

# Circulating Hematopoietic Stem and Progenitor Cells in Aging Atomic Bomb Survivors

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It is not yet known whether hematopoietic stem and progenitor cells (HSPCs) are compromised in the aging population of atomic bomb (A-bomb) survivors after their exposure nearly 70 years ago. To address this, we evaluated age- and radiation-related changes in different subtypes of circulating HSPCs among the CD34-positive/lineage marker-negative (CD34<sup>+</sup>Lin<sup>-</sup>) cell population in 231 Hiroshima A-bomb survivors. We enumerated functional HSPC subtypes, including: cobblestone area-forming cells; long-term culture-initiating cells; erythroid burst-forming units; granulocyte and macrophage colony-forming units; and T-cell and natural killer cell progenitors using cell culture. We obtained the count of each HSPC subtype per unit volume of blood and the proportion of each HSPC subtype in CD34<sup>+</sup>Lin<sup>-</sup> cells to represent the lineage commitment trend. Multivariate analyses, using sex, age and radiation dose as variables, showed significantly decreased counts with age in the total CD34<sup>+</sup>Lin<sup>-</sup> cell population and all HSPC subtypes. As for the proportion, only T-cell progenitors decreased significantly with age, suggesting that the commitment to the T-cell lineage in HSPCs continuously declines with age throughout the lifetime. However, neither the CD34<sup>+</sup>Lin<sup>-</sup> cell population, nor HSPC subtypes showed significant radiation-induced dose-dependent changes in counts or proportions. Moreover, the correlations of the proportions among HSPC subtypes in the survivors properly revealed the hierarchy of lineage commitments. Taken together, our findings suggest that many years after exposure to radiation and with advancing age, the number and function of HSPCs in living survivors as a whole may have recovered to normal levels. © 2016 by Radiation Research Society

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## INTRODUCTION

Hematopoietic functions in atomic bomb (A-bomb) survivors are variably damaged according to the extent of ionizing radiation exposure (1). The initial damage is most obvious in terms of pronounced blood cytopenias that result from radiation-induced death of hematopoietic stem and progenitor cells (HSPCs) (2, 3) and gene mutations in long-lived hematopoietic stem cells (HSCs) (4–8). Several months after irradiation, the hematopoietic system in survivors nearly recovers from the damage (2, 9). However, even more than 60 years after irradiation the A-bomb survivors' proportion of naïve T cells in peripheral blood lymphocytes is decreased in association with age and radiation exposure dose (10–12), whereas the number of white blood cells (WBCs), especially neutrophils, increases with dose (13). With advanced age, lymphopoiesis tends to decline, whereas myelopoiesis generally increases (14, 15). Thus, exposure to A-bomb radiation may accelerate the age-associated shift toward myeloid-dominant hematopoiesis. Furthermore, enhanced myelopoiesis may also be involved in age- and radiation-associated increases in inflammatory responses with a corresponding attenuation of adaptive immunity in A-bomb survivors (12, 16). However, whether aging, in combination with prior radiation exposure, significantly impacts the structure and function of vital HSPC compartments within the hemolymphoid system is unknown.

In the current study, we hypothesized that the effects of A-bomb radiation induced premature aging of HSCs, resulting in reduced numbers and impaired self-renewal and lineage commitment that in turn accelerated loss of lymphoid potential and augmentation of myeloid potential. To test this hypothesis, we performed various numerical and functional hematopoietic analyses of HSPCs circulating in the peripheral blood obtained from A-bomb survivors. These analyses included a cell sorter-based limiting-dilution assay (LDA) using CD34-positive/lineage marker-negative (CD34<sup>+</sup>Lin<sup>-</sup>) cells, which constitute the total HSPC population.

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**TABLE 1**  
**Characteristics of Study Participants**

DS02 dose (Gy)	Age at blood collection (years) <sup>b</sup>						Total
	Males (n = 81)			Females (n = 150)			
	66–73	74–80	81–91	66–73	74–80	81–91	
<0.005 <sup>a</sup>	9	8	17	16	18	33	101
0.005–0.5	6	6	5	9	9	13	48
0.5–1.0	4	2	4	6	6	16	38
1.0+	11	4	5	8	8	8	44
Total	30	20	31	39	41	70	231

<sup>a</sup> Study participants in this dose category were exposed at distances in excess of 3 km from the hypocenter and therefore received doses that are functionally equivalent to zero.

<sup>b</sup> Age at the time of the examinations, which were conducted between July 2011 and December 2013 (mean age: 78 years).

