell lines

We used the following human patient-derived EwS cell lines: TC-71

(DSMZ, Catalog no. ACC-516); TC-EGFP derived from TC-71 cells transfected with the pEGFP-N1 plasmid (Clontech) to stably express Enhanced Green Fluorescent Protein (EGFP) [30]; A673 cells (CRL-1598™, ATCC); patient-derived xenograft (PDX)-EW#7-C, PDX-EW#5-C, and PDX-EW#2-C, which are PDX-derived cell lines established in our laboratory and previously characterized [31,32]. The osteosarcoma U-2 OS cell line (HTB-96™, ATCC) was used for comparison. From A673 cell line, we developed talazoparib-resistant variants (namely A673 RES-TALA) by continuous cell exposure to stepwise increasing concentration of talazoparib (BMN 673) (#S7048, Selleckchem) (IC₅₀ = 0.05 μM) up to the dose of 10 μM (cells resulting to be 200-fold more resistant to the drug than parental cells). To evaluate the impact of EWS::FLI1 on cGAS/STING pathway activation, we used ASP14, a stable cell line generated from A673 EwS cells transfected with doxycycline-inducible shRNA targeting EWS::FLI1 [33], and cultured in complete medium supplemented with 20 μg/ml blasticidin (#R21001, Invitrogen) and 50 μg/ml zeocin (#R250-01, Invitrogen). Cells were exposed to doxycycline hyclate (Supplemental Table 1) at a final concentration of 1 μg/mL for 48 h to induce the silencing of EWS::FLI1 and then exposed to talazoparib at the concentration of 0.05 μM (IC₅₀) for further 72h. To evaluate the effect of STING, transient silencing was performed using short interfering RNA (siRNA) SMARTpool siGENOME Human TMEM173 siRNA (#M-024333-00-0020, GE Healthcare Dharmacon). As a control, siGENOME non-targeting siRNA Control Pools were used (#D-001206-13-50, GE Healthcare Dharmacon). A673 cells (5 × 10⁵) were transfected with siRNAs (40 nmol/L) for 96 h, using siPORT NeoFX (#AM4510, Invitrogen) in accordance with the manufacturer's protocol, ± 72 h of talazoparib treatment. Alternatively, A673 cells (5 × 10⁵) were pretreated with STING inhibitor H-151 (#HY-112693, MedChemExpress) at the concentration of 3 μM for 2 h and then exposed to talazoparib at the concentration of 0.05 μM (IC₅₀) for further 72 h. STING inhibitor H-151 was refreshed every 24 h.

To avoid cross-contamination between cell lines and outgrowth of faster-growing clones in long-term cultures, all cell lines were kept in liquid nitrogen until use. All cell lines were maintained in Iscove's modified Dulbecco's medium (IMDM) (#ECB2072L, EuroClone®) supplemented with 10% Fetal Bovine Serum (FBS) (#ECS0180L, EuroClone®), 100 U/mL penicillin, and 100 mg/mL streptomycin (#P0781–100, Merck Millipore), and incubated at 37°C in a humidified atmosphere containing 5% CO₂ for approximately 8–12 *in vitro* passages (corresponding to 2–3 months) before discarding. When necessary, replicates were started from the same batch of frozen vials. Cells were regularly tested for mycoplasma contamination before starting the experiments and at the end with the MycoAlert Mycoplasma Detection Kit (#LT07–418, Lonza) and authenticated by short tandem repeat PCR analysis using a PowerPlex ESX Fast System kit (CLA service by Eurofins Genomics) (last control July–August 2023).

2.3. In vitro treatments with PARPi

EwS cells were seeded into 96-well plates (40,000 cells/well for PDXs-derived cell lines and 2,500 cells/well for TC-71 e A673) in standard medium and cultured for 24 h before being treated with talazoparib (BMN 673) (#S7048), stenoparib (E7449) (#S8419), pamiparib (BGB-290) (#S8592), olaparib (AZD2281, Ku-0059436) (#S1060) and veliparib (ABT-888) (#S1004) (Supplemental Table 1) for 72 h. Stock solutions of the drugs, all purchased from Selleckchem, were prepared and stored according to the manufacturer's instructions. Where applicable, the cells were also treated with dimethyl sulfoxide (DMSO) (#D2650, Sigma) as a vehicle control. Drug sensitivity was assessed using the TACS® MTT Cell Proliferation Assay kit (#4890-25-K, R&D Systems), according to the manufacturer's instructions.

2.4. Human macrophages preparation and in vitro studies

To study macrophage function, we used THP-1 (TIB-202™, ATCC), a

monocyte cell line from an acute monocytic leukemia patient. To differentiate into macrophages (M0-like), THP-1 cells were plated in 6-well plates (6×10^5 cells/well) and exposed to 150 nM phorbol 12-myristate 13-acetate (PMA) (#16561-29-8, Selleckchem) [34] (Supplemental Table 1) for 24 h. In addition, we used human monocytes isolated from Peripheral Blood Mononuclear Cells (PBMCs) (#CC-2702, Lonza) by positive magnetic separation using CD14 immunomagnetic beads (#130-050-201, Miltenyi), followed by MACS LS column separation (#130-042-401, Miltenyi), according to the manufacturer's recommendations. 1×10^5 CD14⁺ monocytes were plated in 60 mm petri dishes, maintained in IMDM (#ECB2072L, EuroClone) supplemented with 10% FBS (#ECS0180L, EuroClone), 100U/mL penicillin, and 100 mg/mL streptomycin (#P0781-100, Merck Millipore), and incubated at 37°C in 5% CO₂ for 7 days to obtain M0-like macrophages.

M0-like macrophages were exposed (15 min–6 h) to cell supernatants collected from A673 cells treated or not with talazoparib and from A673_RES-TALA before being analyzed by quantitative PCR (qPCR) and western blotting. As positive and negative controls, M0-like macrophages were also exposed to supernatants collected from A673 cells treated with recombinant human (rHu) IFN α (0.3 ng/ml) (#130-093-874, Miltenyi) + rHu IFN β (2 ng/ml) (#130-107-889, Miltenyi) or to rHu anti-IFN α (10 ng/ml) (#hifna-mab1-02, Invivogen) + rHu anti-IFN β (10 ng/ml) (#mabg2-hifnb-3, Invivogen), respectively (Supplemental Table 1).

To test the cytotoxic effect of talazoparib in co-culture conditions with M0-like macrophages, 5,000 CD14⁺-derived M0-like macrophages were seeded in a 96-well plate. After 5 days, A673 cells (2,500 cells) or PDX-EW#5-C (40,000 cells) were added to the pre-coated M0 macrophage plates. Monocultures of M0-like macrophages and EwS cells were used for comparison. After 24 h, talazoparib was added to the medium at IC₅₀. Cell growth was measured after 72 h of treatment using resazurin-based, Alamar Blue™ (DAL1100, Invitrogen) according to the manufacturer's instructions. Briefly, 10 μ L of Alamar Blue™ solution was added to each well, and the plates were incubated for 3 h at 37°C in a humidified 5% CO₂. After incubation, 100 μ L of the solution was transferred to a 96-well plate, and the fluorescence signals were measured at an excitation wavelength of 530–560 nm and an emission wavelength of 590 nm using Infinite M200 photometer (Tecan Group Ltd).

To evaluate the influence of talazoparib on the migration ability of THP-1-derived M0-like macrophages, we performed a motility assay using transwell chambers with 8 μ m pores and polycarbonate filters (3422, Corning Costar). Supernatants from untreated and talazoparib-treated A673 cells, including A673_RES-TALA, served as chemo-attractants in the lower chamber. 1×10^5 macrophages were seeded into the upper chamber. After 2 h, the migrated cells were fixed with methanol, stained with Giemsa (32884, Riedel De Haen, Sigma), and counted.

2.5. *In vitro* phagocytosis assays

5×10^5 TC-EGFP cells were seeded on M0-like macrophages treated with A673 supernatants as described in “Human macrophages preparation and *in vitro* studies”. After 6 h, the macrophages were washed with phosphate-buffered saline (PBS) to remove tumor cells and analyzed by phagocytosis. Macrophages were fixed in 4% paraformaldehyde (PFA) (P6148, Sigma) for 10 min at room temperature and then imaged using a Nikon 90i Eclipse fluorescence microscope (20x NA 0.5, Refractive Index: 1, plan Apo objective) (Nikon Instruments, Italy). Images (1280 x 960 pixels) were captured using a Nikon DS-U2/L2 camera. Transmission and fluorescence images were merged using the NIS Elements A.R. 3.10 software. The phagocytic index was defined as the number of phagocytosed EwS cells per at least 100 macrophages and expressed as a percentage of macrophages with engulfed cells. Cytotoxicity of macrophages against EwS cells was measured by viable cell counting with Trypan Blue dye exclusion (T8154, Sigma). At least three

experiments were performed using macrophages.

2.6. Immunofluorescence

Immunofluorescence was performed on adherent cells grown on coverslips, fixed in cold methanol for 7 min at –20°C or 4% PFA (P6148, Sigma) for 10 min and permeabilized with 0.15% Triton X-100 (#T8787, Sigma) in PBS, blocked with 4% bovine serum albumin (BSA) (#A4503, Sigma)/PBS for 1 h at room temperature and incubated with anti- γ H2AX antibody (1:100) (#9718, Cell Signaling Technology) or anti-cGAS (1:100), (#15102, Cell Signaling Technology). Swine Anti-Rabbit Immunoglobulins Polyclonal Antibody, FITC Conjugated was used as the secondary antibody (1:100) (F005401, Agilent) (Supplemental Table 1). Nuclei were stained with bisbenzimidazole Hoechst 33258 (0.5 μ g/ml) (H3569, Thermo Fisher Scientific). Cell fluorescence for γ H2AX and micronuclei was evaluated using a Nikon Eclipse 90i microscope (Nikon Instruments, Italy). For quantification purposes, γ H2AX spots and micronuclei were quantified from at least 10 random fields (~500 cells), expressed as the percentage of γ H2AX positive cells/field and percentage of cells/field with micronuclei, respectively. Images were acquired and processed using the NIS Elements A.R. 3.10 software. Immunofluorescence images of cGAS localization near micronuclei were acquired with a Nikon A1plus laser-scanning confocal microscope mounted on a Nikon Ni-E microscope and equipped with a Plan Apo λ 60 $\times$ oil-immersion objective (NA 1.4, refractive index 1.515). DAPI and Alexa Fluor 488 signals were collected using 405/488 nm dichroic optics with 452/45 nm and 525/50 nm emission filters, respectively. Excitation was provided by 402nm and 487.2 nm lasers at 1.8% and 7.7% power, respectively. Images were acquired with a resonant scanner and GaAsP detectors, using a 26.82 μ m pinhole, scan zoom 6.0, scan speed 7.5, and 4 $\times$ line averaging. Detector settings were kept constant across acquisitions. Z-stacks were collected with the Ni-E ZDrive over 51 optical sections at 0.1 μ m intervals. Micronuclei were identified based on nuclear morphology, and cGAS fluorescence was quantified in the Alexa Fluor 488 channel using NIS-Elements. For each micronucleus, a circular region of interest (ROI) of 18.74 μ m² was manually defined to include both the micronuclei and the immediately surrounding area. Mean fluorescence intensity of the green signal within the ROI was quantified using the Measure Field and ROI function with the Measure Current Frame option. The analysis was repeated for 10 micronuclei per condition.

