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Isoflavone-Mediated Radioprotection Involves Regulation of Early Endothelial Cell Death and Inflammatory Signaling in Radiation-Induced Lung Injury.

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Abstract

Purpose: Vascular damage and inflammation are limiting toxic effects of lung cancer radiotherapy, which lead to pneumonitis and pulmonary fibrosis. We have demonstrated that soy isoflavones (SIF) mitigate these toxic effects at late time points after radiation. However, the process by which SIF impacts the onset of radiation-induced inflammation remains to be elucidated. We have now investigated early events of radiation-induced inflammation and identified cellular and molecular signaling patterns by endothelial cells that could be modified by SIF to control vascular damage and the initiation of lung inflammation.

Materials and Methods: Histopathological, cellular and molecular studies were performed on mouse lungs from C57Bl/6 mice treated with 10Gy of thoracic radiation (XRT) in conjunction with daily oral SIF treatment given prior and after radiation. Parallel studies were performed *in-vitro* using EA.hy926 endothelial cell line with SIF and radiation. Immunohistochemistry, western blots analysis, and flow cytometry were performed on lung tissue or EA.hy926 cells to analyze endothelial cells, their patterns of cell death or survival, and signaling molecules involved in inflammatory events.

Results: Histopathological differences in inflammatory infiltrates and vascular injury in lungs, including vascular endothelial cells, were observed with SIF treatment at early time points post-XRT. XRT-induced expression of proinflammatory adhesion molecule ICAM-1 cells was reduced by SIF *in-vitro* and *in-vivo* in endothelial cells. Molecular changes in endothelial cells with SIF treatment in conjunction with XRT included increased DNA damage, reduced cell viability and cyclin B1, and inhibition of nuclear translocation of NF- κ B. Analysis of cell death showed that SIF treatment promoted apoptotic endothelial cell death and decreased XRT-induced type III cell death. *In-vitro* molecular studies indicated that SIF+XRT increased apoptotic caspase-9 activation

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and production of IFN β while reducing the release of inflammatory HMGB-1 and IL-1 α , the cleavage of pyroptotic gasdermin D, and the release of active IL-1 β , which are all events associated with type III cell death.

Conclusions: SIF+XRT caused changes in patterns of endothelial cell death and survival, proinflammatory molecule release, and adhesion molecule expression at early time points post-XRT associated with early reduction of immune cell recruitment. These findings suggest that SIF could mediate its radioprotective effects in irradiated lungs by limiting excessive immune cell homing via vascular endothelium into damaged lung tissue and curtailing the overall inflammatory response to radiation.

Keywords

Radiation; soy isoflavones; endothelial cells; lung inflammation

Introduction

Lung cancer remains the worldwide leading cause of cancer death with 25% of non-small cell lung cancer (NSCLC) patients necessitating treatment beyond surgical intervention (Fitzmaurice et al. 2017). Recent development of different fractionation strategies for radiation treatment including accelerating treatment with daily hyperfractionation with smaller fractions (<2Gy), hypofractionation with moderate fractions (2–4Gy) or stereotactic body radiation therapy (SBRT) given in high doses in fewer fractions (>10Gy) are used with concurrent chemotherapy (Roach et al. 2018) (Bradley et al. 2015). Despite these advances in the field of radiotherapy, treatment effectiveness is, in part, complicated by radiation off-target toxicity to thoracic organs, particularly the lung itself resulting in acute pneumonitis and/or progression to pulmonary fibrosis (Ferrero et al. 2015) (Verma et al. 2017). Acute and late toxicities of radiotherapy translate to difficulties in breathing, including reduced lung volume and reduced force vital capacity, which affect the patient quality of life (Ferrero et al. 2015) (Verma et al. 2017).

Radiation-induced pneumonitis is the result of radiation damage to vascular and parenchymal cells that support lung structure and resident immune cells, which the lung mucosa (Bentzen 2006). This multicellular damage initiates an inflammatory response that perpetuates into a chronic inflammatory reaction with continuous activation of stromal cells. (De Ruysscher et al. 2019) (Beach et al. 2018). At later stages, activated stromal cells continue to deposit collagen in the extracellular matrix, resulting in pulmonary fibrosis (Moding et al. 2013). Radiation-induced injury to normal lung tissue substantially compromises radiation treatment planning and in severe cases necessitate premature termination of radiation treatment, in turn jeopardizing treatment regimen efficacy (Ferrero et al. 2015) (Verma et al. 2017). Therefore, it is imperative to develop new approaches that reduce treatment-related lung toxicity while maintaining and improving the therapeutic ratio for advanced NSCLC.

Our previous studies have demonstrated novel radioprotective effects by a class of dietary compounds called soy isoflavones (SIF), which are non-toxic phytoestrogens extracted from soy beans. In xenograft preclinical lung tumor models, SIF were shown to have a dual

capability of increasing radiation damage in the tumor and simultaneously protecting the normal lung from radiation injury. Further murine studies investigating SIF-mediated radioprotection found that treatment of SIF given in conjunction with radiation (XRT) provided radioprotective effects to multiple thoracic organs, including lung (Hillman, Singh-Gupta, Hoogstra, et al. 2013), skin (Hillman, Singh-Gupta, Lonardo, et al. 2013), esophagus (Fountain et al. 2015), and heart tissue (Dominello et al. 2017). These radioprotective effects by SIF, observed at late time points, protected against skin dermatitis, hair follicle damage, respiratory function, fibrosis, vascular damage, immune cell infiltration, and mucosal and vascular smooth muscle cell hypertrophy. Therefore, SIF treatment has the potential to be used as a safe complementary approach to radiotherapy to reduce the dose-limiting toxicity to normal lung tissue in NSCLC.

