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Impact of Head-Only Iron Ion Radiation on the Peripheral LPS Response

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Abstract. *Although there is a large body of evidence indicating that radiation can have a dramatic impact on both immune and brain function, there is very little known about its effect on communication between these critical two systems. In this study, mice were exposed to head-localized irradiation with 5 Gy $^{56}Fe^{26+}$ ions and assessed for immune function. Mice were inoculated with lipopolysaccharide 37-38 days post-irradiation. Subsets of mice were euthanized 1, 7, or 14 days later. Radiation significantly impacted the response to an immune challenge in terms of splenic and circulating leukocyte counts and lymphocyte distributions; the effect was especially pronounced on granulocytes and B-cells ($p<0.05$). However, there were no interactions in spontaneous or mitogen-induced blastogenesis of activated T-cell proportions, brain interleukin-1β, or circulating corticosterone levels. These data demonstrate that head-localized iron ion radiation modified the peripheral response to a potent bacterial component associated with septic shock.*

Studies involving atomic bomb survivors (1) and radiation exposures relevant to space travel (2) suggest that ionizing radiation can dramatically impact a variety of immune parameters. There is also a growing consensus that radiation can affect neurological function (3, 4). However, very little research has been conducted on the influence of ionizing radiation on the communication between these two critical systems. This is surprising as communication between the central nervous system (CNS) and the peripheral immune system are crucial in maintaining homeostasis. Each system compiles, integrates, and responds to an enormous array of external and internal stimuli. Together, these systems co-actively regulate functions ranging from water balance and body temperature to metabolism, fear responses, and sleep (5-7).

The dearth in the literature is particularly apparent for high linear energy transfer (LET) radiation types. However, particle radiation such as protons and carbon ions is increasingly in use clinically (8). In addition to these radiation forms, iron ions ($^{56}Fe^{26+}$) are important to the National Aeronautics and Space Administration (NASA) and other space agencies because they are present in relatively large amounts in galactic cosmic rays (GCR). Although high-LET particles represent about 1% of the total fluence in deep space radiation, they will likely dominate overall biological impact (9, 10).

Fundamentally different from photons, high-charge (Z) high-energy (E) ions (*i.e.*, HZE particles) are densely ionizing, depositing more than 50% of their energy within a ~10 nm core along their linear tracks (11). Up until approximately 10 years ago, facilities able to generate high-LET radiation for use in biological studies were very limited. Due to this limitation, the vast majority of published radiobiological research has involved γ-ray or x-ray radiation. The National Institute of Radiological Sciences in Chiba, Japan, has been a leader in particle radiation studies for many years. Since facilities such as the Loma Linda Proton Treatment and Research Center and the NASA Space Radiation Laboratory (NSRL) have been up and running, these critical questions can be addressed more readily.

In this study, the impact of brain-localized iron ion irradiation on behavior and peripheral inflammatory responses were characterized. Mice were irradiated with 5 Gy brain-localized ^{56}Fe radiation. Four to 5 days later, the mice were assessed for radiation-induced changes in acoustic startle behavior (12). Roughly one month after the behavioral tests, mice were challenged with the bacterial cell wall component, lipopolysaccharide (LPS), and immunological assessments were performed at three time points thereafter.

Materials and Methods

Animals. Eight- to 9-week-old male C57BL/6 mice (N=110; 5-13 non-irradiated mice/time point, 9-13 irradiated mice/time point) were ordered from Charles River Laboratories (Hollister, CA, USA) and delivered directly to the Brookhaven National Laboratory

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(BNL) Animal Handling Facility. Animals were housed in standard shoe-box cages (4/cage) in environmentally controlled rooms (~21˚C, 12-h light-dark schedule, lights on at 0700) and allowed to acclimate for 7-10 days prior to irradiation. Food and water were available *ad libitum*. During the acclimatization period, mice were tattooed and injected with transponders for identification purposes. Briefly, isoflurane (3% for induction, 1% for maintenance, in O_2) was used to anesthetize the animals prior to transponder injection. Male mice were used because the animals underwent behavioral testing prior to immunological assessment and the estrous cycle has been demonstrated to influence many behavioral endpoints (13). Methods and results from this aspect of the experiment have been previously published (12). In summary, the mice were tested with the acoustic startle model during the first week immediately following irradiation. Prior to testing, subsets were given intraperitoneal (*i.p.*) injections of apomorphine (3 mg/kg), haloperidol (1 mg/kg), or vehicle. Within 2 days of behavioral testing, mice were shipped to the Loma Linda University (LLU) animal care facility *via* overnight courier. At 37-38 days post-irradiation, immune assessments were initiated. While there is some potential that the pharmaceutical agents used in the behavioral assessment may somehow have been neuroprotective against radiation and/or LPS effects, both haloperidol and apomorphine have a very short half-life within the blood. Several investigators have reported half lives of less than 1-2 hours for apomorphine (14, 15) and up to 1-2 days for haloperidol (16, 17) in humans, regardless of the route of administration. Because our mice were tested 30 days after the administration of drug, and because the mice were randomized across treatment condition, we believe the use of these drugs had little or no impact on our immune assessment. All protocols were approved by the Institutional Animal Care and Use Committees of both LLU and BNL.

Head-only irradiation. As previously described (12), all mice were irradiated in the Alternating Gradient Synchrotron at BNL (BNL-8). Prior to irradiation, mice were sedated with 3-4% isoflurane and placed into positioning cradles. The head position was stabilized for accurate irradiation with bite bars. Up to four cradles were placed into an anesthesia box designed for the beam line and anesthesia was maintained with 2-3% isoflurane. The head of each mouse was irradiated with a single fraction of 5 Gy $^{56}Fe^{26+}$ (1.046 GeV/nucleon; for a complete characterization of the iron ion beam see reference (18). A collimator was located 5 cm upstream from the center of the mice (1.2 cm diameter aperture, 1.5 cm beam diameter at the head of the mouse), consisting of acrylic (9 cm), aluminum (7.75 cm), and high-density polyethylene (9 cm). Doses were targeted to the center of the brain. Non-exposed control mice were 'sham-irradiated' (*i.e.*, sedated with isoflurane and placed in the exposure boxes). After exposure, mice were returned to their cages and observed until they resumed normal posture and behavior. The high radiation dose used in this study was due to the fact that these mice were initially assessed for behavioral deficits and this was one of the earliest studies in this dose range (BNL-8 was carried out in 2002). Given that little was known at the time about iron irradiation-induced behavioral deficits, and virtually nothing was known about deficits in immune brain communication, we selected 5 Gy as a starting point.

LPS injection. The mice were inoculated 37-38 days post-irradiation. They were injected *i.p.* with 1 mg/kg LPS (*Escherichia coli*, O111:B4; Sigma Chemical Co., St. Louis, MO, USA) in a volume of 0.1 ml 0.9% phosphate buffered saline (PBS). The LPS dose selected is similar to those reported to induce significant immune modulation (19-22). Control mice were injected with PBS only.

Tissue collection and processing. These procedures have been described in previous studies (23-25). Briefly, mice were weighed and subsets were euthanized with 100% CO_2 on days 1, 7, or 14 post-LPS inoculation. Thymus, spleen, liver, and lungs were removed and weighed. Brains were removed and immediately frozen in liquid nitrogen. Relative organ mass (ROM) was calculated: ROM=organ mass (mg)/body mass (g).

Hematological assessment. Whole blood was collected in [K_2]EDTA-containing syringes *via* cardiac puncture. Splenic leukocytes were homogenized into single-celled suspensions. Blood and splenic cells were characterized using an automated ABC Vet Hematology Analyzer (Heska Corp., Waukesha, WI, USA) (26-28). Parameters included: white blood cell (WBC), lymphocyte, monocyte, granulocyte, red blood cell (RBC), and platelet (PLT) counts, hemoglobin (HGB) concentration, 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 corpuscular hemoglobin concentration (MCHC; mean concentration of hemoglobin per RBC), RBC distribution width (RDW), and mean platelet volume (MPV).