2.7. Western blotting

1×10^6 EwS cells/well were cultured for 24 h before being treated with PARPi for 72 h or with doxycycline hyclate (D9891, Sigma) (Supplemental Table 1) for 48 h. 1×10^6 human M0-like macrophages were obtained and cultured as described in the “Human Macrophages Preparation and *in vitro* Studies” section, and were treated for 15 min and 1 h with conditioned media (CM).

Cells were lysed with RIPA buffer (#89900, Thermo Fisher Scientific) containing Pierce™ Protease Inhibitor Mini Tablets, EDTA-free (#A32955, Thermo Fisher Scientific) and Pierce™ Phosphatase Inhibitor Mini Tablets (#A32957, Thermo Fisher Scientific). Membranes were incubated overnight with the following primary antibodies: anti-PARP (1:5000), (#9542, Cell Signaling Technology), anti- γ H2AX (1:1000) (#9718S, Cell Signaling Technology), anti-cGAS (1:2000), (#15102, Cell Signaling Technology), anti-pSTING (1:2000) (#19781, Cell Signaling Technology), anti-STING (1:2000) (#13647, Cell Signaling Technology), anti-pIRF3 (1:1000) (#37829, Cell Signaling Technology), anti-IRF3 (1:1000) (#11904, Cell Signaling Technology), anti-pNFKB p65 (1:1000) (#3031, Cell Signaling Technology), anti-NFKB p65 (1:1000) (#8242, Cell Signaling Technology), anti-FLI1 (1:1000) (#sc-356, Santa Cruz Biotechnology), anti-pSTAT1 (1:1000) (#7649, Cell Signaling Technology), anti-STAT1 (1:1000) (#06-501, Merck Millipore), anti-cFOS (1:1000) (#2250, Cell Signaling Technology), anti-

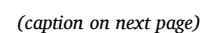


Fig. 1. Talazoparib induces higher cytotoxicity and DNA damage in EwS cells compared to other PARPi. (A) PARPi efficacy correlates with PARP-trapping potency. Graphical representation of PARPi IC₅₀ values in EwS cells (Supplemental Fig. 1B, mean $\pm$ SE of three independent experiments) obtained after 72 h of treatment. (B) Western blotting of PARP1 cleavage and DNA damage measured by γ H2AX induction. A673 and PDX-EW#5-C cells were exposed to IC₃₀ and IC₅₀ doses of PARPi for 72 h (talazoparib: 0.01 μ M–0.05 μ M and 0.003 μ M–0.01 μ M, respectively; stenoparib: 3 μ M–10 μ M; pamiparib: 1 μ M–3 μ M; olaparib: 3 μ M–10 μ M and 1 μ M–3 μ M, respectively; veliparib: 20 μ M–30 μ M and 10 μ M–20 μ M, respectively). Three independent biological replicates were performed. One representative immunoblot is shown. (C) Immunofluorescent evaluation of DNA damage by γ H2AX induction in A673 and PDX-EW#5-C cells treated with the corresponding IC₅₀ dose of talazoparib (0.05 μ M and 0.01 μ M, respectively) for 48 h. Three independent biological experiments were performed. Images and data from one representative experiment are shown. *Left*, representative digital images of γ H2AX positive cells (scale bar, 50 μ m); *right*, histograms show the mean percentage $\pm$ SE of positive cells counted in at least 10 different, random fields (dots). ****p < 0.0001, Mann-Whitney test. (D) Immunofluorescent evaluation of micronuclei (Hoechst 33258 staining) and cGAS expression in A673 and PDX-EW#5-C cells treated with the corresponding IC₅₀ dose of talazoparib (0.05 μ M and 0.01 μ M, respectively) for 48 h. Three independent biological experiments were performed. Images and data from one representative experiment are shown. *Left, top*, representative digital images of micronuclei (arrows) are shown (scale bar, 10 μ m). *Left, bottom*, histograms show the mean percentage $\pm$ SE of micronucleated cells quantified in at least 10 random fields (dots). **p < 0.01; ****p < 0.0001, Mann-Whitney test. *Right, top*, representative volume rendering of Z-scan confocal images showing the immunofluorescent signal for cGAS (green) and Hoechst 33258 nuclear counterstaining (blue) in A673 and PDX-EW#5-C cells, treated with the corresponding IC₅₀ dose of talazoparib (0.05 μ M and 0.01 μ M, respectively) for 72 h. *Right, bottom*, quantification of the cGAS fluorescence intensity quantified within the area including the micronucleus and the immediately surrounding region. The ROI of 18.74 μ m² was manually defined using the Measure Field and ROI function with the Measure Current Frame option. Data represent cGAS mean intensity $\pm$ SE of micronuclei (n = 10). ***p < 0.001; ****p < 0.0001, Mann-Whitney test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cJUN (1:1000) (#9165, Cell Signaling Technology) and anti-GAPDH (1:10000) (#2118, Cell Signaling Technology) (Supplemental Table 1). Anti-rabbit IgG HRP was used as the secondary antibody (1:10000) (NA9340 GE Healthcare), and bands were visualized using SuperSignal West Pico PLUS Chemiluminescent Substrate (34580, Thermo Fisher Scientific). All markers were quantified against GAPDH based on three replicates and reported as ratio of adjusted volume optical density (OD/mm²) by Image Lab v5.1 (Biorad).

2.8. RNA extraction and qPCR

1 $\times$ 10⁶ EwS cells/well were cultured for 24 h before treatment with PARPi for 72 h or with doxycycline hyclate (D9891, Sigma) (Supplemental Table 1) for 48 h.

Total RNAs from EwS cells and M0-like macrophages cultured under different conditions (*in vitro* treatments) was extracted using TRIzol Reagent (15596018, Invitrogen). RNA quality and quantity were assessed using NanoDrop (Thermo Fisher Scientific) analysis. Total RNA from each sample was reverse transcribed into complementary DNA (cDNA) using a High-Capacity cDNA Reverse Transcription Kit (4368813, Life Technologies) according to the manufacturer's protocol. qPCR was performed on a ViiA7 system (Thermo Fisher Scientific). The forward and reverse primers for PCR amplification of the human genes encoding for: *EWS::FLI1*, *PARP1*, *IFN α* , *IFN β* , *IL6*, *TNF α* , *STING*, *CD80*, *CD86* and for the reference gene *GAPDH* are described in Supplemental Table 1. The expression levels of the target genes (RQ) were normalized to that of *GAPDH* and expressed as: $2^{-\Delta\Delta Ct}$, where $\Delta Ct = Ct \text{ target genes} - Ct \text{ GAPDH}$ and $\Delta\Delta Ct = \Delta Ct \text{ sample} - \Delta Ct \text{ calibrator}$ (untreated cells at each time point) [35].

RT² Profiler™ PCR Array Human Cancer Inflammation & Immunity Crosstalk (#PAHS-181Z, Qiagen) was also used. The CT values were normalized based on the Manual Selection of reference genes (*ACTB*). The relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$. Data analysis was performed according to the QIAGEN web portal GeneGlobe software (<http://www.qiagen.com/geneglobe>).

2.9. Cytokine ELISA

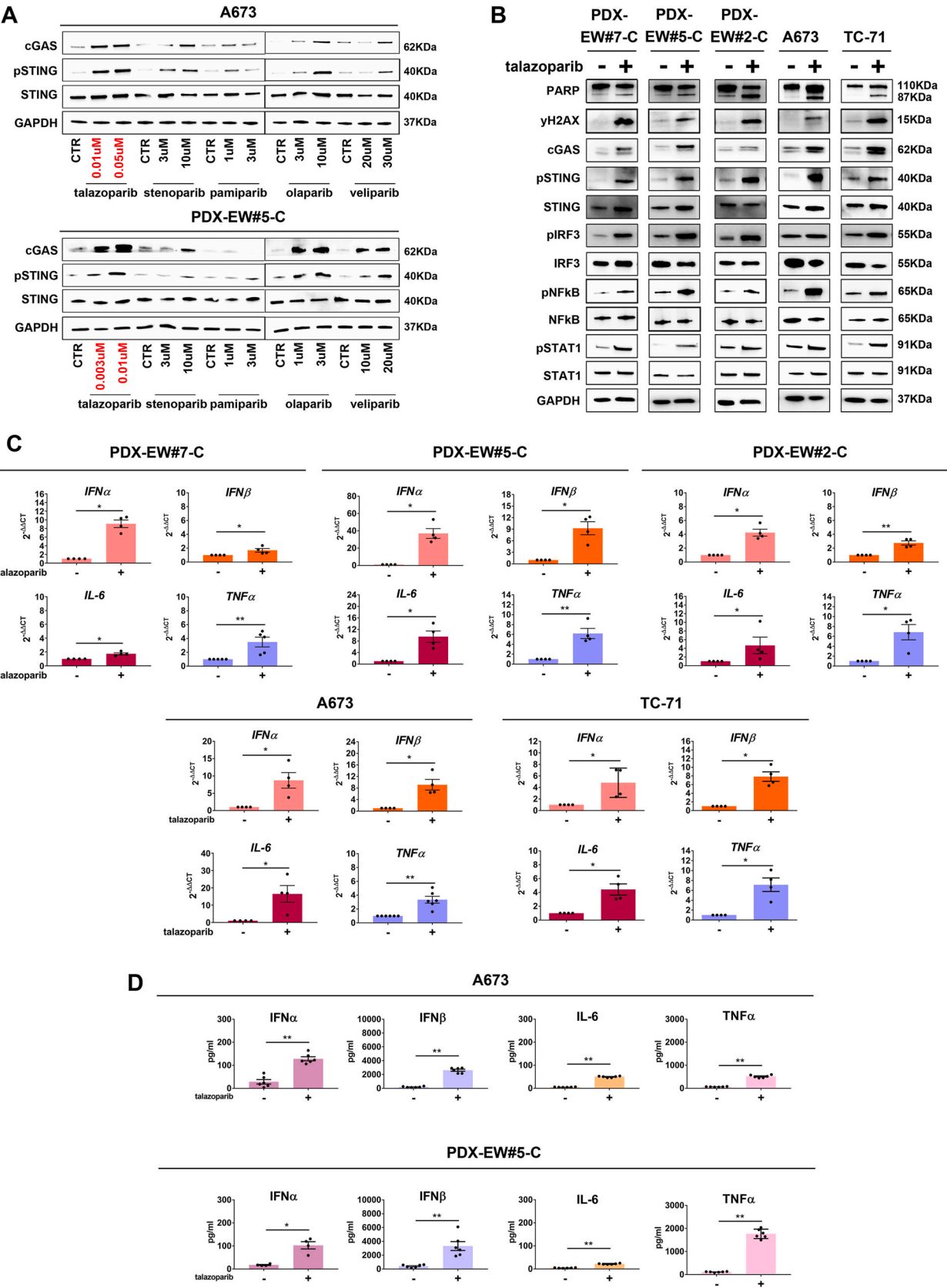
The concentrations of IFN α , IFN β , IL6 and TNF α in the supernatants of A673 cells treated or untreated with talazoparib (96h) were detected using ELISA kits for human IFN- α (#41100-1, Biotechne), human IFN- β (#41410-1, Biotechne), human IL-6 (#D6050B, Biotechne) and human TNF- α (#DTA00D, Biotechne) (Supplemental Table 1) following the manufacturer's instructions. Data are expressed as pg/mL versus control.

