Immune Effects of Spatially Fractionated Radiation Therapy In Triple Negative Breast Cancer

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Abstract

Objectives: Triple negative breast cancer (TNBC) is a heterogenous disease with a high incidence of primary and acquired resistance to immune checkpoint inhibitors (ICIs), due to mechanisms such as decreased CD8+ T cell infiltration in tumors and resistance from the presence of T regulatory cells (Tregs) and myeloid-derived suppressor cells (MDSCs) in the tumor microenvironment. Spatially fractionated radiation therapy (SFRT) is hypothesized to enhance immune system activation through high dose peaks that contribute to antigen presentation as well as sparing of immune cells and vascular access in low dose volumes. Further understanding of SFRT-induced immune activation and suppression is essential to strategically combine SFRT with ICIs to improve local control, reduce toxicity, and augment abscopal responses in metastatic TNBC.

Methods: 4T1 murine breast carcinoma cells were injected subcutaneously into bilateral hindlimbs of adult BALB/c mice. Mice were randomized into groups of 11 and ipsilateral hindlimb syngeneic tumors were irradiated using an SFRT GRID collimator of thickness 3 mm (peak to valley dose ratio (PVDR) 3.3), SFRT GRID collimator of thickness 5 mm (PVDR 3.5), or whole-tumor open field with a dose of 22 Gy compared to a control unirradiated group. The dose of 22 Gy was selected based on clinical SFRT practice and previous preclinical 4T1 data from our group suggesting a dose threshold. The contralateral hindlimb tumors were not irradiated but used for observing distal bystander effects. The GRID collimators made of brass plates were drilled with 5 holes of 2 mm diameter equally spaced in a cross pattern with center-to-center distance of 3 mm, a hole in the center, and a lead shield outside of the irradiation field. Radiation was delivered when serum amyloid A was estimated to be high, to minimize potential confounding immune oscillation effects. The mice were randomized to tumor growth and survival or cytokine measurements and flow cytometry. Bioluminescence imaging was also performed.

Results: The tumor growth curves showed no significant difference between groups, with the whole tumor and GRID treated groups showing an initial growth plateau followed by subsequent rapid growth. Whole tumor treated mice showed higher survival of 5 days, but with side effects of sensitive skin, ruffled fur, and diarrhea compared to the GRID treated and control mice. The GRID treated tumors showed development of necrosis, while the control mice had an increase in tumor volume without necrosis. Mice in the 5 mm GRID cohort had elevated IFN gamma and IL10. Flow cytometry showed a significant increase in exhausted T cells (PD1+, CTLA4+) as well as significantly increased Dendritic Cells (CD155+) within the 5 mm GRID treated tumors and whole tumor treated groups. All groups including the controls showed a high number of M2 macrophages, M-MDSC indicative of an anti-inflammatory tumor microenvironment.

Conclusions: Our findings suggest that SFRT may promote antitumor immunity through high T cell infiltration in both treated and untreated tumors, as well as upregulate CTLA-4 and TIGIT checkpoints, and may therefore synergize the effect of ICIs. However, since SFRT also shows high levels of MDSCs, macrophages, and dendritic cells in both the treated and untreated tumors, this may have prevented any meaningful reduction in tumor growth past an initial plateau once the T cells became exhausted. SFRT was better tolerated than whole-tumor irradiation. A combination of ICIs, SFRT, and drug(s) to minimize MDSCs is a strategy that warrants further evaluation to optimize immune activation and reduce immune suppression to improve checkpoint inhibitor resistance in TNBC.

Introduction

Radiation therapy (RT) can influence systemic disease management by inducing an immune response, including the release of tumor-associated antigens, activation of the cGAS-STING pathway, and upregulation of immune checkpoint molecules[1]. However, RT also has immunosuppressive effects, such as direct killing of effector T cells, as traditionally RT has targeted the entire tumor including immune cells within the tumor microenvironment (TME)[1]. Spatially fractionated radiation therapy (SFRT) targets small tumor volumes with high doses, sparing immune cells and enhancing immune activation.

