Anti-ceramide Antibody Prevents the Radiation GI Syndrome in Mice

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Supplemental Data
Supplemental Figures 1-9 with legends

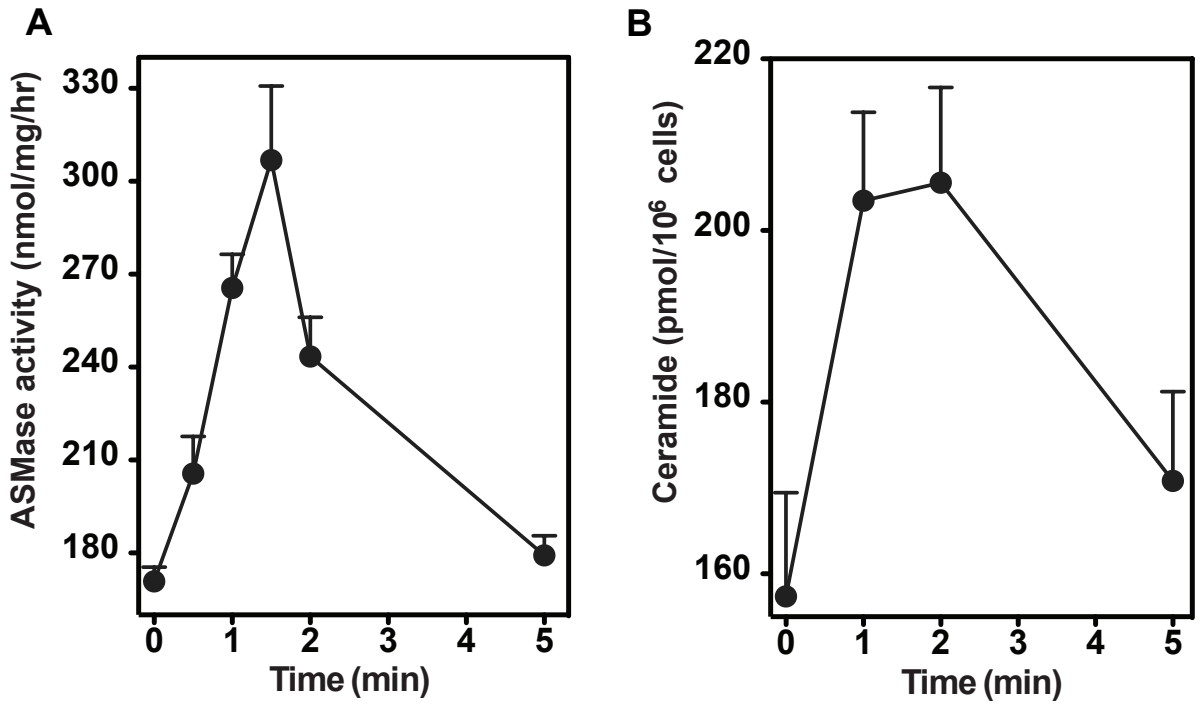

Figure S1. BAEC undergo rapid ASMase activation and ceramide generation following ionizing radiation exposure. BAEC were stimulated with 10 Gy or left untreated and incubated at 37°C for the indicated times. (**A**) ASMase activity was determined using [^{14}C]sphingomyelin as a substrate and collated as described in Methods. (**B**) Ceramide content was measured using the diacylglycerol kinase assay as described in Methods. Data (mean±s.e.m.) are collated from 3 independent experiments performed in triplicate.