2.10. In vivo studies

BALB/c Rag2^{-/-};Il2rg^{-/-} breeders were kindly provided by the Central Institute for Experimental Animals [36]. Mice were bred in the Animal Care Facility of the Laboratory of Immunology and Biology of Metastasis, (P-LL). To support the 3Rs principle by reducing the number of unused animals, we included female immunodeficient BALB/c Rag2^{-/-};Il2rg^{-/-} mice aged 14–38 weeks. Mice within this age range are considered equivalent to young adult humans, and age has been shown not to affect tumor growth in immunodeficient strains [37,38]. Animals were equally distributed across experimental groups. Mice received subcutaneous (s.c.) injection of 5 $\times$ 10⁵ A673, A673_RES-TALA, or 2 $\times$ 10⁶ PDX-EW#5-C cells. Starting from day +6 for A673 and A673_RES-TALA, or from day +16 for PDX-EW#5-C after cell injection, when tumor diameters were measurable, around 5 mm (tumor volume of approximately 40 mm³), the animals were randomized into two groups. At this stage, these EwS models are considered to have established, progressively growing tumors that do not undergo spontaneous regression and typically reach the humane endpoint for tumor size within three weeks [39,40]. The treated group received daily per os (p.o.) oral administration of talazoparib 0.33 mg/kg in DMSO (5%) (D8418, Sigma) - PEG300 (40%) (202371, Merck) - Tween 80 (5%) (P6474, Sigma) - PBS (50%). The control group received p.o. vehicle only. The mice were treated once a day for five days per week for three weeks. Tumor size was measured with calipers. Considering that tumors that grow subcutaneously on the outer side of a hind leg can resemble a hemisphere, tumor volumes were calculated according to the formula of the hemisphere $(4/3\pi r^3)/2$ where r was calculated as the geometric mean of two perpendicular diameters/2. Mouse body weight and tumor volume were measured at least once a week. The experimental humane endpoint was a maximum tumor volume of 2.5 cm³ and all mice were sacrificed as soon as the experimental group (usually vehicle controls) overcame this threshold. To perform a temporal resolution analysis of the *in vivo* TME, six additional mice received s.c. injection of 5 $\times$ 10⁵ A673 cells, starting from day +6 from cell injection, mice were randomized into control and treated group and were sacrificed after 10 days of treatment. At necropsy, the tumors were collected and stored for histopathological and molecular analyses to evaluate the effects of talazoparib on tumor growth and the immune phenotype of tumor cells.

2.11. Immunohistochemistry

Serial 4 μ m-thick paraffin sections from formalin-fixed, paraffin-embedded xenografts, obtained from the *in vivo* experiments, were processed according to standardized immunohistochemistry (IHC) procedures and then immunostained with the following antibodies: anti- γ H2AX (1:100) (#9718S, Cell Signaling Technology), anti-F4/80



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Fig. 2. Talazoparib induces production of type I interferons and inflammatory cytokines through cGAS/STING and NF κ B activation. (A) Western blotting analysis of cGAS expression and STING phosphorylation in A673 and PDX-EW#5-C cells exposed to IC₃₀ and IC₅₀ doses of different PARPi for 72 h (talazoparib: 0.01 μ M–0.05 μ M and 0.003 μ M–0.01 μ M, respectively; stenoparib: 3 μ M–10 μ M; pamiparib: 1 μ M–3 μ M; olaparib: 3 μ M–10 μ M and 1 μ M–3 μ M, respectively; veliparib: 20 μ M–30 μ M and 10 μ M–20 μ M, respectively). Talazoparib induces cGAS activation and STING phosphorylation at significantly lower dosage. Three independent biological replicates were performed. One representative immunoblot is shown. (B) Western blotting analysis of the expression of cGAS/STING and NF κ B signaling pathway mediators in a panel of five EwS cells treated for 72 h with the corresponding IC₅₀ dose of talazoparib (PDX-EW#7-C: 0.16 μ M, PDX-EW#2-C: 0.17 μ M, PDX-EW#5-C: 0.01 μ M, A673: 0.05 μ M, TC-71: 0.01 μ M). Three independent biological replicates were performed. One representative immunoblot is shown. (C) Relative mRNA expression of the cGAS/STING and NF κ B signaling downstream targets evaluated by qPCR. EwS cells were treated for 72 h with the corresponding IC₅₀ dose of talazoparib (PDX-EW#7-C: 0.16 μ M, PDX-EW#2-C: 0.17 μ M, PDX-EW#5-C: 0.01 μ M, A673: 0.05 μ M, TC-71: 0.01 μ M). The mRNA expression is reported as $2^{-\Delta\Delta Ct}$. GAPDH was used as a reference gene. Histograms show the mean $\pm$ SE of at least three independent experiments (dots). * $p < 0.05$; ** $p < 0.01$, Mann-Whitney test. (D) Release of type I IFNs and cytokines in the supernatants of A673 and PDX-EW#5-C cells measured by ELISA assay. Cells were treated for 96 h with the corresponding IC₅₀ dose of talazoparib (0.05 μ M and 0.01 μ M, respectively). Histograms show the mean $\pm$ SE of at least three independent experiments (dots); * $p < 0.05$; ** $p < 0.01$, Mann-Whitney test.

(1:200) (#70076, Cell Signaling Technology) and anti-CD206 (1:500) (#ab64693, Abcam). The avidin–biotin–HRP method was used (VECTASTAIN ABC kit, PK-4001, Vector Lab) (Supplemental Table 1). For morphological analyses, the slides were stained with hematoxylin and eosin. Histological and histomorphometric analyses were carried out with a digital pathology slide scanner with a resolution of 0.5 μ m/pixel (Aperio AT2, Aperio Technologies, Vista, CA) using ImageScope software (v12.4.3) for slide viewing.

2.12. Quantification of IHC staining using QuPath

QuPath (v0.6.x), an open-source software for bioimage analysis, was used [41]. Whole-slide images (WSI) were used for tissue detection and annotation. WSI in .svs format was imported into QuPath v0.6.x. An initial Tissue Detection step was applied to remove the background and isolate the histological sections. The parameters were optimized per stain to ensure accurate detection of tissue boundaries. Manual adjustments were performed when necessary to exclude folds, necrotic areas, or artifacts. Tissue detection, shrinking, and removal of tiny areas were performed using a script provided by Morriss et al. [42]. Color deconvolution was performed to separate the DAB (D5637, Sigma) and hematoxylin channels. Cells were detected using QuPath's Watershed Cell Detection with the following baseline parameters, adjusted per staining (background radius: 8 μ m; median filter radius: 0–2 μ m; sigma: 1.5 μ m; minimum cell area: 10–20 μ m²; maximum cell area: 200–300 μ m²; intensity thresholding in the Optical density sum: 0.01; cell expansion: 2–4 μ m). Parameter adjustments were performed to accommodate heterogeneous nuclear sizes and avoid cell over-segmentation in large macrophages (e.g., F4/80-positive, CD206-positive). For quantification of F4/80 and CD206, staining was quantified using cytoplasmic DAB intensity. Cells were classified as positive if the mean DAB optical density exceeded the detection threshold established for the negative controls. The following outputs were applied: number and density of F4/80-positive cells per ROI, percentage of F4/80-positive cells relative to total nucleated cells, percentage of CD206/F4/80-positive cells. γ H2AX staining was nuclear, therefore a standard nuclear segmentation was used. The classification was based on nuclear DAB optical density. A cell was considered γ H2AX positive when its nuclear DAB mean OD exceeded the threshold defined using untreated control samples.

To ensure reproducibility across samples, all slides within an experiment were processed using identical pipelines and thresholding values were fixed across batches once optimized. For quality control, the ROIs were reviewed independently by two operators and slides with poor staining, tissue detachment, or fold artifacts were excluded a priori.

2.13. Statistical analysis

GraphPad Prism (version 6.0 software) was used for statistical analysis. Statistical analyses were performed using parametric tests for data with normal and symmetric distributions (one-way ANOVA); otherwise, nonparametric tests were used (Kruskal–Wallis and Dunn post hoc tests for unpaired data or two-way ANOVA multiple comparison

with Geisser–Greenhouse correction and Mann–Whitney U test for unpaired two-group data). Values are expressed as mean $\pm$ SE. The data were considered statistically significant at $p < 0.05$. IC₅₀ values were calculated from the linear transformation of dose–response curves using CalcuSyn software (v2.11, Biosoft). Illustrations of this paper were created with Biorender (<https://app.biorender.com/>).