For analyses of T cell and natural killer (NK) cell potential, we performed previously established *in vitro* functional and quantitative assays of circulating T-cell and NK-cell precursors among CD34<sup>+</sup>Lin<sup>-</sup> cells with LDA by co-culturing these cells with OP9-DL1 stromal cells expressing the Notch 1 ligand, Delta-like 1, in a 384-well plate (17, 18). The surface phenotype of the NK-cell progeny generated in the culture represented CD56<sup>hi</sup> CD127<sup>+</sup>CD16<sup>-</sup> thymus-derived (thymic) NK cells. The T cell, but not NK cell, progenitor frequency in CD34<sup>+</sup>Lin<sup>-</sup> cells significantly decreased with donor age in the analysis of in-house volunteers. In the current study, we also used cell sorter-based LDA to quantify cobblestone area-forming cells (CAFCs) and long-term culture-initiating cells (LTC-ICs) generated from CD34<sup>+</sup>Lin<sup>-</sup> cells using co-culture with MS5 stromal cells (19–21). These two HSPC subtypes are believed to be *in vitro* surrogate parameters that reflect self-renewal and the multilineage differentiation ability of HSCs. Furthermore, we also quantified myeloid- or erythroid-committed progenitors in peripheral blood HSPCs by performing granulocyte-macrophage colony-forming unit (CFU-GM) and erythroid burst-forming unit (BFU-E) assays using conventional methylcellulose culture. We evaluated age- and radiation-related changes in these HSPCs in A-bomb survivors using multivariate analysis.

## MATERIALS AND METHODS

### Study Population

Study participants were selected from an A-bomb survivor cohort of 1,705 Hiroshima survivors participating in the Adult Health Study at the Radiation Effects Research Foundation (RERF) in January 2011. The selected study population consisted of two groups of Hiroshima survivors. The first group was exposed to significant doses of 0.005 Gy or more because of their location within 2 km of the hypocenter; the second group was exposed at a distance in excess of 3 km from the hypocenter and as a result would have received doses of less than 0.005 Gy. Of these 1,705 participants, 1,310 participants were selected after exclusion of those with disease and/or treatment histories, as follows. First, 223 participants were unexceptionally excluded because of histories that included infectious diseases, autoimmune diseases, immunodeficiency, hematologic diseases, hematologic malignancies and treatments with radiation, interferon, erythropoietin or a steroid. Next, 172 participants were excluded because of solid cancers that

were diagnosed less than 5 years prior to examination and solid cancers and other diseases that were treated with therapies that affect the immune system, after close investigation with a medical doctor. Of the remaining 1,310 individuals, 1,186 participants who were younger than 90 years of age as of June 30, 2011 were selected. Of these 1,186 participants, a total of 245 individuals were selected using a stratified random sampling method for sex, radiation dose and age category strata. We used the bone marrow dose as the radiation dose, which is the sum of the gamma-ray dose plus 10 times the neutron dose calculated by the recently revised dosimetry system, DS02 (22–25). A sufficient volume of blood was obtained from the 245 individuals and examined for hematopoietic functions between July 2011 and December 2013. The results of 14 of these participants were excluded from the data analysis due to an update of their disease data after blood collection. Finally, data for the proportion of CD34<sup>+</sup>Lin<sup>-</sup> cells in PBMCs were obtained for 231 participants (Table 1).

### Ethics Statement

This study was approved by the Human Investigation Committee of the RERF and was conducted according to the principles expressed in the Declaration of Helsinki. All participants provided written informed consent before examination.

### Cytokines and Antibodies

Recombinant human cKit ligand (KL), Flt3 ligand (FL), interleukin (IL)-3, IL-7 and granulocyte-colony stimulating factor (G-CSF) were purchased from PeproTech® (Rocky Hill, NJ). Erythropoietin was purchased from Invitrogen™ (Carlsbad, CA). Anti-CD3 (clone number: SK7), CD5 (UCHT2), CD7 (M-T701), CD16 (3G8), CD19 (HIB19), CD20 (2H7) and glycophorin A (GA-R2) monoclonal antibodies (mAbs) were purchased from BD Biosciences (San Jose, CA). Anti-CD34 (581 and CD56 (N901) mAbs were purchased from Beckman Coulter Inc. (Brea, CA). mAbs were used for cell surface staining according to the manufacturer's instructions.

### Cell Preparation

Peripheral blood samples were collected from 231 adult health study participants and six in-house volunteers. PBMCs were separated from approximately 7.5 ml of peripheral blood using Ficoll density gradient centrifugation (Lymphocyte Separation Medium 1077, Wako Pure Chemical Industries Ltd., Osaka, Japan).