Flow cytometry. Whole blood and single-cell suspensions of splenic WBC were evaluated using fluorescence-labeled monoclonal antibodies (mAb), a direct-staining 'no lyse-no wash' procedure, and a FACSCalibur™ flow cytometer (Becton Dickinson, Inc., San Jose, CA, USA). The mAb were labeled with fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), allophycocyanin (APC), or peridinin chlorophyll protein (PerCP). For basic lymphocyte phenotyping, a two-tube custom-conjugate mAb mixture (BD Pharmingen, San Diego, CA, USA) allowed characterization of the following populations: $CD45^+$ (30-F11)/$CD3^+$ (145-2C11) – mature T-cells; $CD45^+/CD3^+/CD4^+$ (RM4-5) – T-helper (Th) cells; $CD45^+/CD3^+/ CD8^+$ (53-6.7) – T-cytotoxic (Tc) cells, $CD45^+/B220^+$ (RA3-6B2) – B-cells; and $CD45^+/NK1.1^+$ (PK136) – natural killer (NK) cells. For T-cell activation/proliferation markers, cells were labeled with CD45/CD3/CD25 (PC61)/CD71 (C2) markers. The mononuclear populations (monocytes/macrophages plus lymphocytes) were gated in the side scatter *vs*. CD45 dot plot. At least 5,000 gated events were acquired per tube for analysis *via* CellQuest™ (version 3.1; Becton Dickinson). Population counts were calculated as follows: number of cells in labeled subpopulation/ml=(number of WBC/ml) × (proportion of WBC which are lymphocytes) × (percentage of labeled subpopulation).

Spontaneous and mitogen-induced blastogenesis. For basal proliferation, whole blood and splenic leukocytes were suspended in complete RPMI 1640 medium (Irvine Scientific, Santa Ana, CA, USA) and dispensed into 96-well microculture plates. Immediately after adding 1 μCi of ^{3}H-thymidine (^{3}H -TdR; specific activity=46 Ci/μmol; ICN Biochemicals, Costa Mesa, CA, USA), the plates were incubated for 3 h at 37˚C in 5% CO_2. For mitogen-induced proliferation, splenic leukocytes were dispensed into microtiter plates with medium containing phytohemagglutinin (PHA), concanavalin A (ConA), LPS (*E. coli* serotypes 0111:B4 and 055:B5) or medium with no mitogen. All mitogens were purchased from Sigma Chemical Co. and pre-titrated for maximal response. After a 44 h incubation period, cells were pulse-labeled with 3H-TdR (1 μCi/50 μl/well) for 4 additional hours. In both assays, cells

were harvested (Harvester 96 Mack III-m; Tomtec, Hamden CT, USA) and the radioactivity incorporated into cell DNA was quantified. For basal activity, counts were normalized to 106 cells based on the WBC counts obtained as described above.

Interleukin-1β (IL-1β) in brain. Levels of IL-1β in brain tissue were characterized only on day 1. At the time of sacrifice, brains were excised, drop frozen in liquid nitrogen, and kept frozen at –80˚C. At the time of analysis, tissues were transferred to sterile disposable 1.7 ml homogenizer tubes. Protease Inhibitor Cocktail (Sigma Cat # P2714) was diluted in PBS as described by the manufacturer and 50 µl PBS/Protease Inhibitor Cocktail Stock was added for each 10 mg of wet brain mass. After homogenization, samples were centrifuged at 10,000 × g for 15 min at 4˚C, and the supernatant was aliquoted into fresh, sterile 2 ml cryovials. Thereafter, an enzyme-linked immunosorbent assay (ELISA) was used to quantify IL-1β (Quantikine M Mouse IL-1β Kit; R&D Systems, Minneapolis, MN, USA) as per the manufacturer's instructions.

Corticosterone in plasma. Circulating corticosterone levels were quantified only on day 1 in order to measure early stress response. Whole blood was centrifuged at 5,000 × g for 10 min. Plasma was aliquoted into fresh 1.7 ml microcentrifuge tubes and stored at –80˚C. At the time of analysis, samples were thawed and characterized *via* ELISA (Immunodiagnostic Systems, Ltd., Boldon, UK) as per the manufacturer's instructions.

Statistical analysis. Three-way analysis of variance (ANOVA) was used to determine main effects and interactions (Systat™ software, version 10; Systat Software, Inc., Point Richmond, CA, USA) using radiation dose, LPS challenge, and day of assessment as the independent variables (Dose, Challenge, and Day, respectively). A one-way ANOVA and post-hoc Tukey's tests were run for each time point to characterize differences between individual groups. Outliers as defined by Systat™ were generally excluded from the analysis. A *P*-value of <0.05 indicated significance. Due to the large number of data points presented, we generally did not address trends ($p<0.1$).

Results

Because the primary focus was on the effects of radiation, any effects which were due exclusively to Day or Challenge with LPS are only briefly discussed. However, their effects and interactions are indicated in the figure legends and table descriptions as necessary. In summary, the expected significant main effects and interactions for these two parameters (Day and Challenge) were present in virtually all endpoints with the greatest differences among groups generally occurring on day 1 post-challenge.

Total body and organ mass. As indicated in Table I, there were no main effects or interactions involving radiation on total body mass, nor on the mass of any of the characterized organs. However, when corrected for changes in total body mass (Figure 1), *i.e.*, relative organ mass, there was a strong trend for a Dose X Challenge interaction in the spleen ($p=0.051$) due to a radiation-induced augmentation of the LPS response.

Table I. *Total body and organ masses*.

		0 Gy		5 Gy	
	Day	PBS	LPS	PBS	LPS
Spleen (mg)†	1	84.5±7.1	83.5±4.1	77.8±4.1	94.0±4.7
	7	76.6±4.8	99.2±8.4	80.9±6.0	104.8±6.6[a,c]
	14	83.2±5.6	80.8±6.4	74.6±4.3	79.1±3.3
Liver (mg)‡	1	1576±56	1363±44[a]	563±39[b]	1384±39[a,c]
	7	1571±20	1591±42	1607±44	1643±38
	14	1623±32	1593±54	1605±64	1627±45
Lung (mg)§	1	157.6±5.0	159.5±6.0	157.8±4.0	154.4±2.8
	7	169.0±4.3	168.9±4.8	163.1±3.0	174.4±4.2
	14	164.6±4.1	166.7±2.1	164.5±4.6	169.6±3.0
Thymus (mg)*	1	67.9±8.1	51.9±2.3	64.2±4.1	54.4±3.7
	7	66.1±5.3	57.7±4.4	71.8±2.5	61.8±3.2
	14	62.5±5.0	61.1±5.5	67.3±3.9	67.8±3.6
Total body (g)#	1	26.7±0.6	24.5±0.5	26.9±0.5[b]	24.9±0.4[c]
	7	27±0.3	26.9±0.4	27.1±0.4	27.2±0.4
	14	28.3±0.5	28.4±0.6	28.1±0.5	27.6±0.5

Values represent means±SEM. N=107-110, 5-13/group. †Main effect of Challenge ($p<0.005$) & Day ($p<0.05$); Challenge X Day interaction ($p<0.05$). ‡Main effect of Challenge ($p<0.05$) & Day ($p<0.001$); Challenge X Day interaction ($p<0.005$). §Main effects of Day ($p<0.001$). *Main effects of Challenge ($p<0.005$). #Main effect of Challenge ($p<0.05$) & Day ($p<0.001$); Challenge X Day interaction ($p<0.01$). *Post hoc* Tukeys test ($p<0.05$): a: *vs*. 0 Gy PBS. b: *vs*. 5 Gy PBS. c: *vs*. 5 Gy LPS.

Erythrocytes and platelets. There were no significant main effects or interactions on most of the characterized erythrocyte and platelet parameters (Table II). The exception was a significant 3-way interaction for both MCH and MCHC ($p<0.05$). However, *post-hoc* Tukey analysis did not reveal any statistically significant differences in either the weight or concentration of hemoglobin per RBC among individual groups, suggesting that these interactions may have minimal biological significance.

Circulating leukocyte populations. There were main effects of radiation on total WBC ($p<0.05$) and lymphocyte ($p<0.005$) counts (Figure 2). In both cases, iron ion exposure generally increased counts above those of their non-irradiated counterparts, particularly at the later time points. Although the pattern of changes was similar, there were no significant radiation effects or interactions on monocyte/macrophage counts. In contrast, there was a Dose X Challenge interaction on granulocyte counts ($p<0.01$) due to radiation-induced increase in the LPS response. There were no significant main effects or interactions on the proportion of any major leukocyte population in the blood (Figure 2).