2.14. Ethics approval and consent to participate

This study was approved by the Ethics Committee of the Area Vasta Emilia Centro (CE-AVEC:505/2019/Sper/IO). Written patient-informed consent forms for the establishment of the three PDX models were obtained from a previously approved study (Prot.Gen 0009323 2016/04/22). All animal procedures were performed in accordance with ARRIVE guidelines [43], European Directive 2010/63/UE and Italian Law (DL 26/2014), and experimental protocols were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Bologna and by the Italian Ministry of Health (authorizations 782/2015-PR, 208/2017-PR, 755/2018-PR, 1051/2020-PR and 1139/2024-PR).

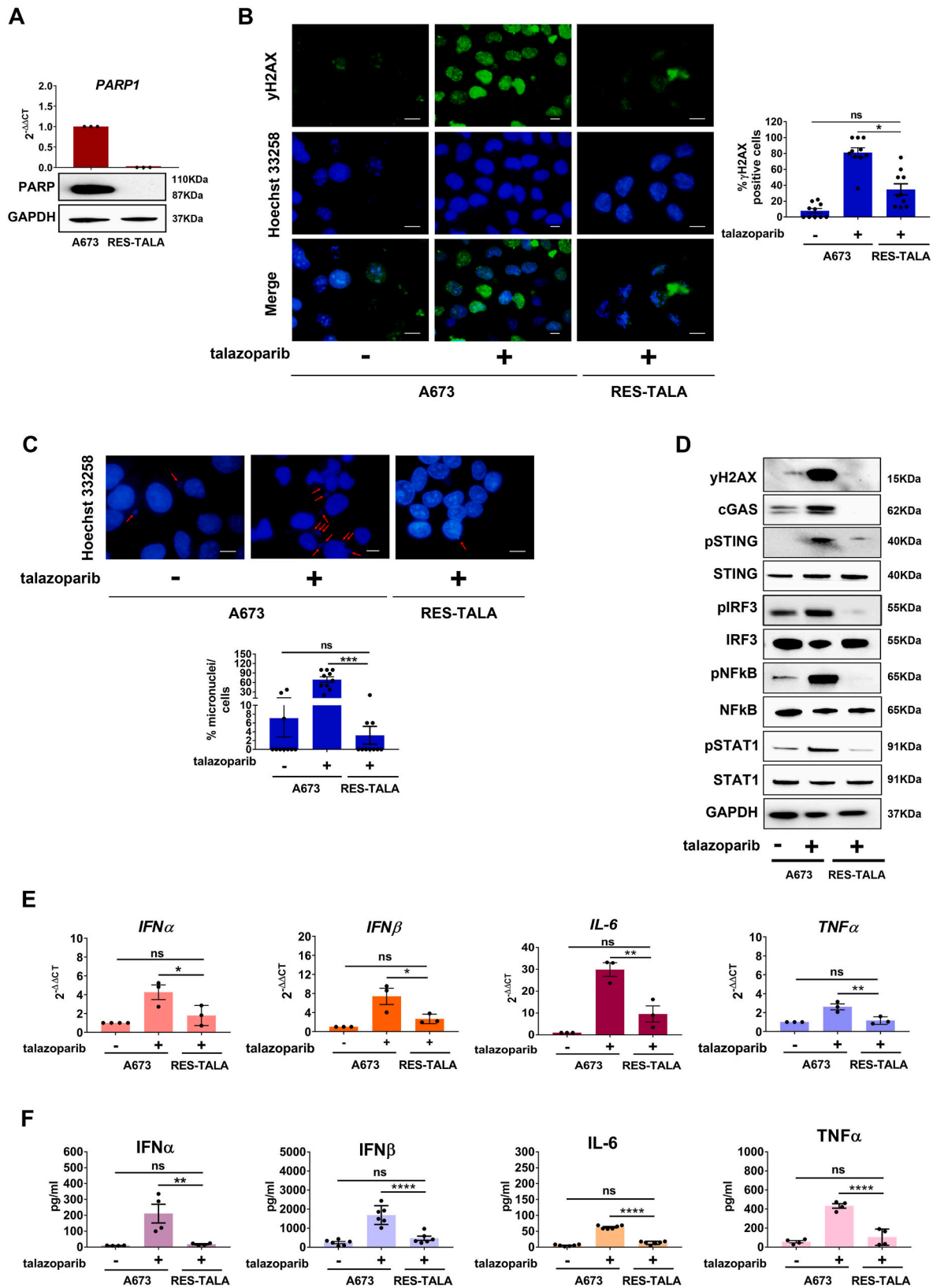
2.15. Data availability

The data generated in this study are available within the article and its supplemental material, or from the corresponding author upon request. PDX-derived cell lines, which are not available through public repositories, are available upon request from the corresponding authors through a material transfer agreement.

3. Results

3.1. In EwS cells PARPi efficacy correlates with PARP-trapping capacity

Besides inhibiting the catalytic activity of PARP enzymes, PARPi differ in their ability to trap PARP1 on DNA and chromatin, with talazoparib having the strongest trapping potential and veliparib the weakest [44–46]. To assess the relevance of PARP trapping in EwS, we tested five clinical PARPi (talazoparib, stenoparib, pamiparib, olaparib, and veliparib) against five EwS cell lines, three of which derived from PDXs. Our results demonstrated that talazoparib showed the greatest activity across all cell lines (IC₅₀ values 0.01 μ M–0.17 μ M), whereas veliparib was consistently ineffective, with IC₅₀ values of about 30 μ M (Fig. 1A and Supplemental Fig. 1). Stenoparib, pamiparib, and olaparib displayed intermediate efficacy with variable responses across cell lines. Notably, the three PDX-derived cell lines were generally more resistant to all PARPi, except talazoparib. Consistent with its high potency, talazoparib induced more robust cleavage of PARP at lowest doses than the other drugs (Fig. 1B), as well as stronger phosphorylation of H2AX at serine 139 (γ H2AX) and the formation of γ H2AX nuclear foci (Fig. 1B and C, Supplemental Fig. 2). In addition, talazoparib treatment led to the accumulation of cytoplasmic micronuclei (Fig. 1D), which is a hallmark of genomic instability. Micronuclei are prone to rupture, releasing DNA



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Fig. 3. Acquired resistance to talazoparib hinders the cGAS/STING/IFNs signaling. (A) *Top*, relative *PARP1* mRNA expression in A673 and A673_RES-TALA cells measured by qPCR, reported as $2^{-\Delta\Delta C_t}$, with *GAPDH* as reference. The histogram shows the mean $\pm$ SE, $n = 3$ (dots). *Bottom*, Western blot of *PARP1* cleavage in A673 and A673_RES-TALA cells. Three biological replicates were performed; one representative immunoblot is shown. (B) Immunofluorescence assessment of DNA damage by γ H2AX induction in A673 and A673_RES-TALA cells treated with talazoparib IC_{50} for 48 h (A673: 0.05 μ M; A673_RES-TALA: 10 μ M). Three independent biological experiments were performed, with images and data shown from one experiment. *Left*, representative images of γ H2AX positive cells (scale bar, 50 μ m); *right*, histograms show the mean percentage $\pm$ SE of positive cells from at least 10 random fields (dots). * $p < 0.05$; ns, not significant, Kruskal-Wallis test. (C) Micronuclei formation in A673 and A673_RES-TALA cells after 48 h of talazoparib IC_{50} treatment (A673: 0.05 μ M; A673_RES-TALA: 10 μ M), stained with Hoechst 33258. Three independent biological experiments were performed. Data from one experiment are shown. *Top*, representative images are shown (scale bar, 10 μ m). *Bottom*, histograms display the mean percentage $\pm$ SE of micronucleated cells, quantified in at least 10 random fields (dots). *** $p < 0.001$; ns, not significant, Kruskal-Wallis test. (D) Western blotting analysis of cGAS/STING and NF κ B pathway mediators in A673 and A673_RES-TALA cells treated with talazoparib IC_{50} for 72 h (A673: 0.05 μ M; A673_RES-TALA: 10 μ M). Three independent biological replicates were performed. One representative immunoblot is shown. (E) Relative mRNA expression of cGAS/STING and NF κ B targets evaluated by qPCR in A673 and A673_RES-TALA cells treated with talazoparib IC_{50} for 72 h (A673: 0.05 μ M; A673_RES-TALA: 10 μ M). Histograms show the mean $\pm$ SE of at least three experiments. * $p < 0.05$; ** $p < 0.01$; ns, not significant, Kruskal-Wallis test. (F) Release of type I IFNs and cytokines in supernatants of A673 and A673_RES-TALA cells measured by ELISA after 96 h of talazoparib IC_{50} treatment (A673: 0.05 μ M; A673_RES-TALA: 10 μ M). Histograms show the mean $\pm$ SE of at least three experiments. ** $p < 0.01$; **** $p < 0.0001$; ns, not significant, Kruskal-Wallis test.

into the cytosol where it can be sensed by cGAS. Accordingly, we found cGAS accumulation around or inside micronuclei after exposure to talazoparib (Fig. 1D). Induction of γ H2AX (Supplemental Fig. 3A) and micronuclei formation (Supplemental Fig. 3C) by talazoparib was strongly inhibited when the expression of EWS::FLI1 was downregulated in ASP14 cells (Supplemental Fig. 3B), an A673 cell variant obtained by stable silencing of the chimera [33], indicating that talazoparib's activity is modulated in an EWS::FLI1-dependent manner. This is in line with previous evidence using other PARPi, such as olaparib [3].

3.2. Talazoparib activates the cGAS/STING pathway and induces innate immune signaling

The increased formation of cytoplasmic micronuclei and accumulation of cGAS in EwS cells treated with talazoparib (Fig. 1D) prompted us to evaluate the cGAS/STING signaling cascade in comparison with other PARPi (Fig. 2A, Supplemental Fig. 2). Activated cGAS catalyzes the production of the immunotransmitter 2'-3'-cGAMP, which in turn activates STING [47]. Following upregulation of cGAS, treatment with talazoparib induced phosphorylation and activation of STING, and subsequent phosphorylation of interferon regulatory factor (IRF)3 at Ser386/Ser396 in all five EwS cell lines (Fig. 2B, Supplemental Fig. 2). IRF3 is a key transcription factor that, upon phosphorylation, translocates into the nucleus to induce type I IFNs (IFN α and IFN β) and interferon-stimulated genes [17,18]. In addition to IRF3 activation, talazoparib also promoted nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) signaling as well as enhanced phosphorylation of STAT1 (Fig. 2B, Supplemental Fig. 2), leading to the induction of pro-inflammatory cytokines, including interleukin (IL)6 and tumor necrosis factor (TNF) α , in addition to type I IFNs (Fig. 2C). Consistently, increased secretion of IFN α , IFN β , IL6, and TNF α was detected in the culture supernatants using ELISA (Fig. 2D). STING silencing by RNA interference or inhibition of STING phosphorylation by H-151 abrogated talazoparib-induced activation of the cGAS/STING/IFNs signaling cascade (Supplemental Fig. 4A and B). Notably, the robust effect of talazoparib was quite specific to EwS, since the activation of the cGAS/STING pathway and the release of IFNs/cytokines were modest if present in osteosarcoma cells (Supplemental Fig. 4C and D).