### Stromal Cell Lines

Mouse stromal cells (MS-5) were kindly provided by Dr. Kazuhiro J. Mori (Niigata University, Niigata, Japan) (26). Generation of the mouse OP9-DL1 stromal cells, which were engineered to express the

green fluorescent protein and mouse delta-like 1, has been previously described elsewhere (27). The MS-5 and OP9-DL1 stromal cells were maintained by culturing in alpha Minimal Essential Medium (MEM; Gibco®; Life Technologies, Grand Island, NY) supplemented with  $4 \times 10^{-6}$  M 2-mercaptoethanol, penicillin-streptomycin and 10 and 20% fetal bovine serum (FBS; GE Healthcare Life Sciences/HyClone™ Laboratories, Logan, UT), respectively, at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>.

#### LDA of CAFCs and LTC-ICs

For progenitor cell culture, MS-5 stromal cells were seeded in wells (50–80% confluent) of a 384-well flat-bottom black plate (Corning). At least 4 h prior to progenitor cell sorting, culture media in each well was replaced with 50 µl alpha MEM supplemented with  $4 \times 10^{-6}$  M 2-mercaptoethanol, penicillin-streptomycin, 12.5% horse serum (Gibco) and 12.5% FBS (GE Healthcare Life Sciences/HyClone). For progenitor cell sorting, PBMCs were stained with allophycocyanin (APC)-conjugated anti-CD34 Ab and phycoerythrin (PE)-conjugated mAbs against lineage markers (anti-CD3, CD14, CD16, CD19, CD20 and CD56 mAbs) for 30 min on ice. One thousand CD34<sup>+</sup>Lin<sup>-</sup> cells were sorted into 80 wells of a 384-well plate at 20, 15, 10 and 5 cells per well (20 wells for each number of cells) with FACSAria™ II (BD Biosciences). After cell sorting, the proportion of CD34<sup>+</sup>Lin<sup>-</sup> cells in PBMCs was determined for further statistical analysis, as described below. LDA culture was maintained at 37°C in a humidified atmosphere with 5% CO<sub>2</sub>. Half of the culture media (25 µl) was changed every week. After five weeks of culture, individual wells were microscopically screened for the presence or absence of cobblestone areas. A well was considered positive when at least six cells in proximity to each other were growing underneath the stroma. Within a day after scoring CAFCs, the culture media was removed and replaced with methylcellulose media as described above. The presence of CFU-GMs and/or BFU-Es in individual wells was determined for scoring LTC-ICs after 14 days.

Progenitor frequencies of CAFCs and LTC-ICs were calculated with online analysis using ELDA software (Linz, Austria) (28), which is available on the Bioinformatics Division home page of the Walter and Eliza Hall Institute of Medical Research (Parkville, Australia). We confirmed the reproducibility of CAFC and LTC-IC assays using Spearman's rank correlation analysis between the first and second measurements among six volunteers CAFC:  $\rho = 0.83$ ,  $P < 0.05$ ; LTC-IC:  $\rho = 1.00$ ,  $P < 0.01$ .

#### LDA of T/NK Progenitors

Procedures and culture conditions for the LDA of T/NK progenitors were described previously (17). Briefly, for progenitor cell culture, OP9-DL1 stromal cells were seeded in wells (50–80% confluent) of a 384-well flat-bottom black plate. Culture media in each well was replaced with 50 µl of phenol red-free alpha MEM containing 20% knock-out serum replacement (Gibco),  $10^{-4}$  M monothioglycerol (Sigma-Aldrich® LLC, St. Louis, MO), 50 µg/ml gentamicin (Sigma-Aldrich), 10 ng/ml KL, 10 ng/ml FL and 10 ng/ml IL-7. Half of the culture media (25 µl) was changed every week. After five weeks of culture, all cells grown in wells were harvested by vigorous pipetting and washed with phosphate buffered saline (PBS) containing 2 mM EDTA, 0.01% NaN<sub>3</sub> and 1% FBS (washing buffer). For detection of T/NK and myeloid lineage progeny, cells were stained with APC-conjugated CD5, PE-conjugated CD7 and PE-Cy7-conjugated CD56 mAbs in washing buffer, as described previously (17). Absolute numbers of each progeny type per well were calculated from the number of events on a flow cytogram. A well with six or more positive events of CD7<sup>+</sup>CD5<sup>+</sup> and CD7<sup>+</sup>CD56<sup>+</sup> cells was designated as a positive well for calculation of progenitor frequencies of T and NK cells, respectively. High reproducibility of the T-cell and NK-cell assay was demonstrated in our previous report (17).

#### CFU-C Assay

CFU-GMs and BFU-Es were assayed with methylcellulose cultures as previously described elsewhere (29). Briefly, PBMCs were plated in 24-well plates at a concentration of  $2.5 \times 10^5$  cells/ml in a 0.25 ml culture containing 1.2% methylcellulose (STEMCELL™ Technologies Inc., Vancouver, Canada) with erythropoietin (6 U/ml), KL (20 ng/ml), G-CSF (20 ng/ml) and IL-3 (20 ng/ml). The methylcellulose cultures were counted after 14 days to determine the number of colonies per well.

