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High-dose radiation preferentially induces the clonal expansion of hematopoietic progenitor cells over mature T and B cells in mouse bone marrow

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SUMMARY

Radiation induces clonal hematopoiesis (CH) involving high-frequency somatic mutations in hematopoietic cells. However, the effects of radiation on clonal expansion of hematopoietic progenitor cells and lymphocytes remain elusive. Here, we investigate CH mutations and T cell receptor (*TCR*) and B cell receptor (*BCR*) sequences within the bone marrow cells of mice 18 months after irradiation (3 Gy) and age-matched controls. Two to six CH mutations were identified in the irradiated mice ($N = 5$), while only one of the four control mice carried a CH mutation. These CH mutations detected in the bone marrow were also identified in the splenic CD11b^{+} myeloid cell population. Meanwhile, the cumulative size of the ten largest *TCR* and *BCR* clones, as well as their clonality, did not differ significantly between irradiated and control mice. Our findings suggest that radiation preferentially induces clonal expansion of hematopoietic progenitor cells over mature lymphocytes in the bone marrow.

INTRODUCTION

Clonal hematopoiesis (CH), characterized by the expansion of genetically identical hematopoietic cell populations, is frequently observed in older adults, affecting approximately 10%–20% of individuals aged ≥70 years. CH is associated with an increased risk of developing hematologic malignancies and non-cancer diseases, such as cardiovascular disease (CVD) (Jaiswal et al., 2014, 2017; Zink et al., 2017). Studies on the atomic bomb (A-bomb) survivors have revealed positive associations between radiation exposure and the risk of developing hematologic malignancies (Ozasa et al., 2012; Hsu et al., 2013) or CVD (Ozasa et al., 2012; Yamada et al., 2004). In addition, investigations into the effects of radiation exposure have revealed a dose-dependent increase in the proportion of chromosomal aberrations in peripheral blood T cells (Kodama et al., 2001). We previously reported identical chromosomal alterations in several A-bomb survivors, suggesting clonal chromosomal aberrations originating from hematopoietic progenitor cells and mature T cells (Nakano et al., 2004; Kusunoki et al., 1995; Hamasaki et al., 2009) alongside specific T cell receptor (*TCR*) rearrangements indicative of clonal T cell expansion (Kusunoki et al., 1993).

Additionally, somatic mutation frequencies in *glycophorin-A* (*GPA*) (Kyoizumi et al., 1996) and *hypoxanthine phosphoribosyl transferase* (*HPRT*) (Hirai et al., 1995) genes increase with radiation dose. *GPA* mutations appear to stem from long-standing hematopoietic progenitor cells (Kyoizumi et al., 1996), whereas *HPRT* mutations in T and B cell colonies can be traced to a single ancestor of hematopoietic cells (Hakoda et al., 1989). Similarly, *in vivo* studies in mice have documented radiation-induced somatic mutations (Matsuda et al., 2023) and chromosomal aberrations (Hamasaki et al., 2023) in stem cell-derived colonies generated from long-term hematopoietic stem cells. However, such changes have not been extensively investigated in mature blood cell lineages, including T and B cells. Recent advances in genome sequencing, including *TCR* and B cell receptor (*BCR*) sequencing, have enabled large-scale investigation of CH and clonal expansion in mature T and B cells. We previously performed whole-exome sequencing (WES) to identify CH in a radiation-induced CH mouse model (induced by whole-body irradiation at 3 Gy) (Yoshida et al., 2022). Evaluation of bone marrow cells after 12–18 months of irradiation revealed that 11 of the 12 irradiated mice—contrary to their non-irradiated counterparts ($n = 6$)—frequently exhibited CH with recurrent mutations, particularly deletions (Yoshida et al., 2022). Most of the mutations in 2 of these 11 CH-bearing mice were confirmed to be derived from hematopoietic stem/progenitor cells based on their presence in colonies expanded from hematopoietic stem or multipotent progenitor cells of the irradiated mice *in vitro* (Yoshida et al., 2022). However, the existence of clonal mature T or B cell populations carrying CH mutations in irradiated mice remains to be elucidated.

