

## Variable Hematopoietic Responses to Acute Photons, Protons and Simulated Solar Particle Event Protons

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**Abstract.** *The goal of this study was to evaluate, for the first time, the response of bone marrow-derived cell populations to protons mimicking a space radiation environment. Materials and Methods: C57BL/6 mice were exposed to 2 Gray (Gy) simulated solar particle event protons (sSPE) over 36 h; energies ranged from 15 to 215 MeV/n and were administered in 10 MeV increments. Acute 2 Gy irradiation with photons ( $\gamma$ -rays) and protons were administered to different groups at 0.7 Gy/min and 0.9 Gy/min, respectively, for comparison with sSPE. The animals were euthanized on days 4 and 21 post-exposure for analyses. Results: Exposure to radiation, regardless of regimen, resulted in immune depression and other abnormalities in cell populations residing in the blood and spleen; the extent of the radiation damage was somewhat dependent upon body compartment and time post-exposure. However, variations were also noted among the three radiation regimens in a number of measurements: relative spleen mass, basal DNA synthesis by leukocytes, white blood cell counts and three-part differentials (lymphocytes, granulocytes, monocytes-macrophages), lymphocyte subpopulations ( $CD4^+$  T,  $CD8^+$  T, B and NK cells) and erythrocyte and thrombocyte characteristics. Conclusion: The data demonstrate that exposure to proton radiation mimicking a solar explosion induces abnormalities in leukocytes, erythrocytes and platelets that may have adverse health consequences. However, the damaging effects of sSPE on leukocytes and platelets were generally less pronounced compared to the other radiation regimens. Results obtained with photons ( $\gamma$ -rays, X-rays) and monoenergetic protons at space-relevant total doses may not necessarily predict biological responses after exposure to a solar particle event.*

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Astronauts on space missions are at risk for exposure to relatively high radiation doses during solar particle events (SPE) that develop rapidly and may last anywhere from a few hours to several days. These emissions from the sun consist predominantly of protons, and the occurrence of individual events is, as yet, unpredictable (1, 2). On average, solar activity has an 11-year cycle during which several large SPE may occur; there were at least 8 such events during 1986 to 1996 (solar cycle 22) that involved proton energies greater than 30 MeV (3). Crew members could receive doses of 2 Gray (Gy), or perhaps even more, during an SPE (3, 4).

Health consequences due to radiation-induced immune modulation in the spaceflight environment are not yet clear. Effective immune defenses consist of complex actions and interactions among cells, tissues, organs, and secreted molecules, and the spaceflight environment can potentially impact each level of this network. Immune depression or dysfunction can increase the risk not only for infectious diseases, but also for other pathologies such as hypersensitivity, autoimmunity and cancer. Furthermore, inflammation and other functions of the immune system are activated under conditions that include physical injury, e.g. wounds and broken bones that may occur during space travel, or while on the surface of Mars or the Moon. Until recently, the majority of studies evaluating radiation-induced biological effects have been performed with photons ( $\gamma$ -rays, X-rays) and isolated cells or tissues in culture. Although many important discoveries have been made, there is still great uncertainty whether the findings can be extrapolated to the intact mammal in which interactive mechanisms are abundant and bystander effects are likely (5).

Studies have documented numerous immune aberrations in astronauts, cosmonauts and research animals following space flight (6-12). In mice evaluated within hours after return from a 12-day mission to the International Space Station (STS-108/UF-1), we found a number of abnormalities in bone marrow-derived cell populations (13, 14). Consistent with these observations is the increased prevalence of latent virus reactivation (e.g. Epstein-Barr virus and varicella-zoster

virus) (15-17) and genitourinary tract infections (18). Research also shows that spaceflight conditions are associated with increased susceptibility to primary infections (19), accelerated proliferation, mutation and virulence of microbes, and increased resistance to antibiotics (20). Since cells of the immune system are especially vulnerable to the effects of radiation, immune dysfunction in the space flight environment could be at least partially due to radiation exposure. A better understanding of proton-induced immune modulation is crucial in order to design appropriate countermeasures for astronauts on extended missions during which health care is likely to be limited.

We previously demonstrated in rodent models that total-body irradiation with protons can induce immune depression and that some abnormalities persist long-term (21-24). The data presented here are part of a larger study in which protons simulating an SPE (sSPE) were utilized, for the first time, to evaluate biological consequences in an intact mammalian system. Scanning magnets were used to deliver protons of variable energies over a 36-hour time period (25). The overriding intent was to obtain data with potential applicability in human health risk assessments.

## Materials and Methods

*Animals and experimental design.* C57BL/6 mice (n=116 total) were purchased at 8-9 weeks of age (Charles River Breeding Laboratories Inc., Hollister, CA, USA) and maintained in ventilated plastic cages (BioZone VentiRack™, BioZone, Inc., Fort Mill, SC, USA) at a maximum of 10 mice/cage under standard vivarium conditions. After a 1-2 week acclimatization period, the animals were assigned to four groups: i) 0 Gy control, ii) photons, iii) protons and iv) sSPE. Euthanasia was performed with 100% CO<sub>2</sub> on days 4 and 21 post-exposure for analyses. There were 12-22 mice/group/time point. The protocol was approved by the Institutional Animal Care and Use Committee (IACUC).

*Simulated solar particle event (sSPE) irradiation.* Mice were transported to the Proton Research Facility at LLUMC and placed individually into plastic cubicles with dimensions that met the minimum requirement for housing mice for >24 h. The cubicles were put into the plastic cages (4 cubicles/cage) within the BioZone VentiRack™ and the mice were exposed to 2 Gy at entry (skin) over a 36-h time period. A total of 48 animals were irradiated simultaneously. Pellets of standard rodent chow and NapaNectar™ gel (SE Lab Group, Cincinnati, OH, USA) were provided *ad libitum*, a 12h:12h light:dark cycle was maintained, and the room was kept within the appropriate temperature range. The beam delivery system simulated the proton energy spectrum and dose rate profile of a solar particle; a large 1989 flare was chosen as the example to simulate (26). Energies ranged from 15 MeV/n to 215 MeV/n and were administered in 10 MeV increments. The beam size was enlarged at the target to 50 cm diameter using a 5.5 mm Pb foil scatterer. Scanning magnets upstream of the Pb foil deflected the beam sequentially at each of 9 spots forming a 3 x 3 array to cover an area of one square meter. Each spot required one beam spill, or one accelerator cycle, which required 2.2 s.

Therefore, the entire area was covered in 9 spills or 19.8 s. The dose to fluence relationship was used to calculate the number of protons required for the desired dose. A manuscript describing the dosimetry and other aspects of beam delivery in detail has been recently published (25).

*Acute irradiation with photons and protons.* As described previously (22, 23, 27), on the day of photon ( $\gamma$ -ray) irradiation, the animals were transported to a retired Eldorado therapy unit (Atomic Energy of Canada, Ltd., Commercial Products Division, Ottawa, Canada) containing a <sup>60</sup>Co source (1.17 and 1.33 MeV, LET=0.267 KeV/ $\mu$ m). Immediately prior to irradiation, non-anesthetized animals were placed individually into rectangular plastic aerated boxes (30 x 30 x 60 mm). A total dose of 2 Gy was delivered in a single fraction at a dose rate of 0.7 Gy/min using a vertical beam. Dose calibration was performed with a Model PRO6-G cylindrical thimble ionization chamber (Capintec, Inc., NJ, USA).

