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Changes in the distribution and function of leukocytes after whole-body iron ion irradiation

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ABSTRACT

High-energy particle radiation could have a considerable impact on health during space missions. This study evaluated C57BL/6 mice on Day 40 after total-body $^{56}Fe^{26+}$ irradiation at 0, 1, 2 and 3 gray (Gy). Radiation consistently increased thymus mass (one-way ANOVA: $P < 0.005$); spleen, liver and lung masses were similar among all groups. In the blood, there was no radiation effect on the white blood cell (WBC) count or major leukocyte types. However, the red blood cell count, hemoglobin, hematocrit and the CD8+ T cytotoxic (Tc) cell count and percentage all decreased, while both the CD4:CD8 (Th:Tc) cell ratio and spontaneous blastogenesis increased, in one or more irradiated groups compared with unirradiated controls ($P < 0.05$ vs 0 Gy). In contrast, splenic WBC, lymphocyte, B cell and T helper (Th) counts, %B cells and the CD4:CD8 ratio were all significantly elevated, while Tc percentages decreased, in one or more of the irradiated groups compared with controls ($P < 0.05$ vs 0 Gy). Although there were trends for minor, radiation-induced increases in %CD11b+ granulocytes in the spleen, cells double-labeled with adhesion markers (CD11b+CD54+, CD11b+CD62E+) were normal. Splenocyte spontaneous blastogenesis and that induced by mitogens (PHA, ConA, LPS) was equivalent to normal. In bone marrow, the percentage of cells expressing stem cell markers, Sca-1 and CD34/Sca-1, were low in one or more of the irradiated groups ($P < 0.05$ vs 0 Gy). Collectively, the data indicate that significant immunological abnormalities still exist more than a month after ^{56}Fe irradiation and that there are differences dependent upon body compartment.

KEYWORDS: particle radiation, total-body irradiation, hematopoiesis, mouse model, spaceflight

INTRODUCTION

The impact of radiation on astronaut health, especially during extended deep-space missions, continues to be a significant concern to the National Aeronautics and Space Administration (NASA). Since current guidelines of the National Council on Radiation Protection and Measurements (NCRP) apply only to low Earth orbit (LEO) missions [1], the need for data on radiation effects beyond LEO is increasing as space exploration proceeds. Exposure to relatively high doses and various forms of radiation during spaceflight beyond LEO is inevitable. Sources include galactic cosmic radiation (GCR), which originates from outside our solar system, and solar particle events (SPEs), which appear sporadically and are unpredictable [2].

During extended deep-space missions to Mars and elsewhere, crew members could receive relatively high doses of radiation [3–5]. The great majority of cosmic radiation is composed of protons (86%) and helium ions (11%). High-charge and high-energy (HZE) ions represent a very small percentage of the various forms of space radiation, i.e. ~1%. Of these, ^{56}Fe is considered to be the most important because of its high linear energy transfer (LET). Although many body systems could be adversely affected, the immune system is especially radiosensitive. Immune depression and/or dysfunction could lead to overwhelming infection and other pathologies, which could increase the risk of mission failure and possibly decrease quality of life after return to Earth. Potential immunological aberrations associated with radiation and other stressors in the spaceflight environment (e.g. alterations in gravitational force, hypoxia and psychological stress of confinement) have been summarized relatively recently [6].

Many studies have found immune aberrations in astronauts, cosmonauts and rodents on various space missions [7–17]. In addition, ground-based studies that simulate space radiation and other stressors have shown significant changes in immune parameters compared with controls [18–28]. The great majority of the space radiation-associated studies have used photons (γ-rays, X-rays) and to a lesser extent also protons. Overall, there is much less knowledge regarding immune effects after exposure to HZE ions. This is important to note because different forms of radiation do not always result in identical outcomes [20, 29–31].

Although progress certainly has been made in understanding the direct and indirect health risks associated with high-charge/high-energy particle radiation during space exploration, the immunological responses are complex and many questions still remain [32]. The current study was one of a series done with ^{56}Fe radiation in murine models in order to confirm and extend our previous findings. In those studies, we found drastic reductions in virtually all characterized immune parameters, with associated changes in *ex vivo* function, four days post-irradiation [33–36]. While most of the population recovered by Day 110–113 [30, 36], there were still some strain-dependent changes in T and B cell populations 30 days post-exposure [34]. Those findings are extensively compared with the present data in the discussion. The major goal here was to determine the effect of whole-body $^{56}Fe^{26+}$ irradiation on leukocyte distribution and function at a relatively long time-point after exposure.

MATERIALS AND METHODS

Animals and total-body irradiation

The Institutional Animal Care and Use Committees of Loma Linda University (LLU) and Brookhaven National Laboratory (BNL) approved this study. The animals were in the BNL-7 run of the NASA radiation health experimental series. C57BL/6 J female mice ($n = 60$) were purchased from Charles River Breeding Laboratories, Wilmington, MA, USA, and shipped directly to BNL at 8–9 weeks of age. After an acclimatization period of ~1 week, non-anesthetized animals were placed individually into well-aerated polystyrene boxes (volume 3 × 3 × 6 cm) and were exposed to iron ions (^{56}Fe, Z = 26, 1 GeV/nucleon, LET = 148.2 keV/μm track average) using the Alternating Gradient Synchrotron (AGS). Additional beam characteristics can be found elsewhere [37, 38]. The radiation was delivered in a single fraction to total doses of 1, 2 and 3 Gy at a dose rate of ~1 Gy/min at beam entry. A 0 Gy control group was treated in an identical manner but without irradiation. Several days later the animals were shipped to LLU. On Day 40 after irradiation, the mice were weighed and then rapidly euthanized in 100% CO_2

Specimen collection and processing

Spleen, thymus, liver and right lung were collected and weighed immediately after euthanasia. Organ mass was normalized relative to body mass using the following formula: Norm. mass = organ mass (mg)/body mass (g). Additional procedures were carried out as previously described [17, 33, 35]. Briefly, blood was collected in syringes containing potassium-ethylenediaminetetraacetic acid (K_2-EDTA; Fisher Scientific, Inc., Pittsburgh, PA, USA) via cardiac puncture. Spleens were placed into 1 ml of complete RPMI 1640 medium (Irvine Scientific, Santa Ana, CA, USA) that included 10% heat-inactivated fetal bovine serum, processed into single-celled suspensions using autoclaved wooden applicator sticks, and then erythrocytes were lysed using a standard procedures. Bone marrow was flushed from the right femur using 1 ml of complete RPMI-1640 medium.

Automated hematological analysis of blood and spleen cells

Whole blood and splenocyte samples (12 μl) were evaluated using an ABC Vet Hematology Analyzer (Heska Corp., Waukesha, WI, USA). For the blood, this included a white blood cell (WBC) count, lymphocyte, monocyte and granulocyte counts and percentages, 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). For the spleen, WBC counts and the numbers and percentages of the three major leukocyte populations were obtained.

Spontaneous and mitogen-induced blastogenesis

To determine spontaneous blastogenesis of cells in blood and spleen, aliquots were diluted with complete RPMI 1640 medium and dispensed into wells of microculture plates. Immediately thereafter, 1 μCi of ^{3}H-thymidine (^{3}H-TdR), specific activity = 46 Ci/mmol (ICN Biochemicals, Costa Mesa, CA, USA) was added, and the plates were incubated for 3 h at 37°C in 5% CO_2. Counts per minute (cpm) for both blood and spleen were normalized to cell count.

The response of spleen cells to three different mitogens, i.e. phytohemagglutinin (PHA), concanavalin A (ConA) and lipopolysaccharide (LPS) (Sigma Chemical Co., St Louis, MO, USA), was also determined. After adjusting to 2 × 10^6 cells/ml in complete RPMI 1640 medium, the cells were dispensed into microtiter plate wells, with and without each of the three mitogens (pre-titrated for maximal response), at 2 × 10^5 splenic leukocytes/0.2 ml/well and incubated for 48 h. During the last 4 h, the cells were pulse-labeled with ^{3}H-TdR at 1 μCi/50 μl/well. The cpms in response to the mitogens were converted to a stimulation index (SI): SI = (cpm with mitogen – cpm without mitogen)/cpm without mitogen.

In both types of assays described above, samples from each mouse were tested in triplicate. The cpm was obtained using a liquid beta-scintillation counter (Beckman Instruments, Inc., Fullerton, CA, USA). Additional details for these procedures have been previously described [39, 40].

