Comparison of intratumor and local immune response between MV X-ray FLASH and conventional radiotherapies

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ABSTRACT

Background/Purpose: Investigating the antitumor effect and intratumor as well as local immune response in breast cancer-bearing mice after MV X-ray ultra-high dose rate radiotherapy (FLASH-RT) and conventional dose rate radiotherapy (CONV-RT).

Materials/Methods: Six-week-old female C57BL/6 mice were inoculated subcutaneously with Py8119 and Py230 breast tumor cells in the inguinal mammary gland and administered 10 Gy abdominal 6 MV X-ray FLASH-RT (125 Gy/s) or CONV-RT (0.2 Gy/s) 15 days after tumor inoculation. Tumor and spleen tissues were obtained at different time points post-irradiation (PI) for analysis of immune cell infiltration using flow cytometry and immunohistochemical (IHC) staining. Intestine tissues were collected 3 days PI to evaluate normal tissue damage and immune cell infiltration.

Results: Both FLASH-RT and CONV-RT significantly delayed tumor growth. Flow cytometry showed increased CD8+/CD3+ and CD8+/CD4+ ratios, and IHC confirmed a similar increased CD8+ T cell infiltration at 2 weeks PI in Py8119 tumor tissues in both irradiation groups. No statistical difference was observed between the irradiation groups in terms of tumor growth and increased T cell infiltration in the tumor. Unexpectedly, significantly smaller spleen weight and substantially higher CD8+/CD3+ and lower CD4+/CD3+ ratios were observed in the spleens of the FLASH-RT group than in the spleens of the non-irradiated control and CONV-RT groups 4 weeks PI. Pathological analysis revealed severe red pulp expansion in several spleens from the CONV-RT group, but not in the spleens of the FLASH-RT group. Reduced intestinal damage, macrophage and neutrophil infiltration were observed in the FLASH-RT group compared with CONV-RT group.

Conclusions: FLASH-RT and CONV-RT effectively suppressed tumor growth and promoted CD8+ T cell influx into tumors. FLASH-RT can induce different splenic immune responses and reduce radiation-induced damage in the spleen and intestine, which may potentially enhance the therapeutic ratio of FLASH-RT.

1. Introduction

Ultra-high dose rate radiotherapy (FLASH-RT) has received considerable attention as a series of studies have shown that FLASH-RT can spare normal tissues from radiation toxicity while retaining tumor repressing capabilities [1–5]. Such phenomenon is referred to as the...
“FLASH effect” and has been proven in different preclinical models, including mouse [1,2,6–8], zebrafish embryos [9,10], canine [11], mini pigs, and cat [12] models. This unique biological response drives research enthusiasm in the clinical applications of FLASH-RT [10,13]. Although the underlying mechanisms of the FLASH effect are unclear, attempts are ongoing to define the involved affecting factors and [3,14,15], identify the triggering conditions of the FLASH effect to take advantage of the unique radiobiological response [8,16], and enlarge the therapeutic window by incorporating other therapy techniques [17]. Immune regulation is critical in the mechanism of RT, but the immune response after FLASH-RT requires further investigation. A recent computational study has suggested that compared to conventional dose rate radiotherapy (CONV-RT), FLASH-RT may prime immune responses, including circulating immune cells [18], tumor microenvironment (TME), and inflammatory responses. Kim et al. found that FLASH-RT increased CD8+ myeloid and CD8+ cytotoxic T cells in a Lewis lung carcinoma model [14]. It was hypothesized that FLASH-RT combined with immune checkpoint inhibitors might enhance the therapeutic efficacy of FLASH-RT [17]. Eggold et al. reported that FLASH-RT increased intratumoral CD8+ T cell infiltration in combination with programmed death-1 (PD-1) immune checkpoint inhibition treatment in both anti-PD-1 antibody (aPD-1)-resistant ID8 model and aPD-1-sensitive UKP10 model of ovarian cancer 17 and 12 days post-RT, respectively [17]. However, the effect of ultra-high dose rate irradiation on peripheral immune cells is unclear. Venkatesulu et al. reported that 35 Gy/s electron depleted CD3, CD4, CD8, and CD19 lymphocyte populations, similar to irradiation at conventional dose rate in both cardiac and splenic irradiation models of lymphopenia [19]. However, the effect of high-energy X-ray FLASH-RT on the splenic microenvironment in tumor-bearing mouse models has not been reported.