Splenic leukocyte populations. There were no significant radiation effects on any of the major splenic leukocyte counts (Table III). A main effect of radiation, however, was noted

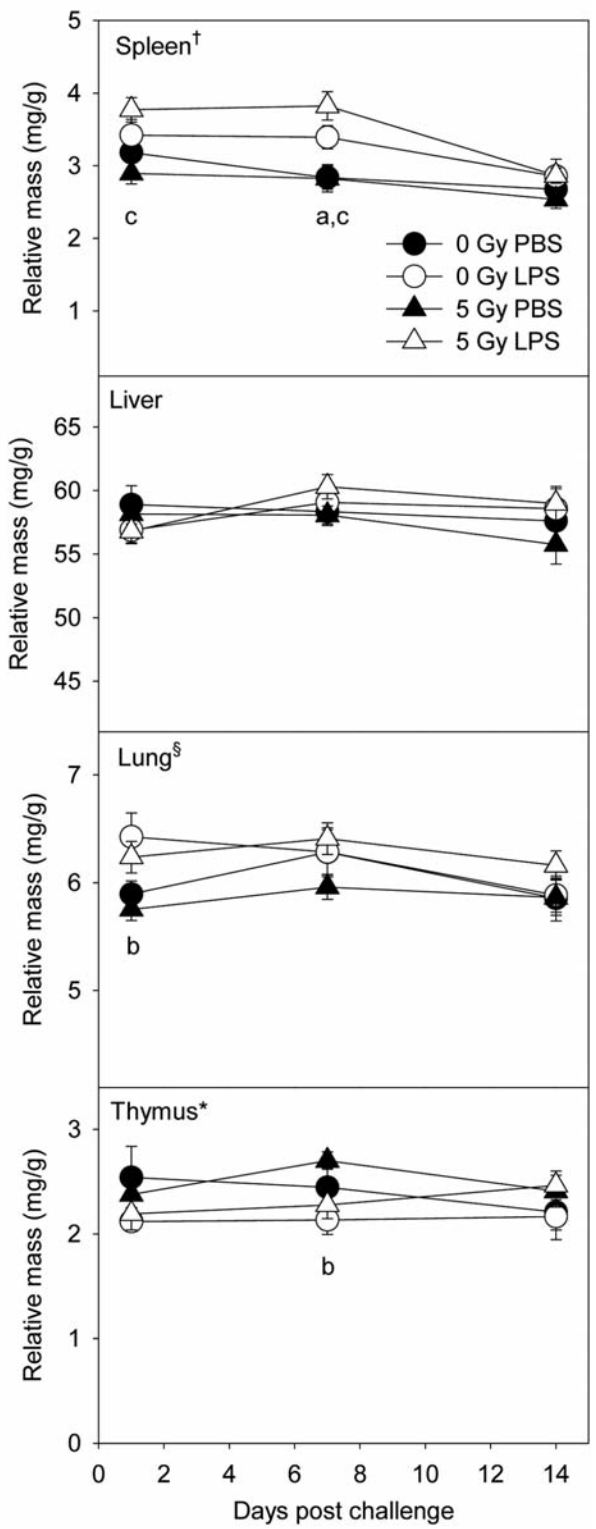

Figure 1. *Relative organ mass. Values represent means±SEM. N=104-109, 5-13/group. †Main effects of Challenge & Day (p<0.001); Dose X Challenge interaction (p=0.051). §Main effects of Challenge (p<0.01). *Main effects of Challenge (p<0.05). Post-hoc Tukeys test (p<0.05): a: 0 Gy PBS vs. 5 Gy LPS. b: 0 Gy LPS vs. 5 Gy PBS. c: 5 Gy PBS vs. 5 Gy LPS.*

Table II. *Erythrocyte and platelet parameters.*

		0 Gy		5 Gy	
	Day	PBS	LPS	PBS	LPS
RBC	1	9.5±0.3	9.5±0.3	9.6±0.1	9.4±0.3
($\times10^9$	7	9.7±0.2	9.3±0.2	9.9±0.1	9.1±0.2c
cells/ml)†	14	9.8±0.2	9.5±0.2	9.9±0.1	9.7±0.2
HGB	1	13.7±0.4	13.8±0.4	14.3±0.2	13.4±0.3
(g/dl)‡	7	13.9±0.2	12.9±0.3	13.3±0.1	12.9±0.3
	14	14.3±0.1	13.9±0.1	14.1±0.1	13.9±0.1
HCT (%)§	1	43.4±1.8	42.5±1.3	43.7±0.7	42.5±1.3
	7	44.0±0.7	42.5±1.0	44.2±0.9	41.1±0.9
	14	44.8±0.8	43.3±0.9	45.0±0.5	44.1±0.6
MCV	1	45.8±0.4	44.9±0.3	45.3±0.2	45.2±0.3
(μm^3)††	7	45.3±0.3	45.4±0.2	45.3±0.2	45.5±0.2
	14	45.7±0.1	45.4±0.2	45.6±0.1	45.1±0.2
MCH (pg)‡‡	1	14.4±0.2	14.6±0.3	14.8±0.2	14.2±0.2
	7	14.3±0.3	13.8±0.3	13.7±0.3	14.2±0.2
	14	14.2±0.2	14.7±0.3	14.1±0.2	14.3±0.3
MCHC	1	31.6±0.8	32.5±0.7	32.6±0.5	31.5±0.6
(g/dl)§§	7	31.5±0.6	30.5±0.6	30.1±0.5	31.3±0.4
	14	31.1±0.4	32.3±0.6	31.0±0.4	31.5±0.5
RDW (%)	1	15.7±0.2	15.2±0.3	15.2±0.2	15.2±0.2
	7	15.3±0.2	15.8±0.3	15.5±0.2	15.5±0.1
	14	15.2±0.2	15.6±0.2	15.2±0.2	15.4±0.2
PLT ($\times10^6$	1	1316±66	853±31[a]	1302±52b	753±26[a,c]
cells/ml)†††	7	1287±60	1503±68	1297±49	1472±66
	14	1168±42	1387±34a	1236±44	1264±46
MPV	1	9.5±0.5	11.7±0.3[a]	9.4±0.2b	11.9±0.4[a,c]
(μm^3)‡‡	7	9.2±0.3	8.8±0.2	9.2±0.2	8.7±0.2
	14	9.1±0.2	8.9±0.2	9.2±0.2	9.4±0.2

Values represent means±SEM. N=105-110, 5-13/group. †Main effects of Challenge ($p<0.005$). ‡Main effect of Challenge ($p<0.005$) and Day ($p<0.001$). §Main effect of Challenge ($p<0.01$). ††Main effect of Challenge ($p<0.05$). ‡‡Main effect of Day ($p<0.05$); 3-way interaction ($p<0.05$). §§Main effect of Day ($p<0.01$); 3-way interaction ($p<0.05$). †††Main effects of Challenge ($p<0.05$) & Day ($p<0.001$); Challenge X Day interaction ($p<0.001$). ‡‡‡Main effects of Challenge & Day ($p<0.001$); Challenge X Day interaction ($p<0.001$). *Post hoc* Tukeys test ($p<0.05$): a: *vs.* 0 Gy PBS. b: *vs.* 5 Gy PBS. c: *vs.* 5 Gy LPS.

on splenic monocyte/macrophage proportions ($p<0.05$) due to a very slight overall proportional decrease (Figure 3). However, there were no significant differences between individual groups in this parameter at any time point. Significant 3-way interactions occurred affecting lymphocyte and granulocyte proportions ($p<0.05$). This appears to be due to early radiation-induced increases in granulocyte proportions in the LPS-treated mice, with corresponding decreases in lymphocytes. This trend had reversed by day 14.