In the present study, we aimed to develop a straightforward approach to evaluate CH originating from each

Table 1. Number of clonal hematopoiesis mutations in bone marrow cells obtained from female C57BL6 mice 18 months after 3 Gy of X-irradiation

Mouse	Autopsy age (month)	Tumor	No. of CH mutations[a] (VAF >0.02 and <0.35)	No. of SNV	No. of deletion mutations[b]
3-Gy irradiated					
F15	20	lymphoma (mesenteric)	25	24	1
F32	20	–	6	3	3
F33	20	–	2	2	0
F35	20	–	6	4	2
F53	20	left eye	3	1	2
F56	20	liver	3	2	1
Control					
F21	20	–	1	1	0
F41 (ref.)	20	–	NA	NA	NA
F42	20	–	0	0	0
F45	20	–	0	0	0

Different superscript letters indicate the statistical differences between the 3-Gy irradiated and control mice obtained using the exact Poisson regression model; the mouse bearing lymphoma (F15) was excluded from the analysis.
VAF, variant allele frequency; ref., the mouse used as the reference.
[a]Significant difference was observed between the total number of CH mutations among 3-Gy irradiated and control mice ($p = 0.0007$).
[b]Significant difference was observed between the deletion mutations in 3-Gy irradiated and control mice ($p = 0.0066$).

hematopoietic progenitor cell and mature T and B cell populations using a different mouse cohort to facilitate translation to human CH studies. We first identified CH mutations and clonal *TCR* and *BCR* sequences in the bone marrow cells of mice exposed to 3 Gy of radiation. Subsequently, we evaluated the frequencies of these mutations in mature myeloid, T, and B cell populations within splenocytes of the mice and distinguished the hematopoietic progenitor cell- and mature T and B cell-derived clones. Our findings suggest that radiation-induced CH is primarily derived from hematopoietic progenitor cells with a myeloid-biased differentiation pattern, providing mechanistic insights into the increased risk of hematologic malignancies and inflammatory diseases in individuals exposed to radiation.

RESULTS

CH mutations occur frequently in the bone marrow cells of high-dose irradiated mice

We successfully detected CH mutations after filtering low-quality sequences and variant calling with MuTect2 (McKenna et al., 2010) using a non-irradiated mouse as a reference, with variant allele frequencies (VAFs) between 0.02 and 0.35 in bone marrow cells. Targeted amplicon sequencing of DNA from the bone marrow, spleen, tail, brain, and thyroid tissues of the mice validated the CH mutations and excluded embryonic mosaic mutations that also appeared in non-hematopoietic tissues, the brain, tail, and thyroid with similar or lower VAFs. The genes harboring the CH mutations are listed in Table S1, along with their relevance to diseases as annotated in the Online Mendelian Inheritance in Man database (OMIM: https://www.omim.org). They include *Larp4b* (downregulated in patients with myeloid leukemia) and *Picalm* (fusion gene to *AF10* involved in leukemogenesis). However, none of the mutated sequences in the CH mutations detected here, including the *Larp4b* or *Picalm* mutation, are listed in the Japanese Society of Hematology Guideline of Hematologic Malignancy Genomic Diagnosis (http://www.jshem.or.jp/modules/genomgl). All mice irradiated at 3 Gy ($n = 6$) exhibited at least two CH mutations (range: 2–25), whereas only one of the four control mice carried a single mutation (Table 1). In one 3-Gy irradiated mouse that developed lymphoma in the mesenteric lymph nodes at the time of bone marrow sampling, 25 CH mutations were detected, including 24 single-nucleotide variants (SNVs) and 1 deletion mutation. Among the remaining five 3-Gy irradiated mice without lymphoma, one had a left orbital tumor and another had a liver tumor. In the irradiated mice without lymphoma, a total of 20 CH mutations

Table 2. T cell receptor sequence data in bone marrow cells obtained from female C57BL6 mice 18 months after 3-Gy X-irradiation

Mouse	Cumulative frequencies of top ten clones (%)[a]	No. of *TCR* sequences with the frequency >0.02[a]	Clonality[a]
3-Gy irradiated			
F15	14.68	0	0.0587
F32	16.01	2	0.0654
F33	19.17	1	0.0935
F35	10.19	0	0.0418
F53	19.62	2	0.0877
F56	11.19	1	0.0540
Control			
F21	16.73	1	0.0668
F41	14.77	1	0.0634
F42	20.59	2	0.0838
F45	21.93	3	0.0820

[a]No significant differences were observed in the cumulative frequencies of top ten clones, the number of *TCR* sequences with the frequency >0.02, or clonality between 3-Gy irradiated and control mice (p = 0.254, 0.297, and 0.683, respectively) analyzed using an exact Poisson regression and a permutation test, respectively.

were detected, including 11 SNVs and 9 deletion mutations. In contrast, the CH mutation detected in the control mouse was an SNV. Statistical analysis revealed significant differences in the total number of CH mutations (p = 0.0007) and the number of deletion mutations (p = 0.0066) between the 3-Gy irradiated and control mice, excluding the lymphoma-bearing mouse (Table 1).