In order to further understand the role of SIF in modulating inflammation after radiation, innate immune cell phenotypes and cytokine profiles of irradiated lungs have been extensively characterized at late time points in preclinical models of XRT-induced lung injury (Abernathy et al. 2015; Abernathy et al. 2017). Most notably, SIF treatment prevents radiation-induced influx of granulocytes/neutrophils and monocyte/macrophages from 4–18 weeks after radiation suggesting that SIF inhibits the progression of inflammation caused by radiation (Abernathy et al. 2015; Abernathy et al. 2017). The goal of the current study was to understand how SIF alters the initiation of XRT-induced inflammation at early time points after radiation exposure to the lung. More specifically, the major objectives were to define early differences in lung pathology after XRT with and without SIF treatment and determine cellular and molecular changes in vascular endothelial cells after XRT and SIF+XRT. Our hypothesis was that SIF+XRT may modulate events of XRT-induced injury to vascular endothelial cells that are involved in initiating and perpetuating inflammation.

At early time points, early histopathological changes were observed in lung vasculature, parenchyma, and lung inflammatory infiltrates which differ between XRT and SIF+XRT. SIF treatment altered the sensitivity of endothelial cells to XRT causing increased DNA damage and inhibition of cell cycle signaling and transcription factors involved in cell growth. Extensive *in-vitro* and *in-vivo* analysis of endothelial cells revealed different patterns of endothelial cell death and associated inflammatory signaling between XRT and SIF+XRT. Whereas apoptotic cell death by SIF+XRT can limit vascular endothelial barrier breakdown, XRT-induced type III cell death promotes vascular damage and vessel wall disruption leading to an uncontrolled inflammatory response with prolonged ICAM-1-mediated immune cell homing to damaged lung tissue. These findings suggest that SIF +XRT changes XRT-induced death of endothelial cells and limits the inflammatory signaling profile that could initiate and perpetuate XRT-induced lung inflammation.

Materials and methods

In vitro cell culture treatment

EA.hy926 modified human umbilical vein endothelial cell line was purchased from ATCC (Manassas, VA) and grown in DMEM containing 10% heat-inactivated fetal bovine serum. Cells were plated at 3×10^3 cells in 6-well plates and cultured for 48 hours prior to treatment with SIF. SIF formulation (Novasoy 400) is a 40% pure mixture of soybean derived-

isoflavones consisting of the 3 major isoflavone phytoestrogens genistein, daidzein, and glycitein (Archer Daniels Midland, Decatur, IL). For cell treatment *in-vitro*, SIF was prepared at 10mM in DMSO and added to cell culture media at a ratio of 3:1000 for final concentration of 30 μ M. An equivalent volume of SIF-free DMSO was added to culture media of control plates. At 24 hours after SIF treatment, photon irradiation was administered at a dose of 8 Gy using a PANTAC Bipolar Series 2 HF 300 (Test Equipment Distributor, LLC) operated at 250kV at a dose rate of 80.0cGy/min. The dose of 8Gy was selected for radiation treatment of EA.hy926 cells based on preliminary dose searching studies showing a significant effect of radiation while retaining sufficient cell viability in order to complete experiments at time points up to 48h.

***In vivo* mouse experimental protocol**

Female C57Bl/6 mice (Envigo, Indianapolis, IN, USA) of 5–6 weeks old were housed and handled in animal facilities accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. The animal protocol was approved by Wayne State University Institutional Animal Care and Use Committee. For mouse treatment, SIF was dissolved 10 μ L DMSO and mixed with 190 μ L sesame seed oil at a 1:20 ratio for gavage administration as previously described (Hillman, Singh-Gupta, Lonardo, et al. 2013). Mice were treated with SIF preparation which was administered by gavage initially at a dose of 12.5mg/mouse/day for 3 days prior to XRT and continued daily post-XRT at a dose of 2.5mg/mouse/day for up to 8 weeks until lung tissue harvest. The rationale for giving a higher dose of SIF for pretreatment was based on optimization of SIF effects determined in previous studies (Hillman, Singh-Gupta, Lonardo, et al. 2013). XRT was delivered at a dose of 10Gy to full thorax of anesthetized mice, positioned in jigs, three mice at a time, under a 6.4-mm lead shield with 3 cut-outs in an aluminum frame mounted on the X-ray machine to permit selective lung irradiation, as previously detailed (Hillman, Singh-Gupta, Lonardo, et al. 2013). The thoracic radiation dose of 10Gy was selected based on dose searching studies performed in our previous studies that was safe for examining radiation-induced lung disease over several months. Photon XRT was performed with a PANTAC Bipolar Series 2 HF 300 operated at 250kV, 10mA with 0.5mm tin + 0.25mm copper + 1mm aluminum filter at a 42.0 cm target to mouse distance. To minimize backscattering of XRT, the bottom of the aluminum frame that holds the jigs was hollowed and the back plate of the jig was thinned to 1.6-mm thickness. Dose to shielded regions was reduced to 1% of the dose in the irradiated field.