Circulating lymphocyte populations. There were significant main effects of radiation on B-cell counts ($p<0.05$), primarily due to increases noted in the irradiated groups on days 7 and 14 (Figure 4). Although radiation had no effect

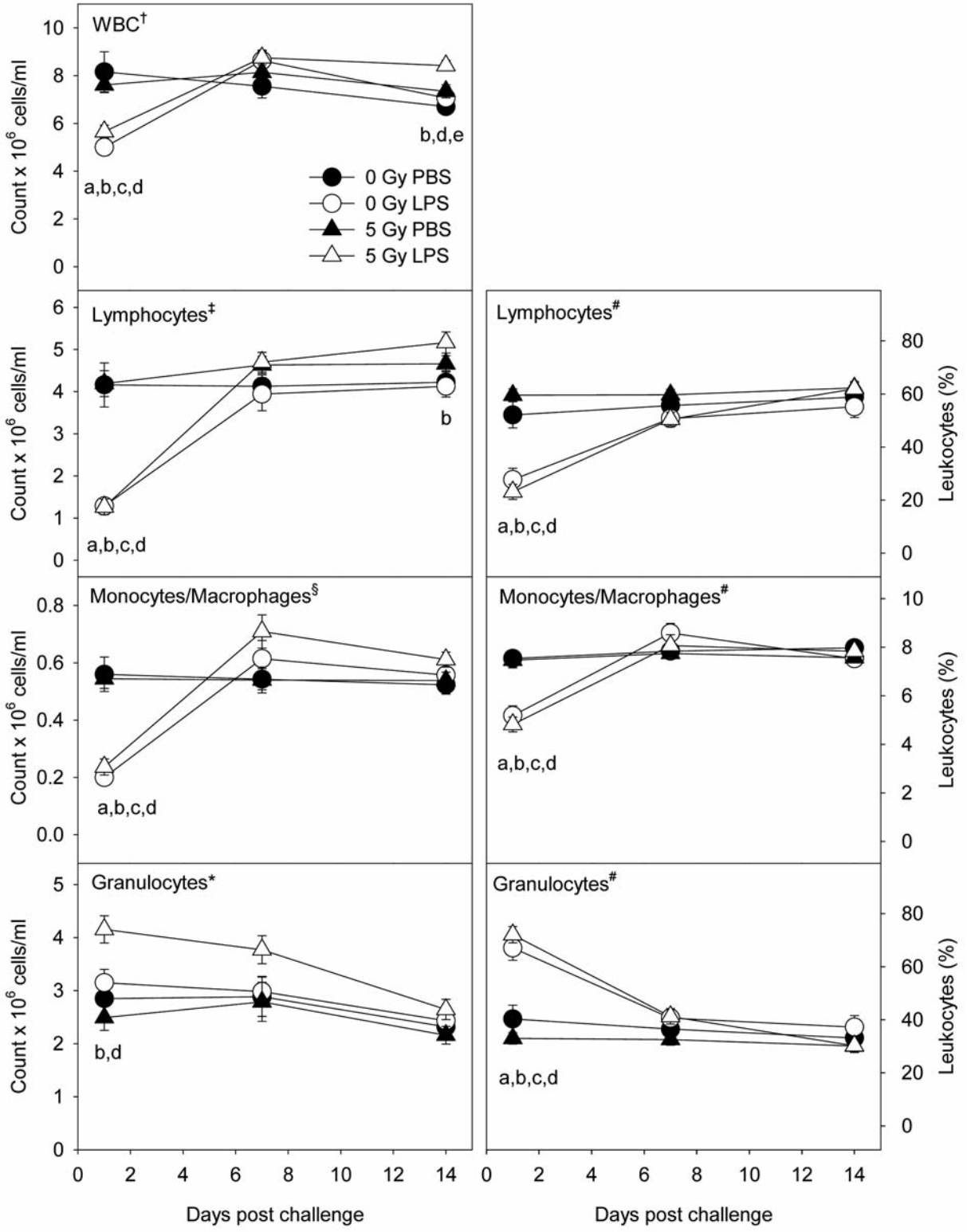

Figure 2. *Circulating leukocytes. Values represent means±SEM. N=102-109, 5-13/group. †Main effects of Dose (p<0.05) & Day (p<0.001); Challenge X Day interaction (p<0.001). ‡Main effects of Dose (p<0.005), Challenge & Day (p<0.001); Challenge X Day interaction (p<0.001). §Main effects of Challenge (p<0.05) and Day (p<0.001); Challenge X Day interaction (p<0.001). *Main effect of Challenge and Day (p<0.001); Challenge X Dose interaction(p<0.01). #Main effects of Challenge & Day (p<0.001); Challenge X Day interaction (p<0.001). Post-hoc Tukeys test (p<0.05): a: 0 Gy PBS vs. 0 Gy LPS, b: 0 Gy PBS vs. 5 Gy LPS, c: 0 Gy LPS vs. 5 Gy PBS, d: 5 Gy PBS vs. 5 Gy LPS, e: 0 Gy LPS vs. 5 Gy LPS.*

Table III. *Splenic leukocyte counts*.

		0 Gy		5 Gy	
	Day	PBS	LPS	PBS	LPS
WBC	1	13.8±1.1	14.0±0.8	13.8±1.0	15.7±0.8
($\times10^6$	7	15.2±1.1	16.4±1.1	15.4±0.9	18.8±1.3
cells/ml)†	14	14.0±0.8	14.8±0.9	12.4±0.7	13.5±0.7
Lymph	1	9.1±0.6	9.2±0.5	9.6±0.6	10.0±0.4
($\times10^6$	7	10.4±0.6	10.2±0.5	10.5±0.4	11.5±0.5
cells/ml)‡	14	9.8±0.5	9.9±0.4	8.9±0.4	9.6±0.4
Mono	1	1.0±0.1	1.0±0.1	1.0±0.1	1.1±0.1
($\times10^6$	7	1.1±0.1	1.3±0.1	1.1±0.1	1.4±0.1
cells/ml)§	14	1.0±0.1	1.1±0.1	0.8±0.1	0.9±0.1
Gran	1	3.7±0.5	3.8±0.5	3.2±0.3	4.6±0.4
($\times10^6$	7	3.7±0.5	4.1±0.3	3.8±0.5	5.3±0.6
cells/ml)*	14	3.2±0.3	3.7±0.5	2.7±0.2	2.9±0.3

Values represent means±SEM. N=107-109, 5-13/group. †Main effect of Challenge ($p<0.05$) & Day ($p<0.001$). ‡Main effects of Day ($p<0.005$). §Main effect of Challenge ($p<0.05$) & Day ($p<0.001$). *Main effects of Challenge ($p<0.01$) & Day ($p<0.01$). *Post hoc* Tukeys test ($p<0.05$): No significant differences between individual groups.

Table IV. *Major circulating lymphocyte proportions*.

		0 Gy		5 Gy	
	Day	PBS	LPS	PBS	LPS
T cell	1	32.0±1.1	38.9±1.7[a]	29.4±0.9[b]	38.1±1.5[a,c]
(%MNC)†	7	28.9±0.9	33.7±1.2[a]	29.7±0.7[b]	32.4±0.7a
	14	31.1±0.9	30.0±0.7	30.1±1.0	28.4±0.7
B cell	1	63.6±1.0	52.3±1.8[a]	67.1±1.1[b]	46.8±1.8[a,c]
(%MNC)‡	7	66.8±0.8	57.9±1.5[a]	66.3±0.7[b]	60.7±0.7[a,c]
	14	64.4±0.9	65.0±0.7	66.0±1.1	67.4±0.8
NK cell	1	4.4±0.7	12.1±0.5	3.5±0.6	20.5±1.3
(%MNC)§	7	4.4±0.3	8.4±0.7[a]	4.0±0.2[b]	7.3±0.7[a,c]
	14	4.1±0.3	5.0±0.3	3.9±0.2	4.2±0.2
Th cell	1	18.4±0.8	22.4±1.0	17.9±0.6[b]	23.3±1.1[a,c]
(%MNC)††	7	16.5±0.7	19.0±0.7[a]	18.1±0.6	18.4±0.4
	14	18.6±0.7	17.1±0.4	17.5±0.8	16.6±0.6
Tc cell	1	11.8±0.2	11.5±0.5	10.1±0.4	10.7±0.3
(%MNC)‡‡	7	11.1±0.3	13.3±0.5[a]	10.9±0.2[b]	12.6±0.4[a,c]
	14	11.4±0.3	11.6±0.4	11.3±0.3	10.8±0.2

Values represent means±SEM. N=98-108, 4-13/group. MNC: Mononuclear cells. †Main effects of Challenge & Day ($p<0.001$); Challenge X Day interaction ($p<0.001$). ‡Main effects of Challenge & Day ($p<0.001$); Challenge X Day ($p<0.001$) & 3-way interactions ($p<0.005$). §Main effect of Dose ($p<0.01$), Challenge ($p<0.001$) & Day ($p<0.001$); Dose X Challenge, Dose X Day, Challenge X Day, & 3-way interactions ($p<0.001$). ††Main effects of Challenge ($p<0.001$) & Day ($p<0.001$); Challenge X Day interaction ($p<0.001$). ‡‡Main effects of Dose ($p<0.001$), Challenge ($p<0.005$), Day ($p<0.005$); Challenge X Day interaction ($p<0.001$). *Post hoc* Tukeys test ($p<0.05$): a: *vs*. 0 Gy PBS. b: *vs*. 5 Gy PBS. c: *vs*. 5 Gy LPS.

on T-cell counts, there was a significant main effect of radiation on CD4:CD8 ratios ($p<0.01$). This was due to the high ratio seen in the irradiated mice.