Differential inflammatory responses, including cytokine activation and leukocyte migration, induced by CONV-RT and FLASH-RT might be related to the potential immune impact on the protective effect of FLASH-RT. For example, a series of preclinical studies have shown that FLASH-RT reduced the expression levels of transforming growth factor-beta, a maker of tissue damage and a key switch for fibrosis initiation [20,21]. A previous study found that FLASH-irradiated BALB/c nude mice had significantly fewer inflammatory blood cells and diminished pro-inflammatory cytokines 4 weeks post-irradiation (PI), which might be beneficial factors in reducing radiotoxicity after FLASH-RT [4]. Therefore, in the current study, we aimed to investigate the intratumor and local immune responses in two transplanted murine breast cancer models using the least explored FLASH-RT beam modality, that is, high energy (MV) X-ray. Our results suggest that tumor control efficiency and change of TME are similar after FLASH-RT and CONV-RT. However, reduced normal tissue toxicity and significantly different local immune responses highlight the potential of improving the therapeutic ratio for FLASH-RT.

2. Methods

2.1. Animal and tumor models

Six-week-old female C57BL/6 mice were purchased from GemPharmatech (Jiangsu, China). The mice received tumor cell inoculation after 1 week of acclimatization. The Institutional Animal Care and Use Committee authorized all procedures for the use and care of animals. The Py230 and Py8119 syngeneic murine breast cancer cells were trypsinized, washed twice in phosphate-buffered saline (PBS), counted, and resuspended in 1:1 Matrigel-PBS before being injected (5 × 10^6 cells/mouse) into the fourth mammary fat pad on the right side of the mice. The tumor volume was calculated using the formula \( V = \frac{(W^2 \times L)}{2} \) from caliper measurements, where \( V \) is the tumor volume, \( W \) is the tumor width, and \( L \) is the tumor length.

2.2. Irradiation setup and dosimetry

Radiotherapy was performed 15 days after tumor inoculation. Mice were anesthetized with 0.2 ml/10 g body weight of tribromoethanol and fixed using a 1.5-cm-thick polymethyl methacrylate holder, which also functioned as a dose build-up layer during X-ray irradiation (Fig. 1a). A prescription dose of 10 Gy to the surface of the abdomen of mice was adopted for both FLASH-RT and CONV-RT. The irradiation field was centered at the tumor center. Gafchromic EBT3 films (Ashland Inc., Covington, KY, USA) were attached to the abdomen of mice to measure the delivered dose (Fig. 1a).

FLASH-RT was performed using the Chengdu THz Free Electron Laser (CTFEL) facility that produces 6 MV bremsstrahlung X-rays [4,5]. A 5-cm-thick lead shield with a 2-cm (craniocaudal) × 4-cm (lateral) window was used for FLASH-RT; and the mice received RT at a source distance (SSD) of 13.5 cm. The time structure and dose rate of the FLASH pulses were measured using a fast current transformer devise, a diamond radiation detector, and a CeBr3 scintillation detector. CONV-RT was performed using a clinical Elekta Infinity linac system (Elekta AB, Stockholm, Sweden) that produced a flattening filter-free 6 MV X-ray beam, and a radiation field of 2 cm × 4 cm was acquired at an SSD of 100 cm.

2.3. Flow cytometry

Tumor tissues and spleens were harvested immediately after euthanasia. Tissues were minced with scissors, digested, and filtrated through 70-µm cell strainers (Fisherbrand, Cat. 22363548) to obtain single-cell suspensions. Cell suspensions were washed, resuspended in staining buffer (PBS with 2 % fetal bovine serum), and stained with the Zombie NIR fixable viability kit (BioLegend, Cat. 423106) to detect live cells and blocked with TruStain FcX® PLUC (anti-mouse CD16/32, BioLegend, Cat. 156603) to prevent nonspecific binding.

Multicolor antibody panels were designed and optimized to identify and characterize leukocyte subpopulations from tumor and spleen samples. The surface markers were stained using fluorescent-labeled antibodies, as listed in the supplementary Table A1. These panels were established for the analysis of mouse tumor and spleen samples. Flow cytometry was performed on a CytoFLEX Flow Cytometer (Beckman Coulter), and data were analyzed using CytExpert software version 2.4.

2.4. Histological analyses

Intestinal and spleen tissues were collected from the mice at the time of euthanasia. Intestinal tissues and spleens were fixed in 10 % neutral buffered formalin and embedded in paraffin, with the intestinal tissues embedded using the Swiss-rolling technique. The embedded intestinal and spleen tissues were sliced into 4 µm thickness and stained with hematoxylin and eosin according to standard procedures (Solarbio, Wuhan, China). These sections were scanned using a KF-PRO-020 whole slide scanner (KFBIQ, Ningbo, China) and analyzed using K-Viewer software (version 1.5.3.1, KFBIQ, Ningbo, China). For the quantification of intestine damage, the most severely damaged segment was considered, and a polylone with a 3-mm perimeter was drawn for analysis. The number of crypts along the polylone was counted, and the length of each crypt was measured; only crypts with more than ten cells were considered to be surviving crypts. The damage of spleen was quantified according to the reference with the red pulp expansion [22,23], and the scores were set as: score 0, no expansion; score 1, minimal expansion; score 2, mild expansion; score 3, moderate expansion; score 4, severe expansion.