#### Blood Cell Counts

The number of WBCs per µl of blood and the percentages of lymphocytes and monocytes in WBCs for each participant were measured with the automated hematology analyzer Sysmex® XN-1000™ (Sysmex, Kobe, Japan). These values were used to calculate the number of PBMCs per milliliter of blood and the actual numbers of CD34<sup>+</sup>Lin<sup>-</sup> cells and HSPC subtypes per milliliter of blood using the following formula: Number of cells of HSPC subtypes per milliliter of blood = number of WBCs per microliter of blood [lymphocytes (%) + monocytes (%)] × CD34<sup>+</sup>Lin<sup>-</sup> cells (%) in PBMCs × HSPC subtypes (%) in CD34<sup>+</sup>Lin<sup>-</sup> cells ×  $10^{-3}$ .

#### Data Analysis

Statistical analyses were conducted on the actual calculated number of HSPCs per unit volume of blood, designated as “count”, as well as the fractional component (percentage) of HSPCs within the CD34<sup>+</sup>Lin<sup>-</sup> cell population, designated as “proportion”. Associations of the count and proportion with sex, age at the time of examination (age) and radiation dose (dose) were investigated using multivariate linear regression. The possibility of nonlinear relationships of age and dose with each HSPC subtype was tested by including quadratic and cubic terms of age and radiation. As a result, only a linear term was selected for both age and radiation with additional confirmation of nonlinearity by comparing the parametric form of the regression curve to the nonparametric smoothing curves of residuals of the multivariate regression models. When we did not find a significant association between each HSPC subtype and radiation, we retained a linear term for radiation in the model, and the association between each HSPC subtype and other variables was evaluated. All tests were two-sided and conducted using statistical package R (The R Foundation, <http://cran.r-project.org>). A partial correlation between the proportions of two HSPC subtypes adjusted for sex, age and dose as controlling variables was analyzed using SPSS 16.01 software (SPSS®, Chicago, IL).

## RESULTS

### HSPC Counts and Proportions

Statistical analyses were conducted on the actual calculated numbers of HSPCs per unit volume of blood, designated “counts”, to assess the association of age and dose with the total capacity of HSPCs to self-renew and differentiate (Table 2). Similar analyses were performed for the fractional component of HSPCs within the CD34<sup>+</sup>Lin<sup>-</sup> cell population, designated “proportions”, to evaluate the age- and dose-related changes in lineage commitment of HSPCs (Table 2). For the CFC assays, we did not use sorted CD34<sup>+</sup>Lin<sup>-</sup> cells but used total PBMCs. However, we confirmed that nearly 100% of both CFU-GMs and BFU-Es in PBMCs resided in the CD34<sup>+</sup>Lin<sup>-</sup> cell population according to preliminary experiments using PBMCs

**TABLE 2**  
**Summary of HSPC Data**

HSPCs	No. of participants <sup>a</sup>	Range <sup>b</sup>	Median	Mean
Count(per ml)				
CD34 <sup>+</sup> Lin <sup>-</sup>	230	78–3014	698	819
CAFCs	227	0.04–303	18.6	29.6
LTC-ICs	227	0.04–291	19.2	30.3
BFU-Es	230	5.60–849	164	200
CFU-GMs	230	3.90–546	72.6	90.9
T cells	221	0.18–74	8.0	11.0
NK cells	221	1.10–103	19.8	24.5
Proportion (%)				
CAFCs	228	0.05–11.4	2.81	3.11
LTC-ICs	228	0.05–12.8	2.84	3.19
BFU-Es	231	1.30–62.4	22.6	23.7
CFU-GMs	231	0.90–39.2	10.3	11.0
T cells	222	0.05–4.6	1.19	1.27
NK cells	222	0.20–9.5	2.96	3.03

<sup>a</sup> Number of individuals whose data were successfully obtained among 231 participants in total. The numbers of participants whose data were missing mainly due to insufficient blood volume or experimental errors during measurements were: 1 for blood cell count, 3 for CAFCs and LTC-ICs and 9 for T and NK cells.

<sup>b</sup> Data with a value of 0 were converted to half the value of the detection limit for each HSPC assay.

obtained from in-house volunteers (median: 98.5%; range: 96.2–100%; n = 3). Therefore, the proportions of CFU-GMs and BFU-Es in CD34<sup>+</sup>Lin<sup>-</sup> cells were obtained by calculation from the numbers of CFU-GMs, BFU-Es and CD34<sup>+</sup>Lin<sup>-</sup> cells in PBMCs. Multivariate analyses with linear regression using age, sex and dose as variables were conducted to assess associations of log-transformed HSPC counts and proportions with these variables.