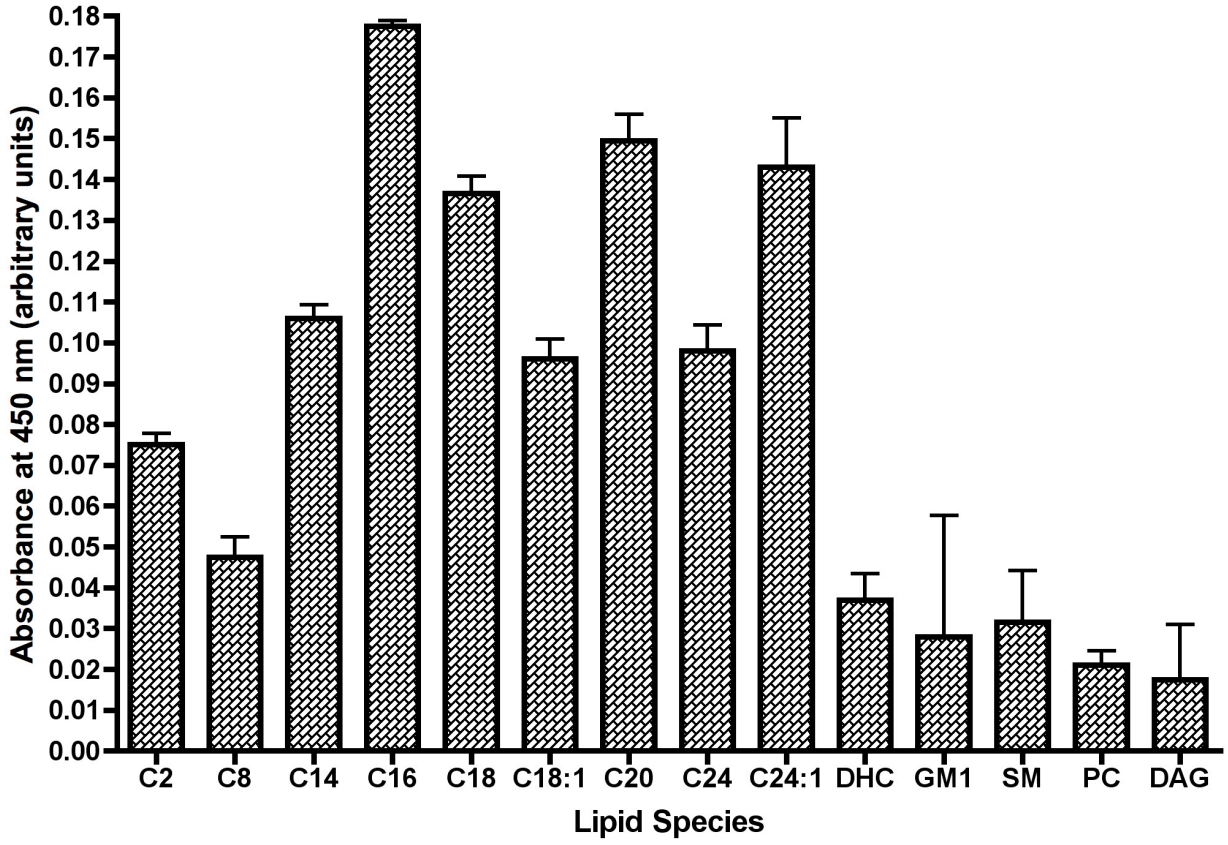

Figure S2. Purified monoclonal 2A2 Ab specifically binds to ceramides. ELISA using ceramides of varying fatty acid chain length (C2-C24) or C16-dihydroceramide (DHC), ganglioside GM1, sphingomyelin (SM), phosphorylcholine (PC) or diacylglycerol (DAG) revealed that 2A2 mouse monoclonal IgM specifically binds ceramide. Data represent mean±s.e.m. of two experiments performed in triplicate.