Histological staining of lung tissue

The lungs were intratracheally instilled with 10% buffered formalin, resected, embedded in paraffin, and sectioned, and stained for H&E or immunohistochemistry (IHC). For IHC, sections were incubated with primary antibodies CD45 (eBioscience, Waltham, MA, USA), CD31 (BD Biosciences, San Jose, CA), MPO (Abcam, Cambridge, UK), and ICAM-1 (Santa Cruz Biotechnology, Dallas, TX) following by biotinylated secondary Abs (Biorad, Hercules, CA) and amplified with the avidin-biotin immunoperoxidase technique (Vector Labs). To quantify MPO staining, MPO+ cells were counted using 10 fields per lung with lungs from 3 mice per treatment group (n = 3) and reported as averages with corresponding standard deviations. To assess cell death *in-situ*, sections were prepared for chromogenic

TUNEL cell death staining according to vendor's protocol (Trevigen, Gaithersburg, MD). Slides were examined using Nikon E800 microscope (Nikon Inc., Melville, NY, USA).

Flow cytometry analysis.

Preparation of single cell suspensions from cell culture.—EA.hy926 endothelial cells were harvested for flow cytometry using 0.25% Trypsin. Cells were spun at 1500 rpm, resuspended in 1% FBS 1x PBS, counted, and processed for flow cytometry staining.

Preparation of single cell suspensions from mouse lungs.—Three mouse lungs from each treatment group were pooled and digested into single cell suspension with 0.4 mg/ml collagenase IV, followed by lysis of red blood cells. Each pooled treatment sample was separated into 3 technical replicates and incubated with Fc receptor-blocking antibody (eBioscience, San Diego, CA). Surface immunostaining of lung cell suspensions included CD31-PE (Invitrogen, Carlsbad, CA) for endothelial cells and CD45-APC (Invitrogen, Carlsbad, CA) for leukocytes, followed by staining with specific markers as below.

Annexin-V staining for cell death.—Fresh, unfixed cells were stained in a calcium-balanced binding buffer at a concentration of 1×10^6 cells/ml using Annexin-V-FITC Apoptosis detection kit (eBioscience, Austria) combined with propidium iodide (for *in-vitro* experiments) or 7-AAD (for *in-vivo* experiments). Different nuclear stains were used based on the combination of fluorochromes selected.

Cells were analyzed by flow cytometry using a BD LSR II flow cytometer (BD Biosciences, San Jose, CA, USA) and quantified using FlowJo v10 software (Tree Star Inc., Ashland, OR, USA).

Immunofluorescence (IF) staining for γ H2AX and NF- κ B

EA.hy926 cells were treated with XRT and SIF, fixed with 4% paraformaldehyde and permeabilized with 0.5% Triton X100. Cells were incubated with primary antibodies for γ H2AX (Millipore, Burlington, MA) and NF- κ B-p65 (Santa Cruz Biotechnology, Dallas, TX) overnight at 4°C, followed by fluorescent secondary antibodies (Invitrogen, Carlsbad, CA) and DAPI nuclear stain. Cells were mounted with ProLong Antifade Diamond Mountant (Thermo Scientific, Eugene, OR) and imaged using a Nikon Eclipse E-800 UV microscope and analyzed using Image-ProPlus version 6.2 software (MediaCybernetics).

Western blot analysis

Cell culture protein extraction.—Whole cell lysates (WCL) were prepared in lysis buffer containing 0.1% Triton, dithiothreitol, and protease inhibitor cocktail tablets (CPIC) (Roche Diagnostics, Indianapolis, IN) for 30min on ice and then spun at 16,000xg for 5min at 4°C. WCL samples were probed for caspase-3 (Santa Cruz Biotechnology, Dallas, TX), caspase-9 (Santa Cruz Biotechnology, Dallas, TX), caspase-8 (Santa Cruz Biotechnology, Dallas, TX), caspase-1 (Cell Signaling Technology, Danvers, MA), ICAM-1 (Santa Cruz Biotechnology, Dallas, TX), and VCAM-1 (Santa Cruz Biotechnology, Dallas, TX). Nuclear (Nuc) and cytoplasmic (Cyto) protein fractions were prepared by using the CellLytic NuCLEAR extraction kit (Millipore Sigma, Merck KGaM, Darmstadt, Germany) and

probed for cyclinB1 (Santa Cruz Biotechnology, Dallas, TX), γ H2AX (Cell Signaling Technology, Danvers, MA), and NF- κ B-p65 (Abcam, Cambridge, UK). Protein extraction from serum-free supernatant media using an adopted methanol-mediated extraction protocol (Shi et al. 2012) was used to probe in conjunction with WCL or Nuc/Cyto fractions for pro-inflammatory molecules Gasdermin D (Santa Cruz Biotechnology, Dallas, TX), HGMB-1 (Cell Signaling Technology, Danvers, MA), IL-1 α (Santa Cruz Biotechnology, Dallas, TX), IFN β (Santa Cruz Biotechnology, Dallas, TX), and IL-1 β (Cell Signaling Technology, Danvers, MA).

Whole lung tissue protein extraction.—Mouse lungs were homogenized in 10% w/v of PBS with CPIC tablets using a gentleMACS tissue dissociator (Miltenyi Biotec, San Diego, CA). The homogenate was centrifuged at 4000 $\times$ g for 5 minutes and the supernatant was isolated as whole lung protein lysate and probed for MPO (Abcam, Cambridge, UK).

Protein analysis by SDS-PAGE and Western Blot Analysis.—Protein samples were loaded (30–50 μ g) and separated on 10% or 15% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membranes. Blots were probed with primary antibodies followed by secondary IgG-HRP antibodies (Biorad, Kidlington, U.K.). Blots were reprobed with loading control antibodies including Actin and LaminB1 followed by visualization using luminol-based ECL chemiluminescent reagent and captured using a Chemidoc MP Imaging System (Biorad, Hercules, CA, USA).