#### Sex Differences

Both counts and proportions of T-cell progenitors in females were significantly higher than those in males ( $P = 0.0067$  and  $P = 0.019$ , respectively) with multivariate analyses (Tables 3 and 4). Similarly, females showed a higher count ( $P = 0.0004$ ) and proportion ( $P = 0.002$ ) of NK-cell progenitors. These results may be concordant with

our findings that the proportions of mature naïve T cells and thymic NK cells (CD56<sup>hi</sup>CD16<sup>-</sup>CD127<sup>+</sup>) in peripheral blood lymphocytes were significantly higher in female than male survivors (data not shown). No other HSPC subtypes showed a significant difference between males and females. Furthermore, no significant sex differences were detected in the age or dose trend for any HSPC subtypes with multivariate analyses (data not shown).

#### Age-Related Changes

We observed a statistically significant, age-dependent decline in counts of all HSPC subtypes (Table 3). These trends are not surprising because the count of CD34<sup>+</sup>Lin<sup>-</sup> cells, which give rise to all populations of HSPC subtypes, decreased significantly with age ( $P = 0.0022$ ; Table 3, Fig. 1). Although the proportions of all HSPC subtypes showed a trend toward a decrease with age, only T-cell progenitors showed statistical significance ( $P = 0.010$ ; Table 4, Fig. 2), suggesting that age-associated attrition was most evident for lineage commitment of HSPCs to T cells. Of note, both T and NK lineage cells are generated from common lymphoid progenitors. However, the proportion of NK-cell progenitors showed only a marginal trend with age.

#### Dose-Related Changes

No significant association with radiation dose was obtained for the counts of CD34<sup>+</sup>Lin<sup>-</sup> cells or any subtypes of HSPCs after adjusting for sex and age effects (Table 3, Fig. 1). As for the association of the proportion of HSPC subtypes with dose, although CAFCs reflecting the self-renewal ability showed a trend toward a decrease with dose, the trend was not statistically significant ( $P = 0.18$ ; Table 4). The proportion of CFU-GMs representing myeloid commitment showed a trend toward an increase with dose, but the trend was not significant ( $P = 0.46$ ). Furthermore, contrary to our expectations, both the count and proportion of T-cell progenitors showed a trend toward an increase with dose, although these increases were not significant ( $P = 0.56$  and  $P = 0.23$ , respectively).

**TABLE 3**  
**Regression Coefficients for Variables Related to HSPC Counts<sup>a</sup>**

HSPCs	Variables			
	Intercept $\alpha$	Sex <sup>b</sup> ( $\beta_1$ )	Age ( $\beta_2$ )	Dose ( $\beta_3$ )
CD34 <sup>+</sup> Lin <sup>-</sup> cells	4.24	0.08; $P = 0.31$	-0.17; $P = 0.0022$	-0.045; $P = 0.37$
CAFCs	5.12	0.20; $P = 0.20$	-0.31; $P = 0.0047$	-0.135; $P = 0.18$
LTC-ICs	5.18	0.14; $P = 0.35$	-0.32; $P = 0.0047$	-0.134; $P = 0.19$
BFU-Es	7.38	-0.06; $P = 0.62$	-0.27; $P = 0.0020$	-0.127; $P = 0.12$
CFU-GMs	6.44	0.12; $P = 0.27$	-0.25; $P = 0.0024$	-0.003; $P = 0.97$
T cells	3.90	0.42; $P = 0.0067$	-0.44; $P = 0.0001$	0.060; $P = 0.56$
NK cells	5.02	0.38; $P = 0.0004$	-0.30; $P = 0.0001$	-0.049; $P = 0.49$

<sup>a</sup> Regression coefficients of the counts of each progenitor for sex, age and dose were obtained using the following formula:  $\text{Ln}(\text{HSPC count}) = \alpha + \beta_1 \times \text{sex} + \beta_2 \times \text{age} + \beta_3 \times \text{dose}$ .

<sup>b</sup> Sex = 1 for male and 2 for female.

**TABLE 4**  
**Regression coefficients for variables related to HSPC proportions in CD34<sup>+</sup>Lin<sup>-</sup> cells<sup>a</sup>**