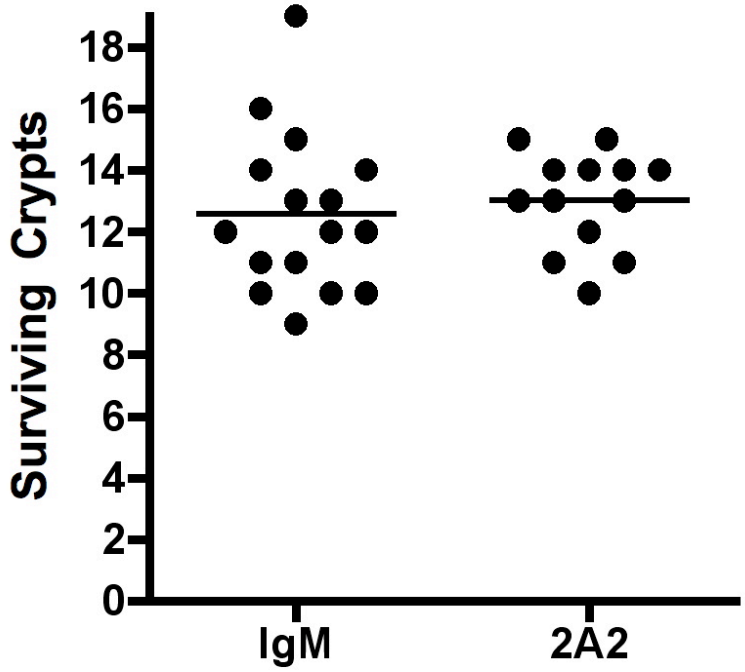

Figure S3. 2A2 Ab does not impact crypt survival in *asmase*$^{-/-}$ mice. Purified monoclonal 2A2 IgM or irrelevant IgM control (1000 μg) were injected intravenously into *asmase*$^{-/-}$ C57BL/6 mice 15 min prior to 15 Gy whole body irradiation. Crypt survival was quantified as in Fig. 2. Data are compiled from 2 mice each, with 5-10 intestinal circumferences analyzed per mouse. Bars represent means. Note: this level of crypt survival is consistent with survival of the small intestines, and highly similar to our published data in *asmase*$^{-/-}$ mice (Rotolo et al., *Int J Radiat Oncol Biol Phys*. 2008; 703: 804-815).

