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Mtf-1 lymphoma-susceptibility locus affects retention of large thymocytes with high ROS levels in mice after γ-irradiation

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Abstract

Mouse strains exhibit different susceptibilities to γ-ray-induced thymic lymphomas. Our previous study identified *Mtf-1* (metal responsive transcription factor-1) as a candidate susceptibility gene, which is involved in the radiation-induced signaling pathway that regulates the cellular reactive oxygen species (ROS). To reveal the mechanism for the increased susceptibility conferred by *Mtf-1* locus, we examined early effects of γ-ray on ROS levels *in vivo* and its difference between *Mtf-1* susceptible and resistant congenic mice. Here, we show the detection of clonally growing thymocytes at 4 weeks after irradiation, indicating the start of clonal expansion at a very early stage. We also show that large thymocytes with higher ROS levels and a proliferation capacity were more numerous in the *Mtf-1* susceptible mice than the resistant mice when examined at 7 days after irradiation, although such tendency was not found in mice lacking one allele of *Bcl11b* tumor suppressor gene. This high retention of the large thymocytes, at a high risk for ROS-induced mutation, is a compensatory proliferation and regeneration response to depletion of the thymocytes after irradiation and the response is likely to augment the development of prelymphoma cells leading to thymic lymphomas.

Keywords: Lymphoma susceptibility; Clonal proliferation; ROS; γ-Irradiation; *Mtf-1*

Fractionated whole-body γ-irradiation (4 × 1.75 Gy at weekly intervals) to mice renders the thymus atrophic by inducing thymocyte apoptosis, and atrophic thymuses develop thymic lymphomas, though the incidence varies among mouse strains [1–3]. Prevention of the lymphoma development was demonstrated by transfer of unirradiated bone marrow cells to irradiated mice, which was presumed to supplement intrathymic pre-T cells [4], within 1 week after the last irradiation but not 1 month or later [5,6]. On the other hand, impaired thymocytes at 2 weeks after irradiation, when transferred to thymus of unirradiated mice, were able to develop into lymphomas [7]. These data suggest the generation of 'prelymphoma' cells in the atrophic thymus at a very early stage.

BALB/c and C57BL/6(B6) mouse strains are highly susceptible to radiogenic thymic lymphomas whereas MSM and C3H strains are resistant [8–10]. Our previous genetic study using BALB/c and MSM strains identified *Mtf-1* (metal responsive transcription factor-1) [11,12] as a candidate susceptibility gene to γ-ray-induced thymic lymphomas [13]. The two different *Mtf-1* alleles, BALB/c encoding the serine-type MTF-1 and MSM encoding the proline-type, exhibited distinct transcriptional activation and responses to ionizing radiation (IR).

Exposure of cells to IR leads to production of reactive oxygen species (ROS) in irradiated cells and their progeny which are thought to be the main cause for the delayed genomic instability [14–16]. In response to physiological

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growth stimulation, the level of ROS also increases and the increase is sufficient for significantly damaging DNA [17–19]. The elevation of ROS activates p53 signaling pathway which in turn controls the ROS level, proliferation, and apoptosis [20,21]. MTF-1, activated by heavy metals such as zinc, is also included in the radiation-induced signaling pathway that responds to and regulates the cellular ROS level [22]. We reasoned that ROS in thymocytes after IR may differ in amount between congenic mice of the different *Mtf-1* genotype. Since ROS is a well-known mutagen contributing generation of more advanced precancerous cells, the number of prelymphoma cells generated may differ between them. In this paper, we examine clonal growth and the ROS levels in thymocytes between susceptible and resistant congenic lines after IR.

Materials and methods

Mice and irradiation. The congenic mouse strain (line-5) of BALB/c background used in this study carried an MSM-derived chromosomal region spanning an approximately 4 Mb interval between *Nds2* and *D4Mit336* on chromosome 4. The mice were obtained by mating of the previously used congenic strain (line-3) [13] with BALB/c after genotyping with nine microsatellite markers within this region. BALB/c mice were purchased from CLEA Japan Inc. (Tokyo, Japan). Mice used in this study were maintained under specific pathogen-free conditions in the animal colony of the Niigata University. Genotyping was carried out with PCR as described previously [13]. Mice were given a whole-body dose of 3 Gy at a dose rate of 1 Gy/min from a broad-beam cesium-137 source when they were at age of 8 weeks. The Ethics Committee for Animal Experimentation of Niigata University approved all experimental procedures involving the mice.