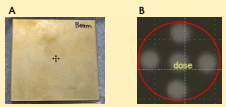
Understanding SFRT-induced immune activation and suppression is crucial for optimizing local control, reducing toxicity, and augmenting abscopal responses. The abscopal effect generated by radiation monotherapy is rare, however, with the development of immunotherapy (IT) strategies incorporating RT with targeted immunomodulators and immune checkpoint blockade, the abscopal effect is becoming increasingly relevant in less immunogenic tumors. Triple negative breast cancer (TNBC) is a heterogeneous disease with varied immune cell compositions and resistance to immune checkpoint inhibitors (ICIs). Strategies to address IT resistance include increasing tumor immunogenicity, antigen presentation, and recruitment of immune effector cells[2]. Examining how SFRT influences the TME can provide insights into overcoming IT resistance in TNBC. This study focused on elucidating the local and systemic immune effects of SFRT in a syngeneic mouse model of TNBC.

Methodology

Cell culture: 4T1 murine breast carcinoma cells were cultured in DMEM with 10% fetal bovine serum and 1% penn/strep, kept in a humidified incubator at 37°C and 5% CO2, and passaged twice weekly.

GRID design and characterization: GRID collimators were designed with 3 mm and 5 mm thick brass plates featuring holes precisely drilled and equally spaced. The plates had 5 holes with 2 mm diameter and 2 mm spacing for center-to-center (Figure 1). The design included a central hole to target the tumor's hypoxic region. Whole-tumor radiation was delivered using brass plates with single holes of 6-10 mm diameter. Irradiation was performed at 250 kV, 16 mA, 40 SSD (source to specimen distance), 3 Gy/min dose rate with an X-RAD 320 small animal irradiator (Precisino X-Ray Inc, North Brandford, CT, USA). Dose profiles were measured using Gafchromic film, and dosimetric parameters were experimentally determined.

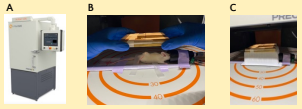
Figure 1. A: Five-hole GRID collimator, B: 5-hole GRID collimator delivered dose on the film



Tumor inoculation: Experiments followed IACUC-approved protocols. Adult BALB/c mice were injected subcutaneously with 3.5 million 4T1 luciferase-transfected cells in the hindlimbs. Tumor size was measured every 2-3 days using calipers, and volume was estimated with the formula (a^2b)/2.

Irradiation procedure: Tumors of 5-10 mm diameter were irradiated using whole-tumor radiation or GRID therapy with collimators (Figure 2). GRID collimators provided dose modulation with high dose peaks and low dose valleys. Irradiation was performed at 250 kV, 16 mA, 40 cm SSD. Mice were positioned to target only the primary tumor, shielding the rest of the body with lead.

Figure 2. A: XRD-320 small animal irradiator, B: GRID collimator positioned to target the ipsilateral hindlimb tumor, C: GRID collimator layered with lead blocking secured over target



Animal experiments: Groups of 11 mice were irradiated using a 5-hole GRID collimator (PVDR 3.3 or 3.5) or whole-tumor radiation at 22 Gy, compared to a control group. PVDR ≥ 3 was used based on a published 4T1 syngeneic murine model[3].

Serum amyloid A immune cycling: To account for immune oscillation, serum amyloid A levels were measured in control mice. Blood samples were collected and analyzed for serum amyloid levels, revealing immune cycling in 3-4 day intervals.

Tumor measurements: Mice were monitored thrice weekly for weight and tumor size measurements. Euthanasia endpoints were determined by tumor volume, ulceration, or day 7 for tumor collection.

Bioluminescence Imaging: In vivo bioluminescence imaging was performed three weeks post-radiation to assess tumor growth. Mice were sedated and injected with D-luciferin before imaging, analyzed using M3 vision software.

Cytokine measurements: A cohort of mice were euthanized on day 7 post-radiation, and blood was collected to analyze IL-10 and INFγ levels using ELISA.

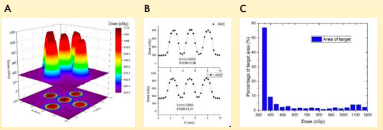
Flow cytometry: Tumors were dissected and mechanically dissociated. Enzymatic dissociation followed by cell straining and centrifugation were performed. Cells were resuspended, stained with fluorophore-conjugated antibodies, and analyzed to assess immune cell populations.

Results

GRID collimator design and peak dose

- The peak dose for delivery of SFRT has been defined most frequently based on 1D analyses of film measurements. In this study we also evaluated the 5-hole GRID collimators with films using 2D analysis and found very small high dose peaks, for example, 2% of the irradiated volume received 138% of the nominal delivered dose of 22 Gy.