Quantification of western blots.—Western blot results were quantified by analyzing band intensity for each treatment group. Values of bands from treatments were calculated based on normalizing to respective loading control and relative to control treatment group. Each western blot band color density was determined using ImageJ software.

Statistical Analysis

Comparisons between means of two treatment groups were analyzed by two-tailed unpaired Student's *t*-test and secondary analysis was performed using ANOVA followed by Tukey's Multiple Comparison Test. ANOVA results were used to confirm the *t*-test significance values. A *p* value of <0.05 was considered statistically significant (*), <0.01 as high significance (**), <0.001 as very high significance (***) or >0.05 as not significant (NS).

Results

Decreased leukocyte infiltration, including MPO+ granulocytes, in lung tissue induced by SIF and XRT.

To assess the initiation and progression of XRT-induced inflammation in lung tissue, immune cell infiltration from 2–60 days post-XRT was shown by staining for CD45+ leukocytes by IHC in lung tissue sections (Fig. 1A). XRT caused a marked infiltration of leukocytes at 7 days which continued at late time points of 30 and 60 days post-XRT while SIF+XRT infiltration was reduced at these late time points (Fig. 1A). Analysis of CD45+ leukocyte infiltration was further quantified using flow cytometry of lung cell suspensions. These data confirmed IHC findings with a greater infiltration of CD45+ leukocytes by 30

days which was prominent at 60 days post-XRT (56.7%) compared to SIF+XRT (49.0%) ($P<0.01$) and control (41.5%) ($p<0.001$) (Fig.1.B). It was also of interest that XRT caused a significant decrease in lung leukocytes at 2 days post-XRT (32.5%) compared to control (42.5%) ($p<0.001$) while this effect was less pronounced with SIF+XRT (38.3%) compared to XRT ($p<0.001$). This observation could suggest a protective role of SIF in early immune cell loss caused by XRT.

There was also a persistent increased infiltration of MPO+ granulocytes as early as 2 days and up to 60 days post-XRT clearly observed by IHC while SIF+XRT lungs have MPO+ cell levels comparable to control (Fig.1C) as quantified in Fig.1D. Western blot analysis of full length and degranulated MPO in lung lysates confirmed an increase in MPO and activated, degranulated MPO following XRT alone but not with SIF+XRT (Fig.1E,F).

Alterations in lung pathology, vessels, and ICAM-1 expression from mice treated with SIF and XRT.

Because a major effect of radiation is injury to tissue vasculature, as we and others have previously demonstrated (Hillman, Singh-Gupta, Hoogstra, et al. 2013; Dominello et al. 2017; Guipaud et al. 2018), we studied the histological and signal molecule changes in the lung vessel structures and endothelial cells obtained from lungs by studying expression of the ICAM-1 pro-inflammatory mediator of endothelial cell activation. At an early time point of 7 days post-XRT, XRT caused damage to vessel and lung parenchyma including extravascular edema (Fig. 2A2) with rounding of cells lining the vessel wall (Fig. 2A5,B), which were identified as CD31+ endothelial cells and not rolling CD45+ leukocytes (Fig. 2B). XRT also caused alveolar septal tissue thickening (Fig. 2A8) and hemorrhages (Fig. 2A11). Lungs treated with SIF+XRT exhibited vessel edema and endothelial cell rounding ((Fig. 2A3,6), but no alveolar septal thickening (Fig.2A9) nor hemorrhages (Fig. 2A12). ICAM-1 staining of endothelial cells by IHC revealed increased numbers of ICAM-1+ endothelial cells at early time points of 2 and 7 days post-XRT compared to SIF+XRT with subtle differences between treatment groups at 30 and 60 days post-XRT (Fig. 2C). Quantification by flow cytometry analysis of CD31+ endothelial cells from mouse lungs revealed that XRT treatment significantly increased the number of ICAM-1+ endothelial cells compared to control at all time points ($p<0.0001$) (Fig. 2D). SIF+XRT treatment also had increased ICAM-1+ cells but at significantly lower levels than XRT at all time points ($p<0.001$).

Proinflammatory signaling mediators in endothelial cells treated with SIF+XRT in-vitro.

Our *in-vivo* studies showed that SIF reduced leukocyte infiltration in lung tissue and expression of ICAM-1 in mouse lung endothelial cells which was caused by XRT. The effects of SIF +/- XRT were further investigated specifically on endothelial cells by assessing expression of adhesion molecules and pro-inflammatory factors as well as NF- κ B which is responsible for the transcription of these molecules. Studies were performed *in-vitro* on EA.hy926 endothelial cell line treated with 8.0Gy XRT and 30uM SIF at 48h post-XRT. An increase in adhesion molecules ICAM-1 and VCAM was observed following XRT compared to SIF+XRT or control (Fig. 3A, S1A1,2). IFN β expression was increased in WCL of XRT and SIF+XRT; however, its release in the culture supernatant was higher in

SIF+XRT compared to XRT (Fig. 3B, S1B2). IL1 β expression was equally upregulated in XRT and SIF+XRT yet proteolytic activation and its release into supernatant was increased in XRT compared to SIF+XRT (Fig. 3C, S1C2).

