Adding liposomal doxorubicin enhances the abscopal effect induced by radiation/αPD1 therapy depending on tumor cell mitochondrial DNA and cGAS/STING

Liquan Wang,1,2 Ren Luo,3,4,5,6,7 Kateryna Onyshchenko,1,5,6,7 Xi Rao,1,6,7 Meidan Wang,1,5 Beatrice Menz,1 Simone Gaedicke,1 Anca-Ligia Grosu,1,6,7 Elke Firat,1 Gabriele Niedermann

ABSTRACT

Background Localized radiotherapy (RT) can cause a T cell-mediated abscopal effect on non-irradiated tumor lesions, especially in combination with immune checkpoint blockade. However, this effect is still clinically rare and improvements are highly desirable. We investigated whether triple combination with a low dose of clinically approved liposomal doxorubicin (Doxil) could augment abscopal responses compared with RT/αPD-1 and Doxil/αPD-1. We also investigated whether the enhanced abscopal responses depended on the mitochondrial DNA (mtDNA)/cyclic GMP-AMP synthase (cGAS)/stimulator of interferon genes (STING)/IFN-β pathway.

Materials/methods We used Doxil in combination with RT and αPD-1 in two tumor models (B16-CD133 melanoma and MC38 colon carcinoma) with mice bearing two tumors, only one of which was irradiated. Mechanistic studies on the role of the mtDNA/cGAS/STING/IFN-β axis were performed using inhibitors and knockout cells in vitro as well as in mice.

Results Addition of a single low dose of Doxil to RT and αPD-1 strongly enhanced the RT/αPD-1-induced abscopal effect in both models. Complete cures of non-irradiated tumors were mainly observed in triple-treated mice. Triple therapy induced more cross-presenting dendritic cells (DCs) and more tumor-specific CD8+ T cells than RT/αPD-1 and Doxil/αPD-1, particularly in non-irradiated tumors. Coincubation of Doxil-treated and/or RT-treated tumor cells with DCs enhanced DC antigen cross-presentation which is crucial for inducing CD8+ T cells. CD8+ T cell depletion or implantation of cGAS-deficient or STING-deficient tumor cells abolished the abscopal effect. Doxorubicin-induced/ Doxil-induced IFN-β1 markedly depended on the cGAS/ STING pathway. Doxorubicin-treated/Doxil-treated tumor cells depleted of mtDNA secreted less IFN-β1, of the related T cell-recruiting chemokine CXCL10, and ATP; coinubation with mtDNA-depleted tumor cells strongly reduced IFN-β1 secretion by DCs. Implantation of mtDNA-depleted tumor cells, particularly at the non-irradiated/ abscopal site, substantially diminished the Doxil-enhanced abscopal effect and tumor infiltration by tumor-specific CD8+ T cells.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Combined radiation and immune checkpoint blockade can elicit an abscopal response of non-irradiated tumor lesions, but strong abscopal responses are still clinically rare.

WHAT THIS STUDY ADDS

⇒ This study shows in mice that triple combination with additional low-dose liposomal doxorubicin strongly enhances the radiation/αPD-1-induced abscopal effect, increasing cross-presenting dendritic cells and tumor-specific CD8+ T cells particularly in non-irradiated tumor lesions, and indicates dependence of the immunogenic doxorubicin effects on the mitochondrial DNA/cyclic GMP-AMP synthase/stimulator of interferon genes/IFN-β axis.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Radiotherapy (RT)+αPD-1+ a low dose of clinically approved liposomal doxorubicin induced superior systemic antitumor T cell immunity, abscopal response, and survival as compared with RT+αPD-1 or liposomal doxorubicin+αPD-1. These data suggest that clinical evaluation of this new triple combination is warranted.

Conclusions These data show that single low-dose Doxil can substantially enhance the RT/αPD-1-induced abscopal effect, with a strong increase in cross-presenting DCs and CD8+ tumor-specific T cells particularly in abscopal tumors compared with RT/αPD-1 and Doxil/αPD-1. Moreover, they indicate that the mtDNA/cGAS/STING/IFN-β axis is important for the immunogenic/immunomodulatory doxorubicin effects. Our findings may be helpful for the planning of clinical radiochemoimmunotherapy trials in (oligo)metastatic patients.

BACKGROUND

Traditionally, radiotherapy (RT) was thought to act solely through direct (DNA


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For numbered affiliations see end of article.

Correspondence to Dr Ren Luo; luorenbu@gmail.com

Dr Gabriele Niedermann; gabriele.niedermann@uniklinik-freiburg.de

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BACKGROUND

Traditionally, radiotherapy (RT) was thought to act solely through direct (DNA
damage-mediated) cytotoxicity. However, preclinical work suggests that certain RT dose/fractionation regimens can be immunogenic, depending on T cells. The induced tumor-specific T cells not only attack irradiated tumors, but can also exert systemic effects on non-irradiated tumors, the so-called ‘abscopal effect’. The abscopal effect is mainly observed in combination with immunotherapy such as immune checkpoint blockade (ICB). However, strong abscopal responses are clinically rare. Improvements are thus highly desirable, and it is, therefore, important to test alternative dual combinations or new triple combinations based on RT/ICB dual combinations in preclinical studies.

To look for ways to enhance the RT/ICB-induced abscopal effect, we investigate triple combinations with chemotherapeutics, and we found that a single, relatively low dose of cisplatin can enhance the RT-induced/αPD-1-induced abscopal effect by inducing expression of the T cell-attracting chemokine C-X-C motif chemokine 10 (CXCL10) in tumor cells. A fundamental discovery was that certain chemotherapeutics and ionizing radiation can be immunogenic by inducing tumor-specific T cells, and that induction of immunogenic cell death (ICD) is a main cause of their immunogenicity. One of the few chemotherapeutic agents currently known to induce ICD is the anthracycline doxorubicin. ICD is characterized by formation of damage-associated molecular patterns (DAMPs). Important DAMPs in tumor cell ICD are cell surface exposure of calreticulin as well as secretion of high mobility group box 1, ATP, and type I IFN (IFN-I). The formation of these DAMPs facilitates the priming of tumor-specific CD8+ T cells by cross-presenting dendritic cells (DCs).