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\text{V} = \frac{(W^2 \times L)}{2} 
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2.5. Immunohistochemical staining

Immunohistochemical (IHC) staining was performed on tumor, intestinal and spleen tissues to quantify changes in immune cells (CD4+ T, CD8+ T, MPO-positive neutrophils, and F4/80-positive macrophages). The sections were then deparaffinized and rehydrated. Endogenous peroxidase activity was blocked with 3 % hydrogen peroxide for 30 min at room temperature. Nonspecific binding was reduced by incubation with 10 % normal goat serum for 30 min. The sections were then incubated with rabbit antibody anti-CD4 (CST, Massachusetts, Cat. 25229, 1:125 dilution), anti-CD8 (Abcam, Cambridge, Cat. ab209775, 1:100 dilution), anti-MPO (Abcam, Cambridge, Cat. ab208670, 1:2000 dilution), or anti-F4/80 (CST, Massachusetts, Cat. 70076; 1:500 dilution) overnight at 4 °C. This was followed by a 45-min incubation with secondary antibodies. Immunoreactivity was revealed using the diaminobenzidine chromogen reaction. The detailed information on primary and secondary antibodies used for IHC staining is summarized in the Supplementary Table A2.

The IHC sections were scanned using a KF-PRO-020 whole slide scanner (KFBIO, Ningbo, China) and then analyzed using the HALO image analysis platform (Indica Labs, Albuquerque, NM, USA), as described in our previous publication [4].

2.6. Terminal deoxynucleotidyl transferase 2’-deoxyuridine 5’-triphosphate nick-end labeling assay

Terminal deoxynucleotidyl transferase 2’-deoxyuridine 5’-triphosphate nick-end labeling (TUNEL) assay was used to detect apoptotic cells in intestinal tissues. A one-step TUNEL apoptosis kit (Green, AF488, Elabscience Biotechnology Co., Ltd., Wuhan, China) was used for staining. Briefly, the intestinal tissues were fixed in 10 % formalin and embedded in paraffin. After deparaffinization and washing, the tissue specimens were incubated with proteinase K at room temperature for 10 min. The sections were then incubated with rabbit antibody anti-CD4 (CST, Massachusetts, Cat. 25229, 1:125 dilution), anti-CD8 (Abcam, Cambridge, Cat. ab209775, 1:100 dilution), anti-MPO (Abcam, Cambridge, Cat. ab208670, 1:2000 dilution), or anti-F4/80 (CST, Massachusetts, Cat. 70076; 1:500 dilution) overnight at 4 °C. This was followed by a 45-min incubation with secondary antibodies. Immunoreactivity was revealed using the diaminobenzidine chromogen reaction. The detailed information on primary and secondary antibodies used for IHC staining is summarized in the Supplementary Table A2.

Whole-slide imaging was performed to view green fluorescence using a Vectra Polaris Automated Quantitative Pathology Imaging System.
Clinical and Translational Radiation Oncology 38 (2023) 138–146

H. Zhu et al.

The dosimetric characteristics of the CTFEL platform are well established and described in our previous publications [4,5], and the detail beam parameter values is provided in supplementary Table A3 according to Vozenny et al. [24] During the FLASH-RT, the delivered macro-pulse consisted of a bunch of micropulses (with full width at half maximum of 5 ps and pulse separations of 18.5 s). The micro-pulse was set at 80 ms to deliver 10 Gy at the entrance surface of the mouse abdomen, which resulted in mean and instantaneous dose rates of 125 Gy/s and 4.6 × 10^5 Gy/s, respectively.

According to the EBT3 film measurements, Py8119 mice received 9.5 ± 0.28 Gy and 9.75 ± 0.15 Gy in FLASH-RT and CONV-RT, respectively. Py230 mice received 9.36 ± 0.23 Gy and 9.81 ± 0.13 Gy in FLASH-RT and CONV-RT, respectively. The relative dose differences between Py8119 and Py230 mice that received FLASH-RT or CONV-RT were both < 5%.