Flow-cytometric analysis and cell sorting. Progeny obtained by mating between the heterozygous congenic mice were irradiated and thymus was isolated at 3, 5, 7, and 14 days after irradiation. Flow-cytometric analysis was performed as previously described [23,24]. In brief, single cell suspensions of thymocytes were prepared from thymus and, $1–2 \times 10^6$ cells were incubated with monoclonal antibodies (mAbs) in phosphate-buffered saline containing 2% fetal calf serum and 0.2% NaN_3 for 20 min at 4 °C. The following monoclonal antibodies were purchased from eBioscience: anti-CD4-FITC or -APC (RM4-5) and anti-CD8-APC (53-6.7). To prevent nonspecific binding of mAbs, we added CD16/32 (93; eBioscience) before staining with labeled mAbs. Dead cells and debris were excluded from the analysis by appropriate gating of FSC and SSC.

ROS levels were determined by incubating the thymocytes with 10 μg/ml dichlorodihydrofluorescein diacetate (DCFH-DA, Molecular Probes, Eugene, OR) for 15 min at 37 °C in the dark. Cells were then placed on ice and kept in the dark until analysis, which was carried out within 30 min. The percent of dead cells was determined by the uptake of propidium iodide (PI) (10 μg/ml) and excluded from analysis. A minimum of 10,000 cells for each sample were analyzed by a FACScan (Becton–Dickinson) flow cytometer. Data were analyzed using the Flow-Jo software (Tree-Star, Inc.).

For BrdU incorporation experiments, we injected mice at 5 days after γ-irradiation at 1.5 Gy intraperitoneally with 100 μl of BrdU solution (10 mg/ml) and thymus was isolated 1 h after. Thymocytes were prepared from the thymus and analyzed with the use of the BD Pharmingen BrdU Flow Kit according to manufacturer's instruction. In brief, cells were suspended at a concentration of $1–2 \times 10^6$ cells/ml, fixed, permeabilized, and incubated with a murine anti-BrdU antibody for 60 min on ice. After washing, cells were incubated with FITC-conjugated goat anti-mouse antibodies for 30 min on ice, washed, and resuspended in PBS containing 20 μl of the 7AAD solution. Cells were resuspended in staining buffer and analyzed by FACScan. As for separation of large and small thymocytes, sorting was carried out on a FACSAria (BD Biosciences).

Nested PCR assay and estimation of deletion frequency. Genomic DNA was isolated from the thymus of mice at 28 days after irradiation. The nested PCR assay for *Bcl11b*/*Rit1* internal deletions was performed as described previously [25]. First PCR was done with outer primers F1 and R1 for 30 cycles, and the second PCR was done for another 30 cycles with inner primers F1-2 and R1-2. Reaction mixtures were then analyzed by electrophoresis on agarose gels. Sequence analysis was performed to confirm that DNA in the band consisted of *Bcl11b*/*Rit1* recombinant molecules.

Western blotting. The large and small thymocytes were separated by flow cytometry and analyzed as described previously [23]. Anti-p53 (#9282) was purchased from Cell Signaling Technology.

Results

Clonally growing thymocytes in mice after irradiation

Fig. 1A shows the number of thymocytes in BALB/c mice at various days after irradiation. The number on average at 5 days after was less than 10% of that in unirradiated mice, and then increased greatly in approximately a half of thymuses at 7 days after. The numbers at 10 and 28 days post-irradiation were similar but generally less than those in unirradiated mice. The proliferation of thymocytes seen from 5 to 10 days after is probably a compensatory reaction of thymocytes to depletion of the cells after irradiation.