Flow cytometry analysis of leukocyte subpopulations

Leukocytes were evaluated for expression of surface markers using fluorescence-labeled monoclonal antibodies (mAbs) (Pharmingen, San Diego, CA, USA), a direct-staining procedure and a FACSCalibur™ flow cytometer (Becton Dickinson, Inc., San Jose, CA, USA). The mAbs were labeled with fluorescein isothiocyanate (FITC), R-phycoerythrin (PE), allophycocyanin (APC), or peridinin chlorophyll protein (PerCP). For all mAb combinations

characterized in the spleen and blood, leukocytes were identified using antibody against cluster differentiation (CD) molecule CD45; lymphocytes/mononuclear cells were distinguished from granulocytes based on CD45 versus side scatter gating. For lymphocytes, percentages (%) were defined as a percent of the total mononuclear cell (MNC) count as defined by the standard CD45 vs Side Scatter MNC gate. Similarly, for CD11b+ granulocytes, percentages were defined as percent of cell counts within the granulocyte gate. Numerical values for leukocyte subsets were calculated using the cell counts obtained with the automated hemocytometer (described above).

In the blood and spleen, T helper (Th) lymphocytes were determined using mAbs against CD3/CD4, whereas mAbs against CD3/CD8 were used to identify T cytotoxic (Tc) cells. In the spleen, CD11b+ granulocytes with and without adhesion makers CD54 and CD62E were identified. Similar procedures were also performed on mononuclear bone marrow cells to determine the percentage with stem cell markers, i.e. CD34 and stem cell antigen 1 (Sca-1). In all cases, a minimum of 5000 events were analyzed per sample using CellQuest^{TM} software version 3.1 (Becton Dickinson).

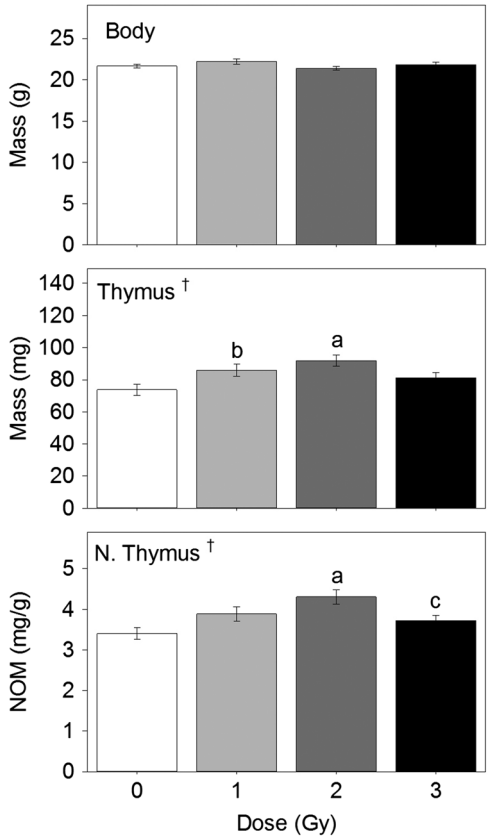

Fig. 1. Body and thymus masses. Values represent means ± SEM. $n = 14–15$ mice/group. N. Thymus: thymus mass normalized to body mass. One-way ANOVA: $^{\dagger}P < 0.005$ for an effect of radiation dose. *Post-hoc* Tukey's: (a) $P < 0.05$ vs 0 Gy, (b) $P < 0.09$ vs 0 Gy, (c) $P < 0.06$ vs 2 Gy.

Statistical analysis

One-way analysis of variance (ANOVA) was performed using radiation dose as the independent variable. Tukey's pairwise multiple comparison test was used in *post-hoc* analysis when indicated. The program used was SigmaPlot for Windows version 13.0 (Systat Software, Inc., Point Richmond, CA, USA). Data are presented as mean ± standard error of the mean (SEM), and a P value of < 0.05 was considered significant; $P < 0.1$ indicated a trend toward significance.

RESULTS

Body and organ masses

Figure 1 shows results for body and thymus masses. There were significant differences in thymus mass alone or when normalized to body mass. The thymus values were consistently higher in the irradiated groups compared with the 0 Gy group (one-way ANOVA: $P < 0.005$), although Tukey's test showed a $P < 0.05$ only for the 2 Gy group versus the 0 Gy group. There were no significant differences between groups in spleen, liver or lung masses, although one-way ANOVA indicated trends for a radiation dose effect ($P < 0.1$) on spleen mass relative to body mass and lung mass alone (Table 1).

Complete blood count

The WBC counts and the three-part differential are presented in Fig. 2. Of the three major leukocyte types, only monocyte numbers were consistently low in irradiated groups (one-way ANOVA: $P < 0.1$), and there was a strong trend for low counts in the 3 Gy

Table 1. Spleen, liver and lung mass alone and normalized to body mass

Organ	Dose (Gy)	Mass (mg)	Norm. mass (mg/g)
Spleen*	0	80.3 ± 2.2	3.7 ± 0.1
	1	89.0 ± 3.1	4.0 ± 0.1
	2	87.4 ± 3.0	4.1 ± 0.2
	3	83.1 ± 2.6	3.8 ± 0.1
Liver	0	1140.7 ± 23.3	52.6 ± 0.7
	1	1175.8 ± 21.0	53.0 ± 0.8
	2	1161.5 ± 22.0	54.3 ± 0.8
	3	1148.8 ± 27.0	52.5 ± 0.7
Lung*	0	88.7± 6.8	4.1 ± 0.3
	1	107.7 ± 5.2[a]	4.9 ± 0.2
	2	100.5 ± 5.5	4.7 ± 0.3
	3	94.2 ± 4.5	4.3 ± 0.2

Values represent means ± SEM on Day 40 after ^{56}Fe irradiation. $N = 15$ mice/group. Values for lung are based on right lung. Norm. mass = organ mass normalized to body mass. One-way ANOVA: $^{*}P < 0.1$ for an effect of radiation dose on spleen Norm. Mass and lung Mass. $P < 0.1$ vs 0 Gy.