Clone size and clonality of the mature T or B cell population in the bone marrow

Using *TCR* and *BCR* sequence analysis, we investigated the clonal expansion of individual mature T and B cells in the mouse bone marrow. In this way, largely expanded clones were detected based on the frequency of identical complementarity-determining region 3 (CDR3) nucleotide sequences (Tables 2 and 3). The mouse with lymphoma had one *BCR* (*IgH V5-D1-J2*) clone with a 0.95 frequency in the total productive *IgH* CDR sequences. Additionally, one *BCR* sequence was identified with a frequency of 0.29 in the control mouse (F21) with one CH mutation (VAF 0.02), and one was identified with a frequency of 0.21 in a 3-Gy irradiated mouse (F35) carrying six CH mutations (VAFs 0.03–0.11). The other *TCR* and *BCR* sequences in the non-lymphoma mice were not necessarily associated with the CH mutations detected via WES in bone marrow cells.

Next, we investigated the effect of high-dose irradiation on *TCR* and *BCR* clone sizes and identified the top ten clones from each mouse's bone marrow DNA (Figure 1). In mice without lymphoma, the cumulative frequencies of the largest clones did not exceed 0.3 or differ based on irradiation. Moreover, the number of sequences with the frequency >0.02, the clonality of *TCR* sequences (Table 2), or those of *BCR* sequences (Table 3) did not differ between the non-irradiated and irradiated mice.

Distribution of CH mutations in splenic myeloid, T, and B cell populations

To evaluate how cells with CH mutations differentiate into mature peripheral hematopoietic cell populations, we assessed the mutation frequencies in the $CD3^+$ T, $CD19^+$ B, $NK1.1^+$ natural killer (NK), and $CD11b^+$ myeloid cell fractions from the spleens of the non-lymphoma mice by targeted amplicon sequencing (Table 4). In most CH mutations, VAFs were higher in the myeloid fraction than in the lymphoid fractions, indicating myeloid-dominant differentiation. Additionally, the VAFs (even ~0.01) of all mutant alleles identified in the myeloid cell population were significant in the targeted amplicon sequencing (more than $10^4\times$ coverage). Hence, the cells carrying these mutations were derived from progenitor cells prior to differentiation into mature myeloid, T, or B cells and were therefore irrelevant to the expansion of *TCR* or *BCR* clones detected in the bone marrow of these mice. In the mice without lymphoma, all mutant clones expanded in the bone marrow and developed prior to *TCR* or *BCR* rearrangement. Moreover, for 19 out of 21 CH mutants (including one from a control mouse), the proportion of $CD11b^+$ myeloid cells was higher than that in total splenocytes (Figure 2), suggesting that the splenic progeny of CH mutants is frequently biased toward myeloid cells.

In the mouse with lymphoma (F15 mouse), the bone marrow cells contained predominantly lymphoblasts and $B220^+$ cells, some of which were $CD11b^+$ while most were negative for surface IgM, CD19, or BP-1 (Figure S1A); the $B220^+$ cells were also positive for cytoplasmic IgM and TdT (Figure S1B). In addition to the large *BCR* clone, these phenotypes indicated that a malignant B cell population was derived from a lymphocyte partially acquiring CD11b and lacking CD19 expression; the VAFs of the CH mutations evaluated in the $CD11b^+$ splenocyte subset of this mouse (Table S2) were moderately correlated (r = 0.50) with those in the bone marrow, which is largely consistent with these phenotypes.

Table 3. B cell receptor sequence data in bone marrow cells obtained from female C57BL6 mice 18 months after 3-Gy X-irradiation

Mouse	Cumulative frequencies of top ten clones (%)[d]	No. of *BCR* sequences with the frequency >0.02[d]	Clonality[d]
3-Gy irradiated			
F15[a]	96.39	1	0.9479
F32	4.56	0	0.0225
F33	13.05	1	0.0539
F35[b]	28.88	1	0.2155
F53	9.36	0	0.0452
F56	16.41	2	0.0944
Control			
F21[c]	37.42	1	0.2896
F41	5.50	0	0.0316
F42	9.45	1	0.0457
F45	14.88	1	0.0591

[a]The mouse exhibited one clone with a frequency of 0.95 in productive gene rearrangements.
[b]The mouse exhibited one clone with a frequency of 0.21 in productive gene rearrangements.
[c]The mouse exhibited one clone with a frequency of 0.29 in productive gene rearrangements.
[d]No significant differences were observed in the cumulative frequencies of top ten clones, the number of *BCR* sequences with the frequency >0.02, or clonality between 3-Gy irradiated and control mice (p = 0.746, 0.906, and 0.667, respectively) analyzed using an exact Poisson regression and a permutation test, respectively; the mouse bearing lymphoma (F15) was excluded from the analysis.