Our previous study showed that there are a considerable number (10^3–10^4) of thymocytes in unirradiated thymus with intragenic deletions of *Bcl11b*/*Rit1*, a tumor suppressor gene for thymic lymphomas [23,25,26], because these deletions are frequently generated by aberrant V(D)J recombination during the thymocyte development [25]. Hence, if a thymocyte with the mutation preferentially proliferates in atrophic thymus following irradiation, the clonal growth should be detectable by examining *Bcl11b* intragenic deletions with nested PCR. DNA was extracted from thymus in mice at 28 days after irradiation and examined with nested PCR (Fig. 1B). The bands of *Bcl11b* intragenic deletions were detected in approximately a half of thymuses under these PCR condition (see Materials and methods). Although the intensities varied, the sizes of the bands were similar to that of the lymphoma DNA diluted 10^2- to 10^3-fold with brain DNA. Assuming that the thymus contained 3×10^7 cells, the number of thymocytes with *Bcl11b* mutations was estimated from the band intensities as approximately 10^5 in a thymus. The result indicates the presence of clones in some of thymuses at 28 days after irradiation, which suggests that the clonal growth of possible prelymphoma cells starts at a very early stage during the lymphoma development.

Detection of ROS in thymocytes in vivo

Radiation has been shown to increase ROS levels in *in vitro* irradiated cells and their progeny [14,15,27]. However, a few studies investigate the *in vivo* effect of IR on the ROS

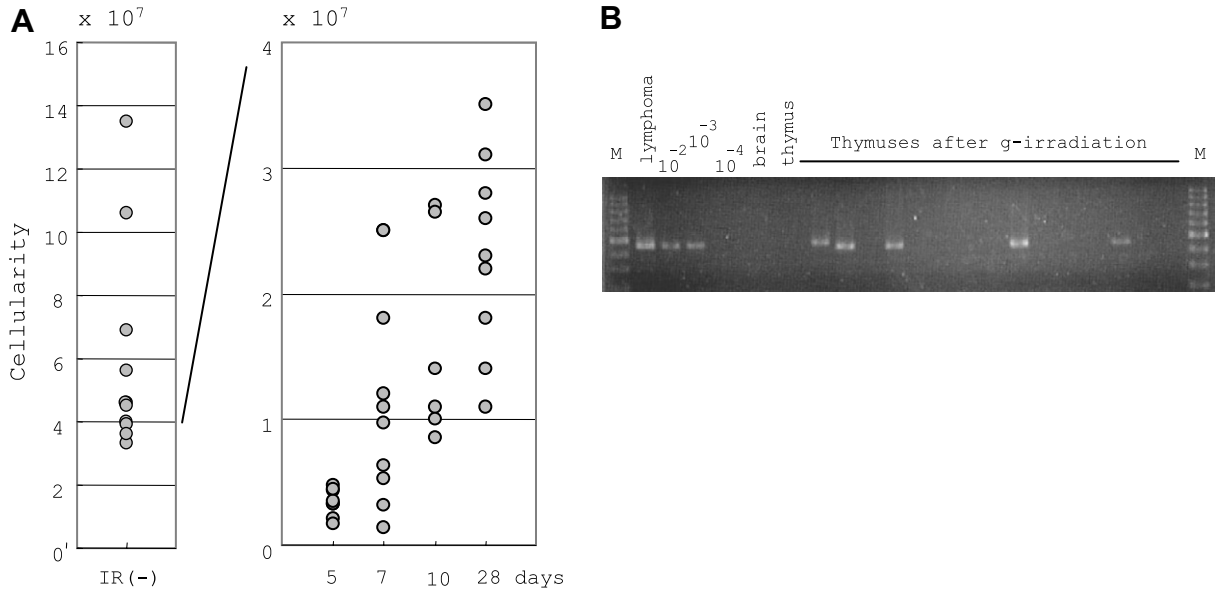

Fig. 1. (A) Cellularity of thymocytes at various times after 3 Gy γ-irradiation. (B) Existence of clonally growing thymocytes in thymuses at 28 days after irradiation. Nested PCR of *Bcl11b* intragenic deletions was performed and the PCR products were compared to the deletion-positive lymphoma DNA which had been 10^2-, 10^3-, and 10^4-fold diluted with brain DNA.

levels. We examined such effects on thymocytes that were isolated from BALB/c mice at 5, 7, 10, and 28 days after the irradiation. Thymocytes were analyzed with flow cytometry after incubation with DCFH-DA, which is a membrane-permeable fluorescent dye and the oxidization of DCFH-DA to DCF by ROS results in increased fluorescence of the dye.