As shown in our previous studies (Singh-Gupta et al. 2011; Abernathy et al. 2017), NF- κ B activation can be indirectly measured by the release of the p65 subunit from I κ B in the cytoplasm and its translocation to the nucleus. Translocation of the p65 subunit is a reliable indicator of NF- κ B activation. IF staining of NF κ B-p65 showed perinuclear accumulation and decreased nuclear translocation of NF κ B-p65 in SIF+XRT (Fig. 3D11,14) compared to XRT (Fig. 3D8,13). This finding was supported by WB analysis with an increase in cytoplasmic NF κ B-p65 concomitant with a decrease in nuclear NF κ B-p65 in SIF+XRT compared to XRT at 48hr post-XRT (Fig. 3E, S1D).

SIF treatment increases endothelial cell damage post-XRT in-vitro

The observation of SIF altering the NF κ B-p65 survival pathway upregulated by XRT led us to document the effect of XRT and SIF+XRT on endothelial cell viability, cell cycle arrest and DNA damage *in vitro*. EA.hy926 endothelial cells have significantly reduced viability in SIF+XRT (4.98×10^6) compared to XRT (7.88×10^6 , $p < 0.001$) and control (1.583×10^7 , $p < 0.0001$) at 48h post-XRT (Fig. 4A). WB analysis showed decreased nuclear cyclin B1 in SIF+XRT compared to XRT (Fig. 4B) as quantified in Fig. 4C. An increase in mono-ubiquitinated γ H2AX was observed in SIF+XRT as well as in SIF alone compared to XRT at 48h post-XRT (Fig. 4D). This was confirmed by immunofluorescence performed to assess γ H2AX (Fig. 4E) showing an average number of γ H2AX foci per cell that was significantly higher in SIF+XRT (10.8) and SIF alone (7.7) compared to XRT (2.3, $p < 0.0001$) and control (1.2, $p < 0.0001$) (Fig. 4F).

SIF treatment shifts XRT-induced endothelial cell death to apoptosis *in vitro* and *in vivo*.

Increased endothelial cell death by SIF+XRT was intriguing and led us to study the patterns of cell death using markers of apoptosis and markers of Type III cell death. Cell death of EA.hy926 endothelial cells or mouse lung CD31+ endothelial cells, isolated from treated mice, was characterized by gating cells for Annexin-V or nuclear stain uptake (PI or 7-AAD) into two cell death patterns of apoptosis (quadrant IV) and type III cell death (quadrant II) (Fig. 5A). At both 24h and 48h post-XRT of EA.hy926 cells *in-vitro*, SIF+XRT increased apoptotic cell death compared to XRT ($p < 0.05$) (Fig. 5B). Conversely, SIF+XRT decreased type III cell death compared to XRT at 24h and 48h post-XRT in EA.hy926 cells ($p < 0.001$) (Fig. 5C).

In-situ TUNEL staining of lung tissue sections revealed increased endothelial cell apoptosis staining in SIF+XRT (brown nucleus, green cytoplasm; Fig. 5D) and increased type III cell death in XRT at 7 days post-XRT (brown nucleus and cytoplasm; Fig. 5D). This was further supported by flow cytometry analysis of lung CD31+ endothelial cells showing increased AnnV+/7-AAD- apoptotic cells compared to XRT alone ($p < 0.05$) (Fig. 5E) while XRT had increased AnnV-/7-AAD+ type III cell death compared to SIF+XRT ($p < 0.01$) (Fig. 5F). These findings emphasized differences between XRT and SIF+XRT which were observed

consistently both *in vitro* using EA.hY926 and *in vivo* on primary endothelial cells isolated from the lungs using sophisticated immunolabeling and flow cytometry.

XRT-induced endothelial cell death-associated signaling is downregulated with SIF treatment

We have shown both *in-vitro* and *in-vivo* that XRT promotes Type III inflammatory endothelial cell death whereas SIF+XRT promotes apoptosis (Fig. 5). Key molecules involved in different forms of cell death and proinflammatory signaling were studied *in-vitro* using EA.hY926 endothelial cells treated with 8.0Gy XRT and 30uM SIF at 48h post-XRT. Activation and cleavage of caspase-3 and pro-apoptotic caspase-9 were increased by SIF +XRT compared to XRT (Fig. 6A,B) whereas pro-apoptotic caspase-8 cleavage was similar following either treatment (Fig. 6C). While there was only slight activation of pyroptotic caspase-1 (Fig. 6D), the pore-forming Gasdermin-D showed increased cleaved subunits in WCL and release of cleavage subunits into culture supernatant in XRT compared to SIF +XRT (Fig. 6E). In support of these findings, release of cleaved pro-IL-1 α and nuclear HMGB1, both DAMP molecules indicative of type III cell death, were elevated in cytoplasmic and supernatant protein fractions of XRT compared to SIF+XRT (Fig. 6F,G).

Discussion

Pulmonary toxicity is a major limitation in the efficacy of radiation therapy for the treatment of NSCLC (Ferrero et al. 2015) (Verma et al. 2017) and other thoracic malignancies (Benveniste et al. 2013). Similarly, abdominal or pelvic radiotherapies are complicated by acute inflammation and progress to chronic fibrosis in gastrointestinal (Hauer-Jensen et al. 2014) and reproductive organs (Morris and Haboubi 2015). In XRT-induced lung disease, XRT causes blood vessel injury that leads to vascular-mediated inflammation, resulting in pneumonitis and progressing to fibrosis (Adamson and Bowden 1983) (Guipaud et al. 2018). Our previous studies have demonstrated the radioprotective effects of SIF when given prior to XRT and continued after XRT (Hillman, Singh-Gupta, Lonardo, et al. 2013). These effects were associated with decreased inflammation, vascular damage, and fibrosis, which were documented at late time points of 4–18 weeks after XRT (Abernathy et al. 2015; Abernathy et al. 2017). In the current study, we have focused on early time point onset of XRT-induced inflammation and its modulation by SIF.