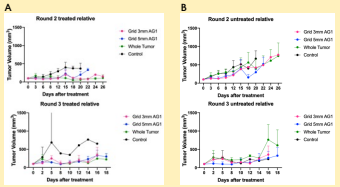
Figure 3. A: 5-hole GRID 3D dose distribution, B: 5-hole GRID dose profiles, C: 5-hole GRID measured differential dose volume histogram



Tumor growth curves, survival, and cytokines

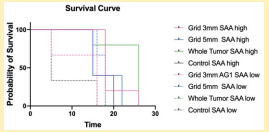
- Growth curves for open-field versus GRID radiotherapy showed no significant difference at 22 Gy peak or uniform dose

Figure 4. A: Tumor growth measurements when SAA levels were high (Round 2) and when SAA levels were low (Round 3) for the radiated ipsilateral hindlimb tumor, B: Tumor growth measurements when SAA levels were high (Round 2) and when SAA levels were low (Round 3) for the contralateral untreated hindlimb tumor



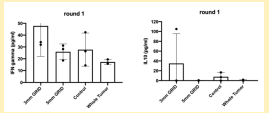
- Mice treated with whole-tumor irradiation (open field) demonstrated higher survival rates (p=0.002) of ; however, they experienced more toxicity.

Figure 5. Probability of survival when SAA levels were high and when SAA levels were low for the GRID treated, whole tumor treated, and control mice



- Seven days after irradiation at high SAA levels, elevated levels of pro-inflammatory IFN-γ were observed across the tumors and relatively low levels of the anti-inflammatory cytokine IL-10.

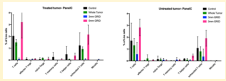
Figure 6. IFN and IL-10 levels after tumors were irradiated when SAA level was HIGH, and mice were euthanized on day 7



Immune compartment differences between GRID and open-field RT

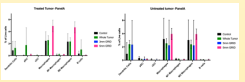
- There was an upregulation of T cells in 5mm GRID-treated tumors, majority of which were exhausted T cells

Figure 7. Flow cytometry T-cell panel for ipsilateral irradiated and contralateral untreated hindlimb tumors with GRID fields, whole tumor irradiation, and controls



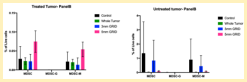
- Upregulation of M2 Macrophages (immune-suppressive, anti-inflammatory, pro-tumorigenic) in both treated and untreated tumors for control, open-field and 5mm GRID cohorts

Figure 8. Flow cytometry myeloid panel for ipsilateral irradiated and contralateral untreated hindlimb tumors with GRID fields, whole tumor irradiation, and controls



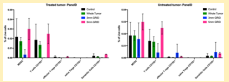
- MDSCs in the tumors were comprised mainly of Monocyte-precursor MDSCs which induce immune suppression through macrophages, dendritic cells and cytokines

Figure 9. Flow cytometry MDSC panel for ipsilateral irradiated and contralateral untreated hindlimb tumors with GRID fields, whole tumor irradiation, and controls



- CTLA4 (CD152)+ T cells are upregulated in 5mm-GRID treated and untreated tumors.
- Dendritic cells have higher CD155/TIGIT expression in 5mm-GRID treated vs 3mm-GRID or open-field treated tumors.

Figure 10. Flow cytometry immune checkpoint panel for ipsilateral irradiated and contralateral untreated hindlimb tumors with GRID fields, whole tumor irradiation, and controls



Conclusions

- SFRT may promote antitumor immunity through high T cell infiltration in both treated and untreated tumors.
- An upregulation of MDSCs and M2 macrophages indicates an immunosuppressive environment across all tumors.
- There seems to be immune checkpoint-mediated suppression in the 5mm GRID treated and contralateral untreated tumors. Pairing with immune checkpoint inhibition may help improve treatment outcomes.
- The 3mm GRID-treated tumors exhibit lower immune cell infiltration, including both regulatory and cytotoxic cells.
- Administering chemotherapy agent that helps deplete Tregs, MDSCs, and M2 macrophages may improve therapeutic efficacy in mice with 5mm GRID-treated or open field-treated tumors.
- Future Directions: Chemoimmunotherapy + SFRT using 5mm GRID

References

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