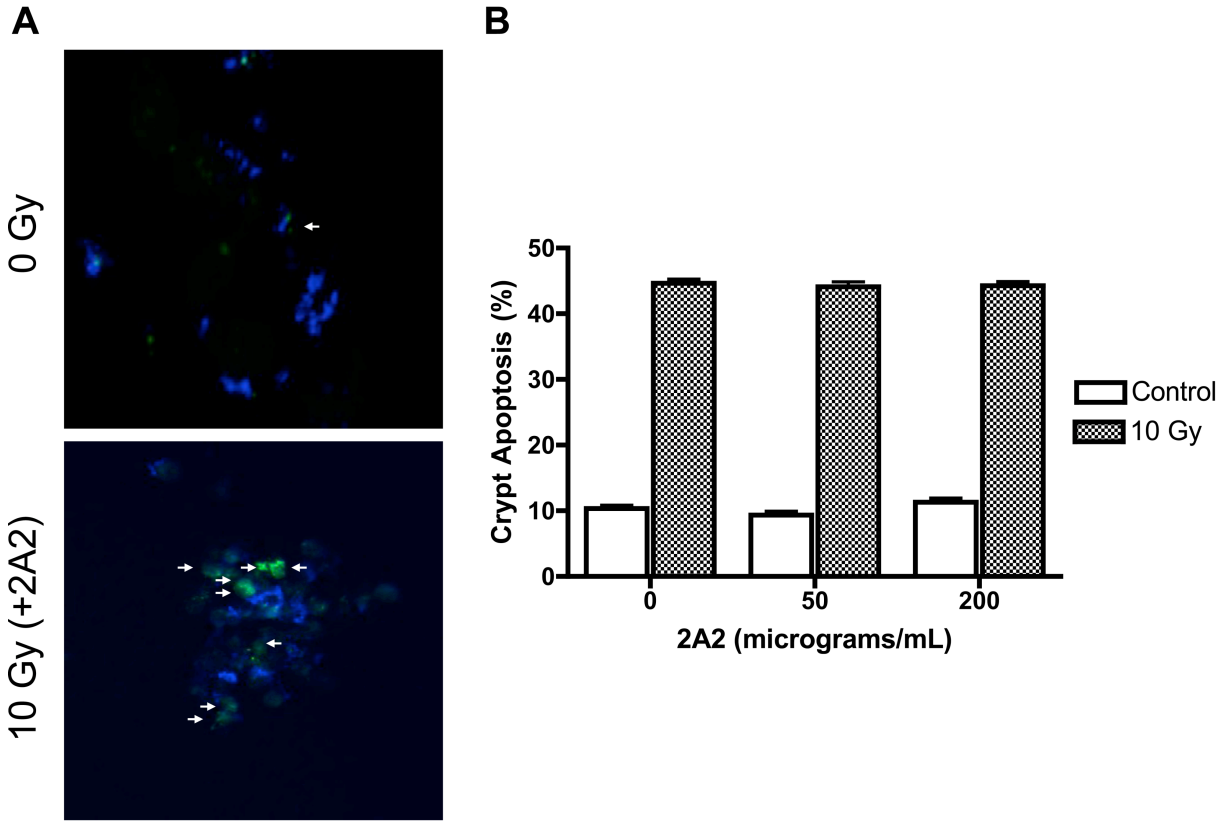

Figure S4. Monoclonal 2A2 Ab does not impact radiation-induced crypt apoptosis *ex vivo*. Crypt epithelial cells were harvested and cultured from 8 week old C57BL/6 mice as described (Sato *et al.*, Nature 459 2009). Briefly, 10 cm of proximal jejunum was harvested, flushed with ice cold PBS, opened laterally, cut into 5 mm segments and incubated in PBS containing 5 mM EDTA for 60 min on ice. Thereafter, samples were washed with PBS and vigorously suspended using a 10-ml pipette for 1 min. Supernatant enriched with villi was decanted, and samples were vigorously suspended for an additional 2 min in fresh PBS. This supernatant was passed through a 100 μM filter, removing residual villi, resulting in a >80% pure crypt fraction revealed by brightfield microscopic analysis. Intact crypts were resuspended in Advanced DMEM/F12 containing 500 ng/ml R-spondin, 100 ng/ml Noggin and 100 ng/ml EGF, and incubated overnight at 37°C in 5% humidity prior to irradiation. Initial radiation dose-response and time-course analyses were performed, and an approximate LD_{50} for crypt apoptosis was determined to be 10 Gy irradiation at 12 h. Note, while crypts were predominantly intact at time of irradiation, staining and washing following fixation resulted in crypt dispersal into a single cell population. (A) Representative images of crypt cells 12 h following 0 Gy (top panel) or 10 Gy (bottom panel) stained with the DeadEnd Fluorometric TUNEL System (Promega Corporation, Madison WI). Blue images represent DAPI-stained nuclei, and green images represent TUNEL-positive apoptotic nuclei. Administration of 2A2 anti-ceramide IgM 15 min prior to 10 Gy had no impact on apoptosis. Cells were fixed with 4% paraformaldehyde, and staining was performed using a protocol modified for suspension cells in which crypts were stained in solution. White arrows

indicate apoptotic nuclei. Original magnification100x (B) The total population of apoptotic crypt epithelial cells, i.e. cells displaying brightly green stained, fragmented nuclei, was quantified using an Axiovert S-100 Zeiss fluorescence microscope. Data represents mean±s.e.m. of a minimum of 150 cells per point, performed in triplicate.

