Appendix B: Supplemental Methods and Materials

Table of Reagents

Name	Company	Catalog Number	Comments
Rabbit anti-Myeloperoxidase	Abcam	ab9535	1:100
Rat anti-F4/80 (BM8)	Abcam	ab16911	1:200
Rabbit anti-CD4 (EPR19514)	Abcam	ab183685	1:200
Rat anti-CD8 (YTS169.4)	Abcam	ab22378	1:250
Rat anti-CD45R (B220)	Thermo Fisher	14-0452-82	1:100
Rabbit anti-Ki67 (SP6)	Thermo Fisher	RM-9106-S1	1:200
Donkey anti-Rabbit IgG Alexa Fluor 488	Thermo Fisher	A21206	1:500
Goat Anti-Rat IgG Alexa Fluor 568	Abcam	ab175710	1:500
Goat anti-rat IgG Alexa Fluor 488	Abcam	ab150157	1:1000
Click-iT EdU Alexa Fluor 488 Imaging Kit	Thermo Fisher	C10337	
In Situ Cell Death Detection Kit, TMR red	Sigma Aldrich	12156792910	

InVivoMAb anti-mouse IL-6R (15A7)	BioXCell	BE0047	
InVivoMAb rat IgG2b isotype control, anti-keyhole limpet hemocyanin (LTF-)	BioXCell	BE0090	

Implantation of the Radiopaque Marker

The radiopaque marker made of bismuth subcarbonate was prepared and implanted as described previously (***). Briefly, mice were anesthetized with inhaled isoflurane at a concentration of 2-2.5% using medical air as a carrier gas. A small, 1 cm abdominal incision was made and the jejunum of the mouse was exposed *in situ.* One side of the radiopaque marker was lightly coated in Medbond Tissue Glue (Stoelting) and the marker was then rapidly applied to the intestine. The peritoneum was sutured with 5-0 absorbable sutures (Ethicon) and the skin was sutured using 5-0 Nylon nonabsorbable sutures (Ethicon). All surgical procedures were conducted aseptically. Mice were administered 10 mg/kg of meloxicam subcutaneously every 24 hours for 72 hours post-surgery. Mice were also administered 1 mg/kg of sustained-release buprenorphine at the time of surgery. Animal weight and survival was monitored from the time of surgery until euthanasia.

Cone Beam CT and Irradiation

Cone Beam CT and irradiation was performed as previously described (***). Using the SARRP Control Interface, a cone-beam CT (CBCT) image was taken with the x-ray operating at 65 kV and 0.5 mA with aluminum filtration. Utilizing Murislice, the surgically implanted

radiopaque marker was visualized and an isocenter was selected for focal irradiation. The robotic stage then moved the animal to the proper location to deliver the dose to the isocenter. 18 Gy was delivered as a single dose using a 5x5 mm collimated beam operating at 175 kV and 15 mA with copper filtration. The dose rate was 1.65 Gy/minute.

H&E Staining

Hematoxylin and eosin staining was completed by deparaffinizing paraffin embedded sections with two washes in xylene for 10 minutes each. This was followed by rehydration in graded alcohols and a rinse in deionized water. Slides were dipped in hematoxylin for 4 minutes and then rinsed with water. The define solution was added for 20 seconds on slides, rinsed with water, kept for 40 seconds in bluing solution, rinsed with water, and finally with eosin for 25 seconds. The slides were dipped 10 times each in 95%, 100% ethanol, and xylene. Cover slips were added automatically using the ClearVue (Thermo Scientific). Frozen sections were treated identically with the exception that the deparaffinization and rehydration steps were not performed.

Masson's Trichrome Staining

2 month samples also were stained using Masson's Trichrome stain to assess collagen deposition[1].

Radiation Injury Scoring

A modified version of a validated radiation injury scoring system was used to assess the morphology of intestinal samples[2]. Mucosal ulceration, epithelial atypia, vascular sclerosis,

ileitis cystica profundal, and lymph congestion were scored (0 to 3) based on severity. Coded slides were evaluated by an independent pathologist blinded to the code.