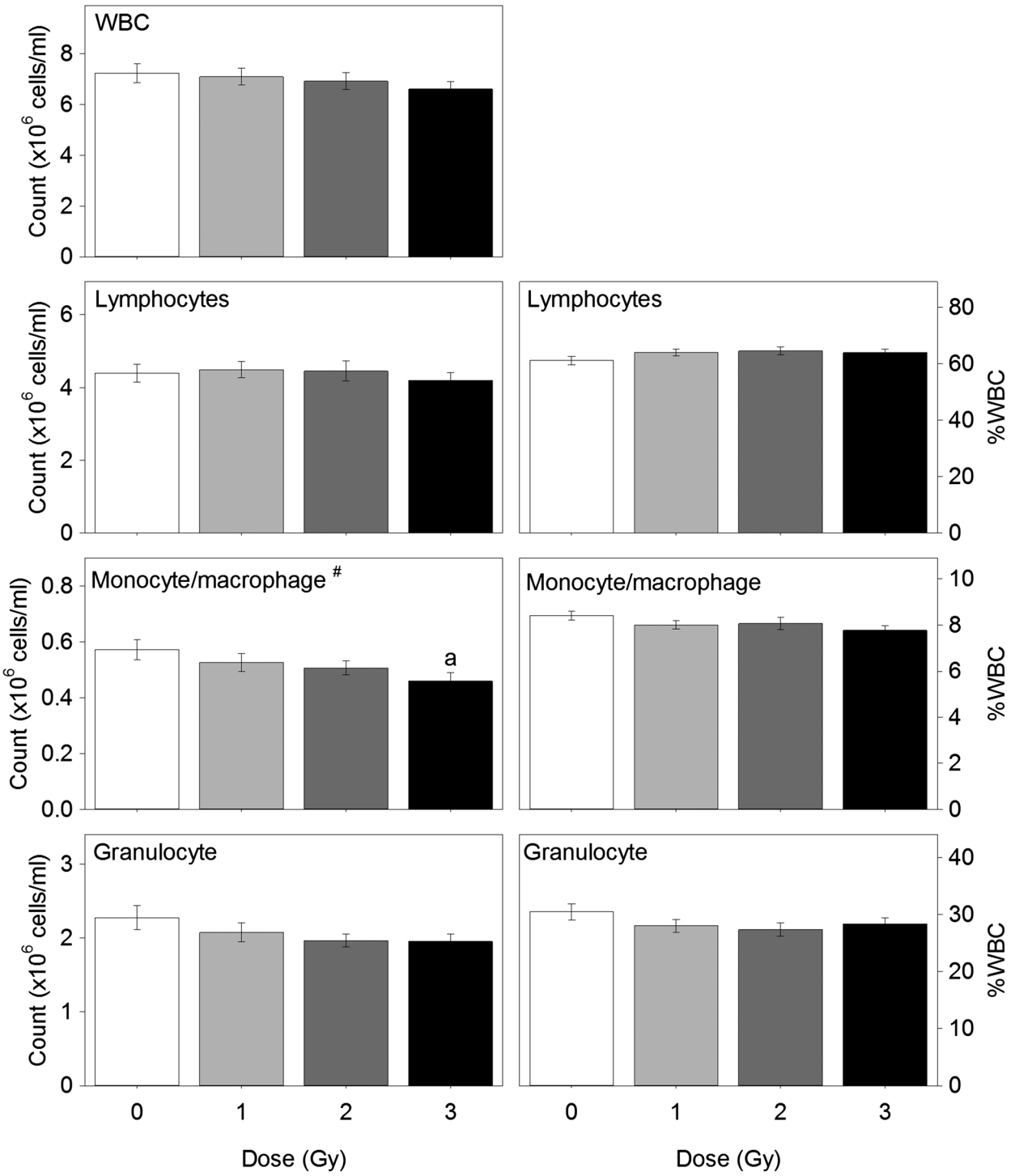

Fig. 2. White blood cell (WBC) counts and major leukocyte types in blood. Data were obtained using an automated hematology analyzer. Values represent means ± SEM. $n = 14–15$ mice/group. One-way ANOVA: $^{\#}P < 0.1$ for an effect of radiation dose. *Post-hoc* Tukey's: (a) $P < 0.06$ vs 0 Gy.

group versus the 0 Gy group ($P < 0.06$). Table 2 shows that the 3 Gy resulted in significantly low RBC counts, HGB and HCT compared with the control group ($P < 0.05$). All of the other evaluated parameters relating to erythrocytes and platelets in the irradiated groups were equivalent to those of the 0 Gy group.

Major leukocyte types in spleen

Figure 3 shows that there was a radiation effect on WBC and lymphocyte counts in the spleen (one-way ANOVA: $P < 0.05$ and $P < 0.005$, respectively). Tukey's test revealed that WBC counts in the 1 Gy group were higher versus the 0 Gy group and that there was a strong trend for high counts in the 2 Gy group ($P < 0.05$ and $P < 0.06$, respectively). This was likely due to the high numbers of lymphocytes in the 1 and 2 Gy groups ($P < 0.05$). A similar pattern was noted for monocyte/macrophage and granulocyte counts, but there was no statistical support. Percentages of these three major leukocyte types were equivalent to control values regardless of radiation (Fig. 3).

Spontaneous and mitogen-induced blastogenesis

The Fig. 4 upper panels show that radiation dose had a significant effect on spontaneous blastogenesis in the blood ($P < 0.05$). A *post-hoc* Tukey's test showed that there were higher cpm in the 2 Gy and 3 Gy groups ($P < 0.05$ vs 0 Gy). In the spleen, however, there were no significant differences or trends between the various groups. This was true for the spleen also with respect to the SI values obtained after mitogen-induced blastogenesis (lower panels of Fig. 4). Although there were steady dose-dependent decreases in PHA- and Con A-induced blastogenesis, this did not reach the level of significance.

Table 2. Red blood cell (RBC) and platelet (PLT) parameters

	0 Gy	1 Gy	2 Gy	3 Gy
RBC ($\times 10^9$/ml)*	9.7 ± 0.2	9.5 ± 0.1	9.5 ± 0.1	9.1 ± 0.2[a]
HGB (g/dl)*	13.8 ± 0.2	13.5 ± 0.1	13.5 ± 0.1	13.0 ± 0.3[a]
HCT (%)**	43.8 ± 0.8	42.7 ± 0.4	42.7 ± 0.3	41.2 ± 0.9[a]
MCV (mm^3)	45.1 ± 0.1	44.8 ± 0.2	44.9 ± 0.2	45.3 ± 0.1
MCH (pg)	14.2 ± 0.1	14.2 ± 0.1	14.1 ± 0.1	14.3 ± 0.04
MCHC (g/dl)	31.6 ± 0.1	31.6 ± 0.1	31.5 ± 0.1	31.6 ± 0.1
RDW (%)	15.3 ± 0.1	15.3 ± 0.1	15.8 ± 0.3	15.5 ± 0.1
PLT ($\times 10^6$/ml)	1010 ± 25	1007 ± 28	1072 ± 77	951 ± 25
MPV (mm^3)	10.4 ± 0.1	10.5 ± 0.2	10.9 ± 0.2	10.5 ± 0.1

Values represent means ± SEM for blood samples on Day 40 after ^{56}Fe irradiation. Data were obtained using an automated hematology analyzer. $N = 14–15$ mice/group. HGB = hemoglobin concentration, HCT = hematocrit, MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, RDW = RBC distribution width, MPV = mean platelet volume. One-way ANOVA: *$P < 0.05$ and **$P < 0.1$ for an effect of radiation dose. *Post-hoc* Tukey's: a $P < 0.05$ vs 0 Gy.

Major lymphocyte types and T cell subsets in blood

Figure 5 shows that radiation had no significant effect on T, B or NK cell counts or percentages. One-way ANOVA, however, did indicate a trend ($P < 0.1$) for a radiation-induced decrease in T cell counts and percentages and an increase in B cell percentage; Tukey's test for %B cells revealed a trend for 3 Gy vs the 0 Gy group ($P < 0.07$).

T cell subset data are presented in Fig. 6. Counts and percentages of CD4+ Th cells were equivalent to normal. However, radiation had a highly significant effect on CD8+ Tc cell counts and percentages (one-way ANOVA: $P < 0.001$). As far as Tc cell counts, the 2 Gy and 3 Gy groups were lower compared with 0 Gy ($P < 0.05$). Tc cell percentages were significantly lower in all irradiated groups compared with the 0 Gy group ($P < 0.05$). This led to a significantly elevated CD4:CD8 ratio compared with the 0 Gy group for all three irradiated groups ($P < 0.05$).

Major lymphocyte types and T cell subsets in the spleen

Figure 7 shows the data for the three major lymphocyte populations. Based on the one-way ANOVA, there was no radiation effect on T cell counts and only a trend was noted for %T cells ($P < 0.1$), with the 3 Gy group having a slightly lower percentage than the 0 Gy group ($P < 0.08$). In contrast, there were significant radiation effects on B cell counts ($P < 0.005$) and percentages ($P < 0.05$). The B cell numbers were higher in the 1 Gy and 2 Gy groups versus the 0 Gy group ($P < 0.05$), and there was a trend for a high count in the 3 Gy group ($P < 0.08$). As far as %B cells, only the 3 Gy group had a higher percentage compared with the 0 Gy group ($P < 0.05$). NK cell counts were equivalent to normal, and there was only a trend for low percentages in the irradiated groups (one-way ANOVA: $P < 0.1$; Tukey's test: $P < 0.08$ for 3 Gy vs 0 Gy).

The results for T cell subsets are shown in Fig. 8. The CD4+ Th cell counts were significantly affected ($P < 0.005$). Tukey's test indicated that the Th cell number was higher in the 2 Gy group ($P < 0.05$), and there was a trend for an increase in the 1 Gy group compared with the 0 Gy group. Th cell percentages, however, were equivalent to normal throughout. In contrast to Th cells, the CD8+ Tc cell counts were affected by radiation dose (one-way ANOVA: $P < 0.05$), but there were no significant differences between irradiated groups and 0 Gy controls. There was, however, a highly significant radiation effect on Tc cell percentages (one-way ANOVA: $P < 0.001$); low proportions were present in all irradiated groups compared with the 0 Gy group ($P < 0.05$). These findings resulted in an elevated CD4:CD8 ratio in the 2 Gy and 3 Gy groups ($P < 0.05$ vs 0 Gy).