For whole-body proton (<sup>1</sup>H<sup>1+</sup>) irradiation, mice were transported to the LLUMC Proton Treatment Facility (21, 24, 28). As for photons, non-anesthetized animals were placed individually into the rectangular boxes immediately before exposure. The surface of the boxes was placed behind a 400 mm by 400 mm polystyrene phantom at a water-equivalent depth of 26.4 mm. Protons were delivered to a total dose of 2 Gy in 0.3 s pulses every 2.2 s at a dose rate of 0.9 Gy/min (entry region of Bragg curve, 230 MeV/n at target, LET=0.4 KeV/ $\mu$ m). Dose calibration was performed using a Markus parallel plate ionization chamber, traceable to the National Institute of Standards and Technology (NIST), at depths corresponding to the center of the mice. The ICRU 59 calibration method was used to convert the ionization signal to dose in water. Up to 6 mice were irradiated simultaneously with either photons or protons.

*Body and relative spleen masses.* Mice were weighed, euthanized, and spleens were excised and weighed. Relative spleen mass (RSM) was calculated as follows: RSM=organ mass (mg)/body mass (g).

*Spontaneous DNA synthesis in blood and spleen.* The procedure for basal DNA synthesis by leukocytes in whole blood collected by cardiac puncture in [K<sub>2</sub>]EDTA-containing syringes and single-celled spleen samples was performed as described elsewhere (23, 29) and is briefly summarized here. Aliquots of each sample were diluted with complete RPMI-1640 medium (Irvine Scientific, Santa Ana, CA, USA) and dispensed into 96-well microculture plates. One  $\mu$ Ci of <sup>3</sup>H-thymidine (<sup>3</sup>H-TdR; specific activity = 46 Ci/ $\mu$ mol; ICN Biochemicals, Costa Mesa, CA, USA) was immediately added, and the plates were incubated for 3 h at 37°C in 5% CO<sub>2</sub>. The counts per minute (cpm), volume tested and cell number/ml were used to normalize the cpm to 10<sup>6</sup> cells.

*Hematological analysis.* White blood cell (WBC), red blood cell (RBC), and platelet (PLT) counts, as well as the three-part differential (lymphocytes, monocytes and granulocytes), were determined in 12  $\mu$ l samples of whole blood with anti-coagulant using the HESKA™ Vet ABC-Diff Hematology Analyzer (Heska Corp., Waukesha, WI, USA). Additional important information included: hemoglobin concentration (HGB), hematocrit (HCT; percentage of whole blood composed of RBC), mean corpuscular volume (MCV; mean volume per RBC), mean corpuscular hemoglobin (MCH; mean weight of hemoglobin per RBC), mean

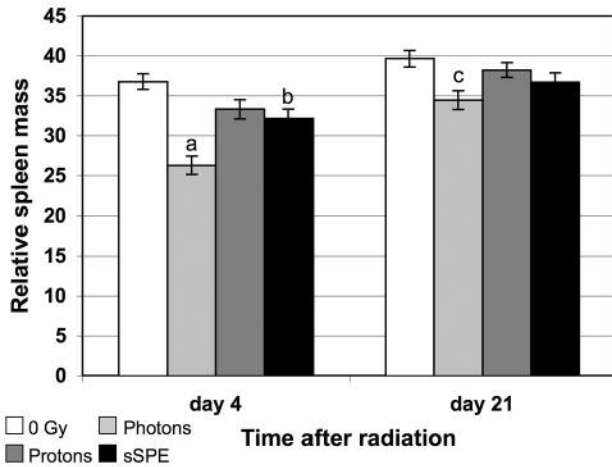


Figure 1. Spleen mass relative to body mass (RSM). Mean  $\pm$  SEM. One-way ANOVA:  $p < 0.001$  for day 4;  $p < 0.01$  for day 21. a,  $p < 0.005$  versus all other groups; b,  $p < 0.05$  versus 0 Gy; c,  $p < 0.01$  versus 0 Gy.

corpuscular hemoglobin concentration (MCHC; mean concentration of hemoglobin per RBC), RBC distribution width (RDW) and mean platelet volume (MPV).

*Leukocyte counts and three-part differentials in spleen.* After erythrocyte lysis, single-celled splenocyte suspensions were evaluated using the ABC Vet Hematology Analyzer (Heska Corp.) (28, 29). WBC, lymphocyte, granulocyte and monocyte-macrophage counts were obtained using 12  $\mu$ l samples.

*Flow cytometric analysis of major lymphocyte populations.* These assessments were carried out on samples of both blood and spleen using a 4-color FACSCalibur™ flow cytometer (Becton Dickinson, Inc., San Jose, CA, USA) as described elsewhere (21, 27, 28). A rapid 2-tube custom-conjugate mixture (PharMingen, San Diego, CA, USA) with monoclonal antibodies including CD3\*FITC, CD4\*APC, CD8\*PE, CD45\*PerCP, NK1.1\*PE and CD19\*APC were used. This analysis together with the numerical data from the automated hematology analyzer provided both numerical and proportional data for T, Th, Tc, B and NK cell populations. Analysis of 5,000-10,000 events/tube was performed using CellQuest™ software (v3.1, Becton Dickinson).

*Statistical analysis.* The data were evaluated using one-way analysis of variance (ANOVA) to determine if a main effect existed for each quantified parameter. The independent variable was group (0 Gy, photons, protons, sSPE) at each time of assessment. Tukey's HSD (honestly significant difference) test was utilized for pair-wise multiple comparisons to obtain means and standard errors (SEM) for each group and to identify significant differences, *i.e.*  $p < 0.05$ , between sets of two groups (SigmaStat™ software, version 2.03, SPSS Inc., Chicago, IL, USA). Due to physical constraints with irradiation procedures, mice in the day 21 acute 2 Gy photon group were processed separately (with appropriate 0 Gy controls). To correct for any day-to-day variability, individual values for these groups were normalized to the means of their respective 0 Gy controls.

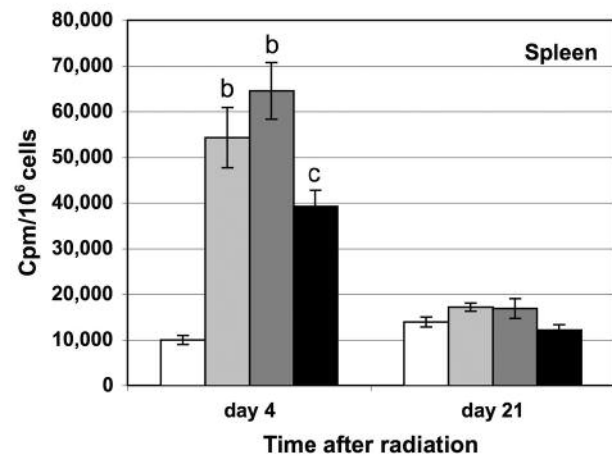
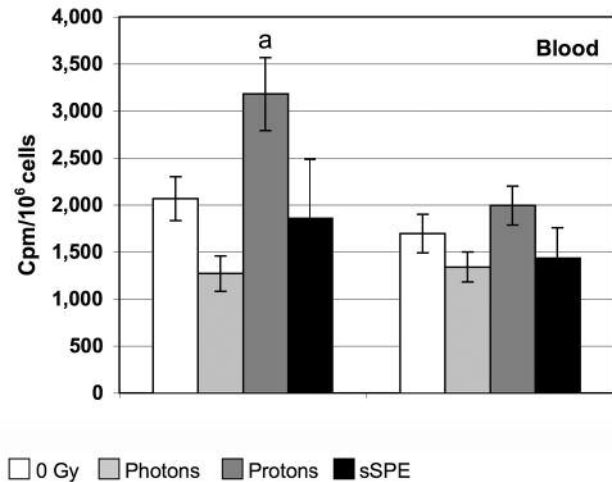


Figure 2. Spontaneous DNA synthesis in blood and spleen leukocytes. Mean  $\pm$  SEM. One-way ANOVA:  $p < 0.01$  for day 4 blood;  $p < 0.001$  for day 4 spleen. a,  $p < 0.01$  versus photons; b,  $p < 0.001$  versus 0 Gy; c,  $p < 0.001$  versus 0 Gy and protons.

