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T-Cell Immunosenescence and Inflammatory Response in Atomic Bomb Survivors

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In this paper we summarize the long-term effects of A-bomb radiation on the T-cell system and discuss the possible involvement of attenuated T-cell immunity in the disease development observed in A-bomb survivors. Our previous observations on such effects include impaired mitogen-dependent proliferation and IL-2 production, decreases in naive T-cell populations, and increased proportions of anergic and functionally weak memory CD4 T-cell subsets. In addition, we recently found a radiation dose-dependent increase in the percentages of $CD25^+/CD127^-$ regulatory T cells in the CD4 T-cell population of the survivors. All these effects of radiation on T-cell immunity resemble effects of aging on the immune system, suggesting that ionizing radiation might direct the T-cell system toward a compromised phenotype and thereby might contribute to an enhanced immunosenescence. Furthermore, there are inverse, significant associations between plasma levels of inflammatory cytokines and the relative number of naïve CD4 T cells, also suggesting that the elevated levels of inflammatory markers found in A-bomb survivors can be ascribed in part to T-cell immunosenescence. We suggest that radiation-induced T-cell immunosenescence may result in activation of inflammatory responses and may be partly involved in the development of aging-associated and inflammation-related diseases frequently observed in A-bomb survivors. © 2010 by Radiation Research Society

INTRODUCTION

Epidemiological studies of A-bomb survivors have suggested a relationship between radiation dose and the mortality or morbidity rates for various noncancer diseases (*1, 2*). Risk estimates made for the years from 1950 to 1997 were found to be elevated for death from all solid cancers combined and also for death from all noncancer diseases combined, with excess relative risks (ERRs) per Gy of 0.47 and 0.14, respectively (*1*). While the estimated ERR for noncancer disease death is clearly low, the death toll has risen to about 32,000, representing approximately 70% of the total recorded deaths in the cohort (*1*). Mechanisms for radiation-related cancer, although not totally understood, are much clearer than those for radiation-related noncancer diseases, about which almost nothing is known. An interesting hypothesis is that radiation effects on the immune system may be involved in part in radiation-related diseases, especially for noncancer diseases.

Advancing age is accompanied by a variety of alterations in the immune system, many of which will tend to increase the susceptibility of elderly people to a wide range of diseases. Thus, for example, age-dependent decreases in T-cell numbers and/or function are almost certain to lead to increases in vulnerability to disease-causing pathogens as well as to several adverse manifestations of chronic inflammation. In A-bomb survivors, dose-dependent increases in morbidity have been associated with a variety of inflammatory diseases, such as chronic liver diseases, thyroid diseases and heart diseases (*2*). To gain further insights into the mechanisms of radiation-related noncancer diseases, we have been focusing on aging- and radiation-related alterations in T-cell immunity. In this paper, we first summarize the long-term effects of A-bomb radiation on the T-cell system, including current study results on regulatory T cells, and then we discuss a possible involvement of attenuated T-cell immunity in the development of diseases frequently observed in A-bomb survivors.

MATERIALS AND METHODS

Blood Donors

A total of 1,035 study subjects were randomly selected from Hiroshima participants in the Adult Health Study (AHS) at the Radiation Effects Research Foundation (RERF) (*3*). For the present study, blood samples from the subjects were obtained with informed consent when they participated in the AHS health examination program between 2006 and 2008. The study protocol has been approved by the Human Investigation Committee of RERF (the RERF Institutional Review Board). Cancer prevalence within the study subjects by dose

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TABLE 1
Characterization of the Study Population

	Age (years)[b]						
	<70		70–79		80+		
Dose (Gy)	Male	Female	Male	Female	Male	Female	Total
<0.005[a]	29	26	73	102	14	97	341
0.005–0.5	11	34	62	86	22	133	348
0.5–1.0	18	23	19	41	19	48	168
1.0–4.0	28	32	29	40	17	32	178
Total	86	115	183	269	72	310	1,035

[a] Individuals in this dose category were exposed at distances in excess of 3 km from the hypocenter and hence received doses that are equivalent to zero.
[b] Age at the time of the examinations that were conducted between 2006 and 2008.

category was 14% at <0.005 Gy, 16% at 0.005–0.5 Gy, 22% at 0.5–1.0 Gy, and 24% at ≥1.0 Gy and tended to be higher in survivors exposed to higher doses, in accord with a recent observation in the AHS population (*4*). The age-, gender- and radiation dose-specific distributions of the 1,035 study subjects are listed in Table 1. Radiation doses are based on Dosimetry System 02 (DS02) estimates (*5*).

Flow Cytometry

Mononuclear cell fractions separated by the Ficoll-Hypaque gradient technique were analyzed by three-color flow cytometry using a FACScan flow cytometer (BD Biosciences, San Jose, CA) as described previously (*6*). For assessment of regulatory T (Treg) cells, approximately 100,000 mononuclear cells were stained with Alexa Fluor® 488-labeled CD25 mAb (Serotec Ltd, Oxford, UK), PE-labeled anti-CD127 mAb (BD Biosciences), and PerCP-labeled CD4 mAb (BD Biosciences). Analyses of naïve and effector/memory cell subsets in the CD4 T-cell population involved staining mononuclear cell fractions with FITC-labeled anti-CD45RA mAb (Beckman Coulter, Inc., Fullerton, CA), PE-labeled anti-CD62L mAb (BD Biosciences), and PerCP-labeled CD4 mAb.

TNF-α Measurement

Of the 1,035 subjects examined for their T-cell subsets, we selected 69 subjects whose DS02 doses exceeded 1 Gy as well as 86 age- and gender-matched subjects who were exposed to less than 5 mGy and analyzed their plasma TNF-α levels. Since the sample selection was originally made for the purpose of examining associations between inflammatory responses and mutant frequency at the glycophorin A locus in erythrocytes (*4*), all subjects were all heterozygous for blood-type MN at that locus. TNF-α levels were analyzed using a highly sensitive enzyme-linked immunosorbent assay kit (Quantikine HS, R&D systems, Minneapolis, MN). The minimum detectable concentration was 0.05 pg/ml.

Data Analysis

Associations of the percentage (Y) of each CD4 T-cell subpopulation with age at time of examination (*age*), gender (*gender*) and radiation dose (*dose*) were analyzed using a multiple regression model (*7*). This model assumes that the percentage of each T-cell subpopulation, or level of TNF-α, is related to each variable in an exponential manner with adjustment for age, gender and other parameters (V) measured for the same individuals:

$$\log Y = \alpha + \beta_1 \times age + \beta_2 \times gender + \beta_3 \times dose + \beta_4 \times V,$$

where *gender* is an indicator of female sex, i.e., *gender* = 0 for male and *gender* = 1 for female, and *dose* is radiation dose in grays. The α is a constant term, and β_1, β_2, β_3 and β_4 are regression coefficients for variables to be estimated. The age term was subtracted by 70 years so that α corresponds to log-transformed percentage of CD4 T-cell subset, i.e., the subset percentage is calculated to be e^{α} (= exponential [α]), for nonirradiated males at 70 years of age. The percentage change of subset percentage was estimated to be $100(e^{10\beta 1} - 1)$ per 10 years increment of age and $100(e^{\beta 3} - 1)$ per 1 Gy radiation dose. All statistical analyses were carried out using the SAS program (SAS Institute Inc., Cary, NC).

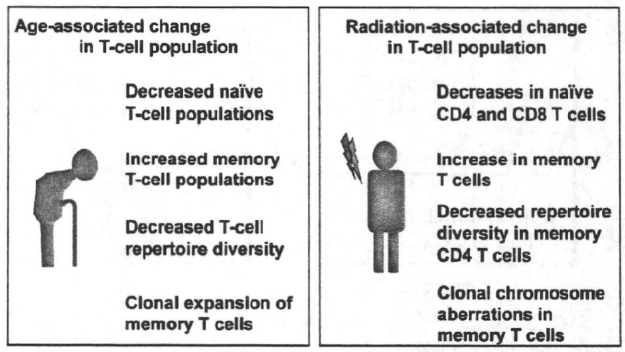

FIG. 1. Age- and radiation-associated alterations in the T-cell system in humans. Left panel: An outline of aging of the T-cell system from a review by Weng (*8*). Right panel: Alterations in the T-cell systems we have observed in association with radiation exposure among A-bomb survivors (*6, 9–12*).

RESULTS AND DISCUSSION

Evidence for T-Cell Immunosenescence from Previous Studies on A-Bomb Survivors

Age-associated changes in T-cell populations are primarily characterized as (1) decreases in cell numbers and functions such as proliferative responses to T-cell receptor (TCR) stimulation and T-helper function in naïve T-cell populations, (2) increases in memory T-cell populations, (3) decreased TCR repertoire diversity along with a reduced antigen recognition, and (4) frequent emergence of an oligoclonally expanded population of memory T cells (Fig. 1, left panel) (*8*). Radiation effects on the human immune system that are in accordance with the age-associated changes in T-cell populations are also depicted in Fig. 1 (right panel). Among A-bomb survivors, we have observed radiation-associated alterations such as (1) decreases in naïve CD4 and CD8 T cells (*6, 9–11*), (2) increases in memory CD4 and CD8 T cells (*6*), and (3) decreased repertoire diversity in memory CD4 T cells (*11*). We also observed (4) clonal chromosome aberrations of memory T-cell origin in heavily exposed survivors, suggesting clonal expansion of memory T cells (*12*). Thus the effects of radiation on T-cell immunity mostly resemble the effects of aging on the immune system.