FLASH-RT showed similar tumor control efficiency to that of CONV-RT

Breast cancer Py8119 and Py230 cell tumor-bearing mice received 10 Gy FLASH-RT or CONV-RT in a single fraction and were euthanized at different time points (Fig. 1b). Two weeks PI, Py8119 tumor-bearing mice were euthanized for reach of the humane endpoint. The final tumor volumes and weights were as follows: control group, 2195.4 ± 629.8 mm^3; CONV group, 1424.2 ± 485.9 mm^3; and FLASH group, 942.4 ± 414.9 mm^3. Py230 tumor-bearing mice were observed for 4 weeks PI, and the final tumor volumes and weights were as follows: control group, 226.7 ± 223.6 mm^3; CONV group, 41.88 ± 22.31 mm^3; and FLASH group, 44.20 ± 30.05 mm^3. FLASH-RT and CONV-RT both significantly delayed tumor growth compared with the non-irradiated control group (Fig. 1c, d), indicating that these two irradiation modalities have equivalent tumor control efficiency.

FLASH-RT and CONV-RT showed increased CD8 + T cells in the breast cancer TME

CD8 + T cell infiltration in tumors is a good predictor of the efficacy of immunotherapies that rely on the tumor-destroying properties of T cells [25]. Recent studies have indicated that FLASH-RT and CONV-RT might prime different immune responses in tumors, and better tumor control efficiency might be achieved due to increased recruitment of T lymphocytes. This has promoted research interest in combining FLASH-RT with immunotherapy to further enhance the therapeutic efficiency [17]. To further investigate the immunomodulation of FLASH-RT or CONV-RT, flow cytometry and IHC staining were performed to determine the immune response in tumor samples. Fig. 2a shows the gating strategy of flow cytometry. The flow cytometry results showed similar CD8+/CD3+, CD4+/CD3+, and CD8+/CD4 + ratios in Py8119 tumor samples among the control, CONV, and FLASH groups 1 week PI (Fig. 2b, d). Significantly increased intratumoral CD8 + T lymphocytes were observed 2 weeks after both FLASH-RT (p = 0.0344) and CONV-RT (p = 0.045) compared with the non-irradiated control group (Fig. 2b), and IHC confirmed the increased CD8 T lymphocyte recruitment after FLASH-RT (p = 0.0149) and CONV-RT (p = 0.0083) 2 weeks PI (Fig. 2f). Flow cytometry analysis also showed significantly reduced B cells and macrophages after both FLASH-RT and CONV-RT compared to those in the control group (supplementary Figure A1). No statistical difference in the tumor immune response was observed between the FLASH-RT and CONV-RT groups.

IHC staining of Py230 tumor samples 4 weeks PI showed that CD8 + T cell infiltration was significantly higher in the CONV-RT group than in the FLASH-RT and non-irradiated control groups. However, both irradiation groups showed less CD4 + T cell infiltration compared to the non-irradiated control group (Supplementary Figure A2).

FLASH-RT reduced the level of splenomegaly and resulted in higher CD8 + T cells, and lower CD4 + T cells in the splenic microenvironment compared to CONV-RT

Interestingly, notably different spleen sizes were observed in Py8119 (Fig. 3a, b) and Py230 (Fig. 3g) tumor-bearing mice in the control, FLASH-RT, and CONV-RT groups. We hypothesized that different treatments in mice might prime the immune system differently. To investigate this, at the time of euthanization, the spleens were collected for weight measurement and flow cytometry analysis.

In the Py8119 tumor-bearing mouse model, the spleen weight was lower in the FLASH-RT and CONV-RT groups than in the control group at 2 weeks PI (Fig. 3a, b). IHC staining showed that splenic CD8 + T cells in the FLASH-RT group were significantly higher than that in the CONV-RT and control groups (Fig. 3c, d). Further, splenic CD4 + T cell was significantly higher in the CONV-RT and FLASH-RT groups than that in the control group at 2 weeks PI (Fig. 3e).

In the Py230 tumor-bearing mouse model, the spleen weight was significantly lower in the FLASH-RT than in the CONV-RT and control groups at 4 weeks PI (Fig. 3g). Flow cytometry analysis showed substantially higher CD8+/CD3 + ratios and lower CD4+/CD3 + ratios in the spleen of the FLASH-RT group than in that of the spleen of the control and CONV-RT groups 4 weeks PI, indicating that FLASH-RT stimulated a systemic immune response (Fig. 3h, i).

Additionally, linear fitting analysis revealed the proportional relationship between the tumor weight and spleen weight (Supplementary Figure A3), indicating that tumor burden resulted in splenomegaly.

According to pathological analysis, one or two spleens of Py8119 tumor-bearing mice in all groups had minimal red pulp expansion and two spleens of Py8119 tumor-bearing mice in the CONV-RT group had severe red pulp expansion. However, damage scores ≥ 2 were not observed in the control and FLASH-RT group (Fig. 3c, f). The results indicated that CONV-RT led to more severe spleen damage, but the difference was not statistically significant.