Granulocytes with adhesion markers in the spleen

These data are presented in Fig. 9. Although the numbers of granulocytes expressing CD11b were similar in all groups, one-way ANOVA indicated a significant radiation dose effect on percentages ($P < 0.05$), most likely due to relatively high values in the 1 Gy group. Tukey's test, however, showed only trends for %CD11b: 0 Gy vs 1 Gy ($P < 0.09$). When the CD11b+ cells were double-labeled with adhesion markers, i.e. either CD54 or CD62E, there were no significant differences or trends in numbers or

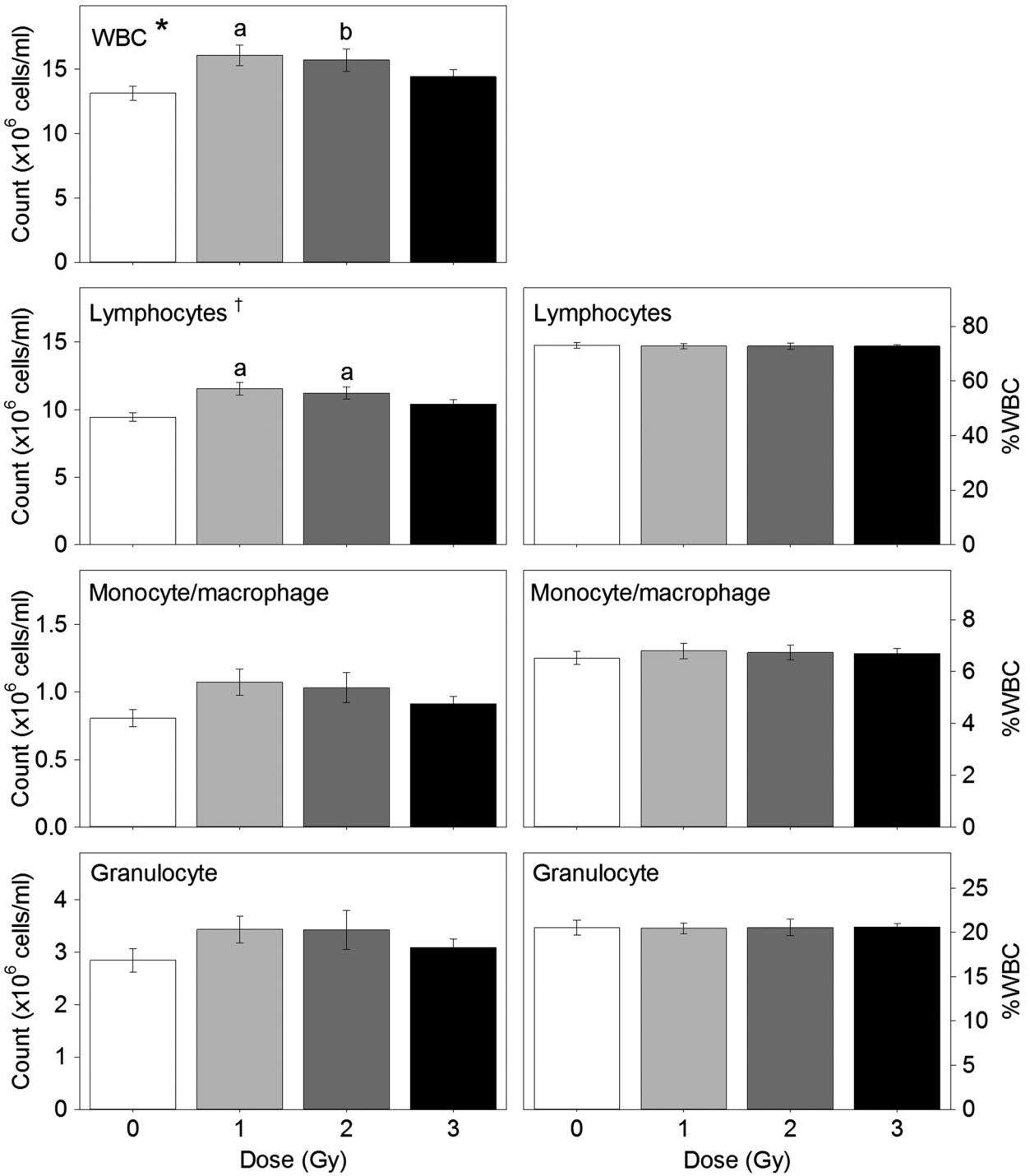

Fig. 3. White blood cell (WBC) counts and major leukocyte types in spleen. Data were obtained using an automated hematology analyzer. Values represent means ± SEM. $n = 14–15$ mice/group. One-way ANOVA: *$P < 0.05$ or †$P < 0.005$ for an effect of radiation dose. *Post-hoc* Tukey's: (a) $P < 0.05$ vs 0 Gy, (b) $P < 0.06$ vs 0 Gy.

percentages between the various groups. Note that mononuclear cells were also tested for the same adhesion markers, but there were no significant differences between or trends among groups (data not shown).

Cells with stem cell markers in bone marrow

Figure 10 shows that there was only a trend for a radiation dose effect on mononuclear cells expressing CD34 ($P < 0.1$). However, significance was obtained for Sca-1 ($P < 0.05$); Tukey's test showed

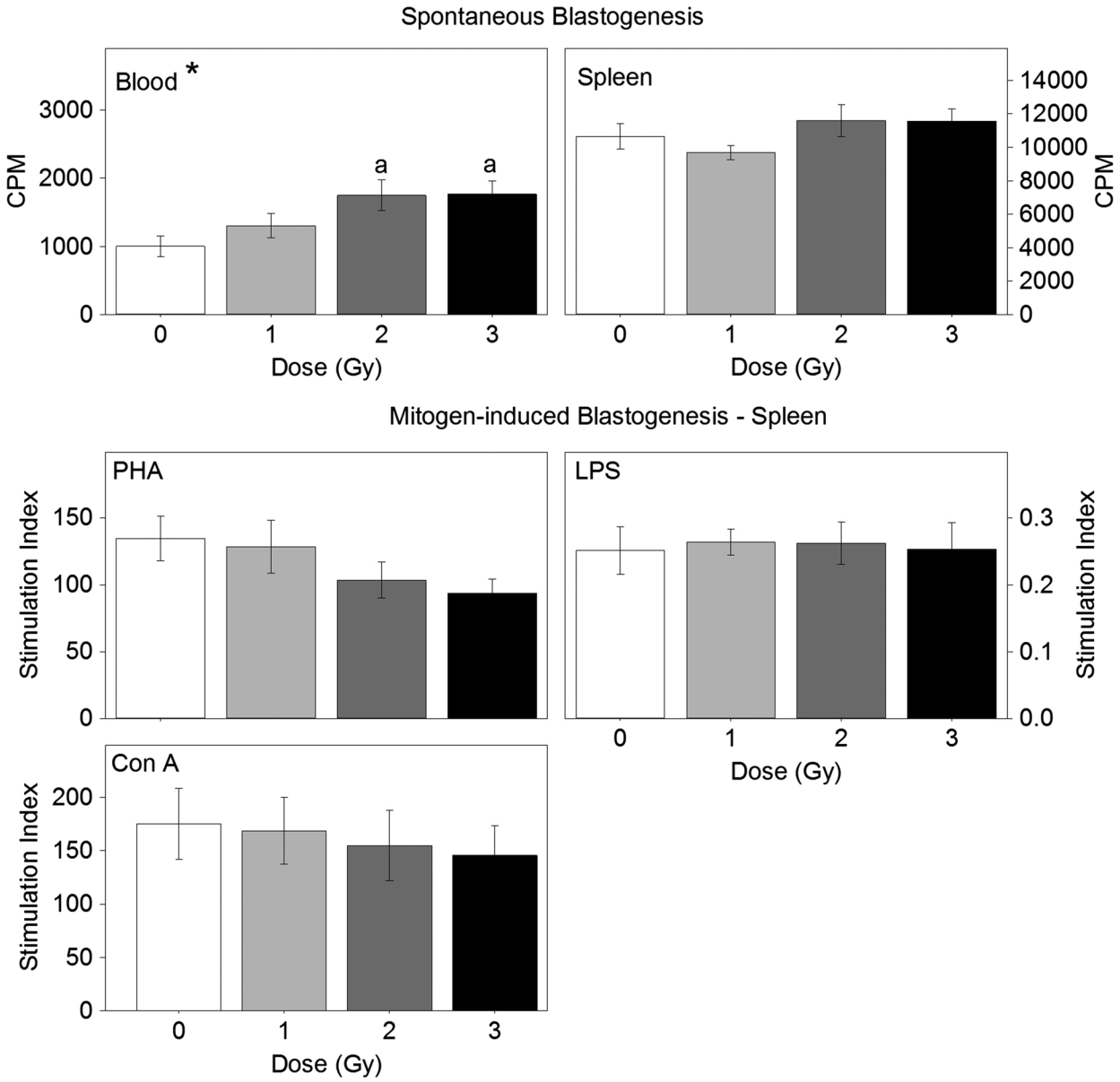

Fig. 4. Spontaneous and mitogen-induced blastogenesis. Data are based on incorporation of ^{3}H-thymidine into cell DNA. CPM: counts per minute. Stimulation index = (CPM with mitogen – CPM without mitogen)/CPM without mitogen. Values represent means ± SEM. n = 14–15 mice/group. One-way ANOVA: *$P < 0.05$ for an effect of radiation dose. *Post-hoc* Tukey's: (a) $P < 0.05$ vs 0 Gy.

that the 2 Gy and 3 Gy groups had lower values compared with the 0 Gy group ($P < 0.05$ and $P < 0.08$, respectively). A significant radiation effect was also present for CD34/Sca-1 cells ($P < 0.005$). The values for these double-positive cells in the 2 Gy and 3 Gy groups were significantly lower than for the 0 Gy controls ($P < 0.05$).