Although other studies have reported that clonal expansion of a subset of memory T cells, $CD28^-$ or $CD57^+$, frequently occurred in older unirradiated

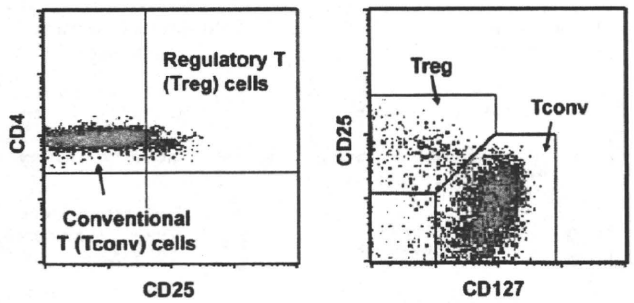

FIG. 2. Flow cytometry patterns of regulatory T (Treg) and conventional T (Tconv) cells that were discriminated with CD25 expression levels alone (left panel) or with both CD25 and CD127 levels (right panel) in the peripheral blood CD4 T-cell fractions from a typical A-bomb survivor.

individuals (*8, 13*), no significant radiation-associated change in percentages of such cells in either CD4 (*14*) or CD8 (*6*) T-cell subsets was observed among A-bomb survivors. To further evaluate the maintenance of T-cell memory in A-bomb survivors, we recently analyzed memory CD4 T-cell subsets using HSCA-2 (recognizing the low-molecular-mass glycoform of CD43), a monoclonal antibody that we established to classify human memory T cells (*15*). We found that functionally weak ($CD43^{middle}$) and anergic ($CD43^{low}$) memory CD4 T-cell subsets dose-dependently increased among A-bomb survivors, suggesting that attrition of the competent memory T-cell population is related to radiation exposure (*16*).

Immunosenescence in the T-cell system may be involved in long-lasting impairments in T-cell functions among A-bomb survivors. In fact, our previous observations indicated that previous radiation exposure dose-dependently induced deleterious effects on T-cell functions in A-bomb survivors. *In vitro* T-cell proliferative responses to PHA (*17*) and alloantigens (*18*) and the frequency of IL-2-producing T cells (*19*) were all found to decrease in association with radiation dose. Also noted were dose-dependent decreases in the proliferative responses of A-bomb survivors' T cells that were exposed to superantigen staphylococcal enterotoxins (*10*). The superantigen responses correlated well with the naïve CD4 T cell percentages, suggesting that the immunosenescence in naïve CD4 T cells that we consistently observed in A-bomb survivors might account in part for their deficits of T-cell proliferative function (*10*). In addition, the other T-cell functions, which were inversely related to radiation, also tended to decrease in association with aging. Taken together, there is accumulating evidence of T-cell immunosenescence being associated with aging and previous radiation exposure in A-bomb survivors and consequently leading to diminished functions in their adaptive immune systems.

Radiation Effects on Regulatory T Cells (Current Study)

$CD4^+$ regulatory T (Treg) cells express CD25 and suppress T-cell activation and function, and their number increases with age (*20*). Our previous measurement of Treg, which were identified by their high CD25 expression level, did not find any effect of age or radiation on these cells, probably due to the difficulty in accurately discriminating Treg from conventional T (Tconv) cells (data not shown). Recently, however, Treg cells were shown to be clearly identified by their $CD25^+/CD127^-$ phenotype (*21, 22*), allowing us to make more accurate measurements. Figure 2 shows representative flow cytometry patterns of Treg cells that were identified by the $CD25^+$ phenotype alone and by the $CD25^+/CD127^-$ phenotype in an A-bomb survivor. We could not find a significant effect of age on Treg cells (Table 2). This is probably due to the advanced ages of our study subjects; all subjects were 60 years old or older. As for radiation effects, there was a dose-dependent increase in the percentages of Treg cells in the CD4 T-cell population. The same trend was suggested for the ratio between Treg and Tconv cells. Those results suggest that A-bomb radiation might direct T-cell immunity toward suppressor phenotypes in relation to immunosenescence. It has been demonstrated that Treg cells have a strong influence in suppressing pathological immune responses in autoimmune diseases (*23*). Clinical studies in A-bomb survivors thus far have found no evidence that supports the idea that there is an increase in autoimmune diseases (*24*). Therefore, the current observation for Treg cell counts is not contradictory to the absence of an excess prevalence of autoimmune diseases in A-bomb survivors.

Radiation Effects on Naïve and Memory CD4 T-Cell Subsets (Current Study)

Naïve and memory T cells are mature T cells that have not previously experienced antigen encounter and that mediate recall responses to second and subsequent exposure to antigens, respectively. Memory T-cell populations are produced by antigen stimulation of naïve T cells and survive for many years and are classified based on the expression of other surface markers including CD62L into central and effector memory T cells and effector T cells with several differences in their functions, migration capacities and proliferation abilities. Our previous examinations of the peripheral blood lymphocyte populations of A-bomb survivors using CD45RO and CD62L as markers showed decreased percentages of naïve CD4 and CD8 T cells and increased percentages of memory CD8 T-cell subsets in a dose-dependent manner (*6*). In the present study, we discriminated naïve and memory CD4 T-cell subsets by using CD45RA and CD62L as markers and assessed the percentages of these subsets in the peripheral blood CD4 T-cell population among A-bomb survivors. We observed a dose-dependent decrease in the percentage of $CD45RA^+/CD62L^+$ (naïve, Tn) cells within the CD4 T-cell population, confirming

TABLE 2
Age, Gender and Radiation Effects on the Percentages of T-Cell Subpopulations in the Peripheral Blood CD4 T-Cell Populations among a Total of 1,035 A-Bomb Survivors[a]

T-cell subpopulation	Estimated percentage for nonirradiated males at 75 years of age	Percentage change of T-cell subpopulation percentage per unit		
		Age (10 years)[b]	Gender[c]	Dose (Gy)[d]
Regulatory T (Treg) and conventional T (Tconv) cells				
$CD25^+/CD127^-$ (Treg)	6.3	1.2 (−1.2, 3.5)[e] $P = 0.34$	−7.3 (−11.7, −5.3) $P = 0.0004$	2.7 (0.1, 5.3) $P = 0.044$
$25.9CD25^-/CD127^+$ (Tconv)	89.7	− 0.8 (−1.2, −0.5) $P = 0.0001$	0.9 (0.3, 1.5) $P = 0.0046$	− 0.1 (−0.5, 0.3) $P = 0.67$
Ratio (Treg/Tconv)	7.0	2.0 (−0.5, 4.6) $P = 0.13$	−8.2 (−12.4, −4.0) $P = 0.0002$	2.7 (−0.1, 5.5) $P = 0.052$
Naïve and memory				
$CD45RA^+/CD62L^+$ (Tn)	25.9	− 18.7 (−23.5, −13.9) $P = 0.0001$	5.6 (−2.2, 13.8) $P = 0.16$	− 5.6 (−10.9, −0.3) $P = 0.040$
$CD45RA^-/CD62L^+$ (Tcm)	44.5	0.4 (−1.6, 2.5) $P = 0.68$	0.3 (−3.0, 3.7) $P = 0.85$	4.5 (2.3, 6.8) $P = 0.0001$
$CD45RA^-/CD62L^-$ (Tem)	19.6	17.3 (13.4, 21.2) $P = 0.0001$	− 2.1 (−8.5, 4.3) $P = 0.51$	2.5 (−1.8, 6.7) $P = 0.26$
$CD45RA^+/CD62L^-$ (Teff)	0.4	8.6 (−3.0, 20.2) $P = 0.15$	− 22.3 (−42.1, −2.6) $P = 0.036$	− 8.0 (−21.1, 5.0) $P = 0.23$

Note. Treg, regulatory T cells; Tconv, conventional T cells; Tn, naïve T cells; Tcm, central memory T cells; Tem; effector memory T cells; Teff, effector T cells.

[a] Percentages of CD4 T-cell subpopulations for age, gender and dose were obtained by the multiple regression model, using the following formula: $\log(\textit{percentage T cells}) = \alpha + \beta_1 \times \textit{age} + \beta_2 \times \textit{gender} + \beta_3 \times \textit{dose}$, where the value showing zero for the percentage of T cells was replaced with 0.01 when the value was transformed into natural log.

[b] Effects of age were estimated for 10 years.

[c] Gender = 0 for male and = 1 for female.

[d] Effects of dose were estimated for 1 Gy.

[e] 95% confidence interval.

our previous findings (Table 2). As for memory T-cell subsets, the percentage of $CD45RA^-/CD62L^+$ (central memory, Tcm) cells appeared to increase with radiation dose, whereas there was no significant radiation effect on $CD45RA^-/CD62L^-$ (effector memory, Tem) or $CD45RA^+/CD62L^-$ (effector, Teff) cells (Table 2). Those results clearly indicate that radiation exposure generated a reduction of Tn cell populations but an increase of Tcm cell populations among A-bomb survivors. Because Treg cells are known to suppress differentiation of resting naïve and memory T cells into effector cells (*24*), we analyzed the associations between the percentages of Treg cells with the $CD25^+/CD127^-$ phenotype and those of Teff cells with the $CD45RA^+/CD62L^-$ phenotype. A multiple regression analysis showed an inverse association between Treg and effector cell percentages ($r = 0.14$, $P = 0.0001$) after adjusting for age, gender and radiation dose. Although it needs to be determined whether the increase in the proportion of Treg cells is actually responsible for a suppression of the effector functions of T cells, elevated Treg cell levels in A-bomb survivors may be partly involved in the diminished T-cell responsiveness that we have observed among the survivors.