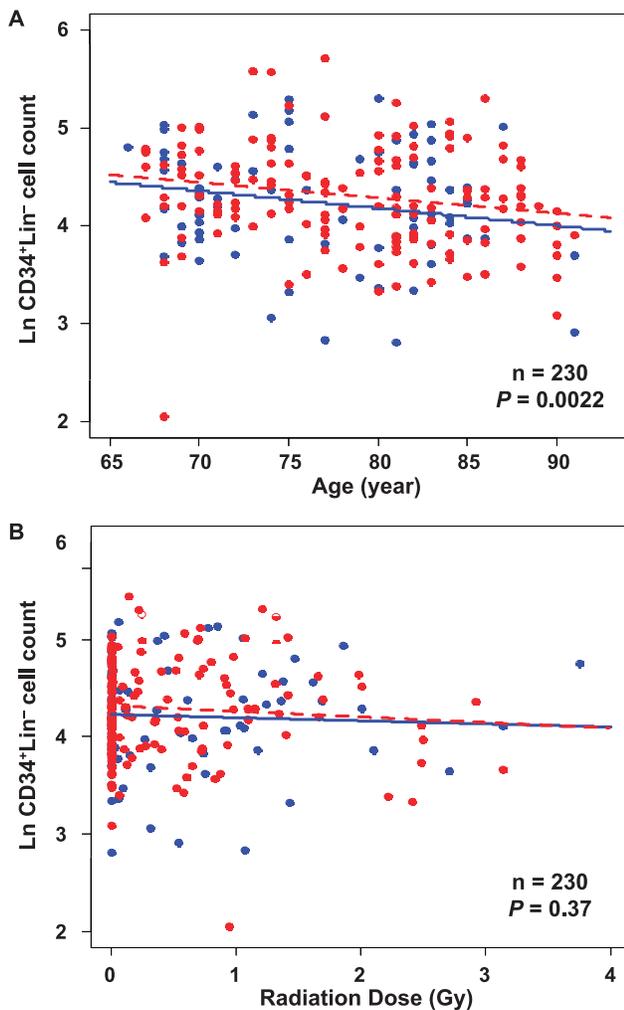
HSPCs	Variables			
	Intercept $\alpha$	Sex <sup>b</sup> ; $\beta_1$	Age; $\beta_2$	Dose; $\beta_3$
CAFCs	0.90	0.11; $P = 0.300$	-0.14; $P = 0.070$	-0.092; $P = 0.18$
LTC-ICs	0.95	0.05; $P = 0.610$	-0.14; $P = 0.066$	-0.091; $P = 0.19$
BFU-Es	3.14	-0.13; $P = 0.170$	-0.10; $P = 0.084$	-0.077; $P = 0.16$
CFU-GMs	2.20	0.05; $P = 0.580$	-0.09; $P = 0.150$	0.039; $P = 0.46$
T cells	-0.31	0.30; $P = 0.019$	-0.24; $P = 0.010$	0.101; $P = 0.23$
NK cells	0.81	0.25; $P = 0.002$	-0.10; $P = 0.092$	-0.006; $P = 0.91$

<sup>a</sup> Regression coefficients of the proportion (percentage) of each HSPC subtype in the total CD34<sup>+</sup>Lin<sup>-</sup> cell population for age, sex and dose were obtained using the following formula:  $\ln(\text{HSPC proportion}) = \alpha + \beta_1 \times \text{sex} + \beta_2 \times \text{age} + \beta_3 \times \text{dose}$

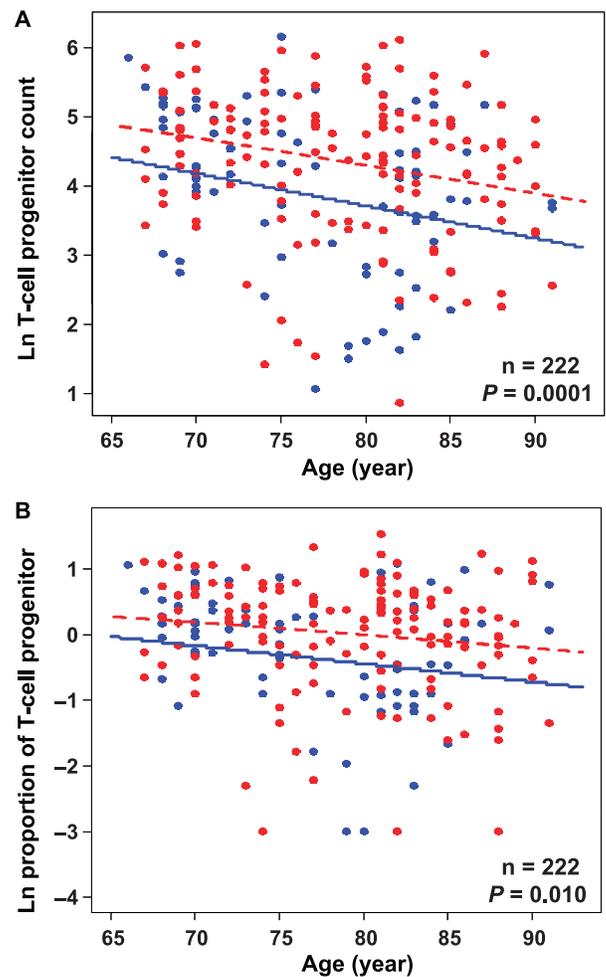
<sup>b</sup> Sex = 1 for male and 2 for female.

