

Supplementary Materials for

**Anti-Ceramide Single-chain Variable Fragment Mitigates Radiation GI  
Syndrome Mortality Independent of DNA Repair**

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## METHODS

**Radiation source:** Radiation was delivered to C57BL/6J mice (The Jackson Lab #000664) using a Shepherd Mark-I unit (Model 68, SN643) operating a  $^{137}\text{Cs}$  source at a dose rate of 2.12 Gy/min. For experiments involving events occurring under 10 min radiation was delivered at 13.1 Gy/min. For protection studies, pre-treatment with anti-ceramide 6B5 scFv or 2A2 Ab was at 15 min prior to irradiation whereas for mitigation studies treatment was at 24h post irradiation.

**Abs used for immunofluorescence DNA repair focus studies:** Primary Abs used include: mouse monoclonal Ab against  $\gamma\text{H2AX-Ser139}$  (Millipore [clone JBW 301], #05-636, dilution 1:1000), mouse monoclonal anti-MDC1 (Millipore #05-1572 [clone P2B11], dilution 1:100), rabbit polyclonal anti-lysozyme Ab (Novus Biologicals #nbp2-61118, dilution 1:1,000). Secondary Abs: F(ab')<sub>2</sub>-goat anti-rabbit or anti-mouse IgG (H+L) cross-adsorbed secondary Ab, Alexa Fluor 488 (ThermoFisher #A-11070 #A-11017) 2 mg/ml were used at a dilution of 1:400.

**Cell culture:** Jurkat T lymphocytes (clone E6-1) were obtained from the ATCC (Rockville, MD). Cells were grown in a 5% CO<sub>2</sub> incubator at 37 °C in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum and 10 mM HEPES (pH 7.4), 2 mM L-glutamine, 1 mM sodium pyruvate, 100  $\mu\text{M}$  nonessential amino acids, 100 units/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin. Apoptosis was measured by morphologic examination following Hoechst 33258 bis-benzimide staining as published (1).

**Survival of mice after whole body irradiation:** Actuarial survival of animals was calculated by the product limit Kaplan-Meier method (2). Terminally sick animals

displaying an agonal breathing pattern were sacrificed by hypercapnia asphyxiation and evaluated by necropsy to determine cause of death. Intestinal specimens were fixed in formaldehyde, and stained with hematoxylin, as described (3). GI damage was diagnosed as cause of death when small intestines displayed denuded mucosa with nearly no villae or crypts apparent or when the mucosa displayed limited mucosal repair (3). Note that for every Kaplan Meier study the batch of commercial C57B/L6 mice (The Jackson Laboratory C#000664) used was first subjected to a control dose survival study to confirm the LD<sub>90</sub> dose as 15Gy. In one of six Kaplan Meier studies reported here however the batch of commercial C57B/L6 mice displayed a slightly lower LD<sub>90</sub> dose of 14.5Gy. For simplicity, data from this study are collated in Fig. 4B with the remaining studies and referred to generically as 15Gy.

***Small intestinal endothelial apoptosis:*** Apoptosis in the small intestinal lamina propria was determined by double staining with TUNEL for apoptosis and immunostaining using a rat Ab against the endothelial cell surface marker MECA-32 (DSHB # MECA-32-s), as described (4).

**DSB repair immunofluorescence focus studies:** Investigations examining DNA repair using focus technology were performed as published by us (5). Briefly, paraffin-embedded tissue sections (3µm) were melted on a heat block, deparaffinized by 3x10 min in xylene, 2x3 min in 100% ethanol, 2x3 min in 95% ethanol, 2x3 min in 70% ethanol, then washed with distilled water and transferred to 1X Phosphate Buffered Saline (PBS). Antigen retrieval was performed in boiled 0.1 M citric acid buffer (pH 6.0) in a Decloaking Chamber (Biocare Medical) at