Radiation had a main effect on Tc-($p<0.001$) and NK ($p<0.01$) proportions, but not on Th or B-cells (Table IV). For the Tc-cells, this was due to slight overall decreases in proportions. However, for the NK cells, this effect was due to large, early proportional increases in the irradiated mice that were particularly pronounced in the LPS-treated animals, resulting in very reliable Dose X Challenge, Dose X Day, and 3-way interactions ($p<0.001$). Although there were no main effects of radiation on B-cell proportions, the changes in this population corresponded inversely with those of NK cells, resulting in a significant 3-way interaction ($p<0.005$).

Splenic lymphocyte populations. There were no significant main effects of radiation on any of the characterized lymphocyte counts in the spleen (Figure 5). However, Dose X Day interactions on T-($p<0.05$), Th-($p<0.05$), Tc-($p<0.05$), and B-($p<0.01$) cell counts did occur. For T-cells, this was due to slight numerical increases in irradiated groups on day 7. However, there were no significant differences among groups in post-hoc Tukey analysis at each time point. A similar pattern of changes was noted in both of the T-cell subsets. For B-cells, this was likely due to early increases in the 5 Gy + LPS groups noted on day 1 followed by a decrease in the 0 Gy + LPS group on day 7. However, as with the T-cells, there were no differences in any group compared to unirradiated, unchallenged controls.

Despite the lack of a significant impact of radiation on either of the T-cell subsets, there was a main effect on the CD4:CD8 ratio ($p<0.05$) due to a slight overall radiation-dependent increase.

Radiation had no significant impact on T-, Th-, or B-cell proportions (Table V). However, there was a main effect of radiation on the percentage of NK cells ($p<0.05$) due to a slight radiation-induced decrease. There was also a strong trend for a 3-way interaction in Tc cell proportions ($p=0.052$) due to a slight, early enhancement in the LPS-challenged irradiated mice.

Splenic activated T-cells. There were no significant effects or interactions involving radiation on any of the activated ($CD25^+$, $CD71^+$) T-cell proportions (Table VI).

Spontaneous blastogenesis. The mean cpm values for both blood and splenic cells are shown in Table VII. There were no significant effects or interactions involving radiation on spontaneous blastogenesis in circulating leukocytes. In contrast, there was a strong trend for a radiation effect in the spleen ($p=0.053$) due to overall increases in the irradiated groups.

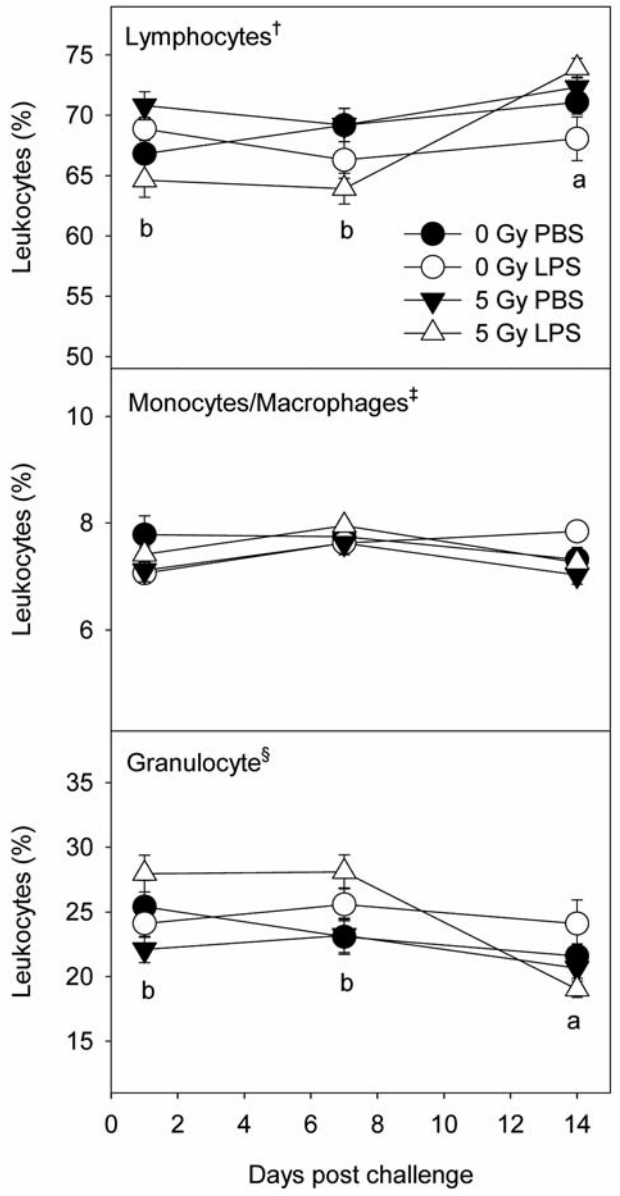

Figure 3. *Splenic leukocyte percentages. Values represent means±SEM. N=105-106, 4-13/group. †Main effects of Challenge (p<0.005) & Day (p<0.001); Dose X Day (p<0.05) & 3-way interactions (p<0.005). ‡Main effects of Day (p<0.05). §Main effects of Challenge (p<0.005) & Day (p<0.001); Dose X Day (p<0.05) & 3-way interaction (p<0.01) s. Post-hoc Tukeys test (p<0.05): a: 0 Gy LPS vs. 5 Gy LPS, b: 5 Gy PBS vs. 5 Gy LPS.*

Mitogen-induced blastogenesis. ConA- and PHA-induced DNA synthesis was determined only for PBS-treated mice, with and without radiation exposure. Radiation dose had no impact on response to either of these mitogens (data not shown). With the two serotypes of LPS, we tested induced blastogenesis in both PBS- and LPS-treated mice. However, there were no significant Dose effects or interactions in either test condition (data not shown).

Brain IL-1β. Whole-brain IL-1β levels were quantified only on day 1 (Figure 6). This was because the maximum change in this cytokine is likely to occur relatively early after LPS injection. Furthermore, given that the irradiation occurred over a month prior to inoculation, any radiation-induced changes in this parameter would likely occur during the peak of the IL-1β response. However, there were no significant effects or interactions involving radiation at this time point.

Circulating corticosterone. Corticosterone levels in plasma were assessed only on day 1 (Figure 7), and there were no significant effects or interactions involving radiation. The rationale for assessing this parameter on day 1 is similar to that for IL-1β.

Discussion

Overall radiation effect. There were no significant main effects of brain-localized irradiation on total body mass or on the masses of any of the excised organs. Similarly, there were generally no significant radiation effects on any of the characterized erythrocyte or platelet parameters. This suggests that the progenitor cells in the bone marrow for these populations, as well as the overall health of the mice at the time points of assessment, remained relatively intact.

Interestingly, 10 days after a head-only exposure to 3-4 Gy protons, we found significant decreases in platelet counts in a rat model (29). This implies that direct exposure of large volumes of the bone marrow is not necessary for changes in this parameter. However, given the life-span of mature platelets (~5-9 days), their adhesion to activated endothelial cells of blood vessel walls, and the temporal nature of reconstitution post-irradiation (30), these early decreases may be partly related to blood flow through the head during irradiation. Furthermore, the difference in platelet counts between our two studies (lack of effect in current study and low counts in the previous study) is likely due to multiple factors, including differences in time of assessment post-irradiation (10 days post-irradiation in the previous study *vs*. 37 days here), animal species, dose delivered, and radiation quality.

The results for circulating leukocyte populations were somewhat surprising; lymphocyte counts were generally higher in mice exposed to brain-localized irradiation. These results also contrast with those of our previous study, where we found early decreases in lymphocyte counts 10 days after head-only exposures (29). These differences in responses across time likely reflect, at least in part, recovery from the initial insult, as well as the other factors mentioned above.