We first addressed radiation-induced immune infiltration and inflammatory disease *in-vivo* in lung tissue of mice treated with SIF and/or XRT. Within a week after XRT, histological changes indicated vascular injury including vessel edema and morphological ‘rounding up’ of CD31+ endothelial cells, which could represent an activation status (Fens et al. 2012). This process was associated with increased infiltration of CD45+ leukocytes, alveolar septa thickening, and hemorrhages at 7 days post-XRT which continued to be prevalent at later time points. These histopathological changes, reflective of inflammatory processes, were not observed with SIF+XRT. SIF treatment restricted immune cell infiltration at 30 and 60 days post-XRT. This restriction included MPO-containing granulocytes and monocytes that would otherwise locally amplify the inflammatory response. Granulocytic neutrophil persistence, or ‘neutrophilia’, is a common hallmark of many lung disease models, including

bacterial (Lefrancais et al. 2018) or viral infection (Kulkarni et al. 2019), sepsis (Ode et al. 2018), idiopathic pulmonary fibrosis (Lee et al. 2018), chronic obstructive pulmonary disease (Uddin et al. 2019), and cystic fibrosis (Pereira et al. 2014). However, for some of these diseases, extensive research has attributed inflammatory initiation to blood vessel dysfunction and vascular changes (Johnson and Matthay 2010).

SIF could influence the role of endothelial cells in modulating lung inflammation and fibrosis after XRT as shown by SIF control of the persistent immune cell infiltration induced by XRT. Therefore, it was of interest to determine if molecular signaling by endothelial cells involved in the recruitment of immune cells was modulated by SIF. Vascular endothelial cells participate in immune cell homing by expression of adhesion molecules, including ICAM-1 and VCAM-1, in order for immune cells to undergo diapedesis from the circulation into damaged tissue (Vestweber 2015). XRT caused an increase in ICAM-1+ lung endothelial cells from 2 to 60 days post-XRT whereas SIF supplemented to XRT significantly reduced ICAM-1+ lung endothelial cells. This effect could suggest a dampening effect by SIF in the recruitment function of endothelial cells from the lung after XRT. A decrease in ICAM-1 and VCAM-1 adhesion molecules was also observed in *in-vitro* in EA.hy926 endothelial cells supporting our findings *in-vivo*.

Adhesion molecules expression are triggered by the release of pro-inflammatory molecules and will subsequently initiate signaling for the recruitment of circulating immune cells (Pober and Sessa 2007; Kreuger and Phillipson 2016; Huber-Lang et al. 2018). Interestingly, *in-vitro* data also showed that while release of other proinflammatory mediators was reduced, SIF+XRT had enhanced release of IFN- β from EA.hy926 endothelial cells. Despite the limited evidence, IFN- β has been shown to decrease activation of adhesion molecules by other cytokines like IL-1 β as was observed with XRT alone (Floris et al. 2002; Kobayashi et al. 2008; Cobelens et al. 2010). Thus, paracrine signaling by IFN- β to adjacent endothelial cells could decrease ICAM-1 expression induced by XRT.

In support of these findings of SIF-mediated reduction of proinflammatory signaling by endothelial cells, we also observed that SIF inhibited XRT-induced NF- κ B nuclear translocation. NF- κ B serves as an upstream regulator for the transcription of proinflammatory mediators, including adhesion molecules and IL-1 β . Inhibition of NF- κ B activation by SIF has been previously demonstrated in our studies on cancer cells and lung tissue (Singh-Gupta et al. 2011; Abernathy et al. 2017). Surprisingly, SIF+XRT showed the same effect on NF- κ B inhibition in endothelial cells in association with increased DNA damage and reduced cellular proliferation. Based on these findings, we surmise that SIF could impact the balance of endothelial cell homeostasis after XRT and the extent to which they undergo cell death.

For the initiation and progression of inflammatory lung disease, vascular endothelial death has emerged as a key contributor in bacterial-induced and sepsis related pneumonitis and severe lung disease (Gibson et al. 1999; Wang et al. 2007; Wu et al. 2011; Qing et al. 2014; Alvarez et al. 2016; Li et al. 2018) similar to XRT-induced lung injury. This process involves the breakdown of the vascular endothelial cell barrier, but the specific type of cell death dictates the progression of the inflammatory process. In these studies, endothelial cell death

was distinguished as either apoptosis (Annexin-V+) or type III cell death (pyroptosis, necrosis, and mitotic catastrophe). Type III cell death involves the release of DAMP's (Paludan et al. 2019) and the release of IL-1 β , in the case of pyroptosis, which promote unresolved, chronic inflammatory disease in the lung. Our *in vitro* and *in vivo* flow cytometry studies showed that SIF treatment given with XRT promoted apoptotic endothelial cell death and decreased XRT-induced type III cell death (Fig. 7). Further evidence was obtained by *in vitro* molecular studies demonstrating that SIF+XRT increased intrinsic apoptosis caspase-9 activation while decreasing pyroptotic cell death activation (gasdermin D), inflammatory DAMP release (HMGB-1, IL-1 α), and the release of active IL-1 β . This is in contrast to the activation and upregulation of these molecules by XRT that are known to induce ICAM-1 expression on endothelial cells and initiates immune cell homing to injured lung tissue (Fig. 7). These data suggest that SIF treatment decreases type III death of endothelial cells while increasing apoptosis and thereby modulates radiation-induced inflammatory lung disease.