Fig. 2A shows examples of the forward scatter and side scatter analysis of unirradiated thymus and thymus at 7 days after 3 Gy irradiation. The proportion of dead cells and debris increased after irradiation. The dead cells and debris-excluded fraction of thymocytes showed significant increase in DCF fluorescence after IR and the increase was higher at 5 days than at 7 days after (upper panels in Fig. 2B). These results suggested an increase in ROS in thymocytes of irradiated mice. The ROS levels of thymocytes at 10 and 28 days after irradiation were similar to those of unirradiated thymocytes (data not shown). In parallel with the increase in ROS, however, the proportion of large cell-sized thymocytes increased in the irradiated samples (lower panels in Fig. 2B). Therefore, the ROS level was separately examined in the large cell-sized and small cell-sized fractions of thymocytes. The large thymocytes were found to have higher levels of ROS than the small thymocytes did (Fig. 2C). Of note is that although the proportion of large thymocytes was very low, the level of ROS in the large thymocytes from unirradiated thymus was as high as that from irradiated thymus. This indicated that the high ROS was the feature of large thymocytes and not due to irradiation.

Properties of small and large thymocytes were investigated by flow-cytometric analysis of cell surface markers. A lower proportion of $CD4^+CD8^+$ double positive cells was found in large thymocytes than in small thymocytes, suggesting that large thymocytes comprised more immature cells than small thymocytes (Fig. 2D). This characteristic of large thymocytes was independent of irradiation status. However, irradiation increased the proportion of immature subsets of thymocytes ($CD4^-CD8^-$ and $CD4^-CD8^+$ cells) in both small and large thymocytes. We next examined BrdU uptake in thymocytes to test the capability for those small and large thymocytes to proliferate (Fig. 2E). Only large thymocytes incorporated BrdU, irrespective of irradiation (data not shown), indicating that the large cells comprise of cycling cells. A high ROS level has been shown to activate p53 tumor suppressor. Hence, the activation was examined by Western blotting. A higher expression of p53 was found in the large thymocytes relative to the small thymocytes (Fig. 2F). Taken together, these results suggest that irradiation increases the number of the immature large thymocytes with high ROS levels and a proliferation capacity. Since ROS can cause DNA damage, the large thymocytes are the likely target for precancerous conversion with clonal expansion.

Higher retention of large thymocytes in susceptible strain than resistant strain

Mtf-1 congenic heterozygous mice of BALB/c(C) background carrying an MSM(M)-derived 4 Mb interval (Fig. 3A) were mated with each other, and progeny were used for flow-cytometric analysis at 7 days after 3 Gy irradiation. We always used unirradiated thymocytes as a control, setting region gates for large thymocytes to range from 15% to 18%. Under the same gate condition, the proportion of large thymocytes was shown to range from 15%