T-Cell Immunosenescence and Inflammation

A link between alterations in T-cell immunity and elevated inflammation among A-bomb survivors is suggested (*24*). We found that plasma levels of inflammatory cytokines such as IL-6, TNF-α and IFN-γ increased with radiation dose (*26*). Kim *et al.* (*27*) reported that treatment of T-cell-deficient nude and *Rag1*-knockout mice with poly I:C led to a lethal cytokine storm, whereas similar doses of poly I:C did not kill wild-type mice, which possess abundant T lymphocytes. They also showed that T cells, either Tn or Treg, were sufficient to control this cytokine response by the adoptive transfer of T cells prior to poly I:C treatment of *Rag1*-knockout mice, which resulted in decreased pro-inflammatory cytokine production. Those results suggest that T cells suppress the cytokine storm that occurs during the initial innate immune response. In A-bomb survivors, there were dose-dependent increases in plasma levels of inflammatory cytokines and CRP, and such enhanced inflammatory responses might be caused by alterations in the T-cell system of the survivors. For example, we found that plasma IL-6 and CRP levels were significantly elevated in the survivors with low percentages of peripheral blood CD4 T cells (*26*). A similar trend was also apparent in the survivors with low percentages of Tn (YK and TH, unpublished observation).

To seek a further link of T-cell immunosenescence to inflammatory responses among A-bomb survivors, in the present study we analyzed associations between percentages of T-cell subsets (that we currently examined) and plasma TNF-α levels. TNF-α concentration has already been measured among a large number of subjects for another study and purpose; 1.50 pg/ml was estimated for nonirradiated males at 75 years of age, and the effects (percentage increases) of age, gender and radiation dose

TABLE 3
Partial Correlation Coefficients between TNF-α Levels and Percentages of T-Cell Subsets in the Peripheral Blood CD4 T-Cell Population

	T-cell subset				
	Treg	Tn	Tcm	Tem	Teff
TNF-α	−0.08 (P = 0.36)	−0.20 (P = 0.014)	0.04 (P = 0.67)	0.23 (P = 0.005)	0.12 (P = 0.15)

Notes. Partial correlation coefficients adjusted for sex, age and radiation dose were calculated after TNF-α levels and percentages of T-cell subsets were naturally log-transformed. Each P value in parentheses is a significant level of partial correlation coefficient between natural log-transformed percentage of the T-cell subset and the log-transformed of TNF-α level. The value showing zero for the percentage of T cells was replaced with 0.01 when the value was transformed into natural log. Treg, regulatory T cells; Tn, naïve T cells; Tcm, central memory T cells; Tem; effector memory T cells; Teff, effector T cells.

on TNF-α concentration were 15% for 10 years, 15% for females relative to males, and 7% for 1 Gy, respectively (*28*). In this study, we remeasured the TNF-α concentration with a subset (155 survivors) of the current study subjects, whose T-cell data are available in Table 2, and we used these data (1.14 pg/ml being estimated for nonirradiated males at 75 years of age) for the calculation of partial correlation coefficients in Table 3. Plasma TNF-α levels were inversely associated with the percentages of Tn but not Treg cells, suggesting an involvement of Tn but not Treg cells in suppression of innate inflammatory responses manifested by increased TNF-α levels (Table 3). This may explain why we observed long-lasting inflammatory responses despite an increase in the relative number of immunosuppressive Treg cells in A-bomb survivors. It was also noted that plasma TNF-α levels were positively associated with the percentage of Tem cells (Table 3). There was a negative correlation between percentages of Tn and Tem cells ($r = -0.71$, $P = 0.0001$) but not between those of Treg and Tem cells ($r = 0.05$, $P = 0.13$). Thus T-cell immunosenescence manifested by a reduction in the relative number of Tn cells might be linked to the development and/or expansion of Tem cells, involving enhanced inflammatory responses.

Implications of T-Cell Immunosenescence in Disease Development among A-Bomb Survivors

We found previously that the CD4 T-cell percentages were significantly lower in survivors with a history of myocardial infarction (MI) than in survivors with no such history (*28*). We also noted that the T cells of survivors with a history of MI tended to be poor responders to several superantigens of *S. aureus* toxins and that these same individuals had proportionally fewer naïve CD4 T cells than survivors with no MI history (*10*). As suggested for a link between alterations in T-cell immunity and inflammation among A-bomb survivors, both IL-6 and CRP levels were significantly higher in survivors with a history of MI than in those without such a history (*26*). We thus believe that the T-cell immunosenescence associated with inflammatory reactions will prove to be a cause of increased risk of cardiovascular disease among A-bomb survivors, in much the same way as seen in other epidemiological studies of unirradiated individuals (*29–31*). However, because it is still possible that the disease itself or some medication might be responsible for such immunosenescence, prospective studies are needed to provide definitive information on the causal link between T-cell immunosenescence and inflammatory diseases.

Noncancer diseases frequently observed in A-bomb survivors include circulatory, respiratory and digestive diseases, especially cardiovascular disease, pneumonia and liver disease (*1, 2*). One possible explanation involves the failure of aging immune systems to control microbial infections, since infections might lead to chronic inflammation and hence to increased susceptibility to such noncancer diseases in heavily exposed survivors, resulting from unregulated and hence long-lasting inflammatory responses. In A-bomb survivors, however, no significant long-term effects of radiation on the antimicrobial functions of blood monocytes or granulocytes (e.g., phagocytosis, *in vitro* migration, etc.) have been noted (*32*). There is a growing interest in the role of pathogen recognition molecules, such as Toll-like receptors (TLRs), in the pathogenesis of chronic inflammatory diseases: TLRs play a key role in the host defense against exposure to microbial pathogens and also in the development and progression of atherosclerotic lesions (*33, 34*). It may be important and possible to conduct an integrated and systematic examination of the A-bomb survivors' innate immune systems in the future, including the analyses of TLR-mediated signaling as well as interaction of the signaling with T-cell immunity.

Conclusions and Perspectives

In summary, A-bomb radiation may have induced T-cell immunosenescence, resulting in attenuation of T-cell-mediated immunity. Such decrements in the T-cell system may cause chronic inflammation and in turn may be partly responsible for cardiovascular disease and other aging-associated diseases of importance. Although there is accumulating evidence of T-cell immunosenescence among A-bomb survivors, how ionizing radiation causes T-cell immunosenescence and how radiation-

induced T-cell immunosenescence interacts with ordinary aging remain to be explained. Mechanistic approaches using appropriate animal models will be necessary to depict a complete picture of radiobiological involvements in host immunological aging. Longitudinal analyses of the changes in the various immunological parameters may provide a suitable vehicle for a better understanding of the interaction between radiation-related and aging-associated immunological changes and for exploring causal relationships between these immunological changes and various noncancer diseases in A-bomb survivors.

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Increased DNA damage in hematopoietic cells of mice with graft-versus-host disease

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ABSTRACT

Patients who received hematopoietic cell transplants have an increased risk for a new malignancy. In addition to genotoxic regimens such as radiotherapy and chemotherapy, graft-versus-host disease (GVHD) is a risk factor for development of new malignancies in long-term survivors. To understand mechanisms underlying this malignant transformation, we evaluated genomic damage in several murine models of GVHD by enumerating reticulocytes containing micronuclei (MN) in the blood after semi-allogeneic (parent-into-F1) hematopoietic cell transplantation. On day 40 after transplantation, MN frequencies were significantly increased in unirradiated (C57BL6 × DBA/2) F1 (BDF1) and (BALB/c × C57BL6) F1 (CBF1) mice that received cells from C57BL6 (B6) donors. MN frequencies were not significantly increased in F1 mice that received cells from DBA/2 or BALB/c donors. Serum levels of tumor necrosis factor-α (TNF-α) were higher after transplantation with B6 donors than with DBA/2 or BALB/c donors. The results indicate that GVHD, without irradiation, can induce genomic damage associated with inflammatory reactions manifested by increased TNF-α levels.

1. Introduction

Second malignancies after hematopoietic cell transplantation (HCT) have been linked not only to DNA-damaging myeloablative treatments, such as ionizing irradiation, but also to graft-versus-host disease (GVHD) [1,2]. The immunological mechanisms for the association of GVHD with malignant transformation remain largely unknown. Current theory postulates that a gene mutation or a chromosomal aberration initiates the first step in a single cell towards malignant transformation and that this initiated cell, with time, clonally proliferates and accumulates further mutations to produce a fully malignant clone. Animal HCT models have suggested that one of the most important cytokines involved in the pathogenesis of GVHD is tumor necrosis factor-α (TNF-α) [3,4]. Tumor promotion activity of inflammatory cytokine TNF-α has also been indicated in several murine models [5,6]. TNF-α may also act as a cell surviving factor and may have endogenous tumor promoter effects by activating nuclear factor-κB (NF-κB) signaling pathways [7,8] that inhibit apoptosis of transformed cells [9–11] and by stimulating protein kinase Cα- and activator protein-1-dependent pathways that enhance clonal proliferation of transformed cells [12].