DISCUSSION

Our previous WES-based CH identification study (Yoshida et al., 2022) showed that all CH mutations were detectable in single-cell colonies propagated *in vitro* from hematopoietic stem cells or multipotent progenitors in the bone marrow of two 3-Gy irradiated mice. This suggests that radiation exposure may frequently induce CH mutations in these hematopoietic progenitor cells. In the current study, CH mutations were identified in all 3-Gy irradiated mice and one non-irradiated mouse. While CH mutations were detectable in $CD11b^+$ splenocytes, they were far less frequent in clonal *TCR* or *BCR* sequences in the 3-Gy irradiated mice. These findings suggest that high-dose radiation exposure more effectively induces clonal expansion in hematopoietic progenitor cells compared to mature T or B cells in the mouse bone marrow.

CH cases with *DNMT3A*, *TET2*, *ASXL1*, *TP53*, and *PPM1D* mutations are frequently observed in older adults (Jaiswal et al., 2014; Zink et al., 2017), while *TP53* and *PPM1D* SNVs are associated with CH cases receiving radiotherapy or chemotherapy (Hagiwara et al., 2023; Bolton et al., 2020; Coombs et al., 2017). However, none of these CH mutations were identified in our present and previous (Yoshida et al., 2022) mouse studies. This discrepancy in results from studies evaluating healthy mice versus patients with cancer is likely due, in part, to the absence or presence of mutations associated with the risk of adverse health conditions before myeloablative treatment. Studies of myelodysplastic syndromes in proximally exposed A-bomb survivors (i.e., survivors exposed to high doses of radiation) revealed mutations in genes distinct from those found in non-exposed cases (Taguchi et al., 2020). These findings suggest that radiation-induced CH may involve a unique spectrum of mutated genes different from those associated with sporadic CH. However, further studies are essential to test this hypothesis by comparing the characteristics of CH mutations between blood samples from patients with cancer who underwent radiotherapy and A-bomb survivors exposed to high-dose radiation without preexisting cancer.

The present study revealed that 3-Gy irradiation did not elicit significant effects on T cell clonality in the bone marrow. This is consistent with a previous study that reported *TCR* sequencing of blood samples in CBA/Ca mice 6 months after 1-Gy irradiation (Candéias et al., 2017). Moreover, radiation reportedly induces thymic deficits, including a long-term decrease in thymocyte production in C57BL6 mice (Xiao et al., 2017). However, the findings of this study suggest that the radiation-induced reduced thymic function may not significantly impact T cell clonality, particularly within the bone marrow. Given that clonal cell expansion has implications for malignancy development, the exclusive clonal expansion from hematopoietic progenitor cells following 3-Gy irradiation may support epidemiological findings suggesting a higher prevalence of hematologic malignancies in myeloid lineages than T cell lineages among high-dose-exposed A-bomb survivors (Preston et al., 2012). Moreover, mice irradiated with high doses during infancy develop immature-type T cell malignancies (Shang et al., 2014; Blyth et al., 2015), distinct from the clonal expansion observed in mature T cells. Additionally, in the current study, radiation exposure did not exert discernible effects on *BCR* clonality after exclusion of the lymphoma-bearing mouse. By tracking CH mutations in splenocyte subpopulations, we identified mutations distributed in B and myeloid cells, indicating an origin from progenitor cells prior to differentiation into mature myeloid, T, or B cells. Taken together, the approaches employed in this study hold promise for the distinctive identification of hematopoietic progenitor- and mature T or B cell-derived CH, contributing to a deeper

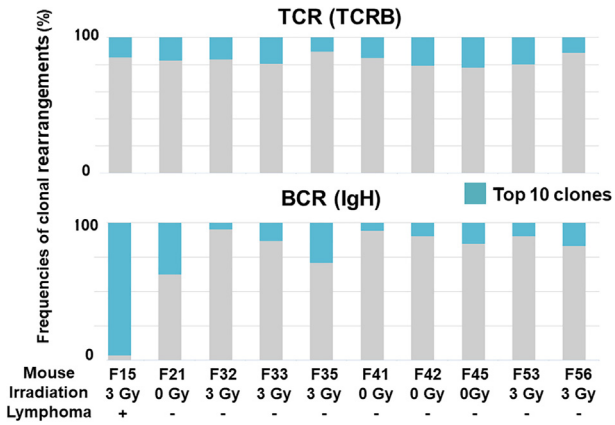

Figure 1. Cumulative frequencies of the most common top ten *TCR* and *BCR* clones in the bone marrow of female C57BL6 mice 18 months after 3 Gy of X-irradiation and age-matched controls