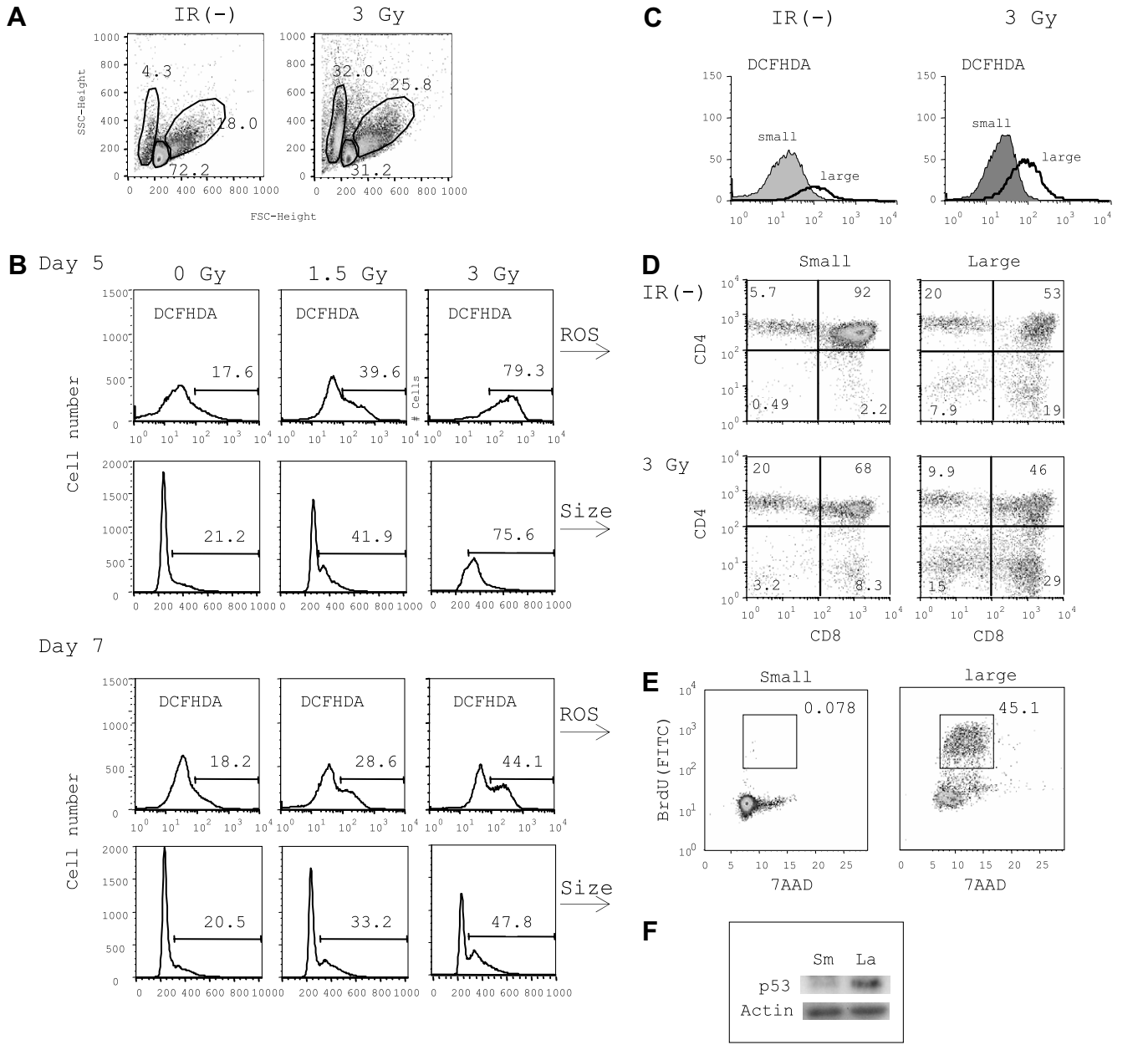

Fig. 2. IR increases the number of large thymocytes with high levels of ROS. (A) Cell size of thymocytes by forward scatter (FSC) versus side scatter (SSC) was analyzed by flow cytometry in unirradiated BALB/c mice and mice at 7 days after irradiation. The percentages of cells are shown in corresponding areas. (B) ROS levels (upper) and the cell size (lower) of thymocytes at 5 and 7 days after irradiation. The horizontal bars indicate percentages of cells in corresponding areas. (C) ROS levels of small and large thymocytes at 7 days after irradiation. (D) Surface expression of CD4 and CD8 on thymocytes was analyzed by flow cytometry in small and large thymocytes at 7 days after irradiation. (E) The measurement of cells incorporated BrdU and total DNA content in small and large thymocytes. The region gate in FACS analysis of large thymocytes indicated that 45.1% of the cells actively incorporated BrdU whereas that of small thymocytes showed that only 0.078% of the cells actively incorporated BrdU. (F) Western blotting indicated an increase of p53 in large thymocytes relative to small thymocytes.

to 60% in irradiated mice. Thymuses were analyzed for the proportion of large thymocytes in mice of the three different *Mtf-1* genotypes (Fig. 3B). We then divided thymuses into two groups, one harboring large thymocytes above the level of 20% and the other harboring large thymocytes below 20%. The former retains radiation influences and can be regarded as a high-risk group with clonally expanding precancerous cells. The latter, on the other hand, seems to be the thymuses well recovered from radiation influences.