EdU Cell Proliferation Assay

Cell proliferation was assessed with the Click-iT EdU Alexa Fluor 488 Imaging Kit (Thermo Fisher). Mice were injected intraperitoneally with 400 µg of 5-ethynyl-2-deoxyuridine (EdU) in normal saline 2 hours prior to euthanasia. OCT embedded tissue sections were fixed in 2% (w/v) paraformaldehyde (PFA) in PBS for 20 minutes at room temperature, washed twice in PBS for 15 minutes each, and treated with 70% ethanol at -20°C. They were then washed three times in PBS for 10 minutes each and blocked in 8% bovine serum albumin (BSA) in PBS containing 0.5% Tween-20 and 0.1%Triton X-100 (PBS-TT) for one hour at room temperature in a humidified staining trough. Sections were washed with PBS-TT for five minutes then the Click-iT reaction mixture was added to each slide for a thirty-minute incubation. Each slide was washed in PBS-TT then counterstained with Hoechst for thirty minutes, washed in PBS-TT, and mounted using Vectashield medium.

Crypt Microcolony Assay

A modified crypt microcolony assay was conducted to assess stem cell survival. The number of EdU positive cells per crypt were counted on a Nikon Eclipse TE2000-U (Nikon) microscope and imaged on a Zeiss microscope (Zeiss) using Zeiss Zen Pro (Zeiss). Regenerating crypts were defined as containing 5 or more EdU positive cells within a lumen. Fifty crypts in the beam track, as identified by the marker location, were counted. Fifty crypts out of the beam track on the same tissue specimen were also counted.

TUNEL Apoptosis Assay

Apoptosis was assessed with the *In Situ* Cell Death Detection Kit, TMR Red (Roche) using TdT mediated dUTP nick end labeling (TUNEL). OCT embedded tissue sections were fixed in 4% (w/v) PFA in PBS for 20 minutes at room temperature. Sections were then washed in PBS for thirty minutes and incubated in 0.1% Triton X-100, 0.1% Sodium Citrate in PBS on ice. The TUNEL reaction mixture was then prepared according to the manufacturer's instructions. Slides were rinsed twice in PBS then incubated with the TUNEL reaction mixture in a humidified staining trough for one hour at 37°C. Slides were washed with PBS and incubated with Hoechst for thirty minutes, washed in PBS, then mounted in Vectashield medium.

The number of apoptotic cells per crypt at the marker implantation site were counted on a Nikon Eclipse TE2000-U (Nikon) microscope and imaged on a Zeiss microscope (Zeiss) using Zeiss Zen Pro (Zeiss).

Immunohistofluorescence

All immunohistofluorescence followed this general protocol with the exception that CD8 required fixation in 100% methanol rather than PFA for 20 minutes and CD4 required acidic antigen retrieval using the Acidic Antigen Retrieval Reagent (R&D Systems) following the manufacturer's instructions. OCT embedded tissue sections were desiccated for thirty minutes after being cut. Sections were then fixed with 2% PFA in TBS for 20 minutes at room temperature. They were rinsed three times in TBS and washed twice for five minutes each in TBS plus 0.025% Triton X-100 with gentle agitation. Sections were then blocked in 10% normal serum from the species in which the secondary antibody was raised with 1% BSA and 0.3 M glycine in TBS for 2 hours at room temperature. The primary antibody was diluted in TBS with 1% BSA and incubated on sections overnight at 4°C. Slides were then rinsed twice for five minutes each in TBS with 0.025% Triton X-100 and the fluorophore conjugated secondary

antibody diluted in TBS with 1% BSA was applied for 1 hour at room temperature. Slides were rinsed 3 times for 5 minutes with TBS, counterstained with Hoechst for thirty minutes, and rinsed with TBS before mounting in Vectashield.

Fibrillar Collagen Quantification

Picrosirius red stained slides were imaged using a polarized light microscope (Nikon). Black and white images were arbitrarily thresholded in ImageJ. The region of interest was gated and the picrosirius positive area was measured in each length of intestine.

Cytokine/Chemokine Analysis

A mouse magnetic bead panel immunology multiplex assay (Millipore) was used to assess cytokine and chemokine expression in small intestinal lysates and serum. This assay was performed according to manufacturer's instructions using a Luminex platform to assess IL6, IL12p40, IL12p70, IL13, IL17, GM-CSF, IFN-g, MIG, and TNF-a. Results were normalized to the total protein concentration as assessed by the DC protein assay (Biorad).

Dosimetry

Dosimetry was performed by measuring EBT2 gafchromic film exposure at depth using solid-water phantom material with a Microtek Artixcam M1 camera. The dose-exposure calibration was done using a cesium irradiator with calibration dose rate.

References

1. Bancroft JD. Theory and practice of histological techniques. Elsevier Health Sciences; 2008.

2. Langberg CW, Sauer T, Reitan JB, Hauer-Jensen M. Relationship between intestinal fibrosis and histopathologic and morphometric changes in consequential and late radiation enteropathy. Acta Oncologica. 1996;35(1):81-7.