To further investigate the role of cGAS/STING/IFN signaling in talazoparib activity, we generated a talazoparib-resistant EwS variant by exposing A673 cells to increasing drug doses. The resistant cell line, designated A673_RES-TALA, exhibited a 200-fold increase in IC_{50} than the parental cell line (10 μ M vs. 0.05 μ M, respectively). Resistant cells lacked *PARP1* expression (Fig. 3A, Supplemental Fig. 2). Consistently, treatment with talazoparib (10 μ M) failed to induce DNA damage, as shown by the lack of an increase in γ H2AX foci and micronuclei formation (Fig. 3B and C). In resistant cells, cGAS expression was absent, STING and IRF3 were not activated (Fig. 3D, Supplemental Fig. 2), and type I IFNs and inflammatory cytokine production were not induced (Fig. 3E and F). Using immune-focused PCR array profiling 84 key genes

mediating tumor-immune crosstalk (RT² ProfilerTM), we confirmed the significant upregulation of interferon-responsive genes (*CXCL9*, *CXCL11*, *STAT1*, *IRF1*) and immunostimulatory cytokines (*IL1 α* , *IL1 β* and *IL15*) in parental A673 cells treated with talazoparib but not in the resistant variant (Supplemental Fig. 5).

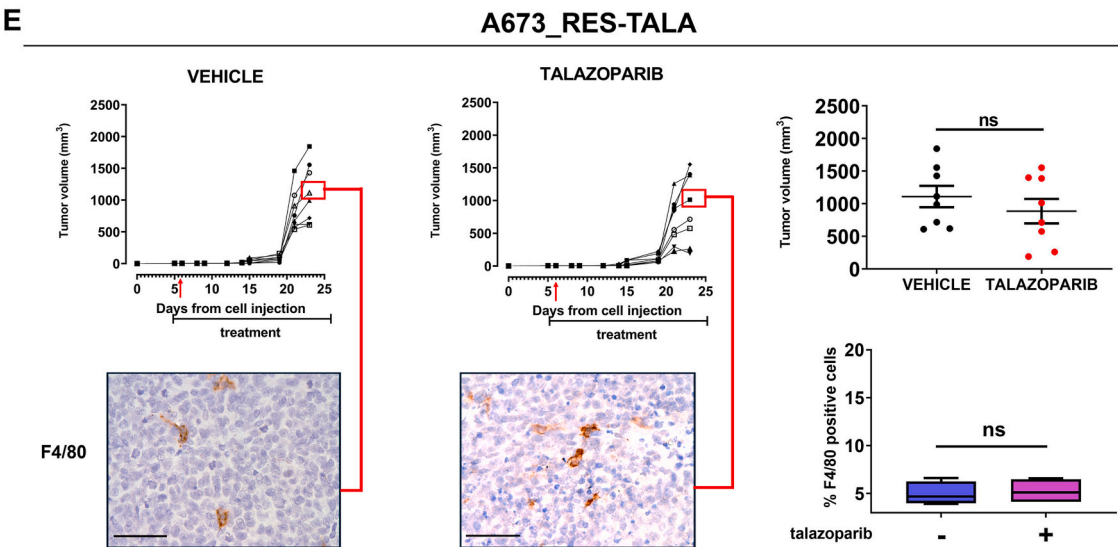
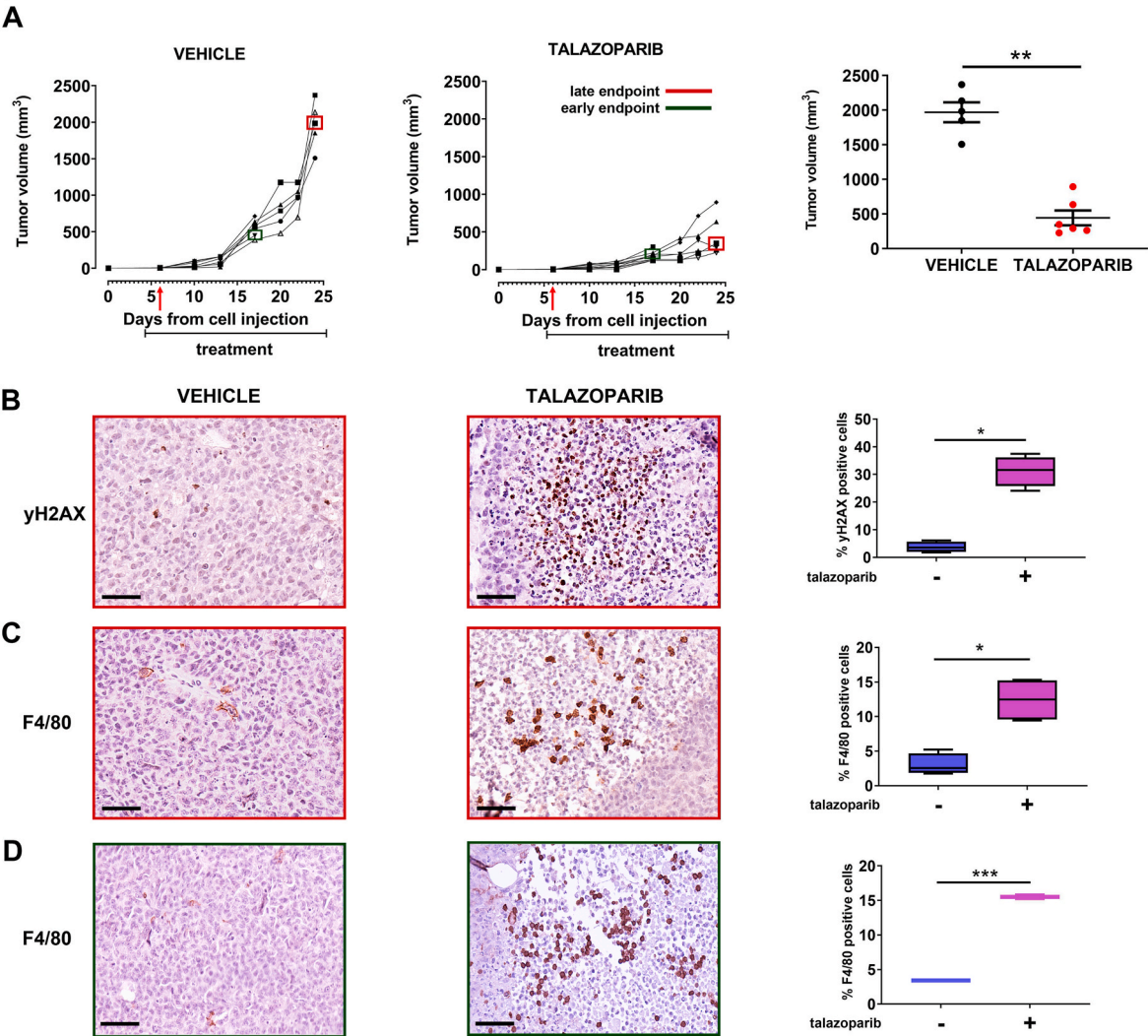
3.3. Talazoparib modulates the immunosuppressive TME that characterizes EwS

The efficacy of talazoparib was confirmed *in vivo* against the human patient-derived A673 cell line and the PDX-EW#5-C cell line (Fig. 4A–D, Supplemental Fig. 6). Mice with tumors of approximately 4–5 mm in diameter were randomized to receive daily oral administration of talazoparib, at the dose of 0.33 mg/kg, or vehicle only (control group). Both models showed a significant decrease in tumor volume (Fig. 4A and Supplemental Fig. 6A). Histologic examination of the treated tumors showed pronounced induction of DNA damage, as evidenced by increased γ H2AX positivity, and increased tumor infiltration with murine F4/80-positive macrophages (Fig. 4B and C and Supplemental Fig. 6B and C). The increased presence of murine macrophages was observed even at an earlier point (10 days post-treatment), when the antitumor effect was just beginning to emerge, further supporting the drug's capacity to “reshape” the immune landscape as a primary therapeutic mechanism (Fig. 4D). Talazoparib treatment of mice bearing resistant xenografts did not inhibit tumor growth, failed to induce γ H2AX foci and did not increase macrophage infiltration compared to controls (Fig. 4E). In untreated A673 and PDX-EW#5 xenografts, the few infiltrating F4/80-positive macrophages frequently expressed CD206, a canonical marker of anti-inflammatory, pro-tumoral M2-like macrophages [48,49]. Treatment with talazoparib markedly reduced CD206-positive macrophages in sensitive tumors but not in resistant xenografts (Fig. 5A). Consistently, M0-like macrophages, derived from *ex vivo* human CD14⁺ monocytes and co-cultured with naïve EwS cells *in vitro* showed increased phagocytic capability when exposed to CM from A673 cells treated with talazoparib but not when exposed to CM from talazoparib-resistant cells (Fig. 5B). This effect was mediated by type I IFNs (Fig. 5B). Notably, the efficacy of talazoparib was significantly increased when EwS cells were co-cultured with M0-like macrophages and exposed to the drug for 72 h (Fig. 5C). Together, these data support a shift in M0-like phenotype toward macrophages with anti-tumor activity when exposed to talazoparib-induced, tumor-derived type I IFNs.