The top ten *TCR* (upper) and *BCR* (lower) clones for each bone marrow DNA sample from female C57BL6 mice 18 months after 3 Gy of X-irradiation ($N = 6$) and age-matched controls ($N = 4$). The blue bars represent the proportion of genes rearranged in the top ten *TCR* and *BCR* clones in the total productive gene rearrangements for each mouse. No significant difference was observed in the percentage of the top ten *TCR* or *BCR* clones between 3-Gy irradiated and control mice without lymphoma.

understanding of the varied long-term outcomes in individuals who develop CH subsequent to A-bomb exposure.

To confirm the absence of radiation-related risk for chronic lymphocytic leukemia (Fujihara et al., 2022), several studies have provided evidence regarding the radiation-induced clonal expansion of mature T and B cells (Nakano et al., 2004; Kusunoki et al., 2003; Neriishi et al., 2003; Iwanaga et al., 2009). This stems from studies exploring the long-term alterations in TCR Vβ repertoires of CD4 memory T cell populations indicating skewed TCR Vβ families in the peripheral blood of radiation-exposed people (Kusunoki et al., 2003). This is further supported by the increased prevalence of clonal chromosomal aberrations in memory T cells (Nakano et al., 2004) and the incidence of multiple myeloma and monoclonal gammopathy of undetermined significance in A-bomb survivors (Hsu et al., 2013; Neriishi et al., 2003; Iwanaga et al., 2009). Moreover, cytomegalovirus infection, associated with clonally expanded CD8 T cell populations in humans (Khan et al., 2004; Sylwester et al., 2005), is also prevalent among all evaluated A-bomb survivors (Hakoda et al., 2006). Nonetheless, our current study effectively differentiated between the clonal expansion of hematopoietic progenitor cells and mature T or B cells by assessing CH mutations in populations differentiated into different cell lineages and identifying T or B cell clones through *TCR* and *BCR* sequencing.

In this study, most mutant clones exhibited predominant myeloid differentiation with minimal T cell differentiation in the spleen (Table 4; Figure 2). Previously, we observed a skewed distribution of CH clones toward bone marrow granulocytes (Yoshida et al., 2022). This biased differentiation of hematopoietic progenitor cells toward myeloid cells could be linked to inflammatory blood phenotypes, such as an elevated myeloid/lymphoid ratio (Yoshida et al., 2019) and a reduced naive CD4 T cell population accompanied by elevated tumor necrosis factor alpha levels (Kusunoki et al., 2010) in survivors of A-bomb exposure. In addition, CH is promoted by inflammatory conditions, such as obesity (Pasupuleti et al., 2023), atherosclerosis (Heyde et al., 2021), and aging (Shlush, 2018), in general human populations. It is, therefore, plausible that the CH identified in the present mouse study resulted in an abundant myeloid cell population in the periphery during post-radiation aging and related inflammatory conditions.

In conclusion, our study provides valuable insights into the impact of high-dose radiation exposure on hematopoietic progenitor cells and mature T and B cell populations in mice. We have demonstrated that radiation exposure induces clonal expansion primarily in hematopoietic progenitor cells, as evidenced by the prevalence of CH mutations in irradiated mice compared to controls. These findings underscore the differential susceptibility of hematopoietic progenitor and mature lymphoid cells to radiation-induced genomic alterations, with implications for understanding the pathogenesis of hematologic malignancies in irradiated individuals. Moreover, our study highlights the importance of further investigations to elucidate the underlying mechanisms driving clonal expansion in response to radiation exposure and its relevance to human health, particularly in the context of cancer treatment and environmental exposure. By elucidating the dynamics of CH development and its association with radiation exposure, our research lays the groundwork for future studies aimed at identifying preventive strategies and therapeutic interventions for radiation-induced hematologic disorders.

Data limitations and perspectives

Despite an increased incidence of immature B cell lymphomas in mice (Tachibana et al., 2020), our study observed only one lymphoma case out of six irradiated mice, the classification of which as either sporadic or radiation-associated lymphomagenesis is challenging. Furthermore, the findings of the present murine-based study do not align with observations of clonal lymphocyte expansion in radiation-exposed humans. These discrepancies may be partially attributed to the small mouse sample size in this study; however, differences in environmental conditions between humans decades ago and contemporary laboratory mice

Table 4. VAFs of CH mutations in bone marrow cells and splenocyte subpopulations from the mice without lymphoma