Doxorubicin is clinically used for treating sarcomas, lymphomas, and carcinomas such as breast and bladder cancer. It is also used clinically encapsulated in liposomes (eg, Doxil). Clinical trials testing the combination of doxorubicin/liposomal doxorubicin and ICB in solid tumors are ongoing (ClinicalTrials.gov: NCT03164993, NCT03598270). Doxorubicin immunogenicity has been demonstrated in vaccination experiments with doxorubicin-pretreated tumor cells, leading to protection of immunocompetent mice against rechallenge with viable tumor cells. Furthermore, doxorubicin treatment of tumor cells has been shown to produce the above-mentioned DAMPs. Accordingly, mice with a single tumor showed a better response when doxorubicin or Doxil was combined with ICB than with the respective monotherapies. These studies also described enhanced DC and CD8+ T cell infiltration of tumors.

IFN-I is crucial for cross-presentation/priming of CD8+ tumor-specific T cells. There are different pathways leading to the induction of IFN-I, depending on either RNA or DNA species. One pathway identified for doxorubicin-induced IFN-I is via the double-stranded (ds)RNA-sensing Toll-like receptor 3. An in vitro study showed that Ataxia Telangiectasia Mutated (ATM) and cyclic GMP-AMP synthase (cGAS) and stimulator of interferon genes (STING) may be involved in IFN-I induction by doxorubicin. The cGAS/STING pathway, a major pathway leading to IFN-I induction, can be triggered by cytosolic genomic DNA (gDNA) or mitochondrial DNA (mtDNA). Doxorubicin has been shown to damage and cause release of mtDNA to the cytosol, thus activating cGAS/STING, but IFN-I was not measured in that study, and doxorubicin-mediated antitumor effects were examined in immunodeficient mice. Thus, overall, it is not yet clear how doxorubicin treatment of tumor cells activates cGAS and STING to induce IFN-I. In particular, it is not yet clear whether and how doxorubicin triggers cytosolic release of gDNA or mtDNA to activate the cGAS/STING/IFN-I pathway and how important this is for antitumor T cell responses and for doxorubicin-induced antitumor effects under conditions of an intact immune system.

Here, we show, in two syngeneic tumor models, that adding a single, low dose of Doxil to RT and anti-PD-1 strongly enhanced the abscopal effect with a high complete abscopal cure rate. This enhanced abscopal effect depended on CD8+ T cells and correlated with infiltration of the non-irradiated tumors by tumor-specific CD8+ T cells and cross-presenting DCs. Using mtDNA-depleted tumor cells as well as cGAS/STING inhibitors and knockout (KO) cells, we demonstrate that both the Doxil-enhanced abscopal effect and doxorubicin/Doxil-induced IFN-I are strongly dependent on mtDNA as well as on cGAS and STING.

MATERIALS AND METHODS

Mice and cell lines
C57BL/6N (RRID: MGI:6198353) mice were purchased from Janvier Labs. To enrich the environment, the mice received cardboard houses, plastic tubes and shredded paper in the cage. There was an acclimatization period of 14 days before the experiments were started. CD133-expressing melanoma cells (B16-CD133) were generated and cultured in RPMI 1640 medium as described previously. MC38 colon carcinoma cells (ENH204-FP) were purchased from Kerafast and cultured in DMEM as recommended by the provider.

Generation of mtDNA-depleted (rho zero, ρ0) cells
B16-CD133 and MC38 cells were treated with Zalcitabine (2'-3'-dideoxycytidine, ddC), a deoxyribonucleoside analog that specifically inhibits mtDNA replication, at 100 nM for 40 days. These cells were supplemented with 50µg/mL uridine and 1mM sodium pyruvate, which are essential for survival with reduced mitochondria. The cells were analyzed for mitochondrial and nuclear DNA content by

quantitative PCR (qPCR), see the mtDNA and gDNA quantification section.

**Generation of cGAS and STING KO (Cgas−/− and Sting−/−) B16-CD133 and MC38 cells**

CRISPR/Cas9 technology was used to generate Cgas−/− and Sting−/− MC38/B16-CD133 cells. Guide RNAs (gRNAs) targeting the mouse Cgas gene (gRNA1: AAGCGGCTCT-GTCTTAGAT; gRNA2: ATATTCTGATGCTCAATCC), and Sting gene (gRNA1: CAGTAGCCAAGTTGTCGTCC; gRNA2: AGCCGTTACCTGCTGCGGCTG) were cloned into pRP(CRISPR)-EGFP/Puro-hCas9-U6 (Vectorbuilder). Transfection was performed with Lipofectamine 3000 (Invitrogen). Two days after transfection, cells were selected with medium containing 2 µg/mL puromycin (ant-pr-1/Invivogen) for 2 days. Cells were expanded and screened for lack of cGAS or STING expression by Western blot.

**Mouse tumor models and treatment**

B16-CD133 and MC38 tumor cell suspensions were mixed with Matrigel (Corning) at 1:1 ratio and injected s.c. into the right flank (primary tumor) and left flank (secondary tumor) of C57BL/6N mice aged 6–8 weeks. For the B16-CD133 model, the mice were implanted with 2×10^6 cells in the right flank, and 4 days later, in the left flank. In the MC38 model, 5×10^9 cells were injected into the right flank, and 3 days later, in the left flank. Tumor sizes were measured with calipers, and the volumes were calculated using the formula: length×width×height. Animals were randomly assigned to treatment groups based on tumor volumes.