3.4. Talazoparib promotes reprogramming of macrophages toward a pro-inflammatory M1-like phenotype in responsive but not resistant EwS cells

To directly test whether talazoparib influences macrophage polarization, we exposed human M0-like macrophages, derived from either THP-1 cells or *ex vivo* CD14⁺ monocytes, to CM from A673 cells treated with talazoparib. Conditioned macrophages displayed a transcriptional profile consistent with M1 polarization, characterized by upregulation

A673



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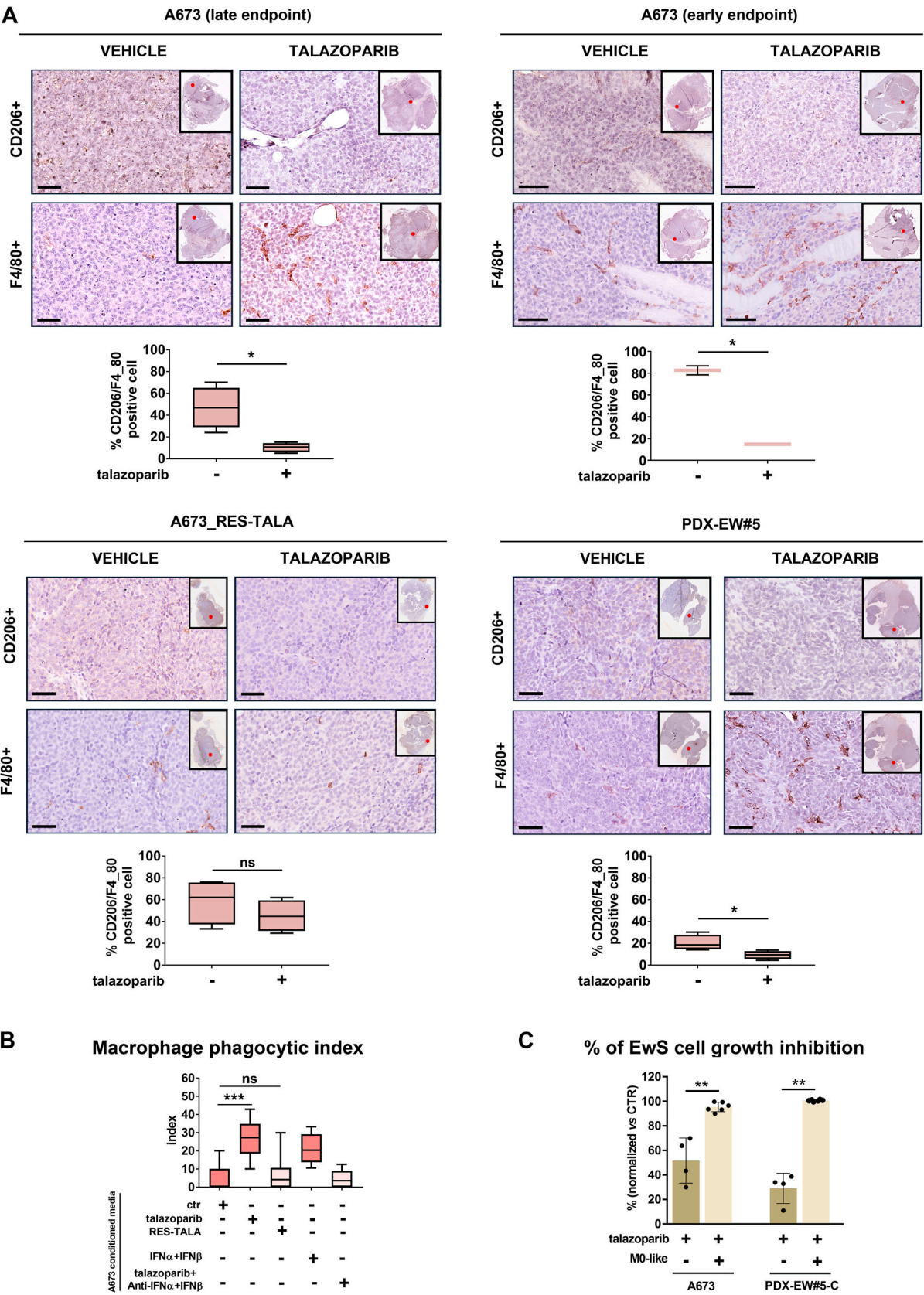
Fig. 4. Talazoparib inhibits tumor growth enhancing macrophage infiltration in sensitive xenografts, but not in resistant ones. (A) Tumor growth inhibition in A673 xenografts after talazoparib treatment. The control group ($n = 7$) received vehicle only, with treatments starting when tumors became measurable (arrows) ($n = 9$). Two of the control group and three mice of the treated one were stopped after 10 days (early endpoint, green). *Left*, growth curves for individual tumors are shown. *Right*, tumor volumes of talazoparib-treated mice *versus* vehicle-treated mice at the end of treatment are shown. Dots represent individual tumor volumes, mean $\pm$ SE displayed. $^{**}p < 0.01$, Mann-Whitney test. (B) DNA damage assessment in xenografts treated or not with talazoparib via γ H2AX staining (late endpoint, red). Digital images were acquired using a pathology slide scanner (0.5 μ m/pixel resolution, Aperio AT2, Aperio Technologies) and viewed with ImageScope software (v12.4.3). The percentage of γ H2AX-positive cells was calculated using QuPath software (v0.6.x). *Left*, representative images of γ H2AX-positive cells (scale bar, 50 μ m). *Right*, histograms show the percentage of γ H2AX-positive cells in the total tumor area from 4 xenografts/group; the box represents the Interquartile Range (IQR) expressed as median (central line) and the upper and lower edges of the box correspond to the first and third quartiles, respectively; the whiskers extend to the minimum and maximum values (min-max). $^{*}p < 0.05$, Mann-Whitney test. (C) Macrophage infiltration in xenografts treated with talazoparib (late endpoint, red). Digital images were obtained using a pathology slide scanner (0.5 μ m/pixel resolution, Aperio AT2, Aperio Technologies) and viewed with ImageScope software (v12.4.3). The percentage of F4/80-positive macrophages was calculated using QuPath software (v0.6.x). *Left*, representative images of infiltrating macrophages (scale bar, 50 μ m). *Right*, histograms show the percentage of F4/80-positive macrophages in the total tumor area of 4 xenografts/group; the box represents the Interquartile Range (IQR) expressed as the median (central line) and the upper and lower edges of the box correspond to the first and third quartiles, respectively; the whiskers extend to the minimum and maximum values (min-max). $^{*}p < 0.05$, Mann-Whitney test. (D) Macrophage infiltration in xenografts after 10 days of treatment with talazoparib (early endpoint, green). Representative murine F4/80-positive macrophages of A673 xenografts treated or not with talazoparib are shown (scale bar, 50 μ m). Digital images were acquired using digital pathology slide scanner with a resolution of 0.5 μ m/pixel (Aperio AT2, Aperio Technologies) and viewed with ImageScope software (v12.4.3). Quantification of different cell components (shown as percentages) on the digital slides were performed using QuPath software (v0.6.x) (for more details see Material and methods section). Histograms show the percentage of F4/80-positive macrophages in the total tumor area of 2 xenografts/group; the box represents the Interquartile Range (IQR) expressed as the median (central line) and the upper and lower edges of the box correspond to the first and third quartiles, respectively; the whiskers extend to the minimum and maximum values (min-max). $^{***}p < 0.001$, Mann-Whitney test. (E) Tumor growth inhibition in A673_RES-TALA xenografts after talazoparib treatment. The control group ($n = 8$) received vehicle only, with treatments starting when tumors became measurable (arrows) ($n = 8$). *Left*, growth curves for individual tumors are shown. *Right*, tumor volumes of talazoparib-treated *versus* vehicle-treated mice at the end of treatment. Dots represent individual tumor volumes, mean $\pm$ SE. ns, not significant, Mann-Whitney test. *Bottom*, macrophage infiltration in A673_RES-TALA xenografts. Representative images of F4/80-positive macrophages (scale bar, 50 μ m) and histograms show the percentage of F4/80-positive macrophages in 4 xenografts/group; the box represents the Interquartile Range (IQR) expressed as the median (central line) and the upper and lower edges of the box correspond to the first and third quartiles, respectively; the whiskers extend to the minimum and maximum values (min-max). ns, not significant, Mann-Whitney test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

of CD80 and CD86 (Fig. 6A and B, Supplemental Fig. 7A and B), enhanced STAT1 phosphorylation and increased expression of the AP-1 transcription factors Fos and Jun, which are known drivers of macrophage activation and M1 polarization [50] (Fig. 6C and D, Supplemental Fig. 8). This effect was strongly attenuated when macrophages were exposed to CM from talazoparib-resistant cells. In addition, talazoparib-CM from sensitive, but not resistant, EwS cells activated the STING pathway and enhanced the expression of type I IFNs in the recipient macrophages (Fig. 6E and F, Supplemental Fig. 8). Mechanistically, macrophage-reprogramming depends on type I IFNs signaling. Exposure of M0-like macrophages to A673 CM treated with rHu IFN α/β at concentrations comparing those secreted by talazoparib-treated EwS cells recapitulated the phenotype, whereas neutralization of type I IFNs by neutralizing antibodies in CM abrogated this effect (Fig. 6A–D, Supplemental Fig. 7A and B and Supplemental Fig. 8). The addition of a STING agonist, 2'-3'-cGAMP did not increase the effects observed when macrophages were conditioned by media of cells treated with talazoparib (Supplemental Fig. 7C), suggesting that talazoparib-induced STING is, perhaps, already at a plateau. Of note, olaparib, the PARPi most widely used clinically [13], failed to induce type I IFNs or inflammatory cytokine expression in EwS cells (Supplemental Fig. 9A). Accordingly, CM from olaparib-treated cells did not alter M0-like gene expression or induce STAT1 activation in macrophages (Supplemental Fig. 9B and C). CM from talazoparib-treated A673 cells, but not from resistant variants, also promoted THP-1 derived M0-like macrophage migration (Supplemental Fig. 9D), mirroring the increase in macrophages observed in the xenografts (Fig. 4C, Supplemental Fig. 6C). This pro-migratory effect was not observed when THP-1 derived macrophages were exposed to the medium from olaparib-treated A673 cells (Supplemental Fig. 9D).

Together, these findings indicate that talazoparib, unlike olaparib, reprograms infiltrating macrophages toward a pro-inflammatory, anti-tumorigenic M1-like state through tumor cGAS/STING/IFNs signaling. This reprogramming is abolished in talazoparib-resistant EwS cells which have lost PARP1 expression and lack cGAS/STING activation.