## Results

*Body and relative spleen mass.* Average body mass was similar among groups at both times of testing. On day 4, body mass ranged from  $19.5 \pm 0.4$  g (photons) to  $20.1 \pm 0.2$  g (sSPE), whereas by day 21 the weights ranged from  $22.1 \pm 0.3$  g (photons) to  $22.9 \pm 0.4$  g (sSPE). There was, however, a main effect of radiation condition on spleen mass, at both time points ( $p < 0.005$ ). Thus, as shown in Figure 1, spleen mass relative to body mass (RSM) was reduced in all irradiated groups on day 4 ( $p < 0.001$  for one-way ANOVA; values for the photon group were lowest and differed significantly not only from those of the 0 Gy, but also from the sSPE group ( $p < 0.05$ ). By day 21, the RSM for the photon-irradiated mice was still lower than for non-irradiated controls ( $p < 0.05$ ).

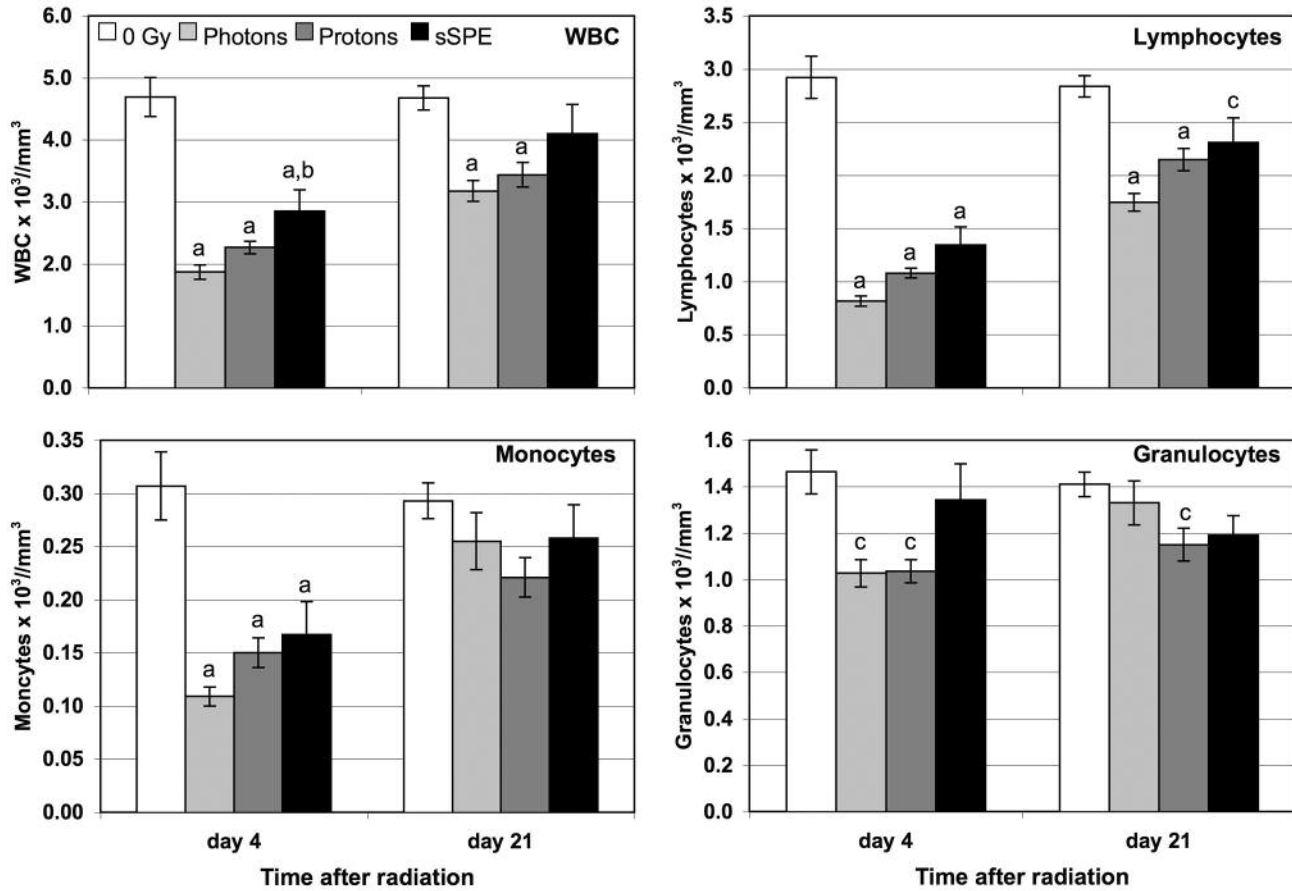


Figure 3. WBC counts and three-part differential in blood. Mean  $\pm$  SEM. One-way ANOVA:  $p < 0.005$  for all cells on day 4;  $p < 0.001$  for WBC and lymphocytes and  $p < 0.05$  for granulocytes on day 21. a,  $p < 0.005$  versus 0 Gy; b,  $p < 0.05$  versus photons; c,  $p < 0.05$  versus 0 Gy.

*Spontaneous DNA synthesis by blood and spleen leukocytes.* Basal synthesis of DNA by leukocytes circulating in blood are shown in Figure 2. On day 4 post-exposure, grouping had a main effect on DNA synthesis in blood cells ( $p < 0.01$ ) and the low level after photon irradiation differed significantly from the relatively high level after proton irradiation ( $p < 0.01$ ) (Figure 2). On day 21, there were no significant differences among groups.

In the spleen (Figure 2), there were significantly reliable effects of radiation condition on leukocyte DNA synthesis ( $p < 0.001$ ). The response was greatly enhanced by radiation, regardless of regimen ( $p < 0.001$  vs. 0 Gy). In addition, the sSPE group had a lower level compared to those receiving acute protons ( $p < 0.005$ ). By day 21, the increased responses were no longer present in any irradiated group.

*Leukocyte counts and three-part differential in blood.* Figure 3 presents the total leukocyte counts and three-part differential in the blood. On day 4, there was a significant effect on the WBC, as well as on each of the major cell

populations ( $p < 0.005$ ). All irradiated groups had lower numbers of WBC, lymphocytes, and monocytes compared to those of the 0 Gy group ( $p < 0.001$ ); the WBC, however, were higher in the sSPE group compared to those of the photon-treated group ( $p < 0.05$ ). Granulocytes were reduced in the photon- and proton-treated groups ( $p < 0.05$ ), but not in the sSPE group, at this same time point.