The primary tumor received two consecutive fractions of local hypofractionated RT (hRT) of 12 Gy (B16-CD133) or three fractions of 8 Gy (MC38) when the primary and secondary tumors reached a volume of 200–350 mm^3 and 75–120 mm^3, respectively (approximately 10 days after primary tumor inoculation). Tumor irradiation was performed using an RS2000 X-ray unit (RadSource). Anesthetized mice were positioned in a custom-made plastic jig with a size-adjustable aperture for the primary tumor; the rest of the mouse body was fully shielded with lead. Weekly i.p. injections of αPD-1 antibody (200 µg; clone RMP1-14, BioXCell) were started on the first day of RT. A 4 mg/kg intravenous injection of Doxil (Caelyx) was given to mice on the first day of treatment. In some experiments, CD8− T cells were depleted by injecting 200 µg/mouse of anti-CD8α antibodies (clone 2.43, ATCC) or anti-CD8β (clone YTS 165.7.7, ECACC European collection of cell cultures) antibodies i.p. 2 days before hRT, on the day of the first hRT, and once weekly thereafter. Survival was defined as the time point after the start of treatment when either the primary or the secondary tumor had reached a size of 2000 mm^3. Rechallenge experiments in cured mice were performed by injecting 1×10^6 live MC38 tumor cells dissolved in 50% Matrigel. The mice were ear-tagged and not grouped together according to treatment group. Therefore, in tumor follow-up and analysis of tissue samples, the experimenter was not aware of the treatment the individual mice had received. However, because the same experimenter performed all experiments and analyses, the experiments were not conducted in a completely blinded fashion.

**Recist V.1.1** was adapted for evaluation of mouse tumors as follows: (1) best relative tumor volume changes from d10 after treatment start to the endpoint of the experiment compared with treatment start were calculated; (2) complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD) are defined as tumor volume change: to minus 100%=disappearance (CR), of at least a 30% decrease (PR), within the range between minus 30% and plus 20% in at least three measurements (SD), and at least a 20% increase (PD), respectively.

**Flow-cytometric analyses**

Flow-cytometric analyses of tumor-infiltrating lymphocytes (TILs) and single-cell suspensions from tumors, blood, and lymph nodes were performed using the following antibodies/MHC tetramers. M8 tetramer-PE (H-2Kb, p15E) (from Baylor College of Medicine) with CD8-AF700 (clone KT15/MBL) were used to detect tonsillar-specific CD8+ T cells. CXCR3-APC-Fire750 (clone CXCR3-173) was from Miltenyi Biotec. MHCII-AF700 (clone M5/114.15.2), CD45-BV510 (clone 30-F11), CD3-PE-Cy5.5 or -FITC (clone 145–2-C11), CD49b-FITC (clone DX5), CD19-FITC (clone 1D3), F4/80-FITC (clone IKB58), CD8-APC (clone 53–6–7), Tim3-BV605 (clone RMT3-23), Ki-67-BV605 (clone 16A8), CD11c-BV650 (clone N418), CD11b-PE-Cy7 (clone M1/70), CD86-BV421 (clone GL1), Ly6C-PerCP-Cy5.5 (clone HK1.4) and CD103-PE-CF594 (clone M290) were from BioLegend. Other antibodies used were: CD101-Pe-Cy7 (clone Moush101) from Invitrogen; Phospho-Histone H2A.X-AF488 (#9719) and Isotype Control-AF488 (#2975) were from Cell signaling. 7-AAD (BD Bioscience), Zombie Red, and Zombie NIR (BioLegend) were used to exclude dead cells. The samples were analyzed using a CytoFLEX S flow cytometer (Beckman Coulter). Data were analyzed by using FlowJo software (V.10.4.0; RRID: SCR_008520).

**Apoptosis and clonogenic survival assays**

For the apoptosis assay, tumor cells were irradiated with 12 Gy, and after 16 hours, cells were harvested and stained with Annexin V and PI using an Annexin V-FITC Kit from Miltenyi Biotec (130-092-052). Thereafter, early apoptotic (annexin V+PI) and dead cells (PI+) were measured by flow cytometry. For the clonogenic assay, 1000 tumor cells were irradiated with 12 Gy and then plated into 6-well plates. Cells were allowed to grow for additional 5 days to form colonies before staining with Giemsa.

**ATP release assay**

Extracellular ATP was assessed using the CellTiter-Glo 2.0 Luminescent Cell Viability Assay. Briefly, 1500/well
B16-CD133 or MC38 cells were seeded in 96-well plates. After 48 hours of incubation with Doxil or hRT treatment, CellTiter-Glo 2.0 Reagent was added to the supernatant (extracellular ATP). Luminescence signals were recorded with a GloMax-Multi Detection System.

**Quantitative reverse transcription PCR**

Total RNA from cells was extracted with the TRIzol Reagent (Invitrogen) and reverse transcribed to complementary DNA (cDNA) by using the PrimeScript first Strand cDNA Synthesis Kit (Takara Bio). Real-time PCR was performed with SVBR Select Master Mix using a Roche LightCycler 480. Data were normalized by the level of Gapdh expression in each individual sample. The $2^{-\Delta\Delta C_{T}}$ method was used to calculate relative expression changes. The primers used are: mouse Cxcl10 forward: GCTGC- CGTATTTCCTGC; mouse Cxcl10 reverse: TCTCATGC- GGCCGTCATC; mouse Gapdh forward: AGGTGGAAGGTGA- CTTC; mouse Gapdh reverse: TGTAGACC ATGTAGGTGAGTCA.

**Cytosolic DNA extraction**

Cytosolic DNA extraction was performed as described. $^{30}$ Briefly, after 48 hours treatment, cell suspensions were divided into two parts at the ratio of 1:3. The smaller part was used for determining total gDNA. The larger part was first lysed in 1 NP-40, then centrifuged at 13000rpm to pellet the insoluble fraction; cytosolic DNA was extracted from the supernatant by using the DNeasy Blood & Tissue Kit (Qiagen).

**mtDNA and gDNA quantification**

After extracting DNA from the cytosolic fraction or from whole cells, qPCR was employed to measure mtDNA and gDNA. Whole cell mtDNA as well as cytosolic mtDNA and gDNA levels were normalized with total gDNA. The primers used were: for mtDNA: mouse Nd1 forward: CCCAGGTACTACCATCATTCAAT; mouse Nd1 reverse: GATGTTTGGGAGATTGTGAGT; mouse Cox1 forward: TTTTACAGCTTCACCATTAGA; mouse Cox1 reverse: CCTACGATATGCGGCAGAATG; mouse Mrps12 forward: ACCCGGGTGCATACGTTAAC; mouse Mrps12 reverse: CCCAGTTTGGGTCTTAGCTG; for gDNA: mouse 18S rRNA forward: CCCCATAGACGAGGAAATT; mouse 18S rRNA reverse: GGGACTTAACTACGCAAGCTT; mouse Gapdh (see above).