Mouse	Irradiation	Mutation		Bone marrow cells	Splenocytes			
		Type	Gene		CD3	CD19	NK1.1	CD11b
F32	3 Gy	Del	*Larp4b*	0.13	0.00	0.00	0.03	0.08
		Del	*Gm904*	0.13	0.00	0.01	0.02	0.04
		Del	*Rnaset2a*	0.06	0.00	0.00	0.03	0.09
		SNV	*Wfdc15b*	0.06	0.00	0.00	0.03	0.10
		SNV	*Med13l*	0.11	0.00	0.00	0.02	0.08
		SNV	*Ankrd27*	0.11	0.00	0.00	0.02	0.10
F33	3 Gy	SNV	*Cep152*	0.08	0.00	0.00	0.03	0.03
		SNV	*Tenm4*	0.07	0.00	0.00	0.02	0.03
F35	3 Gy	SNV	*Trim58*	0.10	0.01	0.03	0.03	0.20
		SNV	*Krt28*	0.07	0.00	0.00	0.00	0.03
		Del	*Tmem251*	0.03	0.00	0.00	0.00	0.03
		SNV	*Serpinb6d*	0.09	0.00	0.02	0.03	0.21
		SNV	*Oas1d-Rph3a*	0.06	0.00	0.01	0.00	0.01
		Del	*Picalm*	0.03	0.00	0.00	0.00	0.02
F53	3 Gy	Del	*Gm9898*	0.20	0.05	0.04	0.06	0.30
		Del	*Il17rb*	0.29	0.05	0.04	0.06	0.33
		SNV	*Snx25*	0.04	0.00	0.04	0.03	0.06
F56	3 Gy	Del	*Epb41l4b*	0.03	0.04	0.07	0.03	0.02
		SNV	*Ero1l*	0.07	0.01	0.01	0.01	0.09
		SNV	*Rfc3*	0.18	0.00	0.00	0.01	0.07
F21	0 Gy	SNV	*Kmt2e*	0.02	0.00	0.00	0.01	0.01

Del, deletion mutation; SNV, single-nucleotide variant.

could also influence the development of lymphocyte clones before and after radiation exposure. Epidemiological studies analyzing *TCR* and *BCR* sequences in A-bomb survivors, together with experiments involving irradiated mice exposed to foreign antigens, such as viruses, may provide insights into the lymphocyte clonalities after radiation exposure.

METHODS

Mice and irradiation

Female C57BL/6 mice (6 weeks old) purchased from Japan Clea Co. (Tokyo, Japan) were housed in autoclaved microisolator cages in a specific pathogen-free facility and fed a sterile standard chow *ad libitum*. Mice (7-week-old; $N = 6$) were subjected to whole-body irradiation with 3 Gy of X-rays using a CP-160 X-ray generator (Faxitron Bioptics, Tucson, AZ, USA; 160 kV peak energy, 6.2 mA, 0.5 mm Al and 0.21 mm Cu filters; 56 kV effective energy) and an AE-1322 dosimeter (Ouyo Giken, Inc., Tokyo, Japan) at a rate of 0.8 Gy/min. Age-matched sham-irradiated mice ($N = 4$) were used as controls. The study design was approved (no. 2020-01) by the Experimental Animal Care Committee of Radiation Effects Research Foundation.

All animal handling procedures were approved by the Experimental Animal Care Committee of the Radiation Effects Research Foundation (no. 2020-01).

Tissue and organ sample collection

Approximately 0.1 mL of venous blood samples were collected from isoflurane-anesthetized mice by orbital sinus bleeding 1 week before and 1, 3, 6, 9, 12, 15, and