FLASH-RT reduced tissue damage and inflammatory response in the small intestine compared to CONV-RT

The average crypt length was markedly longer after FLASH-RT than after CONV-RT (p = 0.0187), indicating less crypt shrinkage after FLASH-RT (Supplementary Figure A4, a-c). Intestinal cell apoptosis quantified using TUNEL staining showed that CONV-RT induced the most intestinal cell apoptosis among all groups 3 days PI (Supplementary Figure A4, d-e).

Furthermore, the proportion of macrophages and neutrophils both decreased in the CONV-RT and FLASH-RT groups compared with the control group. However, the accumulation of neutrophils and macrophages was substantially lower after FLASH-RT compared to CONV-RT, indicating a lower intestinal inflammatory reaction induced by FLASH-RT (Fig. 4, a-c). However, no statistical difference of intestinal CD8 + T...
Fig. 2. Tumor immune response 1 and 2 weeks after ultra-high dose rate radiotherapy (FLASH-RT) and conventional dose rate radiotherapy (CONV-RT). (a) Gating strategy for CD8+/CD3+ and CD4+/CD3+ T cells; (b–d) CD8+/CD3+, CD4+/CD3+, and CD8+/CD4+ ratios obtained with flow cytometry; (e) representative images of IHC staining for tumor samples, with scale bar representing 50 μm; (f–h) percentage of CD8+ and CD4+ T positive cells, and CD8+/CD4+ ratios obtained with IHC sections, n ≥ 6 in each group, *p < 0.05, **p < 0.01, ***p < 0.001. Abbreviations: p, population; FSC-A, forward scatter area; FSC-H, forward scatter height; SSC-A, side scatter area; PI, post-irradiation; IHC, immunohistochemical.
Fig. 3. Spleen weight, pathological analysis, and IHC staining of the spleen. (a) Representative image of the spleen of Py8119 tumor bearing mice 1 week PI; (b) spleen weight of Py8119 tumor bearing mice measured 1 week and 2 weeks PI; (c) Representative images of H&E- and IHC-stained T cell infiltration in spleen sections of Py8119 tumor bearing mice and percentage of (d) CD8+ and (e) CD4+ T positive cells in the spleen of Py8119 tumor bearing mice 2 weeks PI; (f) spleen damage score of Py8119 tumor bearing mice quantified with red pulp expansion. (g) spleen weight of Py230 tumor bearing mice measured 4 W PI; (h) CD8+/CD3+ and (i) CD4+/CD3+ ratios of Py230 tumor bearing mice obtained with FC n ≥ 6 in each group, *p < 0.05, **p < 0.01, ***p < 0.001. Abbreviations: W, week; PI, post-irradiation; IHC, immunohistochemical; H&E, hematoxylin and eosin; FC, flow cytometry. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
cell was observed in all groups, while intestinal CD4+ T cell was found to be significantly decreased after FLASH irradiation (Fig. 4, d-f). The results indicated that compared to CONV-RT, FLASH-RT reduced normal tissue injury and induced a lower intestinal inflammation.

4. Discussion

The unsatisfying early detection rates of many malignancies result in the need for combined therapy, including radiotherapy [26]. With advancements in technology, more accurate and individualized treatments are now available. However, normal tissue toxicity limits further improvements in therapeutic efficiency. FLASH-RT has been reported to be efficient in tumor control while protecting normal tissue. Such a unique radiobiological response promoted substantial research interest in the normal tissue-sparing mechanism and TME alteration of the new treatment modality.

In our study using a murine breast cancer transplant model, we found that both FLASH-RT and CONV-RT effectively suppressed tumor growth and increased CD8+ T cell infiltration in tumors, but no statistical difference was observed between the two treatment modalities. Alleviative splenomegaly and higher splenic CD8+ T cells were observed after FLASH-RT than after CONV-RT, besides, severe spleen red pulp was observed only in the CONV-RT group. Less crypt shrinkage, reduced apoptosis, and lower inflammation response were observed in the small intestine after FLASH-RT compared to those after CONV-RT. Therefore, our results suggest that FLASH-RT and CONV-RT show similar tumor control efficiency and induce similar TME responses. However, reduced toxicity and significantly different immune responses in normal tissues highlight the potential of improving the therapeutic ratio of FLASH-RT.

Previous studies have reported that radiation induces immunological changes in the TME and induces CD8+ T cell infiltration [27,28], which is considered a good predictor of the efficacy of immunotherapies [25]. The function of CD4+ T cells in tumor control is controversial. CD4+ T cells secrete interleukin-2, which enhances CD8+ T cell proliferation and promotes tumor control [29]. However, regulatory T cells (Treg), an important subset of CD4+ T cells, have immunosuppressive effects in tumors [30]. Treg can evade tumor-specific immune responses [31]. Eggold et al. [17] reported that both electron CONV-RT and FLASH-RT could reduce regulatory T cells and increase cytolytic CD8+ T cells in the ovarian cancer TME 17 days PI, and that CD8+ T cell infiltration could be enhanced by a combination of αPD-1 treatment 12 or 17 days PI. In our study, CD8+ T cell infiltration was significantly increased in Py8119 xenograft tumor 2 weeks after both FLASH-RT and CONV-RT. However, significantly higher CD8+ T cells in Py230 xenograft tumor was found in the CONV-RT group than that in the FLASH-RT and control groups. The tumor burden of the two xenograft models might contribute to this disparity. Overall, previous results, along with our results, indicate similar CD8+ T cell responses after CONV-RT and FLASH-RT.