**ELISA**

After treatments of tumor cells or co-culture with bone marrow-derived DCs (BMDCs), supernatants were collected and the IFNβ concentration was measured with the LumiKine Xpress mIFN-β 2.0 kit (InvivoGen) according to the manufacturer’s protocol.

To obtain BMDCs, mice femurs and tibias were flushed with PBS and pipetted vigorously to obtain single cells. Erythrocytes were lysed using eBioscience RBC lysis buffer (Invitrogen). Cells were cultured at $2 \times 10^5$ cells/mL in complete medium supplemented with 500 U/mL GM-CSF and 500 U/mL IL-4. Half of the medium was replaced on day 4 with fresh medium supplemented with granulocyte-macrophage colony-stimulating factor (GM-CSF) and IL-4. Loosely adherent cells were harvested on day 7 and used as BMDCs.

**Western blot analysis**

Cells were lysed in RIPA lysis buffer supplemented with Halt Protease Inhibitor Cocktail (Thermo Scientific). Cell lysates (30 μg) were separated by SDS-PAGE and blotted onto nitrocellulose. Antibodies: Translocase of outer mitochondrial membrane 20, TOM20 (D8T4N; #42406; RRID: AB_2687663), STING (D2P2F; #13647), cGAS (D3O80; #31659), β-ACTIN (13E5; #4970) were purchased from Cell Signaling. Mitochondrial transcription factor A (TFAM)/mtTFA (transcription factor A, mitochondrial) (Cat No GTX112760) was purchased from GeneTex. Anti-TREX1 antibody (C-11) (#sc-133112) was purchased from Santa Cruz Biotechnology. HRP-conjugated secondary antibodies were purchased from Dianova.

**Cross-presentation experiment**

B16-OVA and MC38-OVA cells were gifts from Dr. Vincenzo Cerullo (Helsinki) and Dr. Pedro Romero (Lausanne). $^{31}$ respectively. 1.5×10$^7$ untreated or Doxil and/or RT-treated B16-OVA or MC38-OVA cells were mixed 1:1 with BMDCs in 6-well plates. After 16 hours, the percentage of H-2K$^b$/SIINFEKL on BMDCs was determined by flow cytometry using the H-2K$^b$/SIINFEKL-PE antibody (25-D1.16) from Miltenyi Biotec.

**Statistical analysis**

Previous experience served as the basis for calculations of expected averages and deviations used to calculate sample size to detect an effect with power=0.8. This typically resulted in a minimum sample size of n=5–7, depending on the experiment. The unpaired two-tailed Student’s t-test was used for two-group comparison. The comparison time points for tumor volume measurements are indicated in the figures. One-way analysis of variance with Dunnett (for comparing each group to control group) or Tukey (for comparisons between every two groups) multiple comparison test was used to compare multiple groups. Kruskal-Wallis test was used to compare the medians of percent change of tumor volume between groups with Dunn’s test for multiple comparison correction. Survival data were compared using the log-rank test. A p<0.05 (p<0.05, p<0.01, p<0.001, p<0.0001) was considered significant. All analyses were performed using GraphPad Prism V.7.0 (RRID: SCR_002798).

**RESULTS**

**Combining Doxil with hRT and αPD-1 enhances the abscopal effect**

We investigated whether adding a low dose of Doxil can enhance the hRT/αPD-1-induced abscopal effect in mouse models with bilateral tumors in which the primary
tumor was irradiated, but not the secondary (figure 1A).

For tumor irradiation, we used hRT with either two fractions of 12 Gy or three fractions of 8 Gy because previous preclinical studies by others and ourselves indicated that this is effective for enhancing antitumor T cell responses in conjunction with ICB. Three prime repair exonuclease (TREX)1, which regulates cytoplasmic dsDNA levels, was induced to a similar extent on irradiation with these two hRT regimens in both B16-CD133 melanoma cells and MC38 colon carcinoma cells (online supplemental figure S1). The abscopal models using these cell lines are well established in our laboratory.433 34

Figure 1 Combining Doxil with hRT and αPD-1 enhances the abscopal effect (A) Scheme for treatments. (B) Tumor growth of primary/irradiated and secondary/non-irradiated tumors (B16-CD133 model). (C) Individual tumor growth curves for primary and secondary B16-CD133 tumors in mice treated with RT + αPD-1, Doxil + αPD-1, or triple therapy. (D) Survival of mice. (B–D) n=5–10 mice per group. (E) Tumor growth of primary/irradiated and secondary/non-irradiated tumors (MC38 model). (F) Individual tumor growth curves for primary and secondary MC38 tumors in mice treated with RT + αPD-1, Doxil + αPD-1, or triple therapy. (G) Survival of mice. (E–G) n=5–9 mice per group. Tumor volume data are presented as mean±SEM. P values (ns, not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) were determined by two-tailed t-test (B, E) and log-rank test (D, G). In (B, E) the comparison time points for tumor volume measurement, that is, the final time point at which no mouse of the compared groups had yet reached the experimental end point, are indicated. CR, complete response; hRT, hypofractionated RT; RT, radiotherapy.
These two models show a significant, moderate abscopal effect with hRT + αPD-1, but no complete regression/cure of the non-irradiated tumor and are, therefore, suitable for testing triple therapies that may enhance the RT/αPD-1-induced abscopal effect. The B16-CD133 melanoma model expresses the human stem cell antigen CD133 and is more immunogenic than the B16 wild-type (WT) model. In this model, the primary tumor was irradiated with two fractions of 12 Gy on consecutive days. Systemic αPD-1 antibodies were administered once per week. Triple (hRT/αPD-1/Doxil)-treated mice as well as the Doxil/αPD-1 and the Doxil monotherapy groups received a single low dose of Doxil (4 mg/kg) at the start of the treatment course. This Doxil dose is well below the maximum tolerated dose. Triple-treated mice received the Doxil together with the first RT fraction and the first αPD-1 dose (figure 1A).