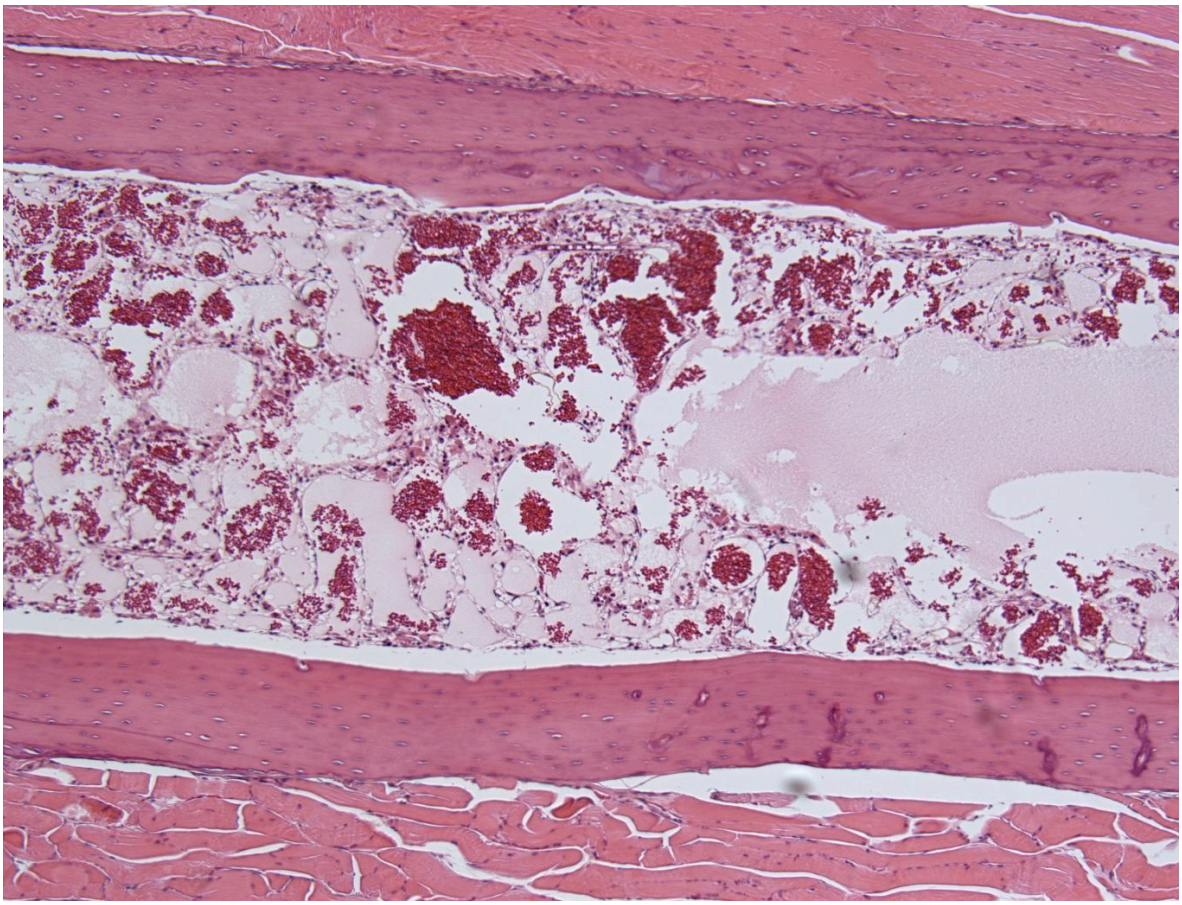

Figure S5. Protection from the Radiation GI Syndrome reveals an accelerated BM aplasia syndrome. While 80% of mice exposed to 15 Gy can be saved by 2A2 anti-ceramide Ab and HSCT transplantation ($3x10^6$ autologous bone marrow cells), 20% die due to engraftment failure as indicated by the above autopsy data. Note moderate hemorrhage and lack of bone marrow hematopoietic elements. Similarly, 40% of mice receiving 2A2 anti-ceramide Ab prior to 16 Gy exposure die from engraftment failure. Original magnification 200x.