In the pathogenesis of a malignancy after HCT, exposure to DNA-damaging agents and chronic inflammation by GVHD likely serve as two important underlying risk factors, with the former causing tumor initiation and the latter causing tumor promotion [13]. On the other hand, inflammation by GVHD *per se* may induce genomic damage in cells that have not been genetically damaged by pretransplant conditioning regimens. Recent studies suggested that TNF-α not only induces anti-apoptotic signals through NF-κB activation, but also causes genomic instability through reactive oxygen species (ROS)-mediated DNA damage [14,15]. In the present study, we investigated whether or not GVHD causes genomic damage, particularly in relation to TNF-α production, using several parent-into-F1 transplantation models in which no DNA-damaging pretransplant conditioning was required to induce GVHD.

It has been suggested that radiation-induced inflammatory response is involved in the emersion of genomic instability in irradiated hosts [16,17]. We have previously analyzed the proportion of reticulocytes containing a micronucleus (MN) as a measure of genomic instability after non-lethal ionizing irradiation in mice. That measure of genomic instability can persist *in vivo* for prolonged periods after irradiation [18]. In this reticulocyte MN assay, the persistence of radiation effects (i.e., long-term genomic instability) can be distinguished from immediate effects on target cells, since reticulocytes have an *in vivo* lifespan as short as a few days [19]. Therefore, we used reticulocyte MN frequencies to evaluate

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genomic damage induced by GVHD in mice. Our present results indicate that GVHD after HCT even without ionizing irradiation can induce genomic damage, possibly through inflammatory effects mediated by TNF-α.

2. Materials and methods

2.1. Mice

A set of female C57BL/6 (B6), DBA/2, and (B6 × DBA/2) F1 (BDF1) mice eight weeks of age were purchased from Japan Clea Co. (Tokyo, Japan). Another set of female BALB/c, B6, and (BALB/c × B6) F1 (CBF1) mice eight weeks of age were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). All mice were housed in autoclaved microisolator cages and fed a sterile regular diet *ad libitum*. All animal handling procedures were approved by the Experimental Animal Care Committee of the Radiation Effects Research Foundation.

2.2. Induction of GVHD

Donor mice 9–16 weeks of age were used for transplantation. GVHD was induced by tail vein injection of either 50×10^6 splenocytes from B6 donors, or splenocytes (90×10^6) and lymph node cells (10×10^6) from DBA/2 donors, into BDF1 recipients 9 weeks of age. Similarly, CBF1 recipients 16 weeks of age were injected with splenocytes (100×10^6) and lymph node cells (12×10^6) from B6 donors, or with splenocytes (100×10^6) and lymph node cells (16×10^6) from BALB/c donors. Negative controls consisted of age- and sex-matched uninjected F1 and parental mice. Survival after HCT was monitored almost daily, and weight changes were assessed weekly. Blood cell chimerisms, reticulocyte MN frequencies, and serum cytokine levels were assessed 40 days after transplantation. Blood was obtained by orbital sinus puncture after ether-anesthesia, and the mice were euthanized by cervical dislocation.

2.3. Assessment of chimerism after transplantation

Heparinized blood was lysed with NH_4Cl buffer, and leukocytes were stained for two-color analysis with fluorescein (FL)-labeled CD3 antibody (BD Biosciences, Franklin Lakes, NJ, USA) and with biotinylated antibodies specific for H-2Kb (BD Biosciences) or H-2Kd (BD Biosciences). Binding of the biotinylated antibody was assessed by staining with streptavidin-conjugated phycoerythrin (PE) (BD Biosciences). The stained cells were subsequently analyzed by a FACScan flow cytometer (BD Biosciences). Bit maps for lymphoid cells and granulocytes were defined by forward- and side-scatter characteristics, and the percent of donor cells within each window was enumerated. Results were corrected for positive control samples and were rounded to the nearest integer. In the lymphoid and granulocyte gates, positive control samples showed 87.1–100% and 82.4–100% staining, respectively.

2.4. Reticulocyte MN assay

Blood reticulocyte MN frequencies were analyzed by flow cytometry with the use of the mouse MN analysis kit MicroFlow^{PLUS} Kit (Mouse) (Litron Laboratories, Rochester, NY, USA), as described previously [18]. Briefly, the blood was mixed with the anticoagulant solution supplied in the kit, and was fixed in absolute methanol at −80 °C and kept in an ultracold (−75 to −85 °C) freezer. After washing the fixed cells, aliquots of each cell pellet were added to tubes containing fluorescein-labeled anti-CD71 and phycoerythrin-labeled anti-CD61 antibodies and RNase A, and then resuspended in cold propidium iodide solution. More than 20,000 reticulocytes gated on $CD71^+/CD61^-$ populations were analyzed by a FACScan to determine MN frequencies. Data analysis was performed using Flowjo software (Tree Star, Ashland, OR, USA).

2.5. Cytokine assay

Serum samples were collected and preserved at −75 to −85 °C until testing. Cytokines, interleukin (IL)-6, IL-10, monocyte chemoattractant protein (MCP)-1, interferon-γ (IFN-γ), TNF-α, IL-12p70) were quantified using the Cytometric Bead Array Mouse Inflammation Kit (BD Biosciences) according to the manufacturer's instructions, with the use of a FACScan. The detection limits in these cytokine assays were 5 pg/ml (IL-6), 17.5 pg/ml (IL-10), 52.7 pg/ml (MCP-1), 2.5 pg/ml (IFN-γ), 7.3 pg/ml (TNF-α), and 10.7 pg/ml (IL-12p70). For data analysis, values under the detection limits were considered as 0 pg/ml. Because measured levels of IL-6, IL-10, and IL-12 were mostly under the detection limits, we did not use the results for these cytokines in this study.

2.6. Statistical analysis

Differences in the MN frequency and cytokine levels between experiment groups were examined with a Mann–Whitney's test, using SPSS (ver. 14.0) software (SPSS, Chicago, IL, USA), because the values were not normally distributed in each group. For all tests, a value of $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. GVHD and MN frequencies in unirradiated BDF1 recipients after HCT from parental B6 donors

In unirradiated BDF1 recipients with GVHD induced by parental B6 splenocytes, weight loss first appeared at two to four weeks after transplantation, and the weight of the transplanted mice was still lower than that of untreated controls 40 days after transplantation (Fig. 1A). On day 33, one of the 22 recipients died. On day 40, we assessed blood cell chimerisms, MN frequencies, and circulating cytokine levels in surviving mice, and compared these values with those of age-matched untreated BDF1 and B6 mice. Percentages of donor cells in blood granulocytes and T cells in the recipient mice were 0–80% (median: 25%) and 1–92% (median: 38%), respectively. Flow cytometry patterns of MN frequency assessment are exemplified in Fig. 2. As shown in Fig. 1B, MN frequencies of the transplanted mice were significantly higher than those of either untreated BDF1 or B6 mice ($P = 0.001$ or 0.005, respectively), indicating that this B6-into-BDF1 transplant model can induce a significant level of genetic damage in hematopoietic cells. In addition, serum TNF-α levels in recipients were significantly higher than in untreated BDF1 controls ($P = 0.001$) but did not significantly differ from those in parental B6 mice ($P = 0.14$, Fig. 1C). There was a positive correlation between MN frequencies and TNF-α levels in the BDF1 recipients, with correlation coefficient (r) of 0.48. On the other hand, we found no significant difference in either IFN-γ or MCP-1 levels among the three groups (data not shown). At 95 days after transplantation, we also found significant differences in MN frequencies and TNF-α levels between BDF1 recipients and untreated BDF1 mice (data not shown). Those results suggest that without irradiation, transplantation of B6 cells into BDF1 recipients can cause elevated MN frequencies in the circulating reticulocyte fraction as well as increased TNF-α levels in the serum.