DISCUSSION

Body mass is considered to be an indicator of overall health. In our study, there was no effect of ^{56}Fe radiation on body mass at the 40-day post-exposure time-point. This is consistent with our previous studies in same strain mice that showed no effect on body mass on Days 4 and 113 after ^{56}Fe irradiation using doses of up to 3 Gy [35, 36]. In other studies using ^{56}Fe, we have found that body mass is dependent on a number of variables such as radiation dose, mouse strain and time of assessment [41]. In a rat model followed for 9 months after exposure to 1–4 Gy ^{56}Fe radiation, body mass was generally lower compared with controls [42, 43].

When evaluating several different organ masses alone and in relation to body mass in our study, only the thymus was significantly affected, i.e. consistently higher mass alone or in relation to

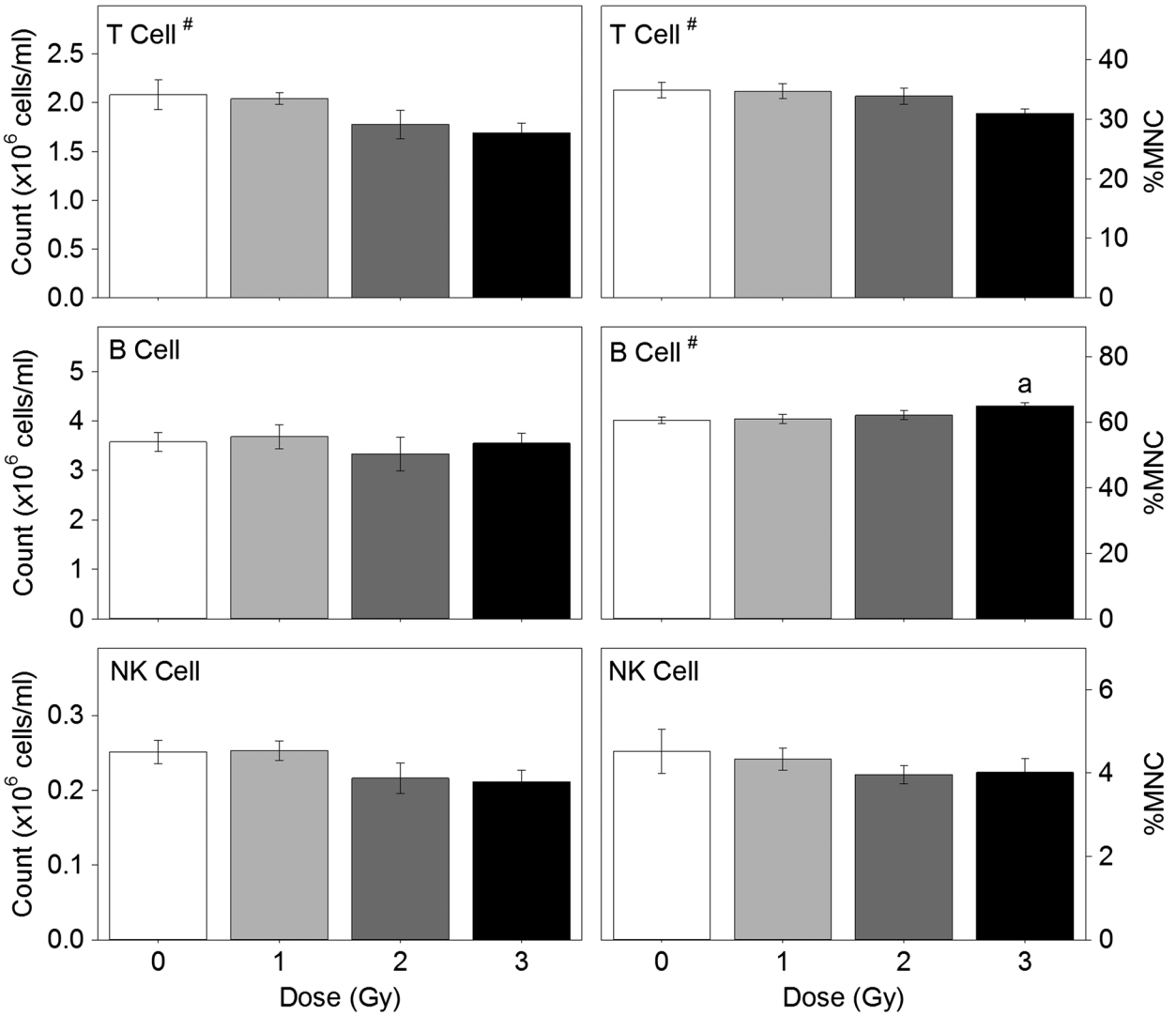

Fig. 5. Major lymphocyte types in blood. Data on T, B and natural killer (NK) cells were obtained using flow cytometry. Values represent means ± SEM. $n = 14–15$ mice/group. MNC: mononuclear cells. One-way ANOVA: $^{\#}P < 0.1$ for an effect of radiation dose. *Post-hoc* Tukey's: (a) $P < 0.07$ vs 0 Gy.

body mass in the irradiated groups. The thymus is the primary site for T cell maturation and plays an important role in the induction of tolerance to self-antigens. Although this organ is most important early in life, it can participate in T cell regeneration under dire circumstances in adults, thus resulting in increased thymic mass [44, 45]. In a previous study using similar doses of ^{56}Fe radiation, we found that thymus and spleen (but not body, liver or lung) masses were significantly decreased in a dose-dependent manner on Day 4 [35].

The complete blood count (CBC) analysis of blood showed no significant radiation effect on WBC count or on the counts or percentages of the three major leukocyte types (lymphocytes, monocytes, granulocytes). There was, however, a strong trend for low monocyte numbers in the 3 Gy group. This was somewhat surprising since monocytes, i.e. important members of the innate immune system, have long been known to be more radioresistant than lymphocytes and granulocytes [46]. A possible reason is that the monocytes may have migrated out of the blood circulation and become localized at a site of injury/inflammation. In a previous study, we found ^{56}Fe dose-dependent decreases in blood WBCs and in all three major leukocyte types on Day 4 post-irradiation, followed by complete recovery in cell numbers by Day 113; monocyte–macrophage percentages, however, were significantly decreased in both blood and spleen [36]. Others have also noted great reduction in WBCs and the three major leukocyte types in mice during 4, 7 and 14 days after 3 Gy ^{56}Fe irradiation [47]. This latter study also demonstrated that subcutaneous injection of androstenediol 30 min after exposure could significantly mitigate the detrimental effect.