Monotherapy with αPD-1 or hRT to the primary tumor did not affect the non-irradiated tumor in this model (figure 1B). hRT/αPD-1 dual therapy induced a substantial abscopal effect but no complete regression of the non-irradiated (abscopal) tumor (figure 1B). Doxil alone did not affect the primary tumor, but suppressed the smaller secondary (figure 1B). hRT/Doxil suppressed the primary tumor, but did not improve the response of the secondary tumor compared with Doxil monotherapy. Doxil/αPD-1 dual therapy, even though it did not control the larger primary as effectively as the hRT-containing treatments, enhanced control of the non-irradiated secondary tumor compared with Doxil or αPD-1 monotherapies. However, triple therapy with hRT + αPD-1 + Doxil was most effective. It enhanced both the abscopal effect (figure 1B, right, figure 1C, online supplemental figure S2A) and survival (figure 1D) significantly compared with all other treatment groups including hRT/αPD-1 and Doxil/αPD-1. Of note, a complete abscopal effect was observed in two-thirds of the triple-treated mice (figure 1B,C and online supplemental figure S2A). In mice with completely cured or almost completely cured tumors, vitiligo-like depigmentation of the fur was observed, including at the sites where the non-irradiated secondary tumors had been located (online supplemental figure S2B).

In the MC38 colon carcinoma model, hRT/αPD-1 also induced a substantial abscopal effect compared with hRT and αPD-1 alone, but complete abscopal responses were not observed (figure 1E,F). In this model, triple treatment was also better than hRT/αPD-1 and Doxil/αPD-1 dual therapies, both in terms of abscopal response and overall survival (figure 1E–G, and online supplemental figure S2C). Complete abscopal responses were only observed in triple-treated mice (figure 1E and F and online supplemental figure S2C). Only one mouse had both primary and secondary tumors permanently healed. Rechallenge with MC38 tumor cells in this mouse did not result in tumor growth, indicating induction of memory T cells (online supplemental figure S2D).

Evaluation of the abscopal effect was also done according to adapted RECIST V1.1 (see the Materials and Methods section). In the B16-CD133 model, the ORR (CR+PR) for the abscopal tumor was 12.5%, 20.0%, and 11.1%, respectively, in double (RT/Doxil, Doxil/αPD-1, RT/αPD-1)-treated mice and 100% in triple-treated mice. In the MC38 model, the ORR for the abscopal tumor was 0% in double (RT/Doxil, Doxil/αPD-1, RT/αPD-1)-treated mice and 44.4% in triple-treated mice, respectively (online supplemental figure S3A,B).

Taken together, our results show that the addition of Doxil to hRT/αPD-1 can substantially improve the hRT/αPD-1-induced abscopal effect and promote the survival of mice in comparison with both hRT/αPD-1 and Doxil/αPD-1. Moreover, complete abscopal responses of non-irradiated tumors usually only occurred in triple-treated mice.

Abscopal tumor regression in hRT/Doxil/αPD-1-triple treated mice depended on CD8+ T cells and correlated with abundance of tumor-specific CD8+ TILs

To investigate the mechanisms underlying the superior response to triple therapy, mice bearing two B16-CD133 melanoma tumors were treated with hRT+αPD1+Doxil and with antibodies to deplete CD8+ cells (figure 2A). As shown in figure 2B–D, the effect of triple therapy on both primary and secondary tumor was reduced when CD8+ cells were depleted by either anti-CD8α or anti-CD8β antibodies. Whereas CD8α is expressed on both CD8+ T cells and DCs, CD8β is only expressed on T cells. Therefore, these depletion experiments demonstrated that primary tumor control depended slightly and the Doxil-enhanced abscopal effect depended substantially on CD8+ T cells. There was a trend toward a stronger reduction in tumor treatment efficacy in CD8α cell-depleted mice, which is in line with the importance of CD8α+ cross-presenting DCs for spontaneous and treatment-induced CD8+ T cell responses.

In accordance with the depletion results, flow cytometry analysis of tumor single-cell suspensions showed that triple treatment caused a significant increase in the numbers of bulk CD8+ T cells and tumor-specific CD8+ T cells in the non-irradiated tumors compared with Doxil/αPD-1 and hRT/αPD-1 treatment (figure 2E,F). Analyzing subpopulations of exhausted TILs showed that the numbers of transitory (effector-like) PD1+TIM3+CD101- tumor-specific CD8+ T cells were also highest in the abscopal tumor of triple-treated mice (figure 2F).

In addition, we analyzed the expression of the C-X-C Motif Chemokine Receptor 3 (CXCR3), which is crucial for T cell immigration into inflamed tumors. As shown in figure 2G, CXCR3+CD8+ T cells were significantly increased in the tumor-draining lymph nodes of triple-treated compared with double-treated (hRT/αPD-1 or Doxil/αPD-1) mice. Similar results were obtained for the peripheral blood, where CXCR3+CD8+ T cells were significantly higher only compared with hRT + αPD-1 (figure 2G).

Together, these results demonstrated that the abscopal antitumor efficacy of the Doxil-containing triple therapy
strongly depended on CD8+ T cells and suggested that tumor-specific CD8+ T cells (including transitory/effector-like cells) contribute to the Doxil-enhanced RT/αPD-1-mediated abscopal effect in triple-treated mice.

Figure 2  Abscopal tumor regression in RT/Doxil/αPD1 triple-treated mice depended on CD8+ T cells and correlated with abundance of tumor-specific CD8+ TILs (A) Scheme for treatments. (B) Tumor growth of primary/irradiated and secondary/non-irradiated tumor in mice bearing B16-CD133 tumors treated with triple therapy (with or without CD8α/β T cell-depleting antibodies). (C) Individual tumor growth curves. (D) Survival of mice. (E, F) CD8+ T cells, M8 tetramer-positive CD8+ T cells, and transitory CD8+ tetramer T cells (PD1+, TIM3+, CD101−) on day 7 in primary and secondary tumors (n = 5 mice/group). (G) Expression of CXCR3 on CD8+ T cells in tumor-draining lymph nodes (TDLNs) and peripheral blood lymphocytes on day 10 (n=5 mice/group). Data are presented as mean±SEM. P values (ns, not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) were determined by two-tailed t-test (B), log-rank test (D), and one-way ANOVA with Tukey’s test (F, G). In (B) the comparison time points for tumor volume measurement, that is, the final time point at which no mouse of the compared groups had yet reached the experimental end point, are indicated. ANOVA, analysis of variance; CR, complete response; RT, radiotherapy; TILs, tumor-infiltrating lymphocytes.