4. Discussion

Our study identified talazoparib as the most effective PARPi in EwS and uncovered an underappreciated mechanism of the drug in the modulation of tumor-immune interactions. Although PARPi were initially developed to exploit synthetic lethality in tumors with defective DNA repair, recent findings demonstrate that PARPi efficacy extends beyond their catalytic inhibition of PARP1, triggering robust local and systemic antitumor immunity involving both adaptive and innate immune responses through a STING-dependent antitumor immune response against BRCA-deficient tumors either in preclinical or clinical conditions [51–54]. In studies conducted mainly on BRCA-deficient ovarian and breast cancers, antitumor activity was observed after treatment with olaparib or niraparib. Instead, in EwS, we observed a clear superior activity of talazoparib *versus* the other PARPi (stenoparib, pamiparib, olaparib and veliparib). Compared to inhibitors with weaker PARP-trapping activity such as veliparib or olaparib, talazoparib induced more robust DNA damage and micronuclei formation, and activated the cGAS/STING pathway toward type I IFNs and inflammatory cytokine production. These results were not observed when osteosarcoma cells were treated with talazoparib, suggesting that talazoparib-induced cGAS/STING activation is dependent on cellular context. Repression of cGAS/STING signaling has been demonstrated in several tumor types [55–57], particularly in chromosomally unstable cells, such as osteosarcoma [58]. In contrast, EwS cells are chromosomally stable, with scarce cytosolic DNA and are characterized by an intact cGAS/STING pathway and normal responses to inflammation. Beyond its cytotoxic effects on EwS tumor growth, talazoparib induces STING-mediated immunostimulatory effects that translated into macrophage repolarization to M1-like macrophages with increased phagocytic capabilities and anti-tumor activities. M1-like macrophage repolarization is mediated by tumor-derived type I IFNs and is accompanied by the activation of STAT1 and AP-1, two key pathways that shape M1-like macrophage behavior, and STING signaling in recipient macrophages. This result was not entirely expected, as EwS cells have been recently reported to robustly express Ectonucleotide Pyrophosphatase/Phosphodiesterase (ENPP1) [59], a transmembrane glycoprotein that suppresses the activation of the cGAS/STING pathway in host



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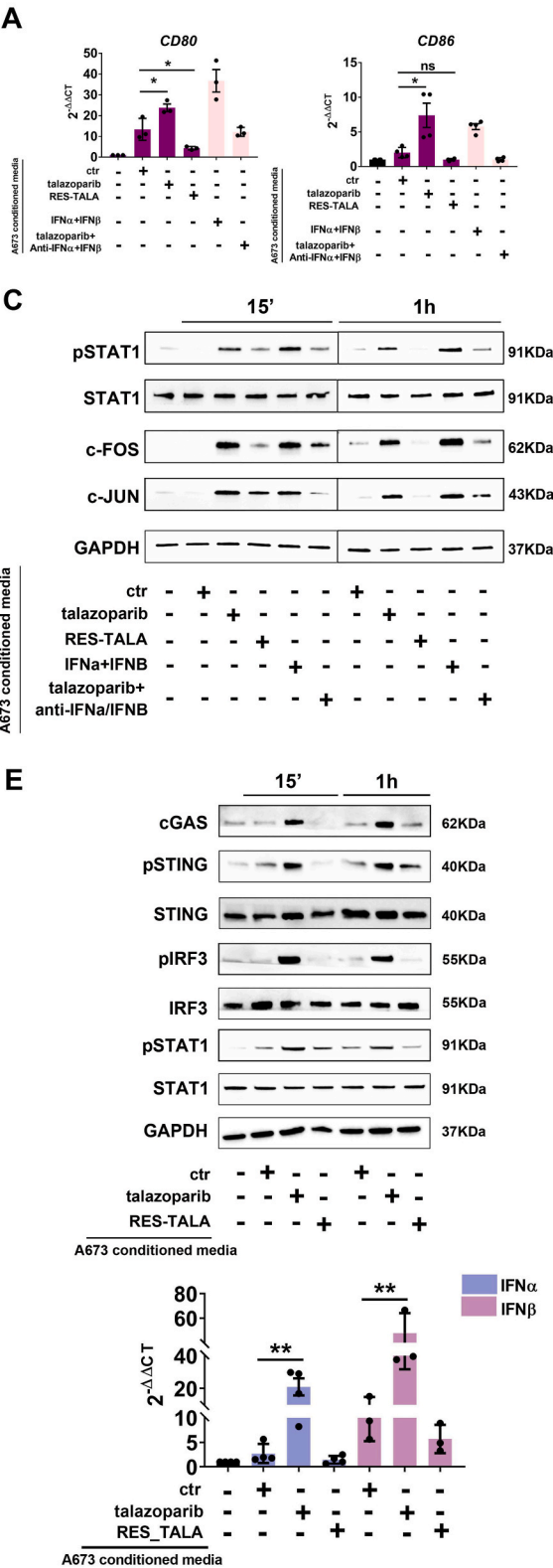
Fig. 5. Talazoparib increases the phagocytic abilities of macrophages. (A) Representative images of infiltrating CD206 or F4/80-positive murine macrophages in A673 (left, late endpoint; right, early endpoint), A673_RES-TALA and PDX-EW#5 xenografts treated or not with talazoparib are shown. Low magnifications are inserted to show global tumors, and the red point indicates the field of view of the 40 $\times$ images displayed for each antigen. Digital images were acquired using digital pathology slide scanner with a resolution of 0.5 μ m/pixel (Aperio AT2, Aperio Technologies) and viewed with ImageScope software (v12.4.3). Histograms show the mean percentage $\pm$ SE of CD206-positive macrophages out of the total infiltrating macrophages (F4/80-positive) in xenografts treated or not with talazoparib. The percentage of CD206 and F4/80-positive macrophages was calculated in the total tumor area using QuPath software (v0.6.x). The box represents the Interquartile Range (IQR) expressed as the median (central line) and the upper and lower edges of the box correspond to the first and third quartiles, respectively; the whiskers extend to the minimum and maximum values (min-max). * $p < 0.05$; ns, not significant, Mann–Whitney test. (B) Phagocytic capability of M0-like macrophages against naïve EwS cells. Phagocytic index increased significantly when macrophages were conditioned for 6 h by supernatants from sensitive but not resistant cells treated with talazoparib or exposed to the same concentrations of exogenous rHu type I IFNs that were detected in the media (rHu IFN α 0.3 ng/ml, rHu IFN β 2 ng/ml, see Fig. 2). The use of neutralizing IFN antibodies (rHu anti-IFN α 10 ng/ml + rHu anti-IFN β 10 ng/ml) reverted the effects. The box represents the Interquartile Range (IQR) expressed as the median (central line) and the upper and lower edges of the box correspond to the first and third quartiles, respectively; the whiskers extend to the minimum and maximum values (min-max). *** $p < 0.001$; ns, not significant, Kruskal–Wallis test. (C) Inhibition of EwS cell growth induced by 72 h talazoparib treatment in the presence or not of human M0-like macrophages. Histograms show the mean $\pm$ SE of at least two independent experiments in triplicates (dots); ** $p < 0.01$, Mann–Whitney test. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

immune cells by degrading extracellular 2'-3'-cGAMP [60,61]. It is likely that type I IFNs release induced by talazoparib in EwS cells overcomes the potential inhibitory effects of ENPP1. Alternatively, other sensors, such as the RNA sensor Melanoma Differentiation-Associated Protein (MDA) may contribute to the detection of aberrant cytoplasmic nucleic acids [62], sustaining the activity of the DNA sensor cGAS and promoting IFN-driven antitumor after drug treatment. However, these mechanisms remain incompletely understood and require further investigation. Studying the EwS cell variant with acquired resistance to talazoparib may provide novel mechanistic insights. In this context, the current study further highlighted the connection between DNA damage and immune responses as a central mechanism of talazoparib anti-tumor activity including its capacity to act as an immune-sensitizing agent reshaping the TME through repolarization of macrophages and induction of an anti-tumor inflammatory environment. We focused on macrophages because they can be reliably studied in immunodeficient mouse models and they are the main cellular immune component of the EwS TME [26–28]. EwS is described as an immune desert, with limited or no infiltration of intratumoral CD4⁺ and CD8⁺ T cells. In addition, the presence of M0- and M2-like cells is associated with worse prognosis [26, 29]. We are aware that PARP1 and PARP2 regulate different aspects of the immune response, modulating both the innate and adaptive immune system [63] and that besides macrophages other immune cell types may contribute to the anti-tumor immune response triggered by talazoparib. For example, in patients with breast cancer carrying germline BRCA1/2 pathogenic variants, neoadjuvant talazoparib significantly increased cytotoxic T-cell density and enhanced tumor immune activation, which was associated with longer recurrence-free survival and improved clinical response [64]. However, no changes in macrophage populations were reported in that study, further highlighting the complexity of PARPi-mediated modulation of immune cell phenotypes and underscoring the need for disease-specific studies to guide the use of immune-modulating agents. In the case of EwS, no natural murine models currently exist, and all attempts to recreate EwS tumorigenesis in mice have thus far failed [65]. Moreover, the use of immunodeficient mice engrafted with human xenografts and reconstituted with human immune components to mimic a functional human immune response still presents inherent limitations, including the long-term survival and functional maturation of both innate and adaptive immune subsets, as well as the difficulty of achieving appropriate HLA matching [66]. Together, these limitations represent a major obstacle to a deeper understanding of EwS and its TME, leaving the contribution of other immune cell populations, such as cytotoxic T cells, to the antitumor immune response induced by talazoparib to be addressed in clinical studies. A phase 1/2 trial of talazoparib in combination with temozolomide and/or irinotecan has been conducted in children and young adults with recurrent or refractory solid malignancies [10,67]. In general, although these 2/3 drug regimens demonstrated some activity in patients with EwS, including one complete response [67], toxicity limited the ability to achieve equivalent patient doses that would match