### Correlation between Proportions of HSPC Subpopulations

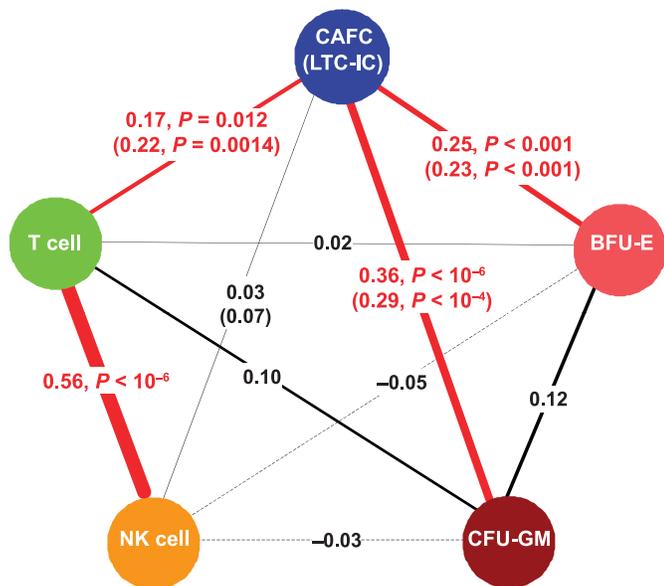
To evaluate the link between lineage commitments of HSPCs, we analyzed the partial correlation between the proportions of different HSPC subtypes using sex, age and



**FIG. 1.** Effect of age (panel A) and radiation dose (panel B) on the count of CD34<sup>+</sup>Lin<sup>-</sup> cells circulating in the peripheral blood of A-bomb survivors. The regression lines for age and the radiation dose were obtained by adjusting for dose or age, respectively according to the formula described in Materials and Methods and were plotted with a blue line for males (blue closed circles) and red line for females (red closed circles).



**FIG. 2.** Effect of age on the count (panel A) and the proportion (panel B) of T-cell progenitors in the peripheral blood of A-bomb survivors. The regression lines were obtained by adjusting for dose according to the formula described in Materials and Methods and were plotted with a blue line for males (blue closed circles) and red line for females (red closed circles).



**FIG. 3.** Partial correlation coefficients between the proportions of HSPC subtypes using sex, age and dose as controlling variables. Positive and negative correlations are indicated by solid and dotted lines, respectively ( $n = 215$ ). Statistically significant correlation coefficients are shown by red lines and numerical numbers with  $P$  values. The line width is proportional to the value of the correlation coefficient. LTC-IC data are shown in parentheses.

dose as controlling variables (Fig. 3). The partial correlation coefficients of both the count and proportion between CAFCs and LTC-ICs were approximately 0.94, because LTC-ICs are believed to be directly derived from the CAFC population. Thus, LTC-IC data are shown in parentheses following CAFC data in Fig. 3. Interestingly, significant correlations were observed between CAFCs (LTC-ICs) and BFU-Es, CFU-GMs and T-cell progenitors, whereas no significant correlations were obtained between any two of the three HSPC subpopulations: BFU-Es, CFU-GMs and T-cell progenitors. Although the correlation coefficient between CAFCs (LTC-ICs) and T-cell progenitors was not sufficiently large to declare a correlation and most correlations were weak, these findings may suggest that CAFCs (LTC-ICs) are upstream of the three lineage progenitors in the differentiation tree. The strongest correlation was observed between T-cell and NK-cell progenitors, because both lineage progenitors are thought to be directly derived from common T/NK progenitors.

## DISCUSSION

Hematopoiesis has been reported to recover autonomously to nearly normal levels within 10 weeks, even after exposure to high-dose radiation as in the case of some A-bomb survivors (2, 9). Nonetheless, radiation-associated excess risks of myeloid leukemia and myelodysplastic syndromes persist in survivors more than 50 years after exposure (30, 31). In our previous studies using humanized mice, compromised human hematopoiesis continued for

more than 10 weeks after irradiation (32, 33). However, no systematic studies have been conducted to assess whether the hematopoietic functions of HSPCs in survivors are compromised about 70 years after radiation exposure. In the current study, we found strong age effects on the numbers of functional HSPCs with advancing age of the survivors whose ages ranged from 66–91 years. This finding suggested that human HSPCs continuously senesce over the entire life. Contrary to expectations, we found no evidence supporting the hypothesis that exposure to A-bomb radiation induces premature aging of HSCs. This may imply that many years after radiation exposure and with advancing age, the number and function of HSCs in the survivors have returned to normal levels, although it is not clear whether circulating HSPCs correctly reflect the hematopoietic functions in bone marrow HSC.