Tumor-infiltrating cross-presenting DCs and CD8+ T cell proliferation are associated with the strong effect of triple therapy

The induction and activation of tumor-specific CD8+ T
cells usually require cross-presenting DCs of the innate immune system, which can present engulfed tumor antigen by MHC class I molecules to CD8+ T cells. Conventional DCs (CD11c−CD11b−), CD8α DCs (lymphoid resident) and CD103+ DCs (migratory) are capable of cross-presentation. CD11b+ monocyte DCs also have this ability. For example, the antitumor immunity of anthracyclines was found to depend on their ability to recruit inflammatory CD11b+CD11c+ monocyte (mo) DCs with cross-presenting activity to the TME. We determined tumor-infiltrating DCs at d3 and d10 after treatment start. Three days after therapy started, we found that Doxil-containing dual or triple therapy and hRT/αPD-1 dual therapy increased tumor infiltration by both CD11b+ CD11c+ DCs (figure 3A) and Ki-67+ (ie, proliferating) CD8α T cells (figure 3B) compared with therapy with αPD-1 alone. The significance level for the difference compared with αPD-1 monotherapy was highest for both DC and proliferating T cell infiltration with triple therapy. At the later time point (d10 after therapy start), more CD103+ (migratory) conventional DCs, that is, classical cross-presenting DCs, were found in both primary and secondary tumor of triple-treated compared with Doxil/αPD-1 or hRT/αPD-1 double-treated mice in the abscopal tumor model (figure 3C). At this time point, mature CD11b+CD11c+ DCs expressing the costimulatory molecule CD86 were also highest in both primary and secondary tumor of triple-treated mice (figure 3C). Furthermore, we have studied cross-presentation functionally in vitro using BMDCs and OVA-expressing B16 melanoma or MC38 colon carcinoma cells. Coincubation of Doxil-treated and/or hRT-treated B16-OVA (or MC38-OVA) tumor cells with BMDCs increased the proportion of BMDCs cross-presenting the OVA-derived SIINFEKL peptide presented by H2Kb (figure 3D). These results showed that the enhancement of the abscopal effect by Doxil was correlated with the number and maturity of DCs, including cross-presenting DCs, in tumor tissue (including the non-irradiated secondary tumor) and that this was associated with increased proliferation of CD8α T cells.

**Doxil-induced IFN-I secretion and the Doxil-enhanced abscopal effect were dependent on cGAS/STING**

We then carried out investigations to find out more precisely how doxorubicin/Doxil may enhance the abscopal effect and to what extent immunomodulatory or even immunogenic effects of Doxil on the secondary (abscopal) tumor play a role. The attraction to tumor tissue and activation of cross-presenting DCs requires DAMPs. The phosphorylation of H2AX at serine 139 (also designated as γH2AX) visualizes DNA damage as we observed here on treatment with RT, Doxil, or both (figure 4A). IFN-I is a crucial DAMP for activating DCs for antitumor immunity. We found that both free doxorubicin and Doxil induced IFNβ1 in both B16-C1D133 and MC38 tumor cells in vitro (figure 4B). By examining the effects of various inhibitors of the cGAS/STING pathway (inhibitors of cGAS, STING, and TANK-binding kinase-1 (TBK1)), we found a strong reduction in the IFNβ1 response of Doxil-exposed or irradiated tumor cells compared with non-inhibitor-treated cells (figure 4C). To corroborate this, we generated Cgas−/− and Sting−/− cells using CRISPR/Cas9 technology (figure 4D). As shown in figure 4E, IFNβ1 induction was also significantly reduced in Cgas−/− and in Sting−/− B16-C1D133 and MC38 cells treated with Doxil or IR.

To elucidate the influence of the cGAS/STING pathway on the Doxil-enhanced abscopal effect, we performed in vivo experiments in the abscopal tumor model where either Cgas−/− or Sting−/− MC38 tumor cells or control nontargeted (Cgas+/Sting+/−) MC38 tumor cells were implanted both at the primary and secondary tumor site. As shown in online supplemental figure S3C, in mice with bilateral non-targeted control MC38 tumors, hRT/αPD-1/ Doxil triple therapy slowed the growth and cured some of the nonirradiated tumors (as in mice bearing parental MC38 WT tumors (see figure 1E)). In contrast, there was a complete abrogation of the abscopal effect in triple-treated mice bearing Sting−/− tumors, and a reduction in mice bearing Cgas−/− tumors with no complete abscopal cures (online supplemental figure S3C and 4F). In both mice bearing either Sting−/− or Cgas−/− tumors, there was no difference in abscopal tumor control between triple-treated and RT/Doxil-treated mice, indicating that additional αPD-1 did not improve the abscopal effect in the absence of cGAS/STING in the tumor cells (figure 4F and online supplemental figure S3E). As shown in online supplemental figure S5 A,B, KO of cGAS or STING did not affect radiation-induced abrogation of clonogenic survival of MC38 tumor cells; likewise, radiation-induced apoptosis was not reduced.

Together, these results demonstrated that Doxil induced cGAS/STING/IFN-I pathway activation in vitro, and the strong therapeutic effect of Doxil-containing triple therapy depended on cGAS/STING in tumor cells in vivo.

**Tumor cell mtDNA was crucial for Doxil-induced IFNβ1, ATP, and Cxcl10 expression by tumor cells and for IFNβ1 production by DCs coinubated with Doxil-treated tumor cells**

As mentioned above, there are two possible sources of cGAS-activating dsDNA species: gDNA from the nucleus (eg, within cytosolic micronuclei), or cytoplasmic mtDNA leaking from mitochondria. The role of the latter in inducing DAMPs by cytotoxic therapies has lately become a growing field of interest. The latter of the role in inducing DAMPs by cytotoxic therapies has lately become a growing field of interest. As shown by qPCR, both cytosolic mtDNA and gDNA were increased in B16-C1D133 and MC38 cells 48 hours after irradiation or Doxil treatment (figure 5A).