Based on flow cytometric analysis, the slight overall radiation-induced increase in circulating lymphocytes

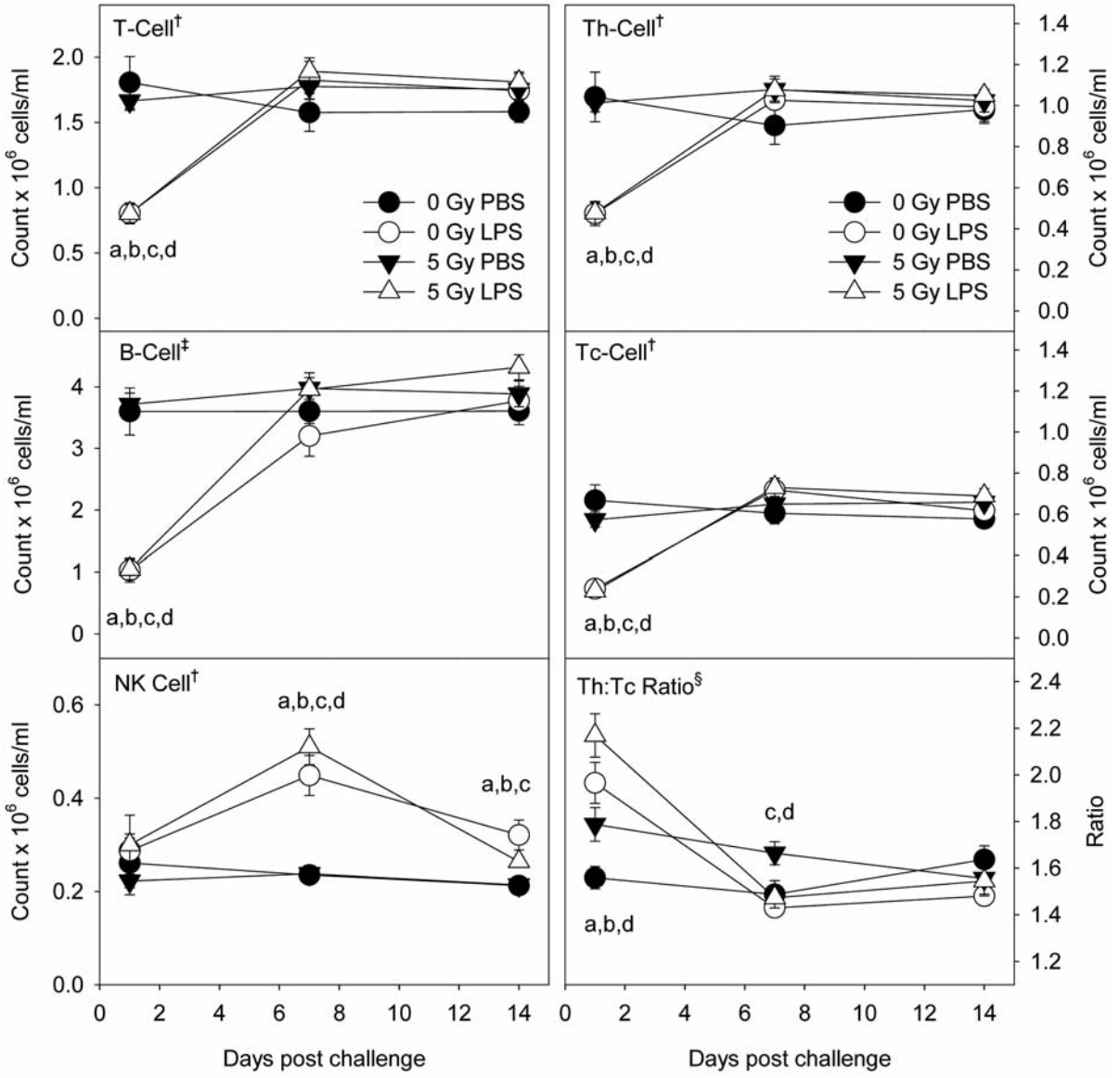

Figure 4. *Major circulating lymphocyte subset counts and Th:Tc ratio. Values represent means±SEM. N=101-107, 5-13/group. †Main effects of Challenge & Day (p<0.001); Challenge X Day interaction (p<0.001). ‡Main effects of Dose (p<0.05), Challenge & Day (p<0.001); Challenge X Day interaction (p<0.001). §Main effects Dose (p<0.01) & Day (p<0.001); Challenge X Day interaction (p<0.001). Post-hoc Tukeys test (p<0.05): a: 0 Gy PBS vs. 0 Gy LPS, b: 0 Gy PBS vs. 5 Gy LPS, c: 0 Gy LPS vs. 5 Gy PBS, d: 5 Gy PBS vs. 5 Gy LPS.*

appears to be primarily due to changes in B-cells. While we have previously reported long-term increases in circulating B-cell counts after whole-body iron ion irradiation (31), this has not been consistent (32). We proposed that this rebound effect could be due to excessively enhanced recovery in B-cell populations, increasing over control levels. However, because in the present study there were no significant radiation-specific differences in the counts of any of the other major leukocyte populations, lymphocyte subsets, erythrocytes, or platelets in the blood, a generalized increase in bone marrow hematopoiesis seems unlikely.

Although the ^{56}Fe beam was collimated, limiting exposures to the brain, tissues outside of the target field still received roughly ~8% of the total dose due to scatter through the collimator (based on dosimetry and modeling performed by Steve Rightnar and Eric Benton, personal communication). Certainly, it cannot be ruled out that this low exposure may have resulted in some degree of hormesis. This concept remains controversial and publications