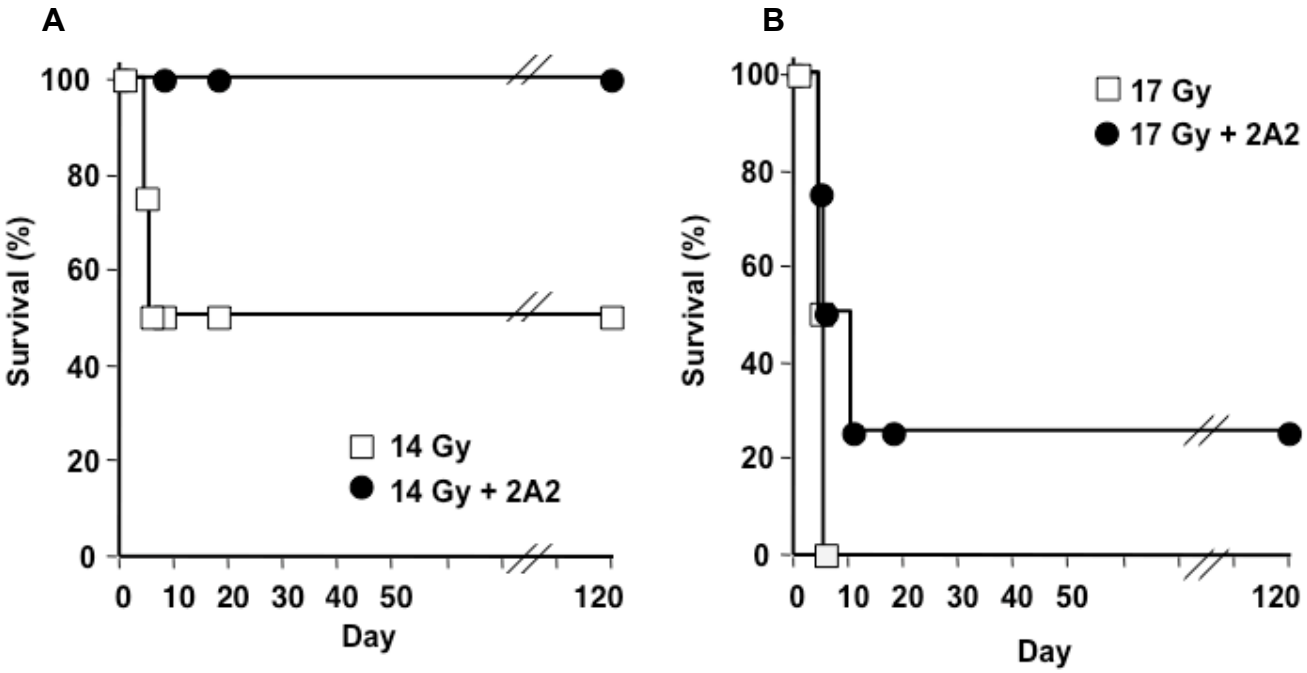

Figure S6. Purified monoclonal 2A2 Ab protects against GI Syndrome lethality up to 17 Gy. Administration of 2A2 Ab 15 min prior to 14 Gy (**A**) or 17 Gy (**B**) increases survival of mice administered HSCT ($3x10^6$ cells). N=4 for each condition in the 14 Gy and 17 Gy studies.