We conclude that SIF treatment could affect the pattern of proinflammatory factors and adhesion molecules on endothelial cells after XRT to inhibit immune cell recruitment (Fig. 7). These findings suggest that SIF could mediate its radioprotective effects in irradiated lungs by modulating vascular endothelial cell fate and limiting excessive vascular-driven immune cell homing and mobilization of circulating immune cells into damaged lung tissue, thereby decreasing the inflammatory response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Leukocyte infiltration in lungs treated with SIF and XRT.

Mouse lungs obtained from control, soy isoflavones (SIF), radiation (XRT) or SIF+XRT were processed for histology or for single cell suspension for flow cytometry analysis at 2, 7, 30, or 60 days post-XRT. **A.** Lung leukocyte infiltration by CD45 IHC showing a greater number of CD45+ cells in lung parenchyma at 30 and 60d post-XRT compared to SIF+XRT. **B.** Flow cytometry assessment of CD45+ leukocytes. Percentages of CD45+ cells of total viable lung cells are presented as mean $\pm$ SD of pooled lung samples ($n = 3$ mice/treatment group tested in triplicate). An increase in CD45+ cells was observed on day 30 and 60 after XRT compared to SIF+XRT. **C.** MPO staining for granulocytes in lung tissue sections by IHC shows a prominent infiltration of granulocytes following XRT compared to SIF+XRT as emphasized in magnified images (100x). **D.** Cell counts of MPO IHC staining per field of lung tissue (10 fields per lung, $n = 3$ mice/treatment group). Greater numbers of MPO+ cells were observed in XRT at all time points compared to SIF+XRT. (Con and SIF lungs are an average of all time points combined; asterisks denote statistical significance relative to control). **E,F.** Western blot analysis and quantification of MPO in pooled lung lysates ($n = 3$ mice/treatment group) detected full-length MPO (59kDa) and activated MPO degranulation product (35kDa) at 7 days post-XRT. Magnifications are 40x (A,C) and 100x (C). Mean values and standard deviations are reported and statistical significance is denoted as follows: NS=Not significant, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

Figure 2. Vascular-associated lung pathology, endothelial cell injury, and ICAM-1 expression in mice treated with XRT and SIF.

Mouse lungs obtained from control, soy isoflavones (SIF), radiation (XRT) or SIF+XRT were processed for histology or for single cell suspension for flow cytometry analysis at 2, 7, 30, or 60 days post-XRT. **A.** Vascular damage observed by H&E staining at 7 days post-XRT. XRT and SIF+XRT cause vessel edema (A2,3, arrows) and endothelial cell rounding (A5,6, arrows). Alveolar septa thickening (A8) and hemorrhages (A11, asterisks) were seen in XRT but not SIF+XRT (A9,12) compared to control. **B.** Lung vessels at 7d post-XRT stained for CD31 and CD45 showing rounding of endothelial cells within vascular wall (arrows). **C.** Lung tissue sections were obtained from mice at 2, 7, 30, and 60 days after treatment with 10Gy XRT and 2.5mg daily SIF. Tissue sections were stained for ICAM-1 by IHC and histological analysis focused on endothelial cell lining of blood vessels to identify ICAM-1+ cells (arrows). XRT showed increased staining of ICAM-1+ endothelial cells at early time points of 2 and 7 days compared to SIF+XRT. **D.** Flow cytometry gating strategy. Pooled lung single cell suspensions were labeled with fluorescent CD31 and ICAM-1 and analyzed by flow cytometry using the outlined gating strategy. **E.** Flow cytometry analysis of ICAM-1+ lung endothelial cells showed elevated levels of ICAM-1+ endothelial cells in XRT compared to SIF+XRT and control at 2, 7, 30, and 60 days post-XRT ($n = 3$ mice/

treatment group tested in triplicate). Magnifications are 40x (A) and 100x (B.,C.). Mean values and standard deviations are reported and statistical significance is denoted as follows: *NS*=Not significant, * $p<0.05$, ** $p<0.01$, *** $p<0.001$, **** $p<0.0001$.

Figure 3. Expression and release of proinflammatory signaling mediators by endothelial cells *in vitro* treated with SIF+XRT.

EA.hy926 cells were treated with 8Gy XRT and 30uM SIF for 1–48h and then processed for whole cell lysates (WCL), cytoplasmic protein fractions (Cyto), nuclear protein fractions (Nuc), and cell media protein defined as supernatant fractions (Sup). **A.** WB analysis of expression of adhesion molecules ICAM-1 and VCAM-1 showed increased levels of both adhesion molecules in XRT compared to SIF+XRT. **B,C.** Analysis of expression and release of IFNβ and IL-1β pro-inflammatory cytokines in WCL and Sup protein fractions showed increase release of IFNβ from SIF+XRT (**B**) while IL-1β release was increased in XRT (**C**). **D.** Immunofluorescence of NFκB-p65 (red) and DAPI nuclear stain (blue) at 48h post-XRT. NFκB-p65 perinuclear accumulation occurs in SIF+XRT (D11,14) but not in XRT (D6,13). Image magnifications at 40x. **E.** Western blot analysis of cytoplasmic and nuclear NFκB-p65 at 1h and 48h post-XRT shows increased cytoplasmic accumulation and reduced nuclear translocation in SIF+XRT compared to XRT at 48h post-XRT. Western blot for LaminB1 for nuclear extract and β-actin for cytoplasmic extract were performed as internal loading controls. Each treatment loading control was used to normalize band intensities for quantification of each treatment group relative to control samples provided in Supplemental Figure S1.