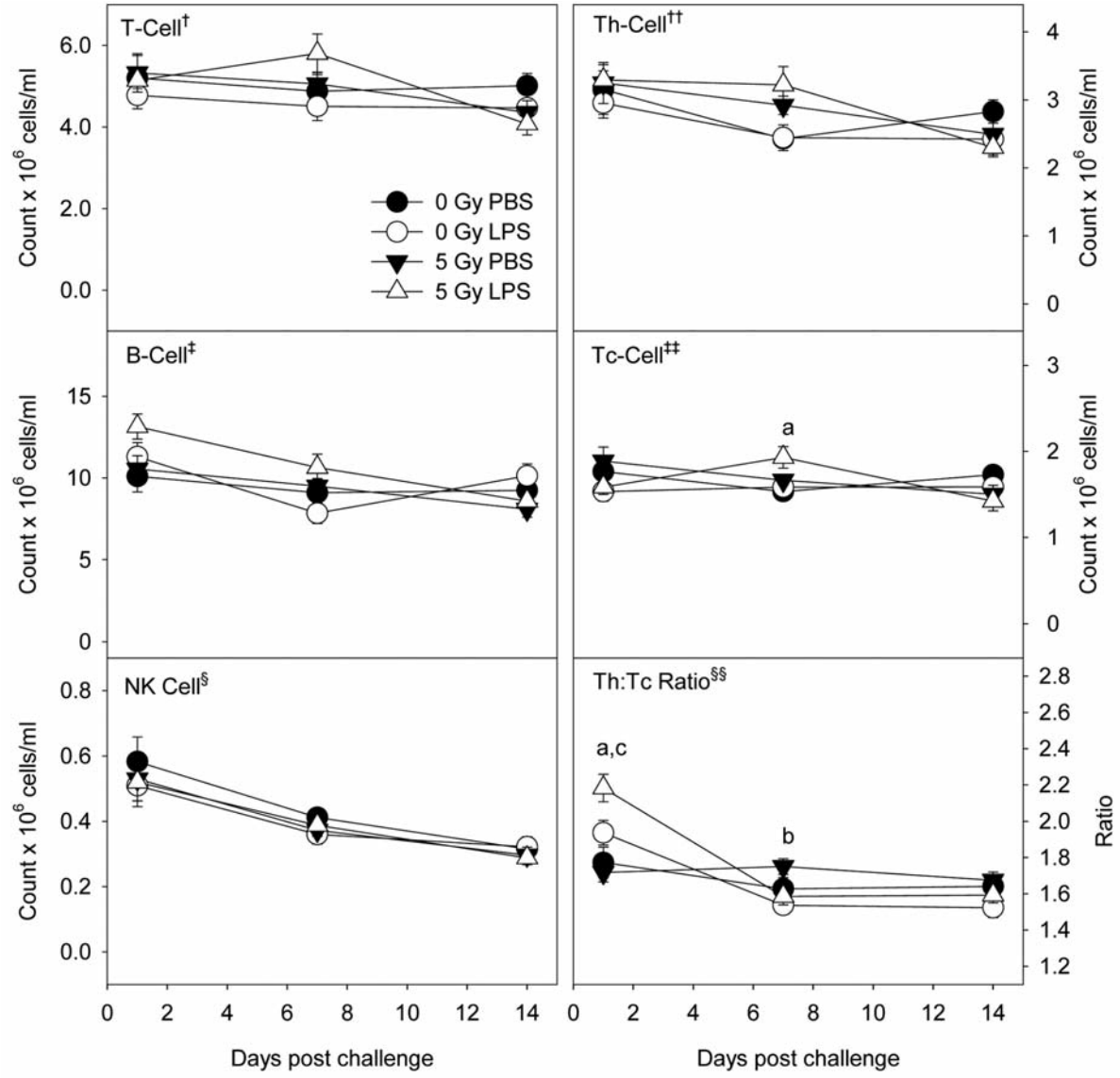

Figure 5. *Major splenic lymphocyte subset counts and Th:Tc (CD4:CD8) ratio. Values represent means±SEM. N=108-109, 5-13/group. †Main effects of Day (p<0.05); Dose X Day (p<0.05). ‡Main effects of Challenge (p<0.05) & Day (p<0.001); Dose X Day interaction (p<0.01). §Main effect of Day (p<0.001). ††Main effect of Day (p<0.001); Dose X Day interaction (p<0.05). ‡‡No main effects; Dose X Day interaction (p<0.05). §§Main effect of Dose (p<0.05) & Day (p<0.001); Challenge X Day interaction (p<0.001). Post-hoc Tukeys test (p<0.05): a: 0 Gy PBS vs 5 Gy LPS, b: 0 Gy LPS vs. 5 Gy PBS, c: 5 Gy PBS vs. 5 Gy LPS.*

specifically addressing ^{56}Fe-induced radioadaptation are, to our knowledge, nonexistent. Furthermore, the reason(s) why a radioprotective or regenerative response should occur in B-cell progenitors and not other lymphocyte populations is unclear. In addition, we believe that any radiation response in the bone marrow or spleen would have been dominated by the response of tissues in the brain, which received an order of magnitude more dose compared to nearby tissues. Similarly, it cannot be ruled out that changes in the periphery are due to direct exposure to the blood cells circulating through the brain during irradiation. However, since leukocyte numbers and function in these mice was assessed 37-38 days after irradiation, directly exposed immunocytes should have already died. Therefore, there must be some other mechanism to explain these long-term changes in immune parameters.

As the exposures in this study were limited primarily to the brain, avoiding major bone marrow reservoirs, some other mechanism beyond bone marrow hematopoiesis is likely in play. One possibility is that the increase in B-cells

Table V. *Major splenic lymphocyte proportions*.

		0 Gy		5 Gy	
	Day	PBS	LPS	PBS	LPS
T cell	1	32.7±0.9	28.9±1.1[a]	32.2±0.7[b]	26.9±0.6[a,c]
(%MNC)[†]	7	33.9±0.7	35.5±0.6	33.9±0.5	34.6±0.4
	14	33.8±0.6	29.8±1.0a	34.2±0.7[b]	31.3±0.8[c]
B cell	1	63.7±1.3	67.9±1.5	64.4±0.8	69.4±0.9[a,c]
(%MNC)[‡]	7	63.2±0.7	61.7±0.5	63.6±0.5	63.1±0.4
	14	63.9±0.6	68.1±1.0[a]	63.5±0.7[b]	66.5±0.7[c]
NK cell	1	3.7±0.4	3.1±0.4	3.4±0.2	3.0±0.3
(%MNC)[§]	7	2.9±0.1	2.8±0.1	2.5±0.1[a,b]	2.4±0.1[a,b]
	14	2.4±0.1	2.2±0.1	2.3±0.1	2.1±0.1[a]
Th cell	1	19.7±0.9	17.8±0.7	19.6±0.5	17.6±0.5[c]
(%MNC)[††]	7	18.7±0.6	19.2±0.5	19.6±0.3	19.1±0.3
	14	19.0±0.5	16.8±0.3[a]	19.9±0.3[b]	17.7±0.4[c]
Tc cell	1	11.2±0.4	9.2±0.2[a]	11.4±0.3[b]	7.9±0.3[a,b,c]
(%MNC)[‡‡]	7	11.5±0.2	12.6±0.4	11.2±0.3[b]	12.2±0.3
	14	11.9±0.2	10.6±0.5	11.8±0.4	11.3±0.3

Values represent means±SEM. N=106-109, 5-13/group. MNC: Mononuclear cells. [†]Main effects of Challenge & Day ($p<0.001$); Challenge X Day interaction ($p<0.001$). [‡]Main effects of Challenge & Day ($p<0.001$); Challenge X Day interaction ($p<0.001$). [§]Main effects of Dose ($p<0.05$), Challenge ($p<0.05$), & Day ($p<0.001$). [††]Main effect of Challenge ($p<0.001$); Challenge X Day interaction ($p<0.005$). [‡‡]Main effects of Challenge & Day ($p<0.001$); Challenge X Day interactions ($p<0.001$). *Post hoc* Tukeys test ($p<0.05$): a: *vs*. 0 Gy PBS. b: *vs*. 5 Gy PBS. c: *vs*. 5 Gy LPS.

Table VI. *Activated T cell proportions*.

		0 Gy		5 Gy	
	Day	PBS	LPS	PBS	LPS
$CD25^+$	1	2.79±0.23	2.74±0.18	2.60±0.13	3.45±0.21[b]
Cells[†]	7	2.93±0.20	2.46±0.16	2.86±0.14	2.34±0.07[a,b]
	14	2.77±0.14	2.32±0.11	2.83±0.09	2.40±0.12
$CD25^+$	1	1.46±0.11	1.69±0.16	1.5±0.08	1.71±0.10
T cell[‡]	7	1.50±0.07	1.58±0.06	1.51±0.05	1.49±0.05
	14	1.52±0.08	1.32±0.07	1.52±0.05	1.49±0.08
$CD71^+$	1	2.87±0.33	2.69±0.33	2.47±0.26	2.56±0.21
Cells[§]	7	1.89±0.49	2.96±0.38	1.58±0.29	2.94±0.28[b]
	14	1.56±0.18	2.32±0.41	1.61±0.15	1.83±0.20
$CD71^+$	1	0.43±0.09	0.24±0.04	0.38±0.05	0.33±0.04
T cells[‡‡]	7	0.30±0.07	0.54±0.08	0.25±0.04[a]	0.56±0.08[a,b]
	14	0.28±0.05	0.39±0.06	0.28±0.02	0.27±0.04

Values represent means±SEM. N=106-109, 5-13/group. [†]Main effects of Day ($p<0.05$); Challenge X Day interaction ($p<0.001$). [‡]No main effects; Challenge X Day interaction ($p<0.05$). [§]Main effects of Challenge & Day ($p<0.001$); Challenge X Day interaction ($p<0.05$). [‡‡]Main effects of Challenge & Day ($p<0.05$); Challenge X Day interactions ($p<0.001$). *Post hoc* Tukeys ($p<0.05$): a: *vs*. 5 Gy PBS. b: *vs*. 5 Gy LPS.

is due to increased proliferation in response to brain-specific antigens released into circulation after exposure. This possibility is consistent with the lack of any radiation impact on *ex vivo* LPS-induced blastogenic response (*i.e.*, there was no significant increase in generalized radiation-dependent DNA synthesis with either LPS serotype). Assessments of antibody production and levels of growth factors that specifically promote B-cell activation, proliferation and/or maturation could help clarify the underlying mechanisms responsible for this phenomenon.

Interestingly, radiation-induced proportional increases in circulating NK cells, with corresponding decreases in the spleen, were also found. As NK cells are known to influence B-cell homeostasis, one possible explanation for the coincident increases in blood B and NK cell populations is the activation of a feedback loop involving both interferon-γ and IL-15 (33). In other words, chronic (albeit slight) elevations in B-cells generating antibodies against brain antigens may have triggered the exodus of NK cells out of the spleen and into the blood in an attempt to drive B-cell counts back to a normal level.

Another piece of evidence pointing in this direction involves subtle changes in T-lymphocyte ratios. Although there were no discernable radiation-dependent changes in $CD4^+$ Th and $CD8^+$ Tc-cell counts in either body compartment, and there was only a slight decrease in blood Tc-cell proportions, we found significant increases in both the blood and spleen in CD4:CD8 ratios. As this elevation in the ratio occurred in both compartments, it is unlikely that the result is a purely statistical anomaly. Rather, it is probable that there were small, diametrically opposed changes in both T-cell populations that only became apparent when characterizing the ratio. Notably, we have consistently found chronic increases in this ratio after whole-body iron-irradiation (31, 32).