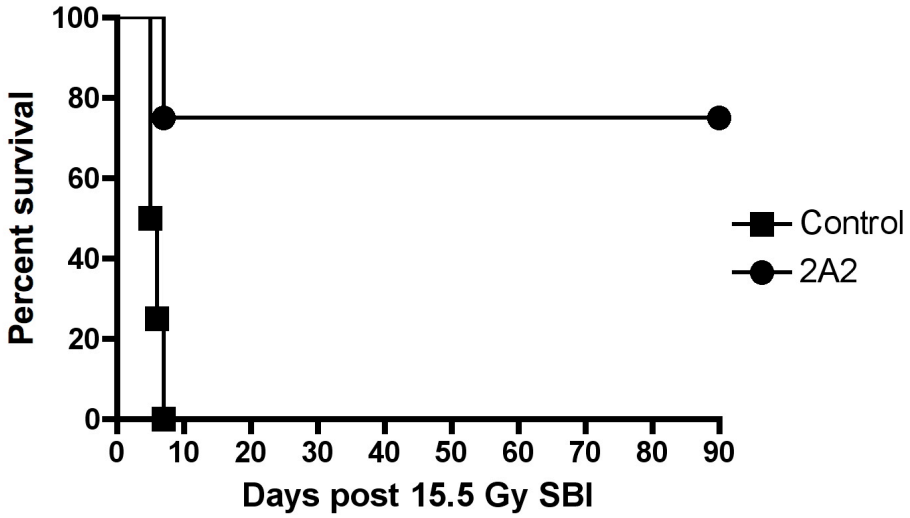

Figure S7. Purified monoclonal 2A2 Ab protects against GI Syndrome lethality following 15.5 Gy subtotal-body irradiation. Male 8-week old C57BL/6 mice were exposed to 15.5 Gy subtotal-body irradiation (Philips MG-324 X-ray unit at a dose rate of 118.3 cGy/min, 50 cm source to skin distance), where the animal's forepaws, head and hind legs were shielded from the irradiation source with a lead jig. 2A2 Ab, administered 15 min prior to irradiation, increased 90-day survival from 0 to 75% of animals ($p<0.01$).

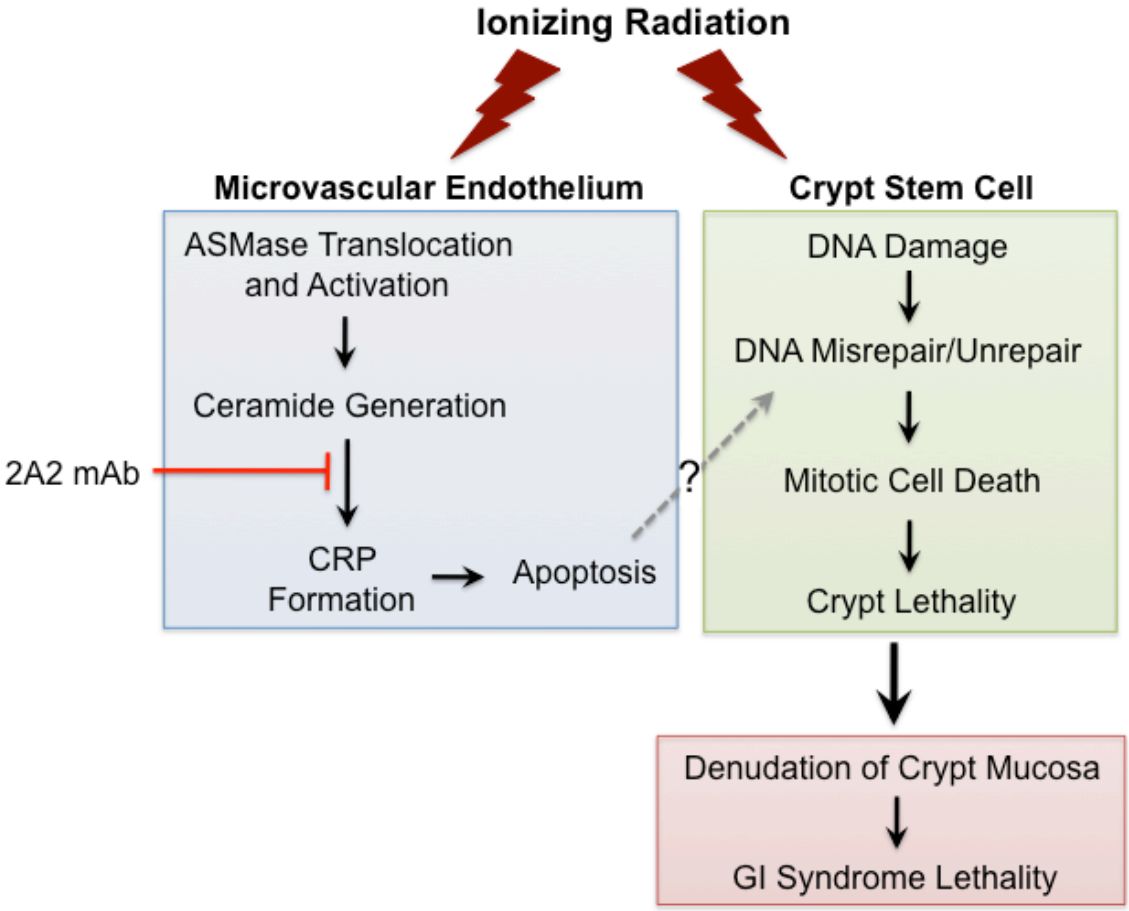

Figure S8. Schematic of 2A2 inhibition of the endothelial component of the Radiation GI Syndrome. Anti-ceramide 2A2 monoclonal antibody (mAb) inhibits CRP formation following high-dose radiation (15-17 Gy) exposure, protecting microvasculature from acute apoptotic injury. Preliminary data indicate ensuing vascular dysfunction attenuates DNA repair in crypt stem cells, promoting stem cell demise. Thus 2A2 inhibition of microvascular endothelial apoptosis attenuates crypt lethality, saving mice from the lethal GI Syndrome.