By day 21 (Figure 3), a reduction in WBC and lymphocytes was still evident in the photon- and proton-treated groups ( $p < 0.005$  vs. 0 Gy). Although lymphocytes were low after sSPE, the effect was less pronounced than in the other two irradiated groups ( $p < 0.05$  vs. 0 Gy). Monocyte numbers were similar among groups and granulocytes were low only in the proton-treated group ( $p < 0.05$  vs. 0 Gy).

As shown in Table I, the numerical changes described above for day 4 resulted in significant proportional decreases in lymphocytes in all irradiated groups ( $p < 0.001$  vs. 0 Gy), whereas the percentages of monocytes and granulocytes were increased ( $p < 0.05$  vs. 0 Gy). By day 21, the cell proportions in the proton-treated and sSPE groups were

Table I. Percentages of major leukocyte populations in blood.

Cell type	0 Gy	Photons	Protons	sSPE
Day 4				
Lymphocytes	63.9±0.9	44.1±1.8 <sup>a</sup>	49.6±0.8 <sup>a,b</sup>	49.1±1.4 <sup>a,b</sup>
Monocytes	7.4±0.2	8.8±0.2 <sup>a</sup>	9.0±0.2 <sup>a</sup>	8.4±0.2 <sup>c</sup>
Granulocytes	28.7±0.8	47.1±1.8 <sup>a</sup>	41.4±0.8 <sup>a,b</sup>	42.6±1.5 <sup>a</sup>
Day 21				
Lymphocytes	63.7±0.7	57.0±0.8 <sup>a</sup>	61.1±0.7 <sup>d</sup>	62.2±1.0 <sup>e</sup>
Monocytes	7.6±0.1	9.5±0.3 <sup>a</sup>	7.7±0.2 <sup>c</sup>	7.9±0.2 <sup>c</sup>
Granulocytes	28.6±0.8	37.5±1.3 <sup>a</sup>	31.2±0.8 <sup>c</sup>	30.0±0.9 <sup>e</sup>

Mean±SEM. Comparisons were made among the four groups for each cell type at each time point. One-way ANOVA:  $p < 0.001$  for main effect of group on all three cell populations on both dates. <sup>a</sup> $p < 0.001$  versus 0 Gy; <sup>b</sup> $p < 0.05$  versus photons; <sup>c</sup> $p < 0.05$  versus 0 Gy; <sup>d</sup> $p < 0.01$  versus photons; <sup>e</sup> $p < 0.001$  versus photons.

similar to 0 Gy controls, but the photon-treated group still had a low percentage of lymphocytes and high percentages of monocytes and granulocytes ( $p < 0.01$  vs. all other groups).

*Leukocyte counts and three-part differential in spleen.* Figure 4 shows that there was a main effect of group on the counts of WBC and all three major leukocyte populations on day 4 ( $p < 0.001$ ). Significant reductions occurred in WBC and lymphocytes in all irradiated groups compared to those of the 0 Gy group ( $p < 0.005$ ). The photon- and proton-treated groups, but not the sSPE group, also had reduced monocytes-macrophages and granulocytes ( $p < 0.05$ ). Furthermore, exposure to photons resulted in greater reduction in monocytes-macrophages and granulocytes compared to that with either protons or sSPE ( $p < 0.05$ ) at the early time point.

On day 21 (Figure 4), a main effect was observed for WBC and all three major leukocyte populations ( $p < 0.01$ ), primarily due to low numbers of these cells in the photon-treated group ( $p < 0.005$  vs. 0 Gy). The proton-treated and sSPE groups had significantly higher levels of WBC and lymphocytes compared to the photon-treated group ( $p < 0.05$ ), whereas monocyte-macrophage and granulocyte counts were similar to the 0 Gy controls.

The numerical changes in the leukocyte populations resulted in altered percentages ( $p < 0.001$  for main effect on all cell types on day 4 and  $p < 0.001$  for monocyte-macrophages on day 21) (Table II). The pattern for the individual cell populations was generally similar to what was observed in the blood, but much less pronounced.

*Lymphocyte populations in blood.* On day 4, there was a main effect of group on both T cell subsets and B cells ( $p < 0.001$ ), but not NK cells (Figure 5). Exposure to either photons or protons resulted in lower levels of CD4<sup>+</sup> Th

Table II. Percentages of major leukocyte populations in spleen.

Cell type	0 Gy	Photons	Protons	sSPE
Day 4				
Lymphocytes	77.5±0.4	73.8±0.5 <sup>a</sup>	74.2±0.5 <sup>a</sup>	75.2±0.7 <sup>b</sup>
Mono-macrophages	4.1±0.1	4.8±0.1 <sup>a</sup>	4.6±0.1 <sup>a</sup>	4.3±0.1 <sup>b,c</sup>
Granulocytes	18.4±0.4	21.4±0.5 <sup>a</sup>	21.2±0.5 <sup>d</sup>	20.4±0.7 <sup>b</sup>
Day 21				
Lymphocytes	75.5±0.6	74.9±0.7	75.5±0.4	76.0±0.6
Mono-macrophages	4.4±0.0	4.6±0.1 <sup>b</sup>	4.4±0.1	4.2±0.1 <sup>c</sup>
Granulocytes	20.1±0.4	20.0±0.4	20.1±0.4	19.7±0.6

Mean±SEM. Comparisons were made among the four groups for each cell type at each time point. One-way ANOVA:  $p < 0.001$  on day 4 for main effect on all three cell types;  $p < 0.001$  on day 21 for monocytes-macrophages. <sup>a</sup> $p < 0.001$  versus 0 Gy; <sup>b</sup> $p < 0.05$  versus 0 Gy; <sup>c</sup> $p < 0.005$  versus photons; <sup>d</sup> $p < 0.005$  versus 0 Gy; <sup>e</sup> $p < 0.001$  versus photons.

cells compared to treatment with 0 Gy, whereas significant reductions in the CD8<sup>+</sup> Tc subset occurred in all three irradiated groups ( $p < 0.05$ ). The effect of sSPE on the T cell subsets was less pronounced than that of photons ( $p < 0.05$ ).

Although considerably diminished by day 21, radiation condition still had a main effect on all lymphocyte subsets on day 21 ( $p < 0.05$ ). CD4<sup>+</sup> Th and NK cell counts were low in the proton-treated group and B cells were low in the photon-treated group ( $p < 0.05$  vs. 0 Gy); CD8<sup>+</sup> Tc cells were low in all irradiated groups ( $p < 0.001$  vs. 0 Gy).

Table III shows that the radiation condition had a main effect on the CD4:CD8 ratio ( $p < 0.001$ ) at both times of analysis. Although all irradiated groups had a high ratio compared to 0 Gy ( $p < 0.05$ ), the greatest increase was found in the photon-treated group that differed significantly also from the proton-treated and sSPE groups ( $p < 0.05$ ). A similar pattern was noted on day 21.

*Lymphocyte populations in spleen.* As shown in Figure 6, there was a main effect of group on CD4<sup>+</sup> Th, CD8<sup>+</sup> Tc, B, and NK cells at 4 days post-irradiation ( $p < 0.001$ ). Although the counts were low for the T cell subsets and B cells in all irradiated groups ( $p < 0.05$  vs. 0 Gy), the greatest depression was seen in the photon-treated group. NK cells were significantly reduced only by photon radiation ( $p < 0.001$  vs. 0 Gy) and were also lower than in the proton-treated and sSPE groups ( $p < 0.05$  and  $p < 0.005$ , respectively).