Eggold et al. also reported that FLASH-RT increased CD4+ T cell infiltration compared to CONV-RT 96 h PI in ID8 ovarian tumor [17]. In our study, photon FLASH-RT and CONV-RT decreased the CD4+/CD3+ ratios in Py8119 xenograft tumor 2 weeks PI and in Py230 xenograft tumor 4 weeks PI. However, radiation treatment modality, detection methods, and tumor models may contribute to the discrepancy in immune cell infiltration. Moreover, immune cell subsets should be clarified and compared because they function differently [32].

Interestingly, in our study, a substantially different splenic immune response was observed. Splenomegaly was observed in the control and
CONV-RT groups. According to data from the Jackson Laboratory, indicated that the normal spleen weight of 26-week-old female C57BL/6j mice is 0.086 ± 0.007 g [33]. However, our data showed that the mean spleen weight of Py8119 and Py230 tumor bearing mice was higher than the normal spleen weight, indicating that tumor burden results in splenomegaly and FLASH-RT reduced the level of splenomegaly.

Flow cytometry analysis showed that a significant increase in the CD8+/CD3 ratio and decrease in the CD4+/CD3 ratio in the spleen of the Py230 tumor-bearing mice in FLASH-RT group compared to those in the CONV-RT group 4 weeks PI. IHC analysis further showed that a similar trends of CD8+ and CD4 + T cell infiltration in the spleen of Py8119 tumor-bearing mice 2 weeks PI. On the contrary, Reijmen et al. reported a significant decrease in CD8+ T lymphocytes and an increase in CD4+ and regulatory T cells in the spleen after fractionated 4 × 3.2 Gy CONV-RT [34]. However, a striking increase in the number of CD137/interferon-γ double-positive CD8+ T cells was observed in the spleen. The different dose fractionation might contribute to this disparity and a more detailed investigation into T-cell population is entailed. [34]. In our study, a non-enlarged splenic weight but higher CD8+ T cell infiltration in both xenograft tumor models might indicate activation of the immune response toward tumor eradication after FLASH-RT, as the splenic immune response also plays an important role in anti-tumor treatment [35].

We also investigated the immune response in the small intestine and found that the proportion of macrophages and neutrophils both decreased after CONV and FLASH compared with the control, which was consistent with our previous report [4]. However, the number of macrophages and neutrophils was significantly lower in the FLASH-RT group than in the CONV-RT group after 10 Gy irradiation at 3 days PI in female C57BL/6 mice. We speculate that all cells may suffer radiation damage at the earlier timepoint of 24 h PI. However, migration of macrophages and neutrophils into the intestine may occur at a later timepoint of 3 days PI. It was reported that macrophages and neutrophils could be recruited or activated by cytokines such as TNF-α and IL-6 [36] Although we did not examine the cytokine response of intestine tissues, our previous study shows that the serum proinflammatory cytokines of TNF-α and IL-6 were significantly higher in the CONV-RT group than that in the FLASH-RT group after 10 Gy irradiation at 6 weeks PI [4]. Additionally, Ong et al. found that radiotherapy-induced sub-acute damage was associated with significantly upregulated IL-1β, IL-6 and TNF mRNA levels in the jejunum and colon [37] Thus, the accumulation of neutrophils and macrophages was substantially lower after FLASH-RT than after CONV-RT, which might indicate a lower intestinal inflammatory reaction induced by FLASH-RT. However, further investigation is needed to confirm this speculation.

This study has some limitations. Since the tumor tissues of the Py230 tumor-bearing mice were small, the total cell number would be insufficient for flow cytometry. Therefore, we only performed flow cytometry using Py8119 xenograft tumor. Additionally, the anti-tumor effects of FLASH-RT and CONV-RT have been mostly investigated using single-fraction irradiation in previous studies [2,17,38], and in our study. Montay-Gruel et al. investigated the glioblastoma tumor control efficiency of different fractionation strategies (4 × 3.5 Gy, 2 × 7 Gy, and 3 × 10 Gy) and reported that FLASH-RT and CONV-RT are equivalent in delaying glioblastoma growth for all tested regimes [39]. Before clinical translation, further studies are needed to elucidate how the FLASH-RT and CONV-RT as well as their associated fractionation strategies affect the inactivation of the subpopulation of cancer stem cells and TME change. Furthermore, more studies are required to explore the exact mechanism towards reduced splenic and intestinal injury in FLASH-RT.