Recent reports suggested that mtDNA is crucial for the induction of IFN-I by RT at least at early time points after irradiation. To study the importance of mtDNA for Doxil-induced IFN-I secretion, we generated tumor cells with strongly reduced mtDNA (p7 cells) by using the compound Zalcitabine (ddC), a nucleoside analog.
Figure 3  Tumor-infiltrating cross-presenting DCs and CD8\(^+\) T cell proliferation are associated with the strong effect of triple therapy. (A) CD11c\(^+\) CD11b\(^+\) DCs on day 3 after treatment start in the B16-CD133 model (n=5 mice/group); the gating strategy is shown for a single-cell suspension from an irradiated tumor of a triple-treated mouse. (B) The percentage of Ki-67\(^+\) (ie, proliferating) cells among CD8\(^+\) T cells in treated tumors on d3 (n=5 mice/group). (C) Migratory CD103\(^+\) cross-presenting DCs and mature CD86\(^+\) CD11b\(^+\)CD11c\(^+\) DCs on day 10 in tumors (n=5 mice/group). (D) Flow cytometric determination of OVA-derived SIINFEKL peptide presented by H-2K\(^b\) on BMDCs co-cultured for 16 hours with OVA-expressing tumor cells pretreated with RT and/or Doxil for 24 hours. Data are presented as means with SEM. P values (ns, not significant; *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001) were determined by one-way ANOVA with Tukey’s test (A, B) or Dunnett test (C, D). ANOVA, analysis of variance; BMDCs, bone marrow-derived DCs; DC, dendritic cell; RT, radiotherapy.
that inhibits the mitochondrial POLG protein and causes a reduction in mtDNA copy number. As shown in figure 5B, treatment of B16-CD133 and MC38 tumor cells with ddC resulted in a strong reduction in the mtDNA content of the tumor cells. In addition, we found reduced expression of the mitochondrial proteins TFAM and Tom-20 (figure 5C). As shown in figure 5D, ddC treatment/reduction of mtDNA in tumor cells significantly
Reduced doxorubicin/Doxil-induced IFN-I production in both B16-CD133 and MC38 cells. Deficiency of the mtDNA-binding mitochondrial membrane protein TFAM can cause release of mtDNA into the cytoplasm and thus prime a cGAS/STING-dependent response. We found that Doxil treatment downregulated TFAM protein expression in both B16-CD133 and MC38 tumor cells (figure 5E).

DCs are believed to be a major source of IFN-I in tumors. Consistent with this notion, we found strong Doxil-mediated induction of IFNβ1 secretion when BMDCs were cocultured with Doxil-treated WT tumor cells (figure 5F). However, this IFN-I induction was strongly reduced when the DCs were cocultured with mtDNA-depleted MC38 and B16-CD133 tumor cells (figure 5F).

Downstream of cGAS/STING and IFN-I activation is the induction of the T cell-recruiting chemokine CXCL10, which was also induced by free doxorubicin or Doxil and reduced in treated mtDNA-depleted tumor cells (figure 5G). Extracellular ATP is important for recruitment, survival, and differentiation of DCs to the tumor microenvironment. As shown in figure 5H, extracellular ATP was strongly reduced in Doxil-treated...
mtDNA-depleted MC38 and B16-CD133 tumor cells compared with WT cells.

Tumor cell mtDNA abundance was crucial for the Doxil-enhanced abscopal effect and for tumor infiltration by tumor-specific CD8+ T cells

Finally, to investigate whether mtDNA abundance is also crucial for the Doxil-enhanced abscopal effect, mtDNA-depleted B16-CD133 tumor cells (obtained by ddC treatment) were implanted at both the primary and the secondary (abscopal) tumor site (\( \rho^0/\rho^0 \), figure 6A).

Even 20 days after implantation, mtDNA-deficient tumors maintained their mtDNA-deficient phenotype (online supplemental figure S4A). As shown in figure 6B and online supplemental figure S2A and S4B, the non-irradiated tumor grew considerably faster on triple therapy (\( \rho^0/\rho^0 \), brown dash line) (ie, the abscopal effect was strongly reduced) compared with mice bearing WT primary and secondary tumors (WT/WT, blue line). No complete abscopal responses were observed anymore in triple-treated mice with mtDNA-deficient tumors. Survival...
of these mice was also worse than that of triple-treated mice with two WT tumors (figure 6C). In mice with two mtDNA-depleted tumors, response of the secondary tumor to triple treatment and to Doxil/αPD-1 double treatment was also not better than that to Doxil monotherapy, also suggesting loss of antitumor T cell immunogenicity (online supplemental figure S4C); in these mice, there was also no difference in abscopal tumor control between triple treatment and the RT/Doxil group (figure 6B). When only the irradiated primary tumor contained mtDNA-deficient tumor cells but the non-irradiated secondary tumor contained WT cells (p0/WT, white line) (online supplemental figure S4D and S4E), the abscopal effect was not significantly reduced on triple therapy compared with mice bearing two WT tumors (WT/WT, blue line); and the abscopal cure rates (5/9 vs 6/9) for the non-irradiated tumors were similar in these two groups. In contrast, when the primary tumor was composed of WT cells and the secondary tumor was composed of mtDNA-depleted cells (WT/ p0, pale blue line), the abscopal effect was strongly reduced (online supplemental figure S4D and S4E). This suggested that the effect of Doxil on the secondary tumor was particularly important for the enhancement of the hRT/αPD-1-induced abscopal effect.

Consistent with an impaired abscopal response, we found evidence for an impaired immune response to mtDNA-deficient tumor cells. As shown in figure 6D,E we observed a lower proliferation of CD8+ TILs at d3 after treatment start in the irradiated tumor; as well as at d7 a lower proportion of tumor-specific CD8+ T cells in peripheral blood and a lower density of tumor-specific CD8+ T cells in both primary and secondary tumor models, Doxil/anti-PD-1 double treatment was not sufficient for CRs of primary and non-irradiated secondary tumors. Additional hRT of the primary tumor (triple treatment) was essential for complete cures of non-irradiated secondary tumors.