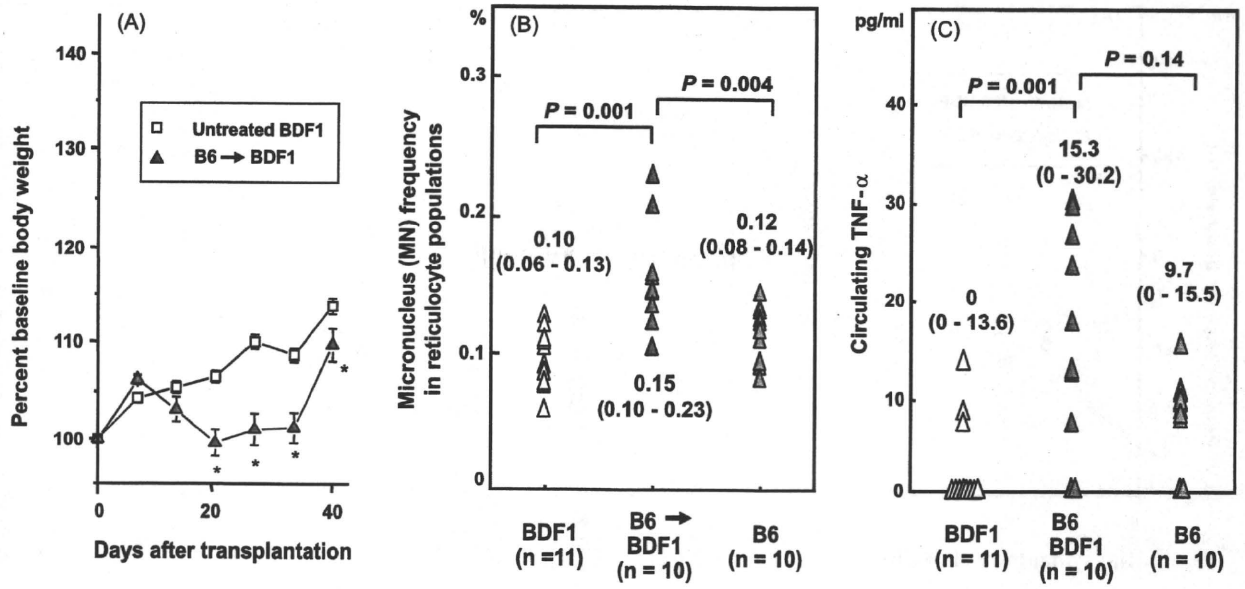

Fig. 1. Reticulocyte micronucleus (MN) frequencies and circulating tumor necrosis factor-α (TNF-α) levels in parental C57BL/6 (B6) mice, untreated (B6 × DBA/2) F1 (BDF1) mice, and BDF1 recipient after hematopoietic cell transplantation (HCT) from B6 donors. (A) Weight changes after transplantation (triangles) and in untreated (squares) BDF1 controls were monitored once weekly. Each symbol represents the mean in each group of mice. Error bars and asterisks denote standard errors (SE) and significant differences between the groups ($P < 0.05$, by t-test), respectively. (B) MN frequencies and (C) TNF-α levels were measured on day 40 after transplantation and plotted for each group. Numbers in the vicinity of the symbols denote the median and range (in parentheses).

3.2. GVHD and MN frequencies in unirradiated BDF1 recipients after HCT from parental DBA/2 donors

In unirradiated BDF1 recipients where GVHD was induced by injection of spleen and lymph node cells from parental DBA/2 donors, weight gain was retarded in comparison to untreated controls at least until day 40 (Fig. 3A). All the transplanted mice survived until 40 days after transplantation. On day 40, MN frequencies in blood samples from transplant recipients did not significantly differ from those of either untreated BDF1 or DBA/2 mice (Fig. 3B). Percentages of donor cells in blood granulocytes and T cells in the recipient mice were 1–36% (median: 9%) and 3–34% (median: 9%), respectively. Serum TNF-α levels were significantly higher in the transplant recipients in comparison to untreated BDF1 controls ($P = 0.033$) but not in comparison to untreated DBA/2 controls ($P = 0.085$) (Fig. 3C). In the DBF1 recipients, TNF-α levels showed no significant correlation with MN frequencies. We found no significant difference in either IFN-γ or MCP-1 levels among the three groups (data not shown). Therefore, unlike transplantation with B6 donors and BDF1 recipients, transplantation with DBA/2 donors and BDF1 recipients did not provide evidence causing genomic instability. This difference between these two transplantation models was associated with a difference in serum TNF-α levels, because TNF-α levels in DBA/2-into-BDF1 recipients were significantly lower than those in the B6-into-BDF1 recipients ($P = 0.039$).

3.3. GVHD and MN frequencies in unirradiated CBF1 recipients after HCT from parental B6 or BALB/c donors

In unirradiated CBF1 recipients with GVHD induced by injection of spleen and lymph node cells from parental B6, weight gain was retarded until at least day 34 (Fig. 4A). All recipients survived until 40 days after transplantation. On day 40, MN frequencies in CBF1 recipients transplanted with B6 cells were significantly higher than those in untreated CBF1 mice ($P = 0.015$). MN frequencies also showed a trend for higher values in CBF1 recipients when compared to age-matched parental B6 controls, but this trend was not statistically significant ($P = 0.11$) (Fig. 4B). Percentages of donor cells in

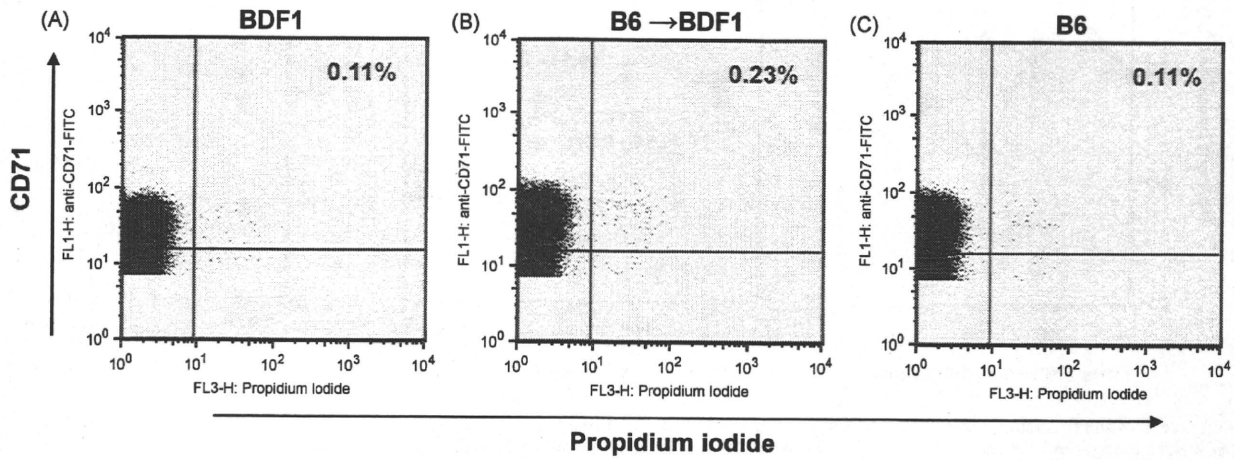

Fig. 2. Typical flow cytometry patterns of reticulocyte MN frequencies in (A) untreated BDF1, (B) BDF1 recipient after HCT from B6 donors, and (C) parental B6 mice. Number in the upper right corner of each cytogram denotes the frequency of reticulocyte MN.

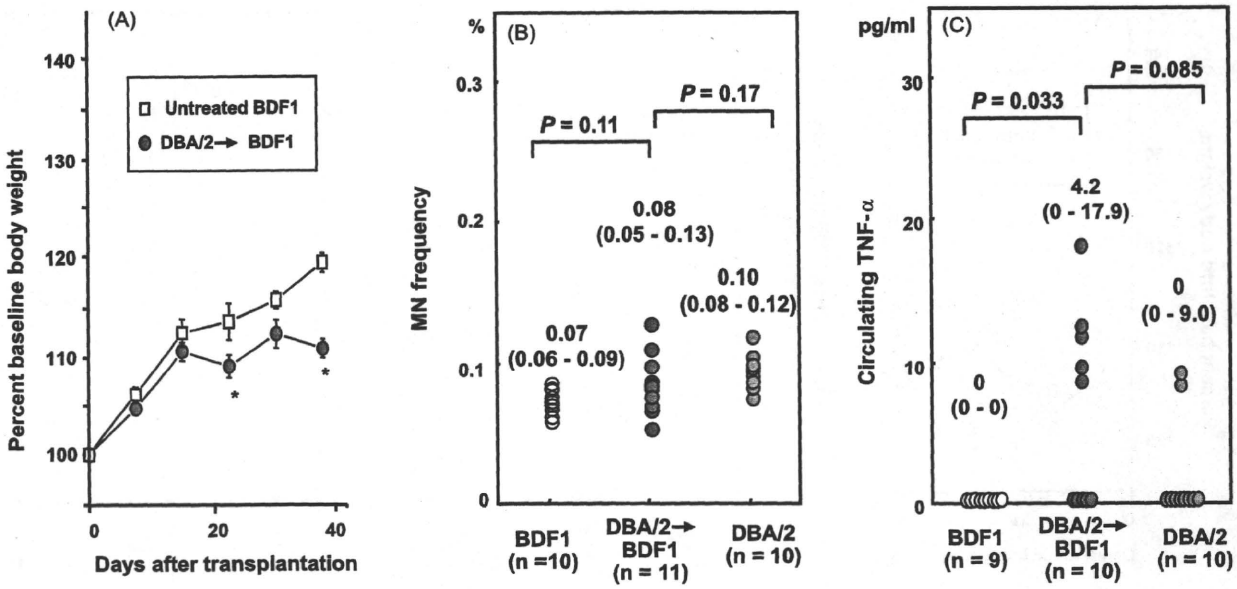

Fig. 3. MN frequencies and circulating TNF-α levels in parental DBA/2 mice, untreated BDF1 mice, and BDF1 recipients after HCT from DBA/2 donors. (A) Weight changes in after HCT (circles) and in untreated (squares) BDF1 controls were monitored once weekly. Each symbol represents the mean in each group of mice. Error bars and asterisks denote SE and significant differences between the groups, respectively. (B) MN frequencies and (C) TNF-α levels were measured on day 40 after transplantation and plotted for each group. Numbers in the vicinity of the symbols denote the median and range (in parentheses).

blood granulocytes and T cells in the recipient mice were 72–100% (median: 100%) and 68–94% (median: 91%), respectively. Circulating TNF-α levels in the B6-into-CBF1 transplant recipients were significantly higher than those in untreated CBF1 mice ($P = 0.029$) (Fig. 4C), and positively correlated with MN frequencies ($r = 0.67$). Transplantation of BALB/c cells into CBF1 recipients did not cause a significant increase in MN frequencies or serum cytokine levels when compared with untreated CBF1 mice. Percentages of donor cells in blood granulocytes and T cells in the recipient mice were 1–21% (median: 9%) and 11–29% (median: 26%), respectively. Therefore, like GVHD in BDF1 mice, transplants from B6 donors caused genomic instability in CBF1 recipients but transplants from BALB/c donors did not.