All HSPC counts showed a highly significant decrease with age, because the counts of  $CD34^+Lin^-$  cells, which give rise to all HSPCs, significantly decreased with age. Although the data are not shown, the proportion of  $CD34^+Lin^-$  cells in PBMCs also significantly decreased with age. Furthermore, we found an age-dependent decrease in the CAFC count, which may indirectly reflect HSC functional status. Therefore, we presume that the number of functional HSCs may also decline with age, although we did not enumerate primitive HSCs according to either the surface phenotype (e.g.,  $CD34^+CD38^-CD45RA^-CD90^+$ ) with flow cytometry and repopulating capacity *in vivo* using humanized mice. These findings may be concordant with a previous study using humanized mice, in which it was reported that the repopulation ability of human bone marrow HSCs decreases with age (15), although the age range of our study subjects was skewed to older ages (66–91 years). However, this previous study also showed that the population size of bone marrow HSCs with specific surface phenotypes increases with age (15). However, because contradictory findings were also observed in human bone marrow HSCs by other researchers (34, 35), the exact features of age-dependent changes in human HSCs are controversial.

An age-dependent decrease in lymphoid potential in HSCs is well recognized and commonly observed in both mice (36–39) and humans (15, 34). We also found that both the count and proportion of T-cell progenitors significantly decreased with age. This suggests that the commitment of HSCs to the T-cell lineage may continuously decline with advanced age even after thymic involution. However, the proportion of NK-cell progenitors showed only a marginal decrease with age in survivors, although both types of lineage progeny are generated from common T/NK progenitors (40). These results are consistent with the findings obtained from our previous study of in-house volunteers whose ages (28–64 years) were younger than those of survivors (17). Clonal analyses of HSPCs from in-house volunteers suggested that bifurcation of T/NK co-progenitors shifts from the T-cell lineage to the NK-cell

lineage with aging. Thus, this biased lineage commitment seems to be imprinted in long-lived HSCs throughout the entire life.

Our previous study demonstrated that the proportion of mature naïve T cells in peripheral blood lymphocytes is decreased in association with radiation dose (11). A reduction in the naïve T-cell pool in the periphery may result from a decline in T-cell production in association with thymic dysfunction. The main causes of thymic dysfunction are thought to involve impairments in both HSPCs and the thymic microenvironment (41). The results of the current study are not consistent with a radiation-induced reduction in the T-cell potential of HSPCs, as mentioned above. Thus, an impaired T-cell potential in HSCs may recover to normal levels over a long period. Regarding thymic function, we have reported that T-cell receptor excision circle (TREC) numbers in both CD4 and CD8 T cells of survivors decrease with age, suggesting an age-dependent reduction in T-cell production levels in the thymus of survivors (42). The TREC numbers showed a significant correlation with the proportion of naïve cells in both the CD4 and CD8 T-cell populations. However, no significant radiation effects on TREC numbers were found in either CD4 or CD8 T cells. Again, this may be because the radiation effect on peripheral TRECs may have been diluted over a long period of time after radiation exposure. Therefore, analysis of archived biological samples including thymus, bone marrow and blood cells shortly after radiation exposure may be required to detect radiation effects on HSCs and thymus function. We are now conducting analyses of the architecture and function of archived thymus specimens embedded in paraffin blocks that were obtained from autopsied survivors.

In the current study, numerical data regarding the different subtypes of HSPCs were obtained with three different methods: CAFc/LTC-IC, T/NK-cell and CFU-GM/BFU-E assays. Interestingly, although these assays used different culture conditions, the correlations between the proportions of HSPC subtypes showed reasonable links among the types of lineage commitment. CAFc (LTC-IC) commitment showed a significant correlation with commitment to BFU-Es, CFU-GMs and T-cell precursors, whereas no significant link was observed between any two of the three HSPC subtypes. These findings suggest that the three lineage progenitors, BFU-Es, CFU-GMs and T cells, are downstream of CAFcs (LTC-ICs) in the differentiation tree, and the commitments of the three lineages are considered to be independent of each other. Furthermore, NK-cell commitment was strongly linked to T-cell commitment. This finding is in good agreement with a previously reported study where at least a portion of thymic NK cells were generated from T/NK bipotential early thymic progenitors (40). Interestingly, no significant link was found between NK cell and CAFc (LTC-IC) commitments, in contrast to the significant link between T cells and CAFcs (LTC-ICs). This suggests that thymic NK-cell

commitment may be independent of T-cell commitment at the stage of divergence from CAFcs (LTC-ICs) and that thymic NK-cell commitment occurs downstream of T-cell commitment in the thymus. These results of commitment linkage analyses appear to be reasonable and may demonstrate the validity of our *in vitro* progenitor assays for the purpose of studying *in vivo* hematopoiesis.

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