Figure 4. Cellular and molecular damage of endothelial cells *in-vitro* induced by XRT and SIF +XRT.

EA.hy926 endothelial cells were treated with 30μM SIF and 8Gy XRT and cultured for 1–48hrs post-XRT. **A.** EA.hy926 endothelial cell viability after 8Gy XRT. At 24h and 48h post-XRT, cell viability was reduced with SIF+XRT compared to XRT. **B,C.** Western blot analysis and quantification of cyclinB1 in cytoplasmic and nuclear protein at 1h and 48h post-XRT. **D.** Western blot analysis of nuclear protein levels of γH2AX (15kDa) and mono-ubiquitinated γH2AX (22kDa) at 48h post-XRT. At 48h post-XRT, mono-ubiquitinated γH2AX was increased in SIF+XRT compared to XRT. **E.** Immunofluorescence detection of γH2AX foci (red) within DAPI nuclear stain (blue). Image magnifications at 40x. **F.** Counts of γH2AX immunofluorescence show an increased staining of γH2AX foci per cell in SIF +XRT compared to XRT and control. Western blot for LaminB1 for nuclear extract and β-actin for cytoplasmic extract were performed as an internal loading control. Each treatment loading control was used to normalize band intensities for quantification of each treatment group relative to control samples. Mean values and standard deviations are reported and statistical significance is denoted as follows: *NS*=Not significant, **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

Figure 5. *In-vitro* and *in-vivo* patterns of endothelial cell death after XRT and SIF treatment. EA.hy926 endothelial cells cultured in vitro or mouse lung CD31+ endothelial cells were analyzed by flow cytometry for annexin-V (Ann-V) and propidium iodide (PI) or 7-AAD DNA stain to determine cell death caused by XRT and SIF. **A.** Gating Strategy for cell death. Quadrant I shows live cells (AnnV-, 7-AAD-/PI-). Quadrant II shows cells undergoing type III cell death that includes pyroptosis, necrosis, and mitotic catastrophe (AnnV-, 7-AAD+/PI+). Quadrant III are dead cells. Quadrant IV are apoptotic cells (AnnV+, 7-AAD-/PI- cells). **B.** *In-vitro* EA.hy926 endothelial cells undergoing apoptosis (Ann-V+, PI-, quadrant IV) were increased with SIF+XRT compared to XRT at 24h and 48h post-XRT. **C.** Type III endothelial cell death (Ann-V-, PI+, quadrant II) was increased in vitro in EA.hy926 cells after XRT compared to SIF+XRT at both 24h and 48h post-XRT. **D.** *In-vivo* TUNEL assay histological detection of cell death was performed on lung tissue and showed increased apoptotic vascular endothelial cell death with SIF+XRT and increased type III cell death in XRT at 7 days post-XRT. Image magnifications at 40x, 100x. **E,F.** *In-vivo* flow cytometry of lung endothelial cells. Lung endothelial cell death was analyzed by flow cytometry on lung single cell suspension from treated mice at 7 days post-XRT. Staining of lung endothelial cells showed increased apoptosis (Ann-V+, 7-AAD-, quadrant IV) with SIF+XRT compared to XRT (**E.**) whereas had increased type III cell death, including XRT pyroptosis, necrosis, and mitotic catastrophe (AnnV-, 7-AAD+, quadrant II) compared to SIF+XRT (**F.**). Experimental groups were in triplicate, pooled samples (*in-vitro*: n = 6 wells/group; *in-vivo*: n = 3 mice/treatment group tested in triplicate). Mean values and standard deviation are reported and statistical significance is denoted as follows: NS=Not significant, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

Figure 6. Cell death associated caspase activation and release of DAMP's by endothelial cells treated with XRT and SIF+XRT.

EA.hy926 cells were treated with 8Gy XRT and 30uM SIF for 48h and then processed for whole cell lysates (WCL), cytoplasmic protein fractions (Cyto), nuclear protein fractions (Nuc), and cell media protein defined as supernatant fractions (Sup). **A.** Analysis of caspase-3 in WCL showed increased activation of pro-caspase-3 in SIF+XRT compared to XRT. **B.** Analysis of pro-apoptotic caspase-9 in WCL showed increased activation of pro-caspase-9 with SIF+XRT compared to XRT alone while **(C.)** caspase-8 in WCL showed similar increased activation of pro-caspase-8 in both XRT and SIF+XRT. **D.** Analysis of pyroptotic caspase-1 in WCL showed mild activation in both XRT and SIF+XRT. **E.** Analysis and quantification of pyroptotic gasdermin-D in WCL and Sup. showed increased cleavage of gasdermin-D in XRT compared to SIF+XRT. **F.** Expression and activation/release of cleaved IL-1α was increased in XRT compared to SIF+XRT as seen in blot and quantification. **G.** Expression and activation/release of HMGB1 was also increased in XRT compared to SIF+XRT. Each blot includes an appropriate internal loading control as LaminB1 (nuclear) and β-Actin (cytoplasmic) which are used to normalize band intensities for quantification of each treatment group relative to control samples provided in Supplemental Figure 2.

Figure 7.
Diagram of pathways involved in endothelial cell death following XRT and SIF+XRT.