5. Conclusion

FLASH-RT was as effective as CONV-RT in suppressing tumor growth. FLASH-RT induced similar intratumor but elevated splenic T cell infiltration, and reduced the level of splenomegaly and intestinal damage compared to CONV-RT.

Ethical Approval and Consent to participate: All procedures for use of animals and their care were approved by the Institutional Animal Care and Use Committee of Sun Yat-sen University Cancer Center (L102042021020R).

Availability of supporting data: Research data are stored in an institutional repository and will be shared upon request to the corresponding author.

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Declaration of Competing Interest

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

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ctro.2022.11.005.

References

[1] Favaudon V, Caplier L, Monceau V, Pouzoulet F, Sayarath M, Fouillard C, et al. Ultrahigh dose-rate FLASH irradiation increases the differential response between normal and tumor tissue in mice. Sci Transl Med 2014;6:245ra93 .
[2] Diffenderfer ES, Verginaud II, Kim MM, Shoniyurov K, Velalopoulos A, Goia D, et al. Design, Implementation, and in Vivo Validation of a Novel Proton FLASH Radiation Therapy System. Int J Radiat Oncol Biol Phys 2020;106:640–8 .
[3] Montay-Gruel P, Acharya MM, Petersson K, Alkhami Y, Yakkala C, Allen BD, et al. Long-term neurocognitive benefits of FLASH radiotherapy driven by reduced reactive oxygen species. Proc Natl Acad Sci U S A 2019;116:10943–51 .
[4] Zhu H, Xie D, Yang Y, Huang S, Gao X, Peng Y, et al. Radioprotective effect of x-ray abdominal FLASH irradiation: Adaptation to oxidative damage and inflammatory response may be benefiting factors. Med Phys 2022;49:4812–22 .
[5] Gao F, Yang Y, Zhu H, Wang J, Xiao D, Zhou Z, et al. First demonstration of the FLASH effect with ultrahigh dose rate high-energy X-rays. Radiat Oncol 2022;166:44–50 .
[6] Zhang Q, Casco E, Li C, Yang Q, Gerweck LE, Huang P, et al. FLASH Investigations Using Protons: Design of Delivery System, Preclinical Setup and Confirmation of FLASH Effect with Protons in Animal Systems. Radiat Res 2020;194:656–64 .
[7] Levy K, Natarajan S, Wang J, Chow S, Egelold JT, Loo PE, et al. Abdominal FLASH irradiation reduces radiation-induced gastrointestinal toxicity for the treatment of ovarian cancer in mice. Sci Rep 2020;10:21600 .
[8] Montay-Gruel P, Petersson K, Jaccard M, Boivin G, Germond JF, Petit B, et al. Irradiation in a Flash: Unique sparing of memory in mice after whole brain irradiation with dose rates above 1000Gy/s. Radiat Oncol 2017;124:365–9 .
[9] Beyerreuter E, Brand M, Hans S, Hildetegy K, Karch S, Lesmann E, et al. Feasibility of proton FLASH effect tested by zebrafish embryo irradiation. Radiother Oncol 2019;139:46–50 .
[10] Bourhis J, Montay-Gruel P, Goncalves Jorge P, Bailat C, Petit B, Ollivier J, et al. Clinical translation of FLASH radiotherapy: Why and how? Radiother Oncol 2019;139:11–7 .
[11] Konradsson B, Arendt ML, Bastholm Jensen K, Borresen B, Hansen AE, Back S, et al. Establishment and Initial Experience of Clinical FLASH Radiotherapy in Canine Cancer Patients. Front Oncol 2021;11:658004 .
[12] Vuoren MC, De Fornel P, Petersson K, Favaudon V, Jaccard M, Germond JF, et al. The Advantage of FLASH Radiotherapy Confirmed in Mini-pig and Cat-cancer Patients. Clin Cancer Res 2019;25:35–42 .
[13] Wu YF, No JH, Breitkreutz DY, Mascia AE, Moeckel R, Bourhis J, et al. Technological Basis for Clinical Trials in FLASH Radiation Therapy. A Review Applied radiation oncology 2021.
Kim YE, Gwak SH, Hong BJ, Oh JM, Choi HS, Kim MS, et al. Effects of Ultra-high dose-rate FLASH irradiation on the Tumor Microenvironment in Lewis Lung Carcinoma: Role of Myosin Light Chain. Int J Radiat Oncol Biol Phys 2021;109:1440–53.