On day 21, a main effect of radiation condition was still evident on both T cell subsets and B cells ( $p < 0.05$ ); for NK cells there was a trend ( $p = 0.052$ ). The CD4<sup>+</sup> Th cells were reduced only after photon radiation ( $p < 0.05$  vs. 0 Gy), CD8<sup>+</sup> Tc cells were low after acute exposure to either photons or protons ( $p < 0.001$  vs. 0 Gy). In contrast, all of these lymphocyte populations in the sSPE group were

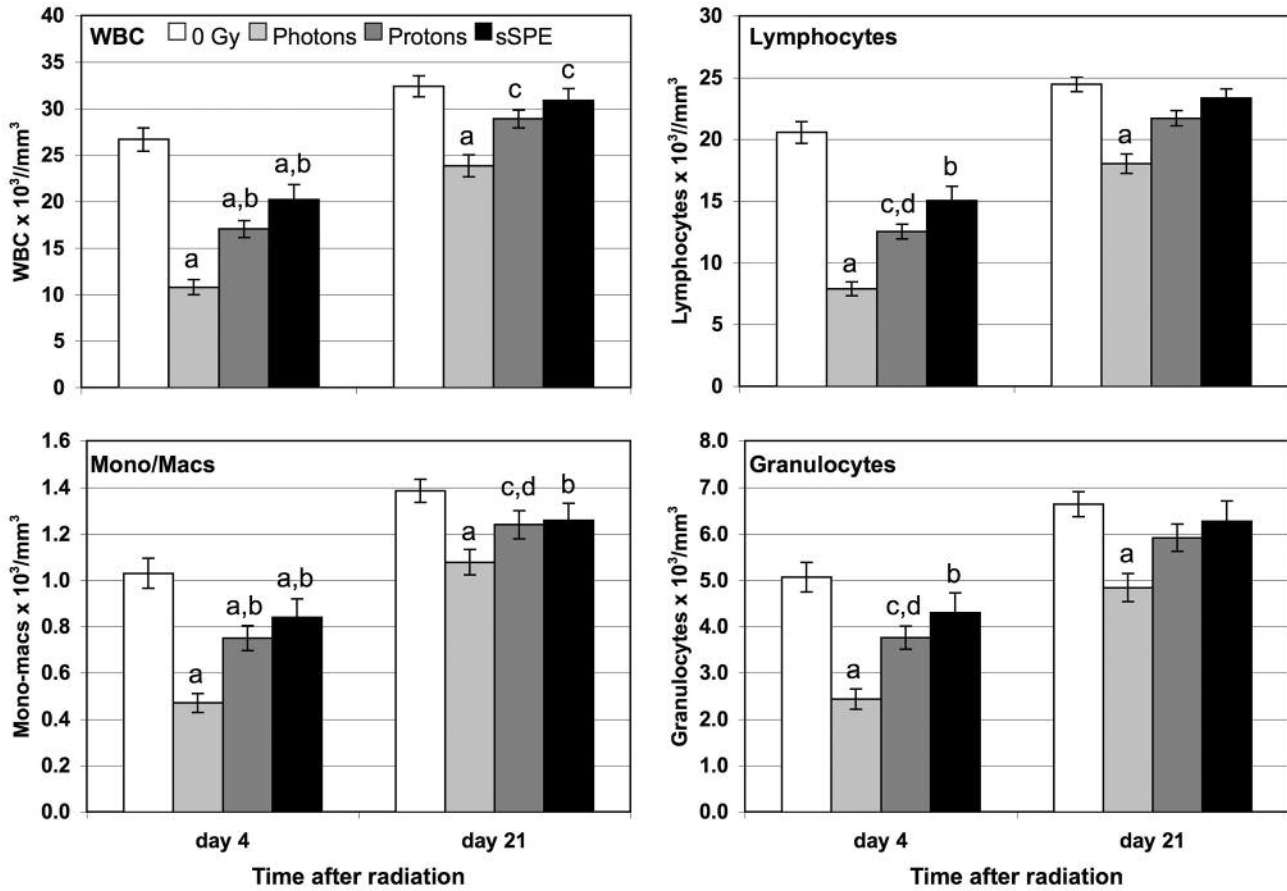


Figure 4. WBC counts and three-part differential in spleen. Mean±SEM. One-way ANOVA:  $p < 0.001$  for all cells on day 4;  $p < 0.001$  for WBC and lymphocytes and  $p < 0.01$  for monocytes-macrophages and granulocytes on day 21. a,  $p < 0.005$  versus 0 Gy; b,  $p < 0.005$  versus photons; c,  $p < 0.05$  vs. photons; d,  $p < 0.05$  versus 0 Gy.

similar to the non-irradiated controls and the levels of the CD8<sup>+</sup> Tc and B cells were higher than in the photon-treated group ( $p < 0.01$ ).

Table III shows that group had a main effect on the CD4:CD8 ratio at both time points ( $p < 0.001$ ). On day 4, the greatest increase was seen in the photon-treated group that differed significantly from the 0 Gy and the other two irradiated groups ( $p < 0.05$ ). By day 21, a main effect of group was still evident ( $p < 0.001$ ). The ratios were elevated in all irradiated groups ( $p < 0.05$  vs. 0 Gy); the greatest difference from normal was seen after proton irradiation ( $p < 0.05$  vs. photon-treated and sSPE groups).

**Erythrocyte counts and characteristics.** In Table IV, it can be seen that there was a main effect of group on all erythrocyte characteristics on day 4 ( $p < 0.005$  for all except MCHC which was  $p < 0.05$ ). RBC counts, HGB, HCT, and RDW were low in the photon-treated group compared to those of the 0 Gy group ( $p < 0.05$ ). Acute irradiation with protons resulted in only high MCV ( $p < 0.05$ ). In contrast, exposure

to sSPE reduced the RBC and increased the MCV, MCH, and MCHC at this time point ( $p < 0.05$ ).

On day 21 (Table IV), there was a main effect of radiation condition on MCV, MCH, and RDW ( $p < 0.001$ ), and MCHC ( $p < 0.05$ ). The photon-treated group had low MCH compared to that in the proton-treated and sSPE ( $p < 0.05$ ) groups and high RDW compared to the 0 Gy group ( $p < 0.005$ ). The proton-treated group had high RDW ( $p < 0.005$  vs. 0 Gy) and the sSPE group had increased MCV, MCH, and RDW compared to the 0 Gy group ( $p < 0.05$ ) and high MCHC compared to the photon-treated group ( $p < 0.05$ ).

**Platelet count and volume.** Figure 7 shows that there was a main effect of group on platelet count and volume ( $p < 0.05$  and  $p < 0.001$ , respectively) on day 4. However, in *post-hoc* Tukeys test, PLT was increased only in the proton-treated group compared to that of the 0 Gy group ( $p < 0.05$ ), whereas MPV was reduced in all irradiated groups ( $p < 0.001$ ). In contrast, the response was reversed by day 21.