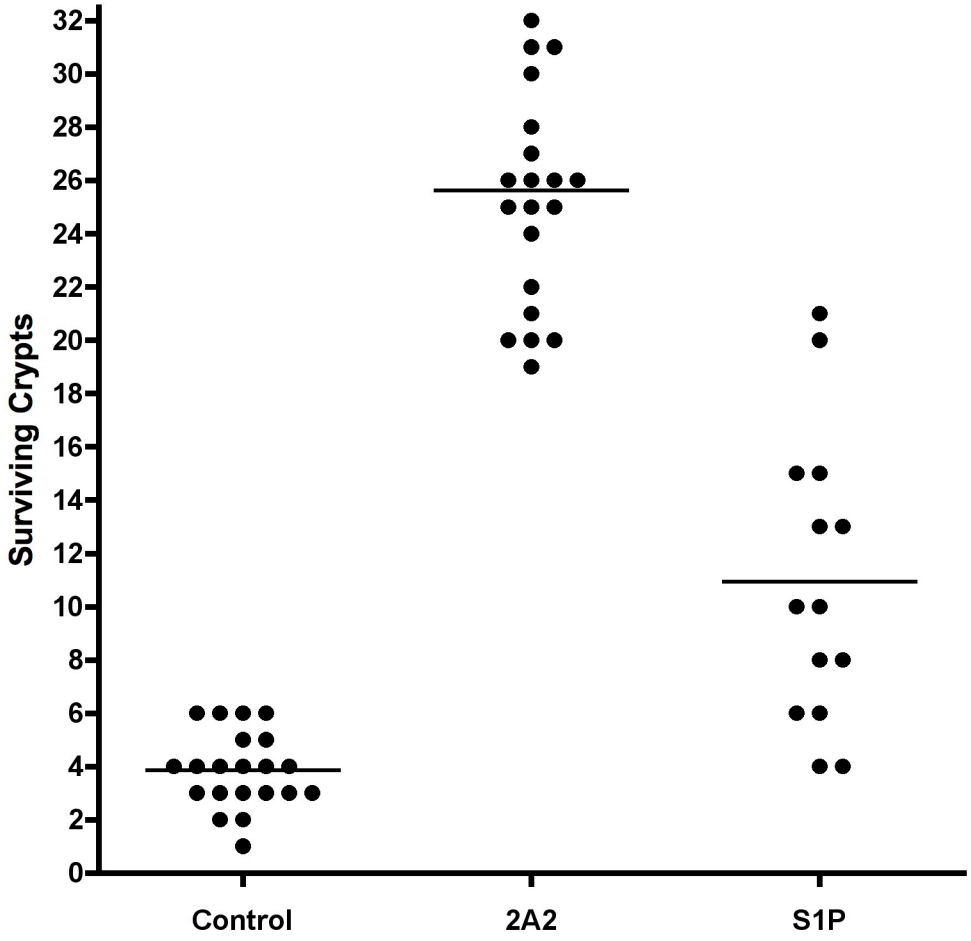

Figure S9. Purified monoclonal 2A2 Ab protects against crypt lethality. 2A2 (1000 μg/25 g mouse) or sphingosine-1-phosphate (S1P, 100 μg/25 g mouse in 0.2 ml PBS containing 5% polyethylene glycol, 2.5% ethanol and 0.8% Tween 80) was administered 15 min prior to 15 Gy whole body radiation. Crypt survival was quantified as in Fig. 2. *$p<0.05$ 2A2 vs. S1P.