Our previous results showed that mice of *Mtf-1* C/C and C/M genotypes are susceptible to γ-ray-induced thymic lymphomas and those of *Mtf-1* M/M genotype resistant [9,13]. A total of 18 mice of C/C and C/M genotype belonged to the former with more than 20% large thymocytes and only four belonged to the latter with less than

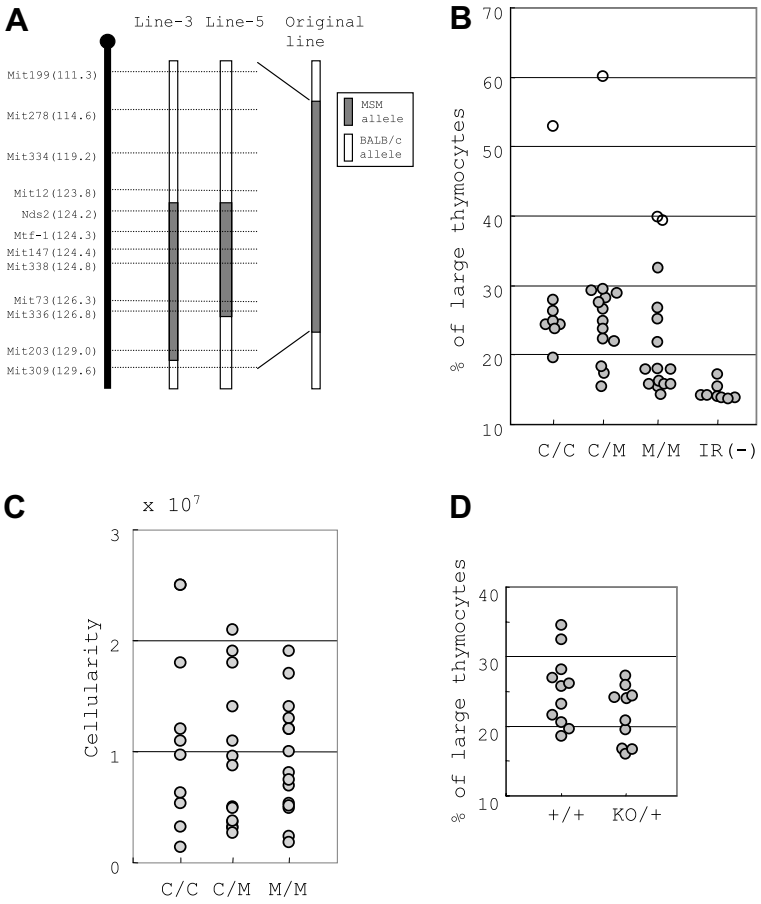

Fig. 3. Higher retention of large thymocytes in susceptible congenic mice. (A) Genetic constitution is displayed of congenic mouse strains (line-3 and line-5) of BALB/c background. The line-5 is a subcongenic line derived from the line-3 mice. Vertical bars represent a chromosomal region with map positions (in Mb from the centromere) and shadowed portions carry an MSM-derived region. (B) Distribution of thymuses with different percents of large thymocytes was analyzed in mice of the three different *Mtf-1* genotypes: C/C, C/M, and M/M genotypes. (C) Cellularity of thymocytes was analyzed in mice of the C/M and M/M *Mtf-1* genotypes at 7 days after γ-irradiation. (D) Distributions of thymuses with different percents of large thymocytes were analyzed in mice of $Bcl11b^{+/+}$ and $Bcl11b^{KO/+}$.