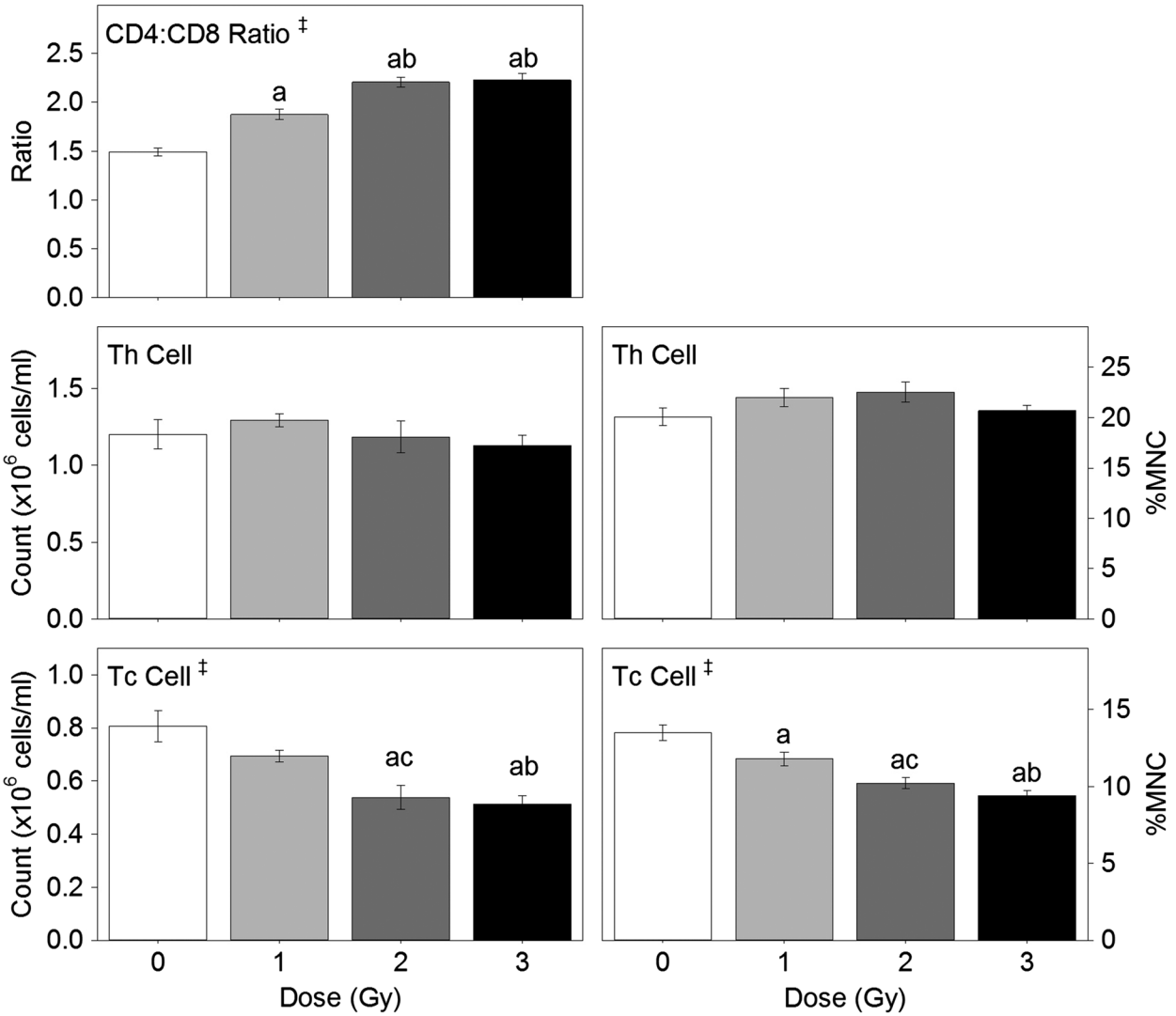

Fig. 6. T cell subsets in blood. Data were obtained using flow cytometry. Values represent means ± SEM for CD4+ T helper (Th) and CD8+ T cytotoxic (Tc) cells. $n = 14–15$ mice/group. MNC: mononuclear cells. One-way ANOVA: $^{‡}P < 0.001$ for an effect of radiation dose. *Post-hoc* Tukey's: (a) $P < 0.05$ vs 0 Gy, (b) $P < 0.05$ vs 1 Gy, (c) $P < 0.06$ vs 1 Gy.

The blood data also showed low RBC counts, HGB and HCT in the 3 Gy group, thereby indicating the possibility of anemia. Since hematopoietic stem cells are precursors to RBCs (as well as WBCs), our bone marrow data showing low levels of cells with hematopoietic stem cell markers are consistent with these findings. Significant depression in all three of these parameters has also been noted on Day 4 after iron ion irradiation, with return to normal by Day 113 [35, 36]. In a comparison of 2 Gy iron, carbon and proton radiation, the ^{56}Fe-irradiated group had the lowest RBC count, HGB and HCT at 110 days post-exposure, although statistical significance was not always obtained [30]. Loss in RBC mass, due at least partly to destruction of newly released RBCs, and anemia have been consistently associated with space missions for many years [48–50]. As recently reviewed, the underlying mechanisms appear to be at least partly linked to neocytolysis under microgravity [51]. Based on these and other reports, the addition of particle radiation exposure, especially during extended deep-space missions, could further exacerbate anemia in astronauts.

Overall, we found that the radiation effect was much greater in the spleen than in blood. Although statistical support was not always obtained, the consistently high numbers of WBCs and of the major leukocyte types (especially lymphocytes) in spleens of the irradiated mice further supports the premise that hematopoiesis was still continuing above a normal level. Data on lymphocyte subpopulations in the blood showed only a trend for a radiation effect on T cells (number and percentage) and B cells (percentage). Further analysis showed significant radiation-induced decreases in CD8+ Tc cell counts and percentages, but no differences among groups in the CD4+ Th cells. These findings led to a greatly increased CD4:CD8 ratio. A very similar pattern was also observed in the spleen, except

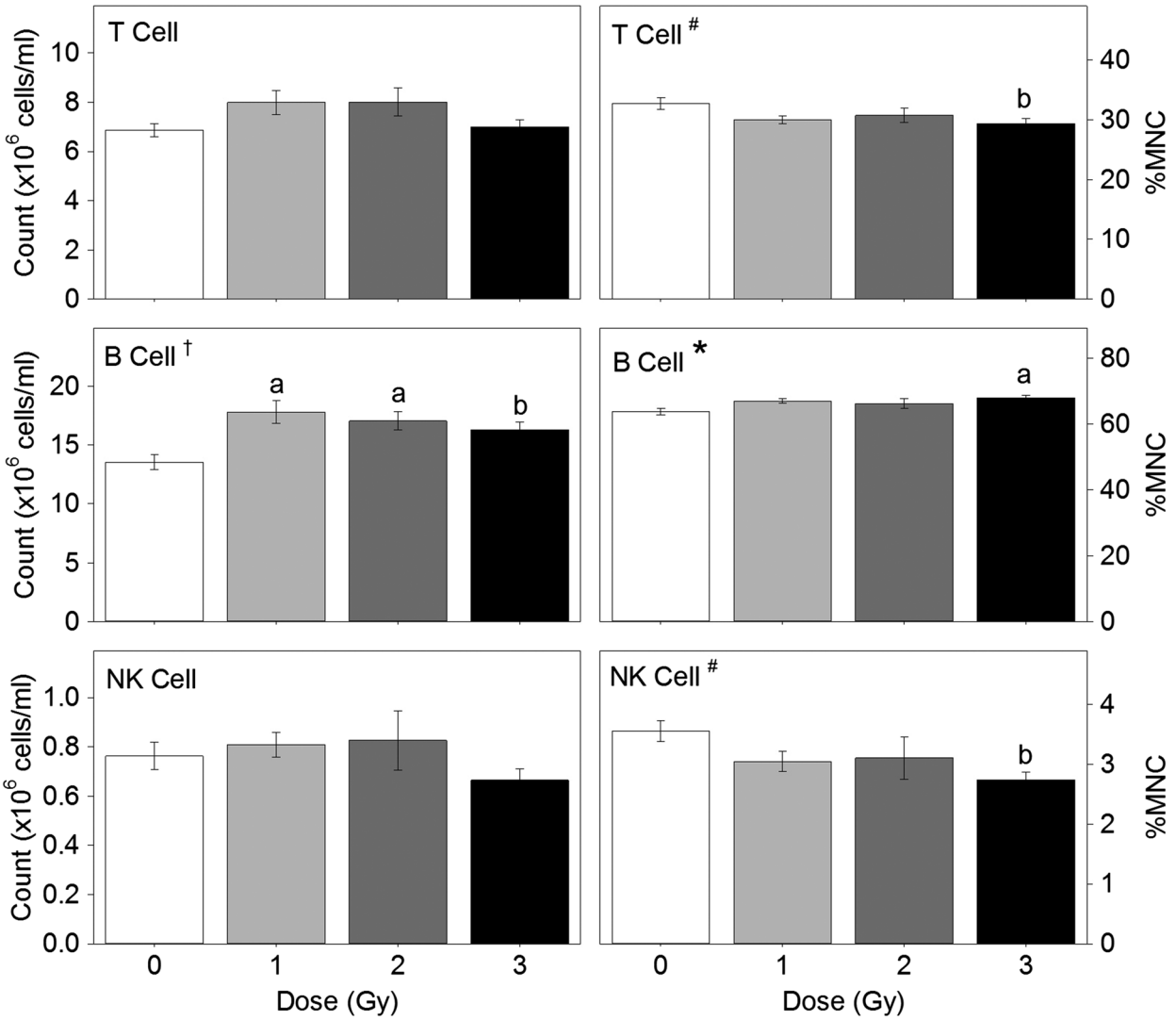

Fig. 7. Major lymphocyte types in spleen. Data on T, B and natural killer (NK) cells were obtained using flow cytometry. Values represent means ± SEM. $n = 14–15$ mice/group. MNC: mononuclear cells. One-way ANOVA: $^{*}P < 0.05$, $^{\dagger}P < 0.005$ or $^{\#}P < 0.1$ for an effect of radiation dose. *Post-hoc* Tukey's: (a) $P < 0.05$ vs 0 Gy, (b) $P < 0.08$ vs 0 Gy.

that there was a radiation-associated increase in B cells, thereby suggesting a continuing need to regenerate these antibody-producing lymphocytes.