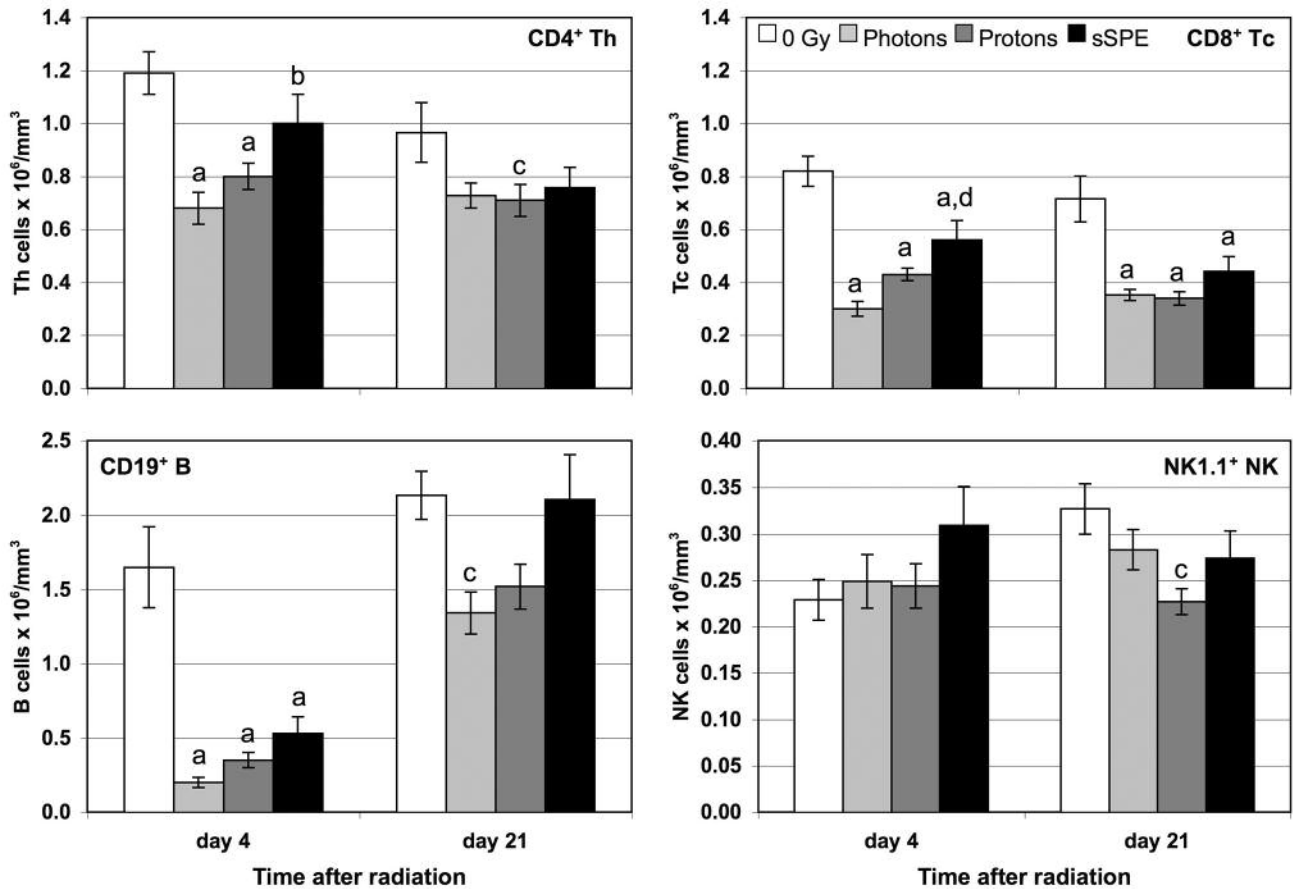


Figure 5. Lymphocyte subpopulations in blood. Mean  $\pm$  SEM. One-way ANOVA:  $p < 0.001$  for all cells except NK on day 4;  $p < 0.05$  for CD4<sup>+</sup> T and NK cells,  $p < 0.001$  for CD8<sup>+</sup> T and  $p < 0.05$  for NK cells on day 21. a,  $p < 0.005$  versus 0 Gy; b,  $p < 0.05$  versus photons; c,  $p < 0.05$  versus 0 Gy; d,  $p < 0.01$  versus photons. One-way ANOVA for NK cells on day 21.

PLT were low in all irradiated groups ( $p < 0.001$ ); MPV was high after photon irradiation ( $p < 0.001$  vs. all other groups) and lower in the sSPE group than in the photon- and proton-treated groups ( $p < 0.05$ ).

## Discussion

Although body mass was not affected by radiation in the present study, decreases in spleen mass relative to body mass (*i.e.* RSM) were noted in the photon-treated and sSPE groups. The decrease is likely to reflect the fact that leukocyte populations are still recovering from radiation exposure. It is important to note that the spleen is the largest lymphoid organ and is increasingly recognized as being important in immune defense. It has a white pulp rich in T and B cells, macrophages lining sinusoids, and produces opsonins and properdin that facilitate destruction of bacteria and other infectious agents. Splenectomy or absence of normal spleen function increases risk for

overwhelming infections (30). A decrease in spleen mass could indicate reduced ability to respond to an immune challenge. In our previous studies, mice returning from a 12-day journey on the space shuttle Endeavour (STS-108/UF-1) had splenic atrophy. Given the relatively low doses of radiation received in low earth orbit, the data suggest that other factors in the space flight environment can adversely affect this organ (13, 14).

Increased DNA synthesis was noted in splenic leukocytes from all irradiated groups on day 4, indicating activation of hematopoietic mechanisms to compensate for cell loss. However, the enhancement was less pronounced after exposure to sSPE than acutely delivered protons. In the blood, increased DNA synthesis was noted only in the proton-treated group. While it is possible that exposure to an sSPE selectively hindered proliferative processes, this scenario seems unlikely. Rather, these findings suggest that the damage caused by sSPE irradiation was either less severe in both body compartments than that experienced by the proton-treated group, or that

Table III. CD4:CD8 T lymphocyte ratio.

Group	Blood		Spleen	
	Day 4	Day 21	Day 4	Day 21
0 Gy	1.48±0.06	1.41±0.03	1.73±0.04	1.59±0.03
Photons	2.35±0.12 <sup>a</sup>	2.21±0.07 <sup>a</sup>	2.30±0.06 <sup>e</sup>	1.84±0.07 <sup>h</sup>
Protons	1.86±0.07 <sup>b</sup>	2.03±0.07 <sup>c</sup>	2.07±0.07 <sup>f</sup>	2.12±0.09 <sup>i</sup>
sSPE	1.85±0.08 <sup>b</sup>	1.77±0.08 <sup>d</sup>	1.85±0.03 <sup>g</sup>	1.81±0.06 <sup>b</sup>

Mean±SEM. Comparisons were made among the four groups for each cell type at each time point. One-way ANOVA:  $p < 0.001$  for main effect of all groups on both dates. <sup>a</sup> $p < 0.005$  versus all other groups; <sup>b</sup> $p < 0.05$  versus 0 Gy and photons; <sup>c</sup> $p < 0.001$  versus 0 Gy,  $p < 0.05$  versus sSPE; <sup>d</sup> $p < 0.001$  versus 0 Gy and photons,  $p < 0.05$  versus protons; <sup>e</sup> $p < 0.001$  versus 0 Gy and sSPE,  $p < 0.05$  versus protons; <sup>f</sup> $p < 0.001$  versus 0 Gy,  $p < 0.05$  versus photons and sSPE; <sup>g</sup> $p < 0.001$  versus photons,  $p < 0.05$  versus protons; <sup>h</sup> $p < 0.05$  versus 0 Gy and protons; <sup>i</sup> $p < 0.001$  versus 0 Gy,  $p < 0.05$  versus photons and sSPE.

effective damage repair was possible during the 36-hour exposure in the sSPE group due to the much lower radiation dose rate (*i.e.* 0.9 Gy/min for acute protons and 0.0009 Gy/min for sSPE). These latter possibilities are generally supported by the consistently greater numbers of WBC, all three major leukocyte populations and the various lymphocyte subsets after sSPE irradiation compared to that with acute protons. Although differences between the two groups were not always significant, the overall trend was fairly consistent. Furthermore, the numerical decreases, especially in the blood, resulted in a proportional shift in favor of cell populations involved in innate immunity, *i.e.* increased percentages of monocytes and granulocytes. The relative sensitivity of the lymphocyte subsets was B > T > NK and CD8<sup>+</sup> Tc > CD4<sup>+</sup> Th cells, regardless of radiation regimen, as noted in our previous studies with  $\gamma$ -rays and protons (21, 27).