125°C for 5 min, cooled down for 20 min at room temperature, washed with distilled water, and transferred to washing buffer containing 0.1% Triton X-100 in 1X PBS for 20 min at room temperature. Blocking with 2% bovine serum albumin acetylated (Sigma Aldrich #B2518) and 10% normal goat serum (ThermoFisher Scientific #PCN5000) in 0.1% Triton X-100 in 1X PBS was for 1h at room temperature. DNA repair foci were probed using murine primary Abs against  $\gamma$ H2AX-Ser139 (Millipore [clone JBW 301], #05-636, dilution 1:1000) and MDC1 (Millipore #05-1572 [clone P2B11], dilution 1:100) overnight at 4°C, followed by anti-mouse IgG (H+L) cross-adsorbed secondary Ab Alexa Fluor 488 (ThermoFisher #A-11070 #A-11017, dilution 1:400). Prolong gold and slow fade anti-fade reagent with DAPI (ThermoFisher Scientific #P36962) were used to protect from photobleaching and quenching of fluorescent signal, respectively.

**Microscopy of DSB repair foci:** Multi-channel fluorescence images were acquired using an upright wide-field Zeiss Axio2 Imaging Microscope with AxioCam MRm Camera (1360x1036 pixels image array) and 40X2 objective of Zeiss Plan-Neofluar 1.3NA oil dic (1083-997). Exposure time was set based on images of intermediate intensity, avoiding over-saturation from brightest foci. Once exposure time was set, it was kept constant within each set of experiments. Microscopy yields blue DAPI staining of nuclear areas, fluorescent focus staining, and overlay of co-localized images. 5 images containing 3-11 crypts/image were randomly selected from each tissue section for quantitation of focus numbers.

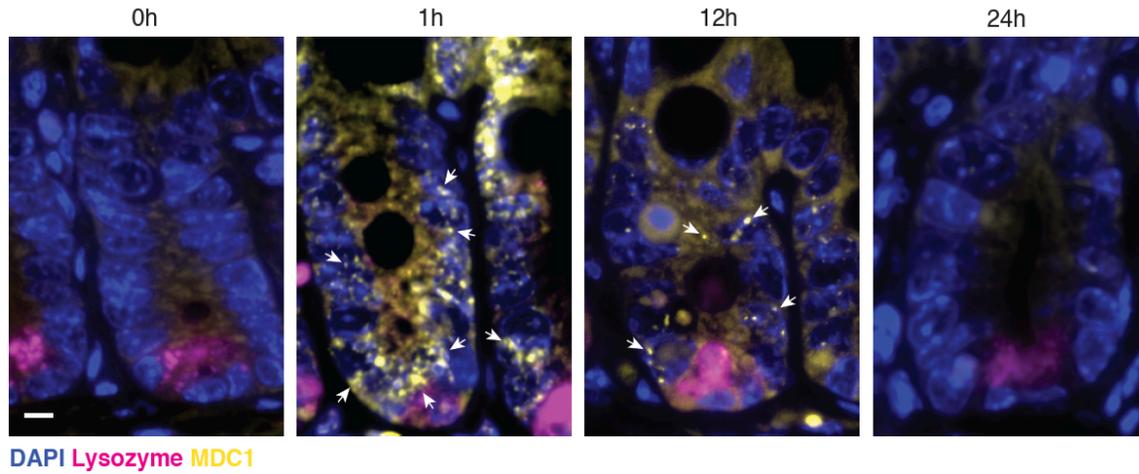
**Crypt Microcolony Survival Assay:** The Microcolony Survival Assay was performed as described by Withers and Elkind (6, 7).

**$\beta$ -galactosidase (lacZ) staining.** 8-10 week old *Lgr5-lacZ* male reporter mice were euthanized after radiation and 2.5 cm segments of proximal jejunum obtained, as published by us (8). Specimens were fixed, stained for the presence of  $\beta$ -galactosidase (lacZ), blocked, sectioned, and counterstained with nuclear fast red as published (8, 9). Numbers of blue *Lgr5*<sup>+</sup> intestinal stem cells were quantified and graphed using ImageJ and Prism9 software, respectively.