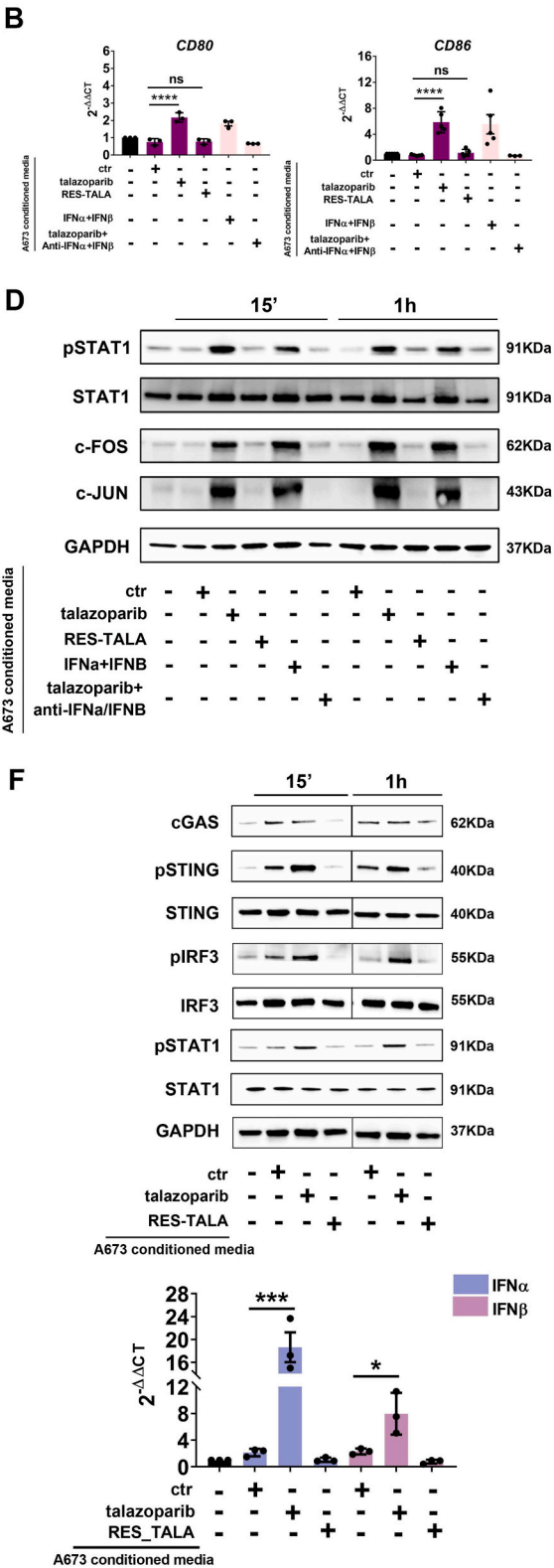
preclinical models. Treated tumors were analyzed for γ H2AX and R-loop accumulation [10], demonstrating the activity of the drug/s, but not evaluated for immune cell infiltration. Therefore, the limited clinical response rate observed for patients with EwS may be due to several reasons, including inadequate drug concentrations and patient's conditions, which for those enrolled in these studies were heavy pretreatment, chemoresistance and compromised immune system. Combining talazoparib with cytotoxic agents may not be the best option, because while improving DNA damage, the combination may impair the potential of talazoparib to establish immune infiltration and inflammatory responses. In contrast, our data supports the rationale for the utilization of talazoparib as an activator of both tumor -intrinsic STING and host STING agonists. In immunocompetent osteosarcoma models, host STING activation was found to be sufficient to promote macrophage-mediated effects even in the absence of tumor STING [58]. In EwS, the addition of a STING agonist to talazoparib in co-cultures of EwS cells with macrophages did not enhance the killing of EwS cells, suggesting that talazoparib induces STING at levels that render exogenous STING-agonist with no additive effect under the tested conditions. Although further investigation in clinical settings is necessary to gain an in-depth understanding of talazoparib-induced immunomodulation, particularly given the lack of adequate preclinical EwS models, our preclinical findings indicate that talazoparib is a promising drug for inducing beneficial innate immune activation in patients with naïve EwS. In addition, its ability to repolarize M0- and M2-like macrophages toward an M1-like phenotype may suggest combination with macrophage-targeting strategies, which are highly valued in macrophage-rich tumors, such as sarcomas, but are still challenging due to macrophage heterogeneity and plasticity [68]. The clinical translation of talazoparib should consider its toxicity [10,67], and the reported association of abnormal cGAS/STING activation with autoimmunity and neurodegeneration [69]. Dissecting the kinetics and persistence of talazoparib-induced immune activation in EwS patients will thus be essential to optimize therapeutic scheduling and perhaps integrate immune checkpoint inhibitors in a setting in which macrophages are no longer an obstacle for immunotherapy.

In conclusion, our findings highlight PARP trapping, rather than enzymatic activity, as the critical determinant of PARPi efficacy in EwS. The study revealed that talazoparib exerts dual anti-tumor activity in EwS: direct cytotoxicity through increased DNA damage and indirect immunomodulation via cGAS/STING-mediated IFNs signaling and macrophage reprogramming. Due to the current limitations of mouse models for EwS, our results can only suggest a role for talazoparib in the activation of innate immune pathways and should not be interpreted as definitive evidence of a broader antitumor immune response. Although further studies using mice with a humanized immune system and other emerging models will be required to fully characterize the contribution of additional immune cell populations, our findings nevertheless support the clinical development of talazoparib for EwS and provide a strong rationale for combining PARP-trapping strategies with macrophage-based immunotherapy to improve outcomes in this aggressive

CD14+ derived macrophages



THP-1 derived macrophages



(caption on next page)

Fig. 6. Talazoparib-mediated cGAS/STING activation induces human macrophage polarization towards M1-like status. (A, B) mRNA expression of *CD80*, *CD86* by qPCR on *ex vivo* human CD14⁺ derived or THP-1-derived macrophages exposed for 3 h to: CM from A673 or A673_RES-TALA cells treated or not with talazoparib (A673: 0.05 μ M; A673_RES-TALA: 10 μ M); CM from A673 treated with rHu IFN α (0.3 ng/ml) + rHu IFN β (2 ng/ml); CM from A673 treated with talazoparib + rHu anti-IFN α (10 ng/ml) + rHu anti-IFN β (10 ng/ml) antibodies, respectively. Relative mRNA expression is reported as $2^{-\Delta\Delta Ct}$. *GAPDH* was used as reference gene. Histograms display the mean $\pm$ SE from at least three experiments. (dots) * $p < 0.05$; **** $p < 0.0001$; ns, not significant, one-way ANOVA test. (C, D) Western blotting of STAT1/c-FOS/c-JUN activation on *ex vivo* human CD14⁺ derived or THP-1-derived macrophages exposed for 15 min or 1 h to: CM from A673 or A673_RES-TALA cells treated or not with talazoparib (A673: 0.05 μ M; A673_RES-TALA: 10 μ M); CM from A673 treated with rHu IFN α (0.3 ng/ml) + rHu IFN β (2 ng/ml); CM from A673 treated with talazoparib + rHu anti-IFN α (10 ng/ml) + rHu anti-IFN β (10 ng/ml) antibodies, respectively. Three biological replicates were performed. One representative immunoblot is shown. (E, F) *Top*, western blot of cGAS/STING signaling pathway mediators on *ex vivo* human CD14⁺ derived or THP-1-derived macrophages exposed for 15 min or 1 h to: CM from A673 or A673_RES-TALA cells treated or not with talazoparib (A673: 0.05 μ M; A673_RES-TALA: 10 μ M). Three biological replicates were performed. One representative immunoblot is shown. *Bottom*, mRNA expression levels of the cGAS/STING signaling downstream targets by qPCR on *ex vivo* human CD14⁺ derived or THP-1-derived macrophages exposed for 1 h to: CM from A673 or A673_RES-TALA cells treated or not with talazoparib (A673: 0.05 μ M; A673_RES-TALA: 10 μ M). Relative mRNA expression is reported as $2^{-\Delta\Delta Ct}$. *GAPDH* was used as a reference gene. Histograms display the mean $\pm$ SE from at least three experiments. (dots) * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, one-way ANOVA test.

pediatric cancer.

Funding sources

The research leading to these results received funding from Fondazione AIRC per la ricerca sul cancro ETS under IG2019-22805 to KS.

During the preparation of this work the author(s) used ChatGPT-4 in order to edit, improve the clarity and fluency of the text, and for grammar checks. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the publication.

CRediT authorship contribution statement

Marianna Carrabotta: Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft, Writing – review & editing. **Maria Cristina Manara:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Lorena Landuzzi:** Data curation, Formal analysis, Investigation, Writing – review & editing. **Elisa Simonetti:** Investigation, Writing – review & editing. **Andrea Nesca:** Data curation, Investigation, Writing – review & editing. **Evelin Pellegrini:** Investigation, Writing – review & editing. **Margherita Maioli:** Methodology, Writing – review & editing. **Sofia Avnet:** Data curation, Investigation, Writing – review & editing. **Francesca Ruzzi:** Investigation, Writing – review & editing. **Francesca Salamanna:** Data curation, Writing – review & editing. **Marco Gambarotti:** Resources, Writing – review & editing. **Pier-Luigi Lollini:** Resources, Writing – review & editing. **Katia Scotlandi:** Conceptualization, Data curation, Funding acquisition, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of competing interest

All authors declare no conflicts of interest.

Acknowledgements

The authors are grateful to the following associations for funding the PhD scholarship of ES at the University of Bologna: Onlus il Pensatore 'Matteo Amtrano,' Aurora Tomaselli, ricerca e prevenzione, Liberi di vivere, 'Luca Righi,' 'Chiara Paradiso' – la forza dell'amore onlus, Associazione Mario Campanacci, and Associazione 'Un sorriso con Luca'. We would like to thank all the patients, parents, and families who agreed to donate their samples for the establishment of the PDX models used in this study.

Glossary

AP	activator protein
BRCA	breast cancer gene
BSA	bovine serum albumin

cGAMP	2'-3'-cyclic GMP-AMP
cGAS	cyclic GMP-AMP synthase
CM	conditioned media
CXCL	C-X-C Motif Chemokine Ligand
DC	dendritic cell
DSB	double strand break
ENPP	Ectonucleotide Pyrophosphatase/Phosphodiesterase
EwS	Ewing sarcoma
HIS	humanized immune system
HR	homologous recombination
HRD	homologous recombination deficiency
IFN	interferon
IL	interleukin
IRF	interferon regulatory factor
MDA5	Melanoma Differentiation-Associated Protein 5
NK	natural killer
PARP	Poly (ADP-ribose) polymerase
PARPi	PARP inhibitors
PBMCs	Peripheral Blood Mononuclear Cells
PBS	phosphate-buffered saline
PDX	patient-derived xenograft
PFA	paraformaldehyde
PMA	phorbol 12-myristate 13-acetate
rHu	recombinant human
STAT	Signal transducer and activator of transcription
STING	stimulator of interferon genes
TAM	tumor-associated macrophage
TME	tumor microenvironment
TNF α	tumor necrosis factor alpha

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.canlet.2026.218704>.

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