In mice with two mtDNA-depleted tumors, response of the secondary tumor to triple treatment and to Doxil/αPD-1 double treatment was also not better than that to Doxil monotherapy, also suggesting loss of antitumor T cell immunogenicity (online supplemental figure S4C); in these mice, there was also no difference in abscopal tumor control between triple treatment and the RT/Doxil group (figure 6B). When only the irradiated primary tumor contained mtDNA-deficient tumor cells but the non-irradiated secondary tumor contained WT cells (p0/WT, white line) (online supplemental figure S4D and S4E), the abscopal effect was not significantly reduced on triple therapy compared with mice bearing two WT tumors (WT/WT, blue line); and the abscopal cure rates (5/9 vs 6/9) for the non-irradiated tumors were similar in these two groups. In contrast, when the primary tumor was composed of WT cells and the secondary tumor was composed of mtDNA-depleted cells (WT/ p0, pale blue line), the abscopal effect was strongly reduced (online supplemental figure S4D and S4E). This suggested that the effect of Doxil on the secondary tumor was particularly important for the enhancement of the hRT/αPD-1-induced abscopal effect.

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On triple treatment, we found higher infiltration of the non-irradiated tumor with CD8+ T cells and cross-presenting DCs compared with Doxil/αPD-1 and RT/αPD-1. This prompted us to investigate the role of IFN-I as a known major DAMP in cross-priming/presentation. cGAS/STING activation by doxorubicin, associated with IFN-I induction, has been found previously. However, no previous study has shown that the mtDNA/cGAS/STING axis is involved in doxorubicin-induced IFN-I induction and that the mtDNA/cGAS/STING/IFN-I axis is involved in CD8+ T cell-dependent antitumor immunity provoked by doxorubicin/Doxil. We provide evidence for these mechanistic scenarios based on the use of inhibitors, cGAS/STING KO and mtDNA-depleted tumor cells in vitro and in abscopal tumor models in vivo. The importance of tumor cell cGAS/STING in RT/Doxil/αPD-1-induced antitumor immunity is consistent with the importance of tumor cell-derived cGAS/
STING in other studies on DNA-damaging agents with/without ICB therapies. However, other studies provided evidence for the importance of the cGAS/STING axis in the myeloid compartment for activating anti-tumor immunity by RT. Our new mechanistic findings may also be important for the use of doxorubicin or encapsulated doxorubicin in chemotherapy or chemoinmunotherapy. Although both free doxorubicin and Doxil can act synergistically with ICB, there is evidence from preclinical monotherapy studies that encapsulated doxorubicin can induce stronger T cell-dependent anti-tumor effects than free doxorubicin. Deficiency of the histone-like mitochondrial protein TFAM has been shown to cause aberrant mtDNA packaging and mtDNA leakage to the cytosol. We observed a decrease in TFAM expression on Doxil treatment. However, whether reduction of TFAM expression is indeed involved in doxorubicin/Doxil-induced mtDNA release from the mitochondria to the cytosol remains to be conclusively demonstrated.

Taken together, we show that Doxil (as a single low dose) can strongly enhance the hRT/αPD-1-induced abscopal effect with a high abscopal cure rate. This was associated with increase in cross-presenting DCs and tumor-specific CD8+ T cells mainly in the non-irradiated tumor and depended strongly on tumor cell mtDNA and cGAS/STING. By a series of mechanistic experiments, we demonstrate that the mtDNA/cGAS/STING axis is crucially involved in doxorubicin-induced IFN-I induction. Together, the in vitro and in vivo experiments...
suggest importance of the mtDNA/cGAS/STING/IFN-I axis in CDS8+ T cell-dependent immunogenicity of doxorubicin/Doxil. The latter is also important for a better understanding of doxorubicin/Doxil’s immunogenicity in general, which may also be relevant for their use in chemotherapy and in chemoimmunotherapy. Our data may help design clinical trials to examine Doxil’s potency to enhance the RT/αPD-1-induced abscopal effect in patients. Therefore, we hope that our publication will help to reduce the gap between preclinically observed abscopal effects and the so far uncommon clinical abscopal effect on RT/ICB-treatment. This discrepancy likely has several reasons, one of which may be insufficient infiltration of non-irradiated tumor lesions by RT/ICB-induced tumor-specific T cells. This could potentially be improved by additional application of low-dose chemotherapeutic agents, preferably immunogenic ones, as we show here. Our data are also interesting in light of recent findings suggesting that mtDNA in tumor tissue may be a prognostic biomarker for cancer patients, and based on our experimental findings, it appears worthy to investigate whether mtDNA abundance in tumors could be a biomarker for patient selection for potentially immunogenic, conventional cytotoxic treatments (eg, doxorubicin, (h)RT, and their combination with ICB).

Author affiliations
1Department of Radiation Oncology, University of Freiburg Faculty of Medicine, Freiburg, Germany
2Department of Radiation Oncology, Harbin Medical University Cancer Hospital, Harbin, China
3Division of Thoracic Tumor Multimodality Treatment, Cancer Center, West China Hospital, Sichuan University, Chengdu, China
4Department of Radiation Oncology, Cancer Center, West China Hospital, Sichuan University, Chengdu, China
5Faculty of Biology, University of Freiburg, Freiburg, Germany
6German Cancer Consortium (DKTK), Partner Site Freiburg, Freiburg, Germany
7German Cancer Research Center (DKFZ), Heidelberg, Germany

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Contributors LW, RL and GN conceived and designed the study. LW and RL acquired, interpreted, and analyzed data. KD, XR, MW, SG and EF performed parts of the experiments. BM was involved in project administration and writing the MS. KO, XR, MW, SG and EF performed parts acquired, interpreted, and analyzed data. All authors edited and approved the manuscript. GN is the guarantor for this article.

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ORCID iD
Gabriele Niedermann http://orcid.org/0000-0001-9508-5497

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