Perstín A, Poirier Y, Sawant A, Tambasco M. Quantifying the DNA-damaging Effects of FLASH Irradiation With Plasmid DNA. Int J Radiat Oncol Biol Phys 2022;113:437–47.

Ruan JL, Lee C, Wouters S, Tuliss ID, Versleegers M, Mysara M, et al. Irradiation at Ultra-High (FLASH) Dose Rates Reduces Acute Normal Tissue Toxicity in the Mouse Gastrointestinal System. Int J Radiat Oncol Biol Phys 2021;11:1250–61.

Eggold JT, Chow S, Melemenidis S, Wang J, Natarajan S, Loo PE, et al. Abdominopelvic FLASH Irradiation Improves PD-1 Immune Checkpoint Inhibition in Preclinical Models of Ovarian Cancer. Mol Cancer Ther 2022;21:371–81.

Zhu X, Xi C, Thomas B, Pace BS. Loss of NRF2 function exacerbates the pathophysiology of sickle cell disease in a transgenic mouse model. Blood, J Am Soc Hematol 2018;131:558–62.

Buonanno M, Grilj V, Brenner DJ. Biological effects in normal cells exposed to FLASH dose rate protons. Radiother Oncol 2019;149:55–62.

Venkatesulu BP, Sharma A, Pollard-Larkin JM, Sadagopan R, Symons J, Nerl S, et al. Ultra high dose rate (35 Gy/sec) radiation does not spare the normal tissue in cardiac and splenic models of lymphopenia and gastrointestinal syndrome. Sci Rep 2019;9:17180.

Cunningham S, McCaulley S, Vairamani K, Shep J, Girdhani S, Abel E, et al. FLASH Proton Pencil Beam Scanning Irradiation Minimizes Radiation-Induced Leg Contracture and Skin Toxicity in Mice. Cancers (Basel) 2021;13:1012.

Braun DA, Wu CJ. Tumor-Infiltrating T Cells: A Portrait. N Engl J Med 2022;386:992–4.

Jackson-Laboratory. https://www.jax.org/de/-/media/jaxweb/files/jax-mice-and-services/b6j-data-summary.xlsx.

Sharabi AB, Lim M, DeWeese TL, Drake CG. Radiation and checkpoint blockade immunotherapy: radiosensitisation and potential mechanisms of synergy. Lancet Oncol 2015;16:e498–509.

Lin L, Kane N, Kobayashi N, Kono EA, Yamashiro JM, Nichols NG, et al. High-dose per fraction radiotherapy induces both antitumor immunity and immunosuppressive responses in prostate tumors. Clin Cancer Res 2021;27:1505–15.

Boo R, Sherman LA. CD4+ T-cell help in the tumor milieu is required for recruitment and cytolytic function of CD8+ T lymphocytes. Cancer Res 2010;70:8368–77.

Campbell DJ, Koch MA. Phenotypical and functional specialization of FOXP3+ regulatory T cells. Nat Rev Immunol 2011;11:119–30.

De Rosa V, Di Rella F, Di Giacomo A, Matarese G. Regulatory T cells as suppressors of anti-tumor immunity: role of metabolism. Cytokine Growth Factor Rev 2017;25:15–25.

Braun DA, Wu CJ. Tumor-Infiltrating T Cells—A Portrait. N Engl J Med 2022;386:992–4.

Reijmen E, De Mey S, De Mey W, Gevaert T, De Ridder K, Locy H, et al. Fractionated Radiation Severely Reduces the Number of CD8+ T Cells and Mature Antigens Presenting Cells Within Lung Tumors. Int J Radiat Oncol Biol Phys 2021;111:272–83.

Mager LF, Burkhard R, Pett N, Cooke NC, Brown K, Ramay H, et al. Microbiome-derived inosine modulates response to checkpoint inhibitor immunotherapy. Science 2020;369:1481–9.

Tang J, Yan Z, Feng Q, Yu L, Wang H. The roles of neutrophils in the pathogenesis of liver diseases. Front Immunol 2021;12:625472.

Ong ZY, Gibson RJ, Bowes JM, Stringer AM, Darby JM, Logan RM, et al. Pro-inflammatory cytokines play a key role in the development of radiotherapy-induced gastrointestinal mucositis. Radiation oncology 2010;5:1–8.

Velalopoulou A, Karagounis IV, Cramer GM, Kim MM, Skourof G, Goia D, et al. FLASH proton radiotherapy spares normal epithelial and mesenchymal tissues while preserving sarcoma response. Cancer Res 2021;81:4808–21.

Montay-Gruel P, Acharya MM, Jorge PG, Petit B, Petridis IG, Fuchs P, et al. Hypofractionated FLASH-RT as an effective treatment against glioblastoma that reduces neurocognitive side effects in mice. Clin Cancer Res 2020.