Our findings are consistent with previous reports on variations in the radiosensitivity of the major T cell subsets (CD8 > CD4) using γ-rays [52–54], protons [55] and ^{56}Fe [36]. Some radiation-associated differences in lymphocyte subpopulation response may be at least partly dependent on the rodent model used [34, 42]. Since CD8+ Tc lymphocytes kill cells that are virally infected or transformed to a potentially malignant phenotype, low numbers certainly could compromise immune resistance during space missions. In addition, recent reports indicate that upon activation these cells differentiate into subpopulations (e.g. Tc2, Tc9, Tc17, CD8+ Treg cells) that, when not controlled properly, could lead to immunopathologies such as allergies and autoimmune diseases [56–58].

Although hematopoiesis appeared to be upregulated in both the spleen and thymus, analysis of bone marrow consistently showed a low percentage of cells expressing stem cell markers (CD34, Sca-1). CD34 is expressed on early progenitor cells involved in hematopoiesis, but has also been reported to be present on highly functional endothelial cell progenitors [59] and as an important facilitator of cell migration [60]. Sca-1 is a very common marker used to identify hematopoietic stem cells in bone marrow, but is also expressed on some cells in a variety of tissues that may serve as tissue-resident stem and progenitor cells [61].

There is very little information regarding the impact of ^{56}Fe on hematopoiesis. A mouse study that included lethal doses found that ^{56}Fe radiation caused accelerated and more severe hematopoietic toxicity compared with γ-rays and protons [29]. Interestingly, this latter study also found that ^{56}Fe had selective enhanced toxicity to

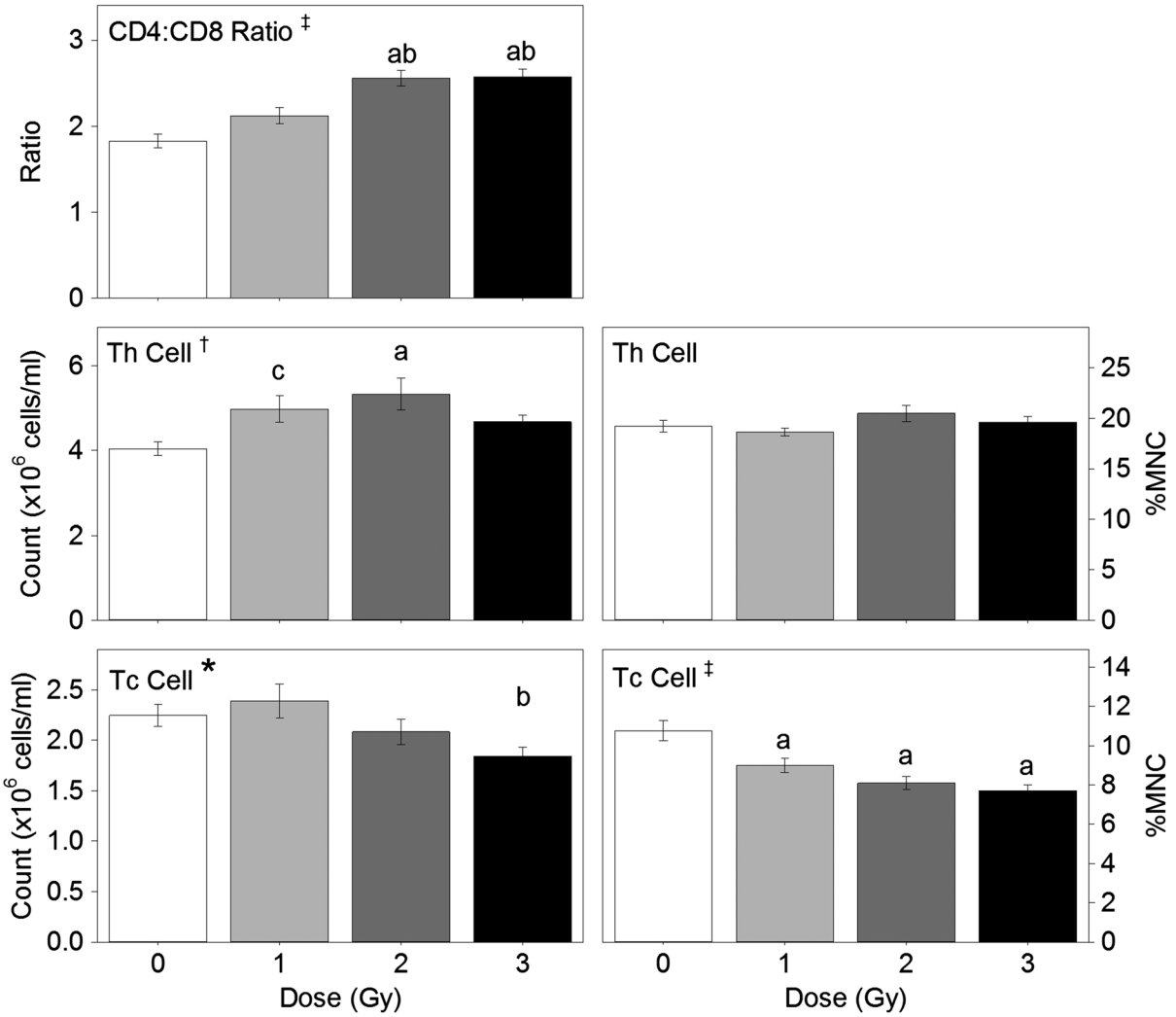

Fig. 8. T cell subsets in spleen. Data were obtained using flow cytometry. Values represent means ± SEM. $n = 14–15$ mice/group. MNC: mononuclear cells. One-way ANOVA: $^{*}P < 0.05$, $^{\dagger}P < 0.005$ or $^{\ddagger}P < 0.001$ for an effect of radiation dose. *Post-hoc* Tukey's: (a) $P < 0.05$ vs 0 Gy, (b) $P < 0.05$ vs 1 Gy, (c) $P < 0.09$ vs 0 Gy.

bone marrow progenitor and stem cells because intestinal crypt cells did not show increased toxicity related to ^{56}Fe exposure. Based on a study of bone marrow cell phenotypes in mice shortly after a 13-day mission in space, the results suggested that the spaceflight mice had more differentiated cells within the very large and granular population compared with ground controls [16] More research is obviously needed on the status of hematopoiesis and tissue regeneration in various body compartments under spaceflight conditions.

Spontaneous blastogenesis for cells in the blood was consistently higher in the irradiated groups, with statistical significance obtained for 2 Gy and 3 Gy versus 0 Gy. This indicates ongoing DNA synthesis in cells entering the blood circulation from the bone marrow. In the spleen, however, spontaneous blastogenesis was equivalent to normal, regardless of radiation, thereby suggesting no great need for leukocyte regeneration at the 40-day post-irradiation time-point. We have previously found that enhanced spontaneous blastogenesis after whole-body ^{56}Fe irradiation is more pronounced within a few days after exposure [33, 41].

Since measuring lymphocyte response to mitogens is a common and effective way of screening for cellular immunodeficiency [62], three different mitogens were selected for the recent study. PHA, a lectin found in plants, is a classical activator of T cell proliferation, regardless of antigen specificity. ConA, another plant lectin, stimulates T cell subsets that include precursors to suppressor T cells [63]. LPS, a B cell activator, is a molecule found on the surface of Gram-negative bacteria such as *Escherichia coli*. In the irradiated groups, spleen cell ability to respond to the T cell mitogens PHA and ConA was relatively low and response to LPS was slightly elevated, but statistical support for any SI differences versus 0 Gy was lacking. In a previous study, we found significant depression in response to PHA and ConA, but not LPS, on Day 4 after exposure to either 2 Gy or 3 Gy ^{56}Fe [33].