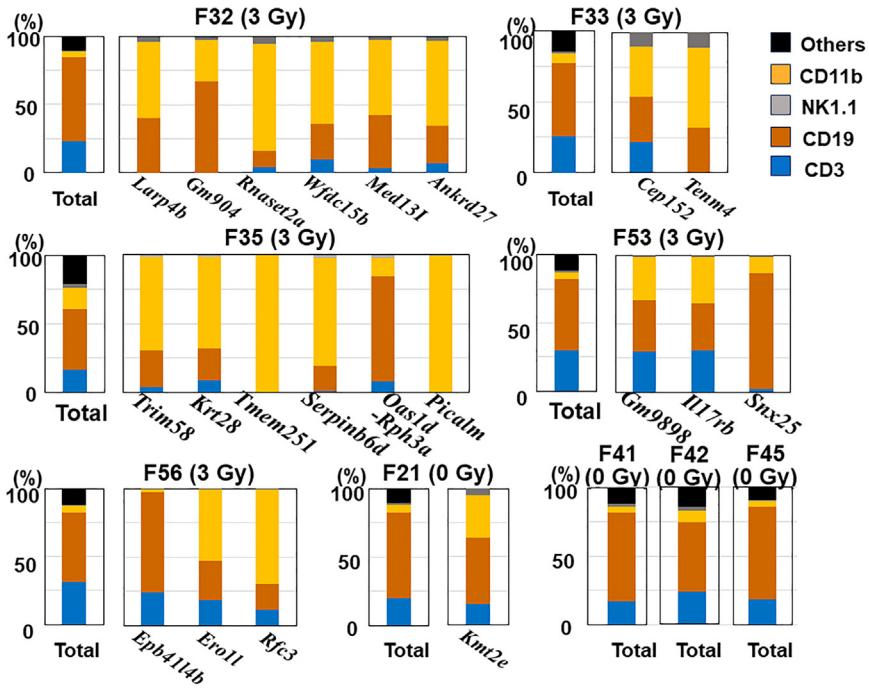

Figure 2. Estimated percentages of CD3$^+$, CD19$^+$, CD11b$^+$, and NK1.1$^+$ cells in the spleen and their ratios in CH-mutant splenocytes of female C57BL6 mice 18 months after 3 Gy of X-irradiation and age-matched controls

Percentages of CD3$^+$ (blue), CD19$^+$ (brown), CD11b$^+$ (orange), and NK1.1$^+$ (gray) cells and the other cells (black) in total splenocytes were analyzed by the FlowJo software based on the data obtained from splenocyte sorting on a FACSAria II cell sorter (representative flow cytometry patterns are shown in Figure S2). The percentages in each mouse are represented by a bar graph indexed as total. Bar graphs indexed as gene names represent proportions (%) of each cell fraction in the total of CD3$^+$ (blue), CD19$^+$ (brown), NK1.1$^+$ (gray), and CD11b$^+$ (orange) cells with a CH mutation, which was calculated by multiplying the number of cells in each cell subset analyzed by the FlowJo software multiplied by the variant allele frequency (VAF) in each cell subset (shown in Table 4) multiplied by 2 (assuming that all analyzed cells are diploid) and further divided by a total of the numbers of CD3$^+$, CD19$^+$, NK1.1$^+$, and CD11b$^+$ cells.

18 months after irradiation. The mice were then euthanized by isoflurane anesthesia, and splenocytes and bone marrow cells were isolated from the spleen and femur, respectively. These cells were resuspended in fetal bovine serum (FBS; HyClone, Cytiva) containing 10% dimethyl sulfoxide in cryotubes and cryopreserved in liquid nitrogen until DNA extraction or sorting experiments. The cryopreserved cells were thawed rapidly in a water bath at 37°C, washed twice with cold RPMI1640 medium (Nacalai Tesque, Inc., Kyoto, Japan) containing 10% FBS, and applied for DNA extraction or sorting experiments. Tail, brain, and thyroid tissue sections were obtained from cadavers frozen at −80°C and used for targeted amplicon sequencing. We also used mesenteric lymph nodes obtained from the cadaver of an irradiated mouse that had mesenteric lymphoma at the time of necropsy.

Cell sorting

Splenic CD3$^+$ T cells, CD19$^+$ B cells, NK1.1$^+$ NK cells, and CD11b$^+$ myeloid cells were sorted after exclusion of DAPI-stained dead cells and Ter119$^+$ erythroid cells, using a FACSAria II cell sorter (BD Biosciences, Franklin Lakes, NJ, USA). The gating strategies for cell sorting are shown in Figure S2, with representative flow cytometry patterns of the splenocyte subsets of F41. All antibodies used are presented in Table S3. The sorted cells were then used for targeted amplicon sequencing.

Phenotype analyses of bone marrow cells

The smear of bone marrow cells from the mouse with lymphoma was fixed with methanol (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), stained with 1 mL of Giemsa solution (FUJIFILM Wako) and 3 mL of PBS (pH 6.8), and visualized using an OLYMPUS DP28 digital microscope camera equipped with cellSens Standard software (Olympus Corporation, Tokyo, Japan). Cell surface expression of CD3, CD19, B220, IgM, BP-1, and CD11b and intracellular expression of IgM and TdT were analyzed by flow cytometry using MACSQuant Analyzer 10 (Miltenyi Biotec, Bergisch Gladbach, Germany) and the FlowJo software (TreeStar, Ashland, OR, USA). The FIX & PERM Cell Fixation & Cell Permeabilization Kit (Thermo Fisher Scientific, Waltham, MA, USA) was used according to the manufacturer's instructions. The gating strategies are shown in Figure S2, and the antibodies are listed in Table S3.