4. Discussion

We evaluated genomic instability in several parent-into-F1 transplantation models that could induce GVHD. Elevated MN frequency was observed in both BDF1 and CBF1 recipients with parental B6 donors but not in recipients with DBA/2 or BALB/c donors. Differences in types of GVHD may be at least partly involved in the different susceptibility to genomic dam-

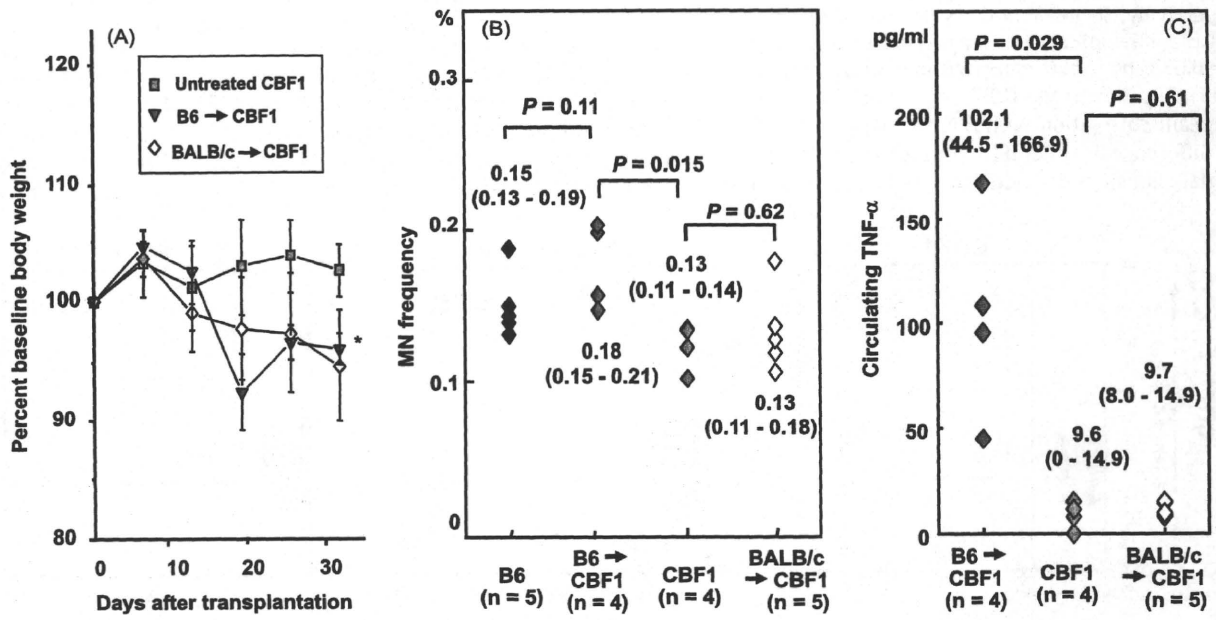

Fig. 4. MN frequencies and circulating TNF-α levels in parental B6 and BALB/c mice, untreated (BALB/c × B6) F1 (CBF1) mice, and CBF1 recipients after HCT from B6 or BALB/c donors. (A) Weight changes in CBF1 mice untreated (squares) and after HCT from B6 (triangles) or BALB/c (diamonds) donors were monitored once weekly. Each symbol represents the average in each group. Error bars denote SE, and the asterisk indicates a significant difference between CBF1 recipients on day 34 after HCT from B6 donors as compared to untreated CBF1 controls. (B) MN frequencies and (C) TNF-α levels were measured on day 40 after transplantation and plotted for each group. Numbers in the vicinity of the symbols denote the median and range (in parentheses).

age among the transplantation models. It is well known that B6-into-BDF1 transplantation induces acute GVHD associated with type-1 immune responses, whereas DBA/2-into-BDF1 transplantation induces chronic GVHD mediated by type-2 immune responses [20–23]. Similar differences in types of GVHD have been reported for B6-into-CBF1 and BALB/c-into-CBF1 models, respectively [24]. Such disease-type differences may also explain the reason why we observed elevated TNF-α levels in both BDF1 and CBF1 recipients with B6 donors but not in those with DBA/2 or BALB/c donors. The level of TNF-α production is genetically controlled, and H-2^b strains express higher levels of TNF-α than H-2^d strains [25]. Thus, TNF-α production appears to be higher when GVHD is caused by B6 donor cells as compared to DBA/2 or BALB/c H-2^d donor cells.

Type-1 immune responses involve inflammatory cells that are potent sources of reactive oxygen species causing DNA-damaging effects to bystander cells [26,27]. In many cell systems, TNF-α signaling is associated with an increase in oxidative stress. In the present study, levels of circulating TNF-α appeared to increase in GVHD mice with elevated MN frequencies, suggesting a possible role of TNF-α in causing MN induction. In support of this hypothesis, TNF-α has chromosomal damaging effects in some systems [14,15]. In addition, an increase in oxidative stress has been noted for hematopoietic cells, including blood erythrocytes and leukocytes, in the B6-into-CBF1 GVHD model [28]. Therefore, TNF-α production in the disease process may be a contributing cause of genetic damage at least in the hematopoietic system of mice with GVHD, although we base this suggestion on correlations and not on results of mechanistic experiments. In further support of that hypothesis, however, we recently found that MN frequencies were dose-dependently elevated in both BALB/c and B6 mice treated with potent TNF-α inducers, including Poly (I-C) and lipopolysaccharide (K.H. & Y.K., manuscript in preparation).

In B6-into-BDF1 model, granulocytes derived from the marrow of F1 recipients were not largely replaced by those derived from parental donors during the first 40 days after transplantation. In addition, the reticulocyte MN frequency was not associated with the level of granulocyte chimerism among the unirradiated F1 recipients (data not shown). We believe that the same holds true for erythroid cells. We found that expression of H-2 class I molecules on reticulocytes was too low to prove the point (data not shown), and we have concerns that assays based on polymorphic mRNA transcripts expressed in reticulocytes (e.g., α-globin) [29], might lack quantitative agreement with actual cell counts. Although the level of chimerism in reticulocytes remains to be determined, it is likely that inflammation by GVHD *per se* induced MN not only in donor cells, but also in recipient-derived cells. The appearance of GVHD-related genomic instability in donor hematopoietic cells might explain the well-documented, but infrequent development of leukemia in donor cells after allogeneic HCT [30–33].

The differences in MN frequencies between GVHD mice and controls appeared to be small as compared to those in MN frequencies between cells exposed and unexposed to genotoxic agents *in vitro*. This may be due largely to the host defense mechanisms that effectively eliminate genetically damaged cells. However, the differences (1.4–1.5-fold increase) that we observed in the present study are almost compatible to the level of genomic instability (1.3–1.6-fold increase) detected with same method in mice one year after whole-body irradiation with 2.5 Gy [18], and amply sufficient to evaluate *in vivo* genetic effects in terms of solid cancer risk. A recent meta-analysis showed a significant 1.45-fold increase of MN frequency in untreated cancer patients (95% confidence interval: 1.28–1.64), compared with a cancer-free population [34]. Evaluation of biomarkers indicating inflammation and genomic instability, and correlation with follow-up data may reveal important clinical associations. Recent advances in high-throughput and automated systems for detecting genetic damage could facilitate such studies [35].

Our study is limited by the reliance on a single method for assessment of genomic damage. The reticulocyte MN assay, however, has the advantages of high sensitivity and validated applicability for measuring genotoxicity *in vivo*. With this assay, acute effects of radiation doses as small as 0.1 Gy are detectable in mice [18]. Assessment of double strand breaks through phosphorylated histone H2AX (γH2AX) focus formation is highly sensitive as an indicator of double strand breaks induced *in vitro*, but the use of this assay for measurement of double strand breaks *in vivo* has not been validated [36]. The flow cytometric version of this assay and the comet assay are both less sensitive than MN frequency for measurement of double strand breaks *in vivo* (K.H. & Y.K., unpublished observation). Our study is also limited by the assessment of genomic damage only in hematopoietic cells. Although we have not shown that GVHD could also induce genomic damage in the recipient tissues affected by GVHD, including the skin and intestine, Faber et al. [37] reported increased microsatellite instability (MSI) in buccal and colon epithelial tissues after allogeneic HCT, but not after autologous HCT. The genetic damage reflected by MSI differs from the double strand breaks that induce MN, but these genomic instabilities are both related to oxidative stress and are thought to be involved in the development of malignancy [38].

In summary, we provide evidence of genomic instability as reflected by reticulocyte MN frequencies in certain strains of F1 mice with GVHD. Analysis of results with different parental donors suggests that type-I inflammatory responses manifested by increased TNF-α levels may be involved in the DNA-damaging process. More detailed understanding of the mechanisms underlying such genomic instability following allogeneic HCT might help to develop approaches for preventing second malignancies after HCT.