As tissues in the mouth and jaw are well known to be exquisitely radiosensitive (34), radiation-induced inflammation in these tissues may explain the change in ratio. However, while there have been reports of similar changes in CD4:CD8 ratios in the blood of patients with chronic periodontitis, this result has been inconsistent and controversial (35-37). Furthermore, the beam was collimated such that only the brain received the full dose of radiation. While it is possible that the jaw received a slightly higher dose due to localized scatter from mineralized tissues such as bone, it is unlikely that this dose was substantial enough to overshadow the effects due to direct exposure to the brain.

Production of glucocorticoids by the adrenal cortex is a normal feedback mechanism by which immune responses, including those of T-cells, are down-regulated. Although we did not find any changes in circulating corticosterone levels, it cannot be ruled out that the aberrant pattern in T-cell subsets noted here was not due to radiation-induced changes in hormone homeostasis. CD4:CD8 ratios have been shown

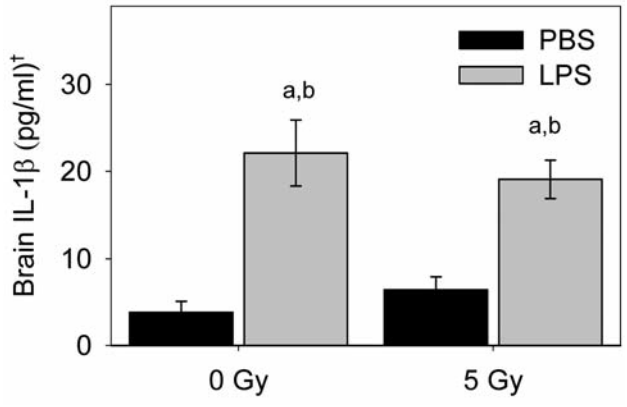

Figure 6. *Brain IL-1β. The level of this cytokine was quantified only on day 1. Values represent means±SEM. N=26, 4-10/group. †Main effect of Challenge (p<0.001). Post-hoc Tukeys test (p<0.05): a: vs. 0 Gy PBS, b: vs. 5 Gy PBS.*

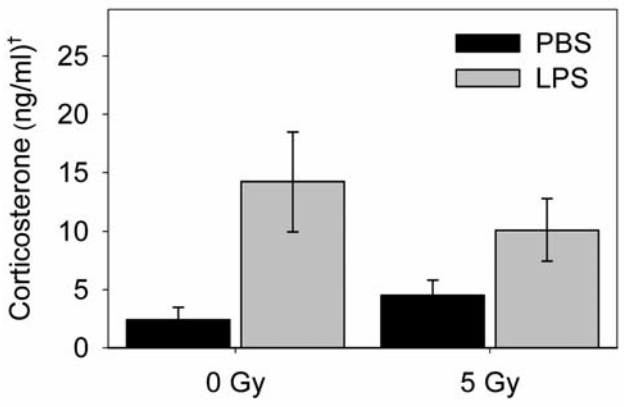

Figure 7. *Circulating corticosterone. Corticosterone level in plasma was quantified only on day 1. Values represent means±SEM. N=26, 4-8/group. †Main effect of Challenge (p<0.005). Post-hoc Tukeys test (p<0.05): Although there were trends for differences between LPS and non-LPS treated groups, none of these reached statistical significance.*

Table VII. *Spontaneous blastogenesis.*

		0 Gy		5 Gy	
	Day	PBS	LPS	PBS	LPS
Blood†	1	982±129	2,248±296[a]	917±105[b]	1,965±73[a,c]
	7	1,294±179	1,026±96	1,043±66	1,062±77
	14	1,304±130	1,342±118	1,618±131	1,239±104
Spleen‡	1	23,324±1,800	28,736±4,524	19,472±2,279	24,148±2,265
	7	12,671±1,319	16,905±841	9,963±1,023[b]	15,222±1,453[c]
	14	33,980±2,731	40,418±1,170	35,113±2439	33,903±2,926

Values represent means±SEM in counts per minute (cpm). N=86-92, 3-12/group. †Main effects of Challenge & Day ($p<0.001$); Challenge X Day interaction ($p<0.001$). ‡Main effects of Challenge ($p<0.01$) & Day ($p<0.001$). *Post hoc* Tukeys test ($p<0.05$): a: *vs.* 0 Gy PBS. b: *vs.* 0 Gy LPS. c: *vs.* 5 Gy PBS.

to increase in patients with growth hormone deficiencies (38) and radiation has long been known to impact growth and endocrine function (39-43).

Given the dose used in this study, another likely rationale involves inflammation in the brain and a compromised blood-brain barrier. Certainly, this has been shown to be the case in other, non-radiation models. For example, nearly identical findings for T-cell subset ratios were reported in rats using a cerebral artery occlusion ischemia model (44). Furthermore, radiation causes both inflammation (45-48) and blood-brain barrier breakdown (49). Perhaps not coincidentally, a leaky blood-brain barrier would be consistent with a radiation-induced increase in antibody production against brain-specific antigens.

Impact of radiation on LPS response. When normalized to total body mass (to minimize the impact of differential growth and sickness/LPS-induced changes in eating behavior), there was a strong trend for a radiation-induced enhancement of the LPS response in the spleen on days 1 and 7. There was a similar pattern of changes in granulocyte counts in both the blood and spleen (though the main effects reached significance only in the blood). Finally, there were clear (and significant) Dose X Challenge interactions in splenic granulocyte and lymphocyte percentages where radiation appears to have accelerated and enhanced the LPS response, resulting in a shift away from lymphocytes toward granulocytes at the early time points. These phenomena are likely related.

LPS is known to trigger an increase in granulocyte production in the bone marrow, thus resulting in an efflux of these cells into the circulation and, ultimately, into lymphoid tissues such as the spleen (50). This migration of granulocytes into the spleen, combined with LPS-induced sickness behavior (and the corresponding decrease in total body mass), resulted in an increase in spleen mass relative to body mass.

While relatively radioresistant in terms of survival, granulocyte and macrophage functions are enhanced following irradiation, *i.e.*, increased oxidative burst capacity and inflammatory cell infiltration to sites of injury occur (51). It is possible that radiation 'primed' granulocytes circulating through the brain or its near vicinity during exposure, resulting in an augmented response to a subsequent challenge with LPS. However, as these mice were challenged with the mitogen 37-38 days post-irradiation and granulocytes are generally short-lived (less than a week) (52), this scenario seems unlikely.

More likely, radiation-induced inflammation in the brain led to the activation of brain-immune communication pathways, *i.e.*, the hypothalamus-pituitary-adrenal (HPA) axis and/or the sympathetic nervous system (SNS). Activation of these pathways have long been known to influence peripheral immune populations (53). Another possibility is that immunosuppressive activity of T regulatory cells was compromised even though this T-cell subset is relatively radioresistant in terms of survival (54).

Although we did not find any radiation-induced changes in whole-brain IL-1β levels, there may have been localized changes in this inflammatory cytokine that were masked when quantifying concentration in the entire brain. Increased levels of IL-1β and other pro-inflammatory cytokines have been reported in rat hypothalamus, thalamus, and hippocampus within hours after irradiation (55). In contrast to the present study, however, the animals were partial-body irradiated, with the head being shielded. Furthermore, previous research has shown that expression of inflammatory cytokines in the brain after irradiation tend to be cyclically enhanced (48).

Similarly, there were no changes in circulating corticosterone levels one day after LPS inoculation in the irradiated mice, suggesting that the brain-localized exposure did not impact overall HPA activity, *i.e.*, there was no shift in homeostasis. However, we cannot rule out any effect due strictly to SNS activity. Additional investigation is required to verify this mechanism (*e.g.*, histological analysis of cytokine and *cfos* expression in the brain and circulating catecholamine levels). Furthermore, since induction of stress hormone production during the acute-phase response to LPS is very rapid and some hormones reach peak levels within one to a few hours (56), the time of assessment is likely to be an important factor.

In conclusion, brain-localized iron ion irradiation alone had its greatest impact on B-cells. Indirect evidence suggests that this may be due to inflammation in the brain, breakdown of the blood-brain barrier, and increased proliferation of activated B-cells during antibody production against brain specific self-antigens. Additionally, the irradiation also impacted the response to LPS, particularly with respect to granulocytes. This, we speculate, may be due to inflammation in the brain and activation of SNS pathways. Interestingly, activation of the HPA/SNS pathways has recently been associated with changes in acoustic startle behavior (57). As the mice in the present study also demonstrated a reduction in startle behavior after irradiation (12), this represents another, albeit circumstantial, piece of evidence for HPA/SNS involvement.

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