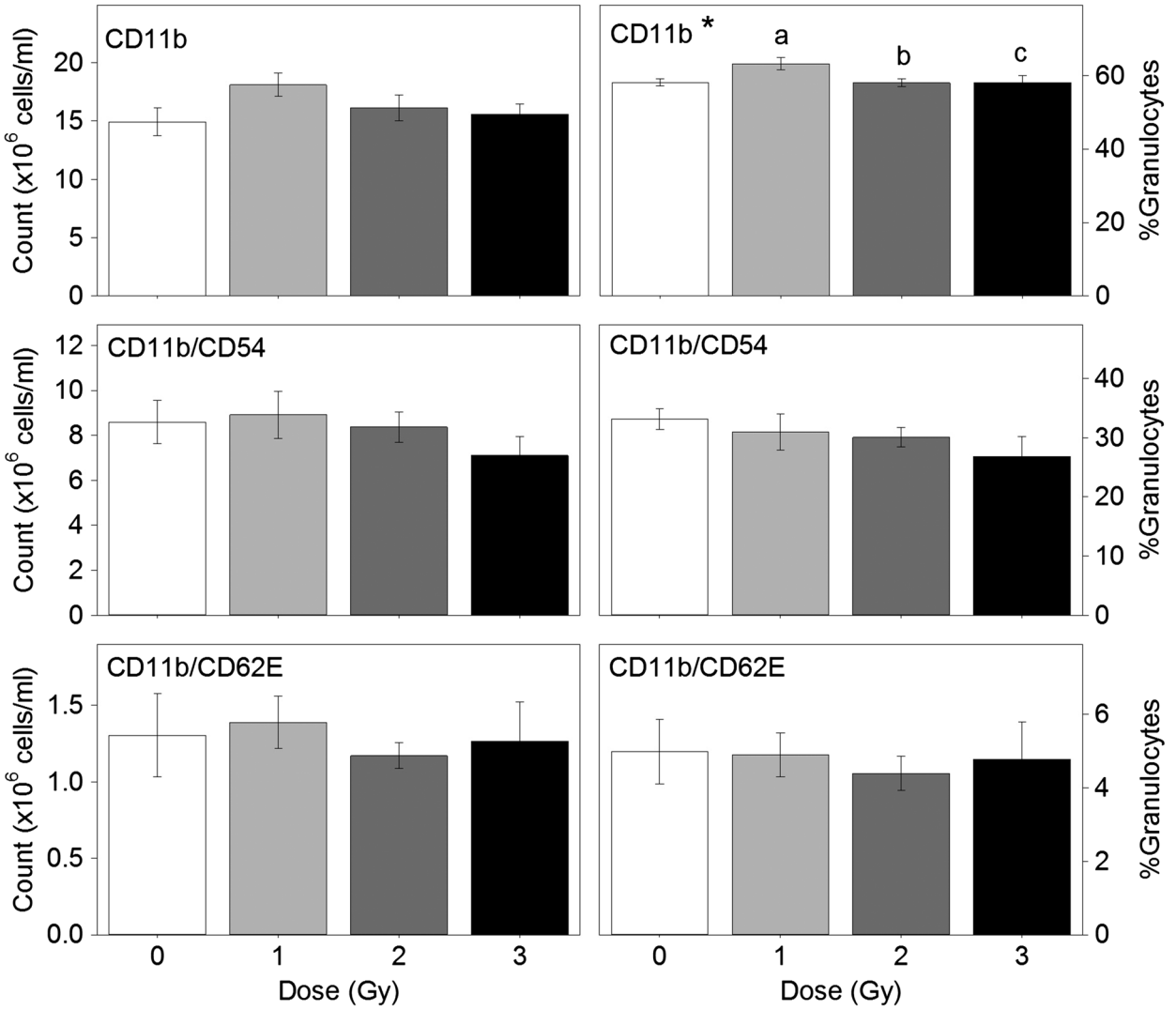

Fig. 9. Adhesion markers on granulocytes in spleen. Data were obtained using flow cytometry. Values represent means ± SEM. $n = 7–8$/group. One-way ANOVA: *$P < 0.05$ for an effect of radiation dose. *Post-hoc* Tukey's: (a) $P < 0.09$ vs 0 Gy, (b) $P < 0.07$ vs 1 Gy, (c) $P < 0.08$ vs 1 Gy.

Based on these and other studies [41], it appears that ^{56}Fe radiation in the spaceflight environment may have no long-term effect on mitogen-induced responsiveness. However, abnormalities in these types of responses due to spaceflight conditions have been reported. For example, *in vitro* activation of human peripheral blood lymphocytes has been reported to be severely depressed during spaceflight [64]. Also, a recent study using ConA and several toll-like receptor (TLR) agonists found aberrations in the response of mouse splenocytes after return from the STS-135 mission in space [14]. Concern regarding immune dysfunction that leads to increased risk for infections, especially during extended missions, remains high.

Expression of adhesion markers by granulocytes is important in leukocyte communication and migration to sites of damage. Neutrophils, by far the most abundant granulocyte, have a very short half-life in the blood circulation. In the spleen, however, there are marginated or slowly transiting pools of these cells within the vascular compartment [65]. In the current study, radiation-associated trends were noted in the percentage of CD11b+ single-label granulocytes in the spleen (slight increase in the 1 Gy group and slight decreases in the 2 Gy and 3 Gy groups vs 0 Gy). CD11b is a trans-membrane protein (also known as Mac-1 and CR3) that binds to complement fragment C3bi. CD11b, as well as other adhesion molecules, are expressed not only by granulocytes but also by monocytes/macrophages, dendritic cells and some lymphocyte populations [66, 67].

There was no radiation effect when CD11b+ cells were double-labeled with CD54, which is the intercellular adhesion molecule-1 (ICAM-1) that binds to leukocyte function antigen-1, or when double-labeled with CD62E, which is involved in trans-endothelial cell migration. In a previous ^{56}Fe study, we found the main effects of dose on CD11b+ and CD54+ cell proportions in the spleen, but

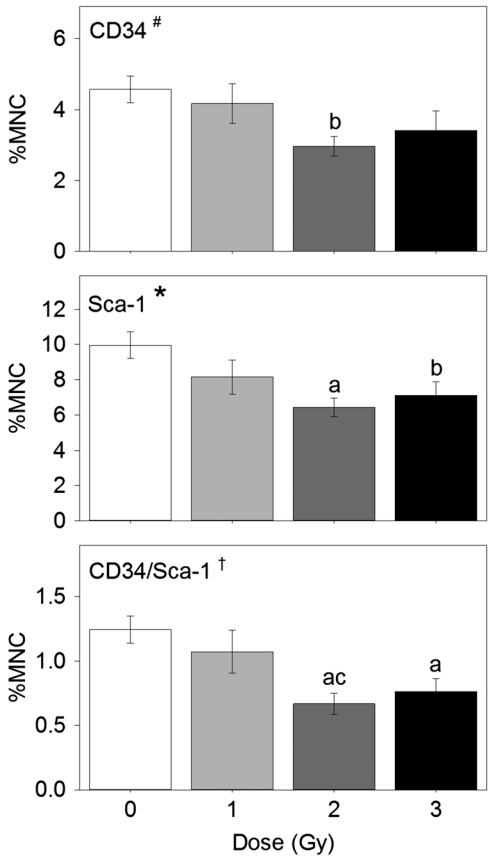

Fig. 10. Stem cell markers in bone marrow. Data were obtained using flow cytometry. Values represent means ± SEM. $n = 15$ mice/group. One-way ANOVA: $^{*}P < 0.05$, $^{\dagger}P < 0.005$, $^{\ddagger}P < 0.001$ or $^{\#}P < 0.1$ for an effect of radiation dose. *Post-hoc* Tukey's: (a) $P < 0.05$ vs 0 Gy, (b) $P < 0.08$ vs 0 Gy, (c) $P < 0.09$ vs 1 Gy.

the effect was greatest on Day 4 [41]. Nonetheless, more ^{56}Fe research in this area should be done.

The impact of spaceflight on adhesion markers has been previously noted. We found changes in genes encoding adhesion molecules and the extracellular matrix in lungs of mice shortly after return from a 13-day mission in space, i.e. STS-118 [68]. In a study of astronauts that assessed soluble adhesion markers 10 days prelaunch, immediately after landing and 2–4 days after flight found dilution of soluble iCAM-1 and E-selectin; soluble P-selectin was affected by flight duration [69]. In another study of astronauts who flew aboard 10 different shuttle flights found changes in a number of adhesion markers, e.g. CD11a, CD54, soluble ICAM-1 and soluble E-selectin [70]. The data in this latter study suggested that spaceflight could result in reduced leukocyte–endothelial adhesion.

In conclusion, our data show aberrations in a variety of immune parameters at 40 days after whole-body ^{56}Fe irradiation. Implications regarding immune defense status and long-term effects remain to be determined. More research is definitely needed on samples obtained during spaceflight as well as from ground-based studies that include multiple forms of radiation to more closely mimic exposure during flight. A better understanding of the complex actions and interactions of stressors in the spaceflight environment will increase the chance for effective countermeasure development.

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

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