Conflict of interest statement

The authors declare no competing financial interests.

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Memory CD4 T-cell subsets discriminated by CD43 expression level in A-bomb survivors

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Abstract

Purpose: Our previous study showed that radiation exposure reduced the diversity of repertoires of memory thymus-derived cells (T cells) with cluster of differentiation (CD)- 4 among atomic-bomb (A-bomb) survivors. To evaluate the maintenance of T-cell memory within A-bomb survivors 60 years after radiation exposure, we examined functionally distinct memory CD4 T-cell subsets in the peripheral blood lymphocytes of the survivors.

Methods: Three functionally different subsets of memory CD4 T cells were identified by differential CD43 expression levels and measured using flow cytometry. These subsets consist of functionally mature memory cells, cells weakly responsive to antigenic stimulation, and those cells functionally anergic and prone to spontaneous apoptosis.

Results: The percentages of these subsets within the peripheral blood CD4 T-cell pool all significantly increased with age. Percentages of functionally weak and anergic subsets were also found to increase with radiation dose, fitting to a log linear model. Within the memory CD4 T-cell pool, however, there was an inverse association between radiation dose and the percentage of functionally mature memory cells.

Conclusion: These results suggest that the steady state of T cell memory, which is regulated by cell activation and/or cell survival processes in subsets, may have been perturbed by prior radiation exposure among A-bomb survivors.

Keywords: *A-bomb, CD4, immunological memory, CD43, flow cytometry, T cell*

Introduction

In humans, immunological memory resides in and is controlled by long-lived lymphocytes, with immunologic memory being maintained at an appropriate level by a constant proliferation of memory thymus-derived cells (T cells) (Dutton et al. 1998). Once subjected to antigenic stimulation, memory T cells tend to divide repeatedly, thus giving rise to greatly expanded clonal populations which may persist for very long periods of time (Maini et al. 1999). Clonally expanded T-cell populations are frequently observed not only in healthy aged persons (Posnett et al. 1994, Fitzgerald et al. 1995, Wack et al. 1998) but also in virally-infected individuals (Eiraku et al. 1998, Silins et al. 1998) and in patients with autoimmune diseases of various types (Fitzgerald et al. 1995, Musette et al. 1996, Waase et al. 1996).

In general, the peripheral blood pool of memory T cells with cluster of differentiation (CD)- 4 appear not to have been significantly affected by radiation exposure among atomic-bomb (A-bomb) survivors. However, there are significant dose-dependent deficits in the naïve T-cell pools (Kusunoki et al. 1998, 2002, Yamaoka et al. 2004). Further, clonal populations originating from peripheral T cells have been identified in blood samples from some of the A-bomb survivors primarily by tracking specific T-cell receptor (TCR) genes and/or chromosome aberrations in memory T-cell populations (Kusunoki et al. 1993, Nakano et al. 2004). In this regard, we have recently reported that the extent of deviation in the TCR repertoire of memory CD4 T cells significantly increased as the intensity of radiation exposure increased (Kusunoki et al. 2003). It seems reasonable, therefore, to assume that A-bomb radiation

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induced the expansion or shrinkage of particular memory T-cell clones, concomitant with a reduced capacity to maintain fully diverse repertoires of helper T-cell memory.

Previously, we have reported that human memory CD4 T cells can be discriminated into three functionally different subsets (M1, M2, and M3) using the human stem cell-associated (HSCA)- 2 monoclonal antibody (mAb) that recognises a sialic acid-dependent epitope on the low molecular mass (~115 kDa) glycoform of CD43 (Ohara et al. 2002, Kyoizumi et al. 2004). The M1 subset consists of functionally mature cells whose CD43 expression is relatively high. The M2 subset expresses moderate levels of CD43, and responds weakly to TCR-mediated stimuli. The M3 subset exhibits relatively low levels of CD43 and is anergic to TCR-mediated stimuli, and prone to spontaneous apoptosis.

In this study, we evaluated the extent to which T-cell memory function is retained in A-bomb survivors by examining the relationships between these memory CD4 T-cell subsets, ageing, and radiation exposure.

Materials and methods

Blood donors

An A-bomb survivor cohort was randomly selected from a group of Hiroshima participants in the Adult Health Study (AHS) at the Radiation Effects Research Foundation (RERF) (Kodama et al. 1996).

For the present study, blood samples of 1132 survivors were obtained, with informed consent, from survivors who participated in the AHS between 2004 and 2008. This study protocol has been approved by the Human Investigation Committee of RERF. We excluded 216 subjects (19% in total subjects) who had been diagnosed with cancer from the current study. Cancer prevalence by dose category was 16% at <0.005 Gy, 21% at 0.005–0.5 Gy, 30% at 0.5–1.0 Gy, and 35% at ≥ 1.0 Gy, and tended to be higher in survivors exposed to higher doses, in accord with a recent observation in the AHS population (Kyoizumi et al. 2005). The age, gender and radiation dose of the remaining 916 survivors whose lymphocyte samples were subjected to data analysis in our study are listed in Table I. Radiation doses are based on the Dosimetry System 2002 (DS02) estimates (Cullings et al. 2006).

Table I. Age, gender, and radiation dose distribution of study population.

Dose (Gy)	Age (yrs)[b] category						
	60–69 yrs		70–79 yrs		≥80 yrs		
	Male	Female	Male	Female	Male	Female	Total
<0.005[a]	27	25	58	84	17	94	305
0.005–0.5	13	33	54	66	22	107	295
0.5–1.0	19	18	20	33	9	49	148
1.0–4.0	28	25	28	35	14	38	168
Total	87	101	160	218	62	288	916

[a]Individuals in this dose category were exposed at distances in excess of 3 km from the hypocenter, and hence received doses that are substantially equivalent to zero. [b]Age at the time of the examinations that were conducted between 2004 and 2008.

Flow cytometry

Mononuclear cell fractions separated by the Ficoll-Hypaque gradient technique were analysed by three-colour flow cytometry using a FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). Fluorescein isothiocyanate (FITC)-labelled HSCA-2 mAb was prepared as described previously (Kyoizumi et al. 2004). PerCP-labelled CD4 mAb and phycoerythrin (PE)-labelled CD45-related O (CD45RO) mAb were purchased from BD-PharMingen (San Diego, CA, USA) and Caltag Laboratories (Burlingame, CA, USA), respectively. Three different memory CD4 subsets were defined: $CD45RO^+$ cells that expressed higher (M1), intermediate (M2), and lower (M3) levels of CD43. For each donor specimen, the window for the M2 subset was set in a range where CD43 level was from ½- to 2-fold of the mean CD43 intensity for $CD45RO^-$ cells, and the windows for the M1 and M3 subsets were set just to the right and left sides of the M2 window, respectively (Figure 1). Note that this method of memory CD4 T-cell subset discrimination was established in a previous study (Ohara et al. 2002) in which functional and phenotypical differences among these subsets were characterised, using a gating procedure (i.e., that involved internal standardisation of fluorescence intensities) that avoided the effects of inter-experimental variability. The percentage of cells in the range of each subset was obtained in a total CD4 T-cell population.

Data analysis

Associations of the percentage of each memory CD4 T-cell subpopulation (*percentage*) with age at time of examination (*age*), gender (*gender*), and radiation dose (*dose*) were analysed using a multiple regression model (Armitage et al. 2002). The method assumed that the percentage of each T-cell subpopulation related to each explanatory variable in a log linear manner:

$$\log(percentage = \alpha + \beta_1(age - 70) + \beta_2 gender + \beta_3 does,$$

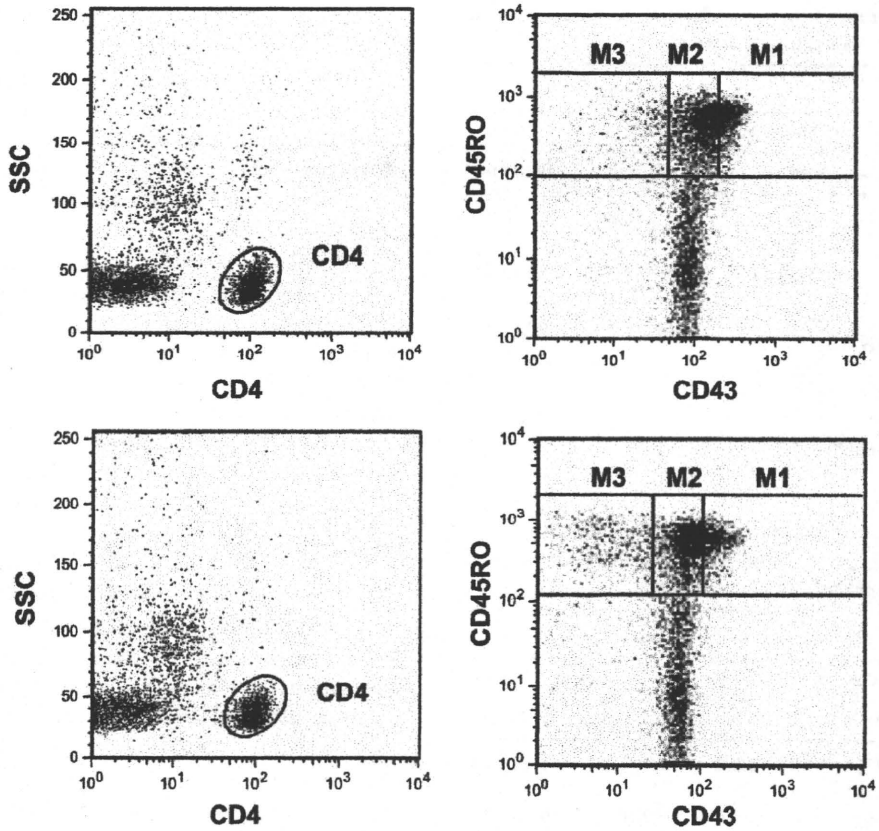

Figure 1. Flow cytometry patterns of CD4 T cells in the peripheral blood of 79-year-old females whose estimated radiation doses were zero (upper) and 0.525 Gy (lower). Peripheral blood mononuclear cells (about 2×10^5) were stained with FITC-labelled CD43 (HSCA-2) mAb, PE-labelled CD45RO mAb, and PerCP-labelled CD4 mAb. CD4 T cells were gated based on side light scattering (SSC) and CD4 intensity (left) and analysed for their expression of CD43 and CD45RO (right). Percentages of memory CD4 T-cell subsets in a total CD4 T-cell population of the unexposed were 24.1 for M1, 30.4 for M2, and 4.8 for M3, and those of the exposed were 17.2 for M1, 35.1 for M2, and 10.6 for M3.