Deep WES and mutation validation

DNA samples were extracted from bone marrow cells of irradiated ($N = 6$) and control mice ($N = 4$) using a QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany). DNA samples were divided into four parts and subjected to WES, target amplicon sequencing, and *TCR* and *BCR* sequence analyses. DNA sequencing libraries were prepared by a third-party company (Macrogen Japan, Tokyo, Japan) using the SureSelect XT Reagents (Agilent Technologies, Santa Clara,

CA, USA). WES was conducted on an Illumina NextSeq platform (San Diego, CA, USA) with a deep depth (500× coverage) to identify CH mutations with a low VAF of 0.02. Sequence reads (fastq files) were mapped using the Burrows-Wheeler Alignment tool onto a mouse reference genome (mm10) and sorted using SAMtools (Li and Durbin, 2009a; Li et al., 2009b). PCR-duplicate reads were identified and removed using the MarkDuplicates (Picard) tool (McKenna et al., 2010). SNVs or small insertions or deletions were identified and assessed for quality in the sequence data using MuTect2 (McKenna et al., 2010), using a non-irradiated control mouse as a reference. Specifically, only CH mutations with a VAF of 0.02–0.35 were selected, while suspected germline variants were excluded based on a high VAF > 0.35. Variants supported by $\leq$ 5 reads or detected in most mice were removed from further analyses to reduce artifacts.

The frequencies of identified CH mutations were validated using the DNA samples from bone marrow cells by target amplicon sequencing (Illumina MiSeq, an average depth of 30,000 or more) with specifically designed primers to amplify gene regions containing the mutated sequences. Nextera XT Index Kit (Illumina) was used to attach dual indices and sequencing adaptors in the sequencing library preparation. The cell lineage specificity of the CH mutations was also assessed for bone marrow cells and splenocytes, as well as the tail, brain, and thyroid tissues. Specifically, amplicon sequencing of DNA from these tissues was performed to determine whether the CH mutations are hematopoietic cell specific or embryonic mosaic.

TCR and BCR sequence analyses

DNA samples from bone marrow were sent to Adaptive Biotechnologies (Seattle, USA); *TCR* and *BCR* sequence analyses were performed using PCR amplification of rearranged *Tcrb* and *IgH* genes. The amplified library was then loaded on an Illumina HiSEQ sequencer system (Illumina) and identified with the rearranged VDJ sequence spanning the length of the CDR3 region (Adaptive Biotechnologies). Adaptive's processing pipeline was applied to each sample. Redundant sequencing (average of 10 copies per template) allowed simple clustering to remove PCR and sequencing errors. The inline synthetic template controls were then used to remove residual PCR bias for each template (Carlson et al., 2013). The numbers of *TCR* and *BCR* sequence reads examined for the female C57BL6 mice are shown in Table S4.

Statistical analysis

Differences in the number of CH mutations and deletions between the irradiated and control mice were assessed based on an exact Poisson regression using SAS 9.4 (SAS Institute, Inc., Cary, NC, USA) after excluding one 3-Gy irradiated mouse with lymphoma. The diversity was obtained by dividing the number of distinct CDR3 sequences by the number of total CDR3 sequences in inframe CDR3 sequences, and the clonality was defined as the inverse of normalized Shannon's entropy (Stewart et al., 1997). These immunological variables were calculated in the TCRB immunoSEQ Analyzer by Adaptive Biotechnologies. Differences in the cumulative frequencies of top ten *TCR* clone sequence data and clonalities between the irradiated and control groups were assessed using a permutation test excluding one 3-Gy irradiated mouse with lymphoma. The correlation between VAFs of bone marrow cells and $CD11b^+$ splenocytes from the lymphoma mouse (F15) was calculated with Spearman's correlation coefficient. All permutation tests, the calculation of the Spearman's correlation coefficients, and additional statistical analyses were performed using R 4.3.1.

RESOURCE AVAILABILITY

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Materials availability

No material available.

Data and code availability

Data supporting the conclusions are available in the manuscript and its supplemental information. The accession number for the whole exome sequence data reported in this paper is DDBJ: DRA018081. Source data are also provided in this paper.

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AUTHOR CONTRIBUTIONS

K.Y. and Y.K. designed the research and conducted the experiments. Y.S., K.H., T.T., A.U., and M.M. analyzed the data. K.Y., A.U., T.T., M.M., S.K., and Y.K. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DECLARATION OF GENERATIVE AI AND AI-ASSISTED TECHNOLOGIES IN THE WRITING PROCESS

The authors do not use generative AI in scientific writing.

SUPPLEMENTAL INFORMATION

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