In contrast to leukocytes, sSPE irradiation resulted in more abnormalities in erythrocyte properties compared to 0 Gy controls than after acute proton irradiation at both time points of assessment. Studies of spaceflight personnel during and after missions in space have found decreased hemoglobin, low iron, conformational abnormalities in hemoporphyrin, altered fluidity of RBC membranes and other changes that could adversely affect oxygen transfer (31, 32). Our data suggest that the anemia frequently reported to occur during space flight (33) may be exacerbated in crew members exposed to a substantial dose of radiation during an SPE. Thus far, the anemia associated with voyages in space has been primarily attributed to a rapid and selective hemolysis of the most immature circulating RBC, *i.e.* “neocytolysis,” a condition that occurs when the level of erythropoietin drops below a critical minimum level (33, 34).

Table IV. Erythrocyte counts and characteristics.

Test	0 Gy	Photons	Protons	sSPE
Day 4*				
RBC	8.0±0.4	6.0±0.3 <sup>a</sup>	7.1±0.2	6.7±0.4 <sup>a</sup>
HGB	12.3±0.6	9.4±0.6 <sup>a</sup>	11.0±0.3	10.6±0.6
HCT	36.8±1.9	27.9±1.6 <sup>a</sup>	32.9±0.8	31.4±1.7
MCV	45.9±0.1	46.2±0.2	46.6±0.2 <sup>a</sup>	46.6±0.1 <sup>a</sup>
MCH	15.4±0.1	15.6±0.1	15.6±0.1	15.8±0.1 <sup>a</sup>
MCHC	33.4±0.1	33.8±0.2	33.4±0.1	33.9±0.1 <sup>b</sup>
RDW	13.5±0.1	13.1±0.1 <sup>c</sup>	13.6±0.1	13.7±0.1
Day 21**				
RBC	8.3±0.1	8.3±0.1	8.3±0.1	8.4±0.1
HGB	12.7±0.2	12.6±0.1	12.7±0.1	12.9±0.1
HCT	37.9±0.6	38.0±0.4	37.8±0.4	38.7±0.4
MCV	45.5±0.1	45.8±0.1	45.9±0.1	46.3±0.1 <sup>d</sup>
MCH	15.2±0.1	15.0±0.1 <sup>c</sup>	15.4±0.1	15.5±0.1 <sup>a</sup>
MCHC	33.4±0.1	32.9±0.1	33.4±0.1	33.5±0.2 <sup>e</sup>
RDW	13.8±0.1	14.3±0.1 <sup>d</sup>	14.2±0.1 <sup>d</sup>	14.3±0.1 <sup>d</sup>

Mean±SEM. Comparisons were made among the four groups for each cell type at each time point. RBC (red blood cell count, 10<sup>6</sup>/mm<sup>3</sup>), HGB (hemoglobin, g/dl), HCT (hematocrit, %), MCV (mean corpuscular volume, fl), MCH (mean corpuscular hemoglobin, pg), MCHC (mean corpuscular hemoglobin concentration, g/dl), RDW (red blood cell distribution width, %). One-way ANOVA: \* $p < 0.005$  for main effect of group for all parameters except MCHC which was  $p < 0.05$ ; \*\* $p < 0.001$  for MCV, MCH and RDW;  $p < 0.05$  for MCHC. <sup>a</sup> $p < 0.05$  versus 0 Gy; <sup>b</sup> $p < 0.05$  versus protons; <sup>c</sup> $p < 0.05$  versus protons and sSPE; <sup>d</sup> $p < 0.005$  versus 0 Gy; <sup>e</sup> $p < 0.05$  versus photons.

Platelet numbers were significantly increased only in the proton-treated group on day 4, at which time low MPV was observed in all irradiated groups. By day 21, the number of these cells was low in all irradiated groups and MPV was increased only in the photon-treated group. This pattern of changes is somewhat different from that seen in either erythrocytes or leukocytes and may at least partly reflect differences in rate of renewal and storage capacity (35). Platelets function in blood clotting and wound healing. A number of studies indicate that response to wounding and injury may be impaired in space (36, 37). Our data support the possibility that a major physical trauma may be especially difficult to manage in spaceflight personnel who have also been exposed to an SPE.

Collectively, the different responses seen among each of the distinct cell populations is indicative of their radiosensitivity, *i.e.* their cycling kinetics, DNA repair capacity, and other innate characteristics. In addition, hematopoietic progenitors are heterogeneous in their ability to repair damage and repopulate after exposure to ionizing radiation (38). The occurrence of either cell death or successful damage repair after radiation exposure is, indeed, dependent upon a balance of many factors including quality and number of energy depositions, time interval between