20%. In contrast, six mice of M/M genotype were belonged to the former and nine to the latter. This difference between susceptible mice and resistant mice is statistically significant (χ^2 test, $P = 0.0089$), indicating the tendency of susceptible strains retaining large thymocytes more than the resistant strain. We noted that the four thymuses possessing more than 40% of large thymocytes were very atrophic (see the open circles in Fig. 3B). On the other hand, no significant difference in the cellularity was observed between mice of *Mtf-1* C/C and C/M genotypes and mice of M/M genotype (Fig. 3C). Analysis of thymocytes with different ROS levels showed a similar pattern of different distributions (data not shown).

Bcl11b-knockout heterozygous mice are more susceptible to γ-ray-induced thymic lymphomas than *Bcl11b* wild-type mice (Kamimura et al., accompanying paper). Thus, analyses of large thymocytes were carried out on *Bcl11b* wild-type and heterozygous mice (Fig. 3D). The proportion of large thymocytes ranged from 16% to 40% in irradiated mice and no significant difference was observed between *Bcl11b* wild-type and knockout heterozygous mice. These results suggest that *Mtf-1* susceptibility locus and *Bcl11b* tumor suppressor gene contribute to the development of radiogenic thymic lymphomas by distinct mechanisms.

Discussion

This paper examines clonal growth of thymocytes after irradiation, early effects of IR on cell behavior *in vivo* and its difference between *Mtf-1* susceptible and resistant congenic mice for γ-ray-induced thymic lymphomas, to reveal the basis for the increased susceptibility given by *Mtf-1* locus. Here, we show thymocytes clonally growing at 28 days after irradiation, indicating the start of clonal expansion at a very early stage. We also show that susceptible mice tended to have more of proliferating immature large thymocytes with higher ROS levels than resistant mice when examined at 7 days after irradiation. This increase of large thymocytes may be a compensatory reaction of thymocytes to depletion of the cells after irradiation. Of particular importance is that the mode of the

compensatory reaction differed between susceptible and resistant mice. The high proportion of the large thymocytes is likely to contribute to lymphoma susceptibility because they are vulnerable to ROS-induced mutation. The importance of the cell death and complementary proliferation for carcinogenesis is also provided by the study on hepatocellular carcinogenesis [28].

Genetic factors underlying cancer susceptibility are best treated as quantitative trait loci [29,30] and in most cases the susceptibility does not operate in the gain-or-loss mechanism which is for the oncogenes and tumor suppressor genes conferring cancerous phenotype of the cell [31,32]. An individual mouse with a predisposing genotype may not develop cancer, while mice with the predisposing genotype will on average exhibit a higher incidence of cancer over those without. Indeed, *Mtf-1* susceptible mice developed thymic lymphomas at the incidence of approximately 70% whereas resistant mice exhibited that of 40% [9,13]. Susceptibility genes may predispose by affecting the level of genomic insult to the cancer target cells (that is, the initiation frequency), the promotion of tumor cell proliferation and growth, and the number of the target cells. This study showed that the *Mtf-1* susceptible gene contributes to the persistence of large thymocytes after irradiation but not to the total number of thymocytes. These suggest that the *Mtf-1* susceptibility affects the number of the large thymocytes, the candidate target cells for malignant conversion. This may be supported by the fact that the *Mtf-1* susceptibility gave the higher incidence of thymic lymphomas but did not affect the latency [9,13]. In contrast, KO heterozygosity of the *p53* and *Bcl11b* genes gave both the higher incidence and the shorter latency probably due to the pre-existence of one hit of mutation ([33]; Kamimura et al., unpublished paper).

In the present study, differences were found in radioresponses of thymocytes in the congenic mice of BALB/c background carrying an MSM-derived 4 Mb region between *Nds2* and *D4Mit336*. Therefore, the difference can be ascribed to a gene or genes in this region and the *Mtf-1* gene in this interval is obviously a strong candidate for the susceptibility gene. At present, the mechanism of *Mtf-1* alleles for affecting the retention of large thymocytes is unclear. In addition, there are many genes within this interval other than *Mtf-1*. Therefore, the observed phenotypic difference may not solely be attributable to the *Mtf-1* polymorphism. The relationship between *Mtf-1* and the susceptibility remains to be further investigated.

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