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**Fig. S1 Representative immunofluorescence images of MDC1 staining of small intestines from control and irradiated mice at the indicated times post 15Gy WBR.** Scale 20  $\mu\text{m}$ . Several examples of MDC1 foci are indicated by arrows. Note that when 6B5 anti-ceramide scFv is delivered at 24h post radiation MDC1 foci are fully resolved.

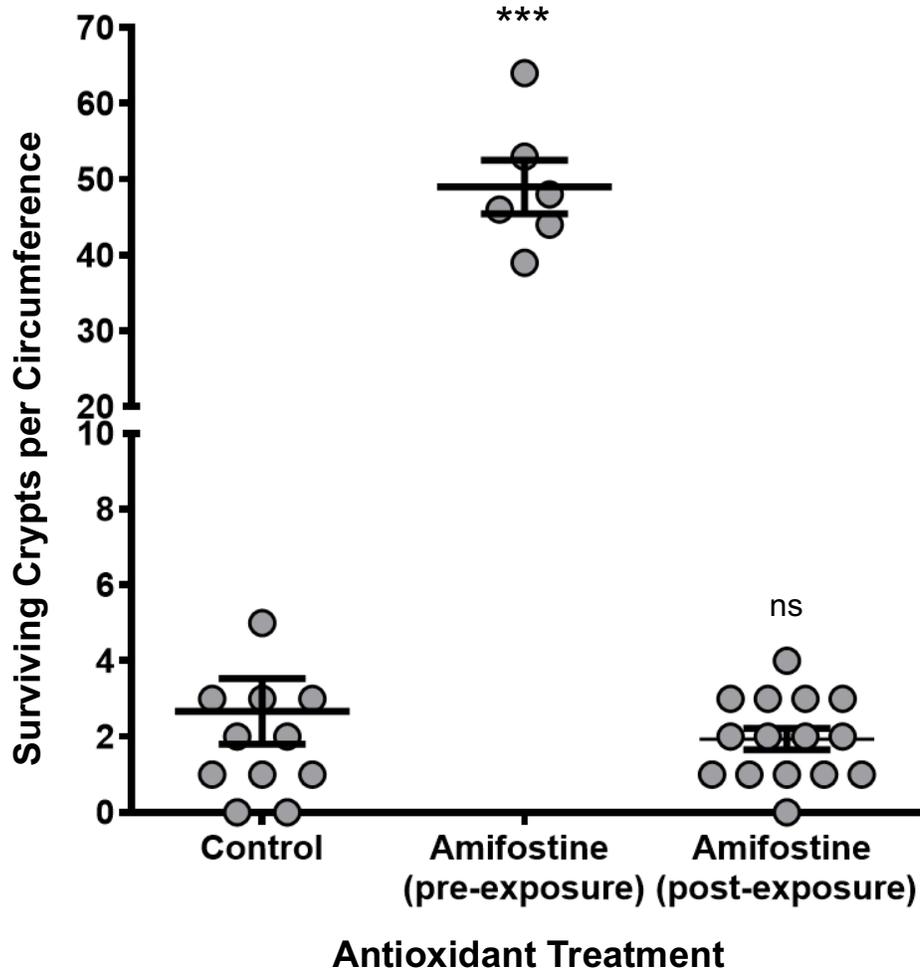


Fig. S2 Quantification of crypt survival following administration of the antioxidant amifostine (WR2721). Amifostine (400 mg/kg by intraperitoneal injection) was administered 15 min prior to or 24 hours following 15Gy whole body radiation. Crypt survival was assessed at 3.5 days post irradiation by the Microcolony Assay of Withers and Elkind. \*\*\*  $P < 0.001$  versus control, unpaired t test.