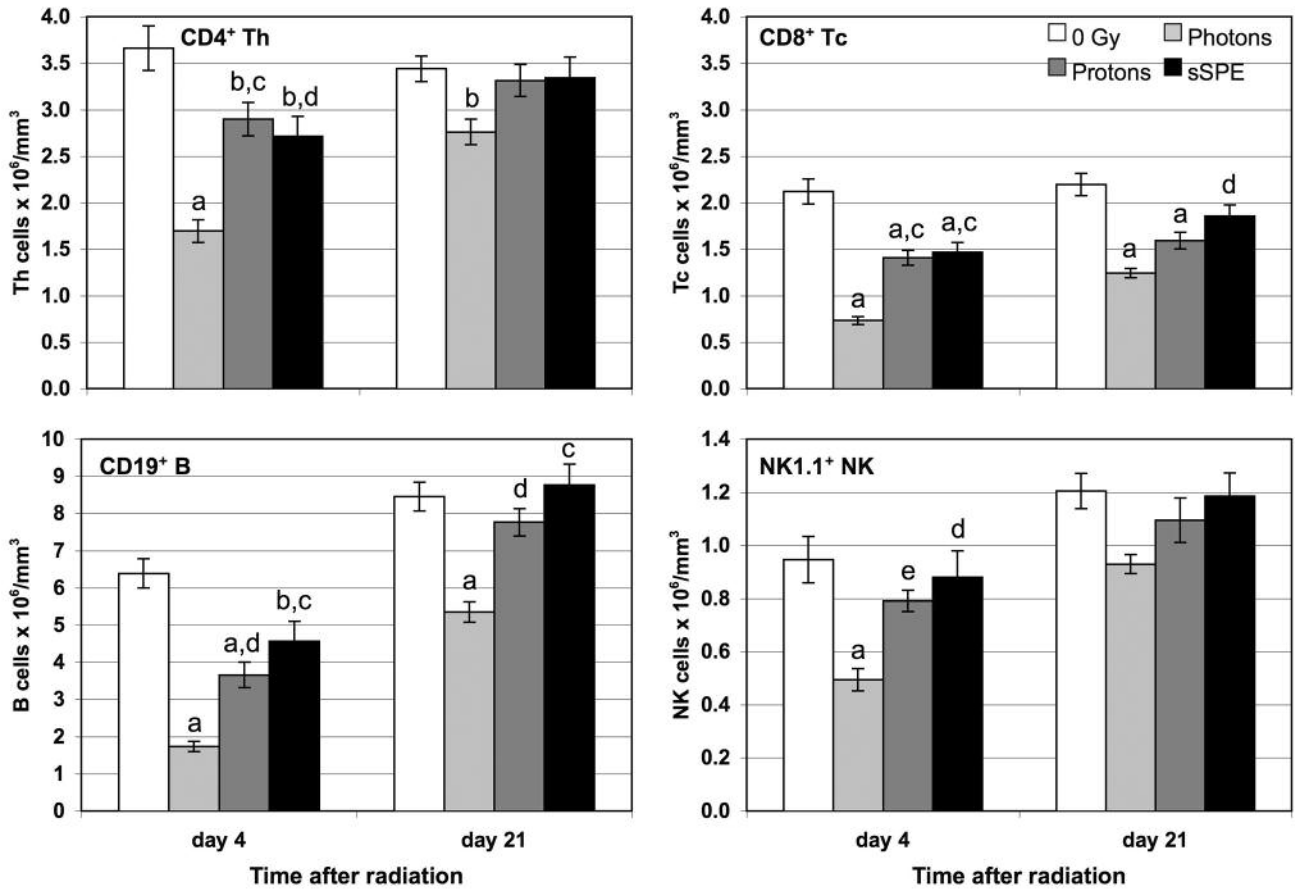


Figure 6. Lymphocyte subpopulations in spleen. Mean  $\pm$  SEM. One-way ANOVA:  $p < 0.001$  for all four cell types on day 4;  $p < 0.001$  for CD8<sup>+</sup> Tc and B cells, and  $p < 0.05$  for CD4<sup>+</sup> Th cells ( $P = 0.052$  for NK cells) on day 21. a,  $p < 0.001$  versus 0 Gy; b,  $p < 0.05$  versus 0 Gy; c,  $p < 0.001$  versus photons; d,  $p < 0.01$  versus photons; e,  $p < 0.05$  versus photons.

the depositions, responses of the specific body site to damaging events, and systemic responses activated as a result of localized damage (39). The differences observed after sSPE and acute proton exposure can be attributed to several factors. The SPE simulation was based on a large SPE that occurred in 1989 (26) and consisted of protons with a dose rate profile that spanned 36 hours and included 21 energies ranging from 15 MeV/n to 215 MeV/n in 10 MeV increments. This regimen differed significantly from our acute proton radiation exposure, which was delivered within a few minutes at a single energy of 230 MeV at the target. Greater energy transfer, and thus also a greater biological effect, would be expected with low energy protons. This was reflected in erythrocyte, but not leukocyte and platelet, characteristics at the times of assessment. Another possibility is that the lower energy protons did not travel all the way through the sSPE-irradiated mice and, therefore, internal organs such as the spleen received a lower total dose. For example, the stopping distance of 15

MeV protons in water (*i.e.* body equivalent) is only about 0.25 cm. Finally, repair mechanisms likely played a significant role in the animals exposed to sSPE. As noted previously, activation of repair processes likely occurred during the 36-hour exposure, repairing damage shortly after it occurred. In the acute exposures, the damage was likely more drastic and sudden and, thus, overwhelmed any available repair mechanisms.

The data also show that acute photon radiation generally induced the greatest variation from normal and that the values sometimes differed significantly from those in the proton-treated group. For example, splenic WBC counts were lower, whereas the CD4:CD8 T cell ratio in blood was higher in the photon- than in the proton-treated group at the early time point. At the later time point, the proton-treated group had the most abnormal CD4:CD8 ratio in the spleen (*i.e.* significantly higher than any of the other groups). The reason for these differences is not clear, but may be attributed at least partly to differences in the

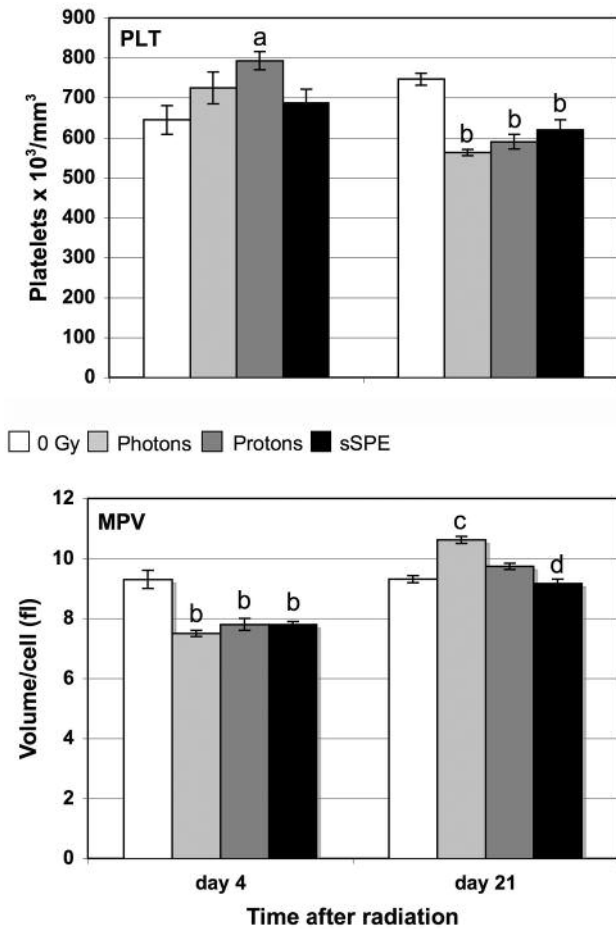


Figure 7. Platelet characteristics. Mean  $\pm$  SEM. One-way ANOVA:  $p < 0.05$  for PLT and  $p < 0.001$  for MPV on day 4;  $p < 0.001$  for PLT and MPV on day 21. a,  $p < 0.05$  versus 0 Gy; b,  $p < 0.001$  versus 0 Gy; c,  $p < 0.001$  versus all other groups; d,  $p < 0.05$  versus photons and protons.

physical characteristics of the two forms of radiation. In addition, the time course of damage expression may be somewhat dependent upon radiation quality, as noted in our previous studies (23, 28).

### Conclusion

This is the first study to demonstrate that protons mimicking a large solar explosion can affect certain cell populations differently compared to acutely delivered monoenergetic protons and  $\gamma$ -ray photons. The focus was on leukocytes in two major body compartments, the spleen and peripheral blood, which reflect the status of progenitors in the bone marrow. Analyses of erythrocytes and thrombocytes, that are also bone marrow-derived cells, were included. Although there were similarities in response to the

three radiation regimens, there were also significant differences. In most measurements of leukocytes and platelets, exposure to sSPE induced less perturbation and better recovery compared to the other two radiation regimens. The findings also indicate that data obtained from studies using either photons ( $\gamma$ -rays, X-rays) or protons at space-relevant total doses may not necessarily be predictive of hematopoietic responses after exposure to an SPE.

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