where *gender* is an indicator of female sex, i.e., *gender* = 0 for male and *gender* = 1 for female, and *dose* is radiation dose in grays. The α is a constant term, and β_1, β_2, *and* β_3 are regression coefficients for variables to be estimated. The age term was subtracted by 70 years so that α corresponds to log-transformed percentage of CD4 T-cell subset, i.e., the subset percentage is calculated to be e^{α} (=exponential [α]), for non-irradiated males at 70 years of age. The % change of subset percentage was estimated to be $100(e^{10\beta_1}-1)$ per 10 years increment of age, and $100(e^{\beta_3}-1)$ per 1 Gy radiation dose. This regression analysis in the log linear manner was applied to evaluate the association of the percentage of memory subset within the CD4 T-cell population or CD45RO-positive memory CD4 T-cell population with age or radiation dose.

Results

Figure 1 shows the flow cytometry patterns of memory CD4 T-cell subsets within blood lymphocyte specimens of two age-matched women whose estimated exposure doses were zero and 0.525 Gy, respectively. Crude mean of percentage of each memory T-cell subset within the CD4 T-cell population was shown by age category and by dose category in Tables II and III, respectively. Table IV shows the association of the percentage of each memory T-cell subset with age and radiation dose, in terms of a multiple regression model. The percentage of memory cells (identified and enumerated by CD45RO-positivity) within the CD4 T-cell population appeared to significantly increase with age ($p < 0.0001$), and also with radiation dose ($p = 0.0060$). There was no difference in the percentage of CD45RO-positive memory cells between males and females (data not shown). As for memory T-cell subsets (M1, M2, and M3), the percentage of each subset in the CD4 T-cell population appeared to significantly increase with age ($p < 0.0001$); but again, these percentages did not differ between males and females (data not shown). The percentages of M2 ($p = 0.0001$) and M3 ($p = 0.0096$) subsets were found to significantly increase with radiation dose.

Table II. Crude means of the percentages of memory subsets in the CD4 T-cell population by age category.

	Age category		
	60–69 yrs	70–79 yrs	≥80 yrs
Subset	Mean 65.5 yrs	75.7 yrs	84.8 yrs
CD45 RO (total memory)	48.8 (1.10)[a]	52.2 (0.85)	58.1 (0.87)
M1 (mature, fully competent)	17.3 (0.62)	18.7 (0.49)	22.4 (0.56)
M2 (immature, poorly competent)	26.1 (0.57)	27.4 (0.43)	29.3 (0.45)
M3 (death prone, anergic)	5.3 (0.14)	6.1 (0.17)	6.4 (0.14)

[a]Standard error in parentheses.

Table III. Crude means of the percentages of memory subsets in the CD4 T-cell population by dose category.

	Radiation dose category			
	<0.005 Gy	0.005–0.5 Gy	0.5–1.0 Gy	1.0–4.0 Gy
Subset	Mean 0.0 Gy	0.20 Gy	0.75 Gy	1.74 Gy
CD45 RO	53.3 (0.93)[a]	53.1 (0.99)	55.0 (1.49)	54.6 (1.13)
M1	19.8 (0.57)	20.1 (0.57)	20.0 (0.88)	19.3 (0.72)
M2	27.4 (0.47)	27.2 (0.51)	29.0 (0.75)	29.0 (0.57)
M3	6.1 (0.18)	5.8 (0.14)	6.0 (0.21)	6.3 (0.20)

[a]Standard error in parentheses.

Table IV. Association of the percentages of memory subsets in the CD4 T-cell population with age or dose (multiple regression analysis)[a].

	% change of subset percentage per unit	
Subset	Age (10 years)[b]	Dose (1 Gy)[c]
CD45RO	10.8	4.3
	(8.0, 13.5)[d]	(1.3, 7.3)
	$p < 0.0001$	$p = 0.0060$
M1	14.6	1.3
	(10.1, 19.1)	(−3.7, 6.2)
	$p < 0.0001$	$p = 0.61$
M2	7.3	5.8
	(4.7, 10.0)	(2.9, 8.7)
	$p < 0.0001$	$p = 0.0001$
M3	10.6	4.9
	(7.2, 13.9)	(1.2, 8.6)
	$p < 0.0001$	$p = 0.0096$

[a]Representative memory subset percentage (95% confidence interval) for non-irradiated males at 70 years of age was calculated to be 15.3 (13.9, 16.7) for M1, 24.8 (23.6, 26.0) for M2, and 5.1 (3.9, 6.4) for M3. Note that there was no significant difference in the percentage of total CD45RO-positive memory cells and that of each memory T-cell subset between males and females. [b]Effects of age were estimated for 10 years. [c]Effects of radiation dose were estimated for 1 Gy. [d]95% confidence interval.

These radiation dose-related changes of memory T-cell subsets observed within the CD4 T-cell population may also involve comparable changes within memory subsets of the CD45RO-positive CD4 T-cell population (Table V). The percentages of M1 and M2 subsets in the memory CD4 T-cell population appeared to significantly increase and decrease with age ($p = 0.0085$ and $p < 0.0001$), respectively. In association with radiation dose, there was a statistically significant decrease in the percentage of M1 subset within the CD45RO-positive memory CD4 T cell population ($p = 0.039$). The ratio of the M1 subset to the combined M2 and M3 subsets also significantly decreased with radiation dose ($p = 0.043$), in contrast to a significant increase in this ratio with age ($p = 0.0030$).

Discussion

Our previous study (Ohara et al. 2002) has clearly shown functional differences among M1, M2, and M3 memory T-cell subsets: Cells in the M1 subset have greater capacity to respond to recall antigens (such as tuberculosis purified protein derivative and tetanus toxoid) and to secrete interferon-γ and IL-4 than cells in either of the other subsets; the M2 subset is comprised of memory-type cells that are less mature than cells of the M1 subset, in terms of not only their memory cell function (i.e., recall antigen reactivity and cytokine-producing ability), but also in terms of their chromosomes' telomere length (longer telomeres); and the M3 subset, in contrast to the M2 subset, consists of cells that are anergic to TCR-mediated stimuli and prone to apoptosis. Therefore, an increase in the proportion of these functionally less competent T-cell subsets (i.e., M2 and M3) may

Table V. Comparable changes of memory subsets within CD45RO-positive memory CD4 T-cell population with age or dose (multiple regression analysis).

	% change of subset percentage per unit	
Subset	Age (10 years)[a]	Dose (1 Gy)[b]
M1	3.5	−3.0
	(0.9, 6.1)[c]	(−5.8, −0.2)
	$p = 0.0085$	$p = 0.039$
M2	−3.2	1.5
	(−4.6, −1.8)	(−0.6, 3.0)
	$p < 0.0001$	$p = 0.059$
M3	−0.2	0.6
	(−3.4, 3.0)	(−2.9, 4.1)
	$p = 0.91$	$p = 0.73$
Ratio [M1/(M2+M3)]	6.1	−4.5
	(2.1, 9.0)	(−8.8, −0.1)
	$p = 0.0030$	$p = 0.043$

[a]Effects of age were estimated for 10 years. [b]Effects of radiation dose were estimated for 1 Gy. [c]95% confidence interval.

not be beneficial to the individual in terms of immunological memory to previously encountered foreign antigens. Such preferential expansion of M2 and M3 subsets may also imply an insufficient maturation of antigen-primed CD4 T cells to the fully memory-competent M1 subset within the individuals' immune system. A hypothesis on memory CD4 T-cell differentiation pathways is depicted in Figure 2. After antigen exposure, naive T cells may undergo repeated cycles of cell division and transformation into the premature memory stage M2 cells. The conversion of M2 cells into the fully functioning mature memory stage M1 cells also requires population doublings following antigen exposure. Replication of M1 cells in response to recall antigens is largely responsible for the maintenance of memory functions. M3 cells, by contrast, are likely to be cells that are approaching senescence, and may arise from fully mature M1 cells that have lost survival signals such as cytokine signalling. We can also suspect that premature M2 cells are directly transformed into death-prone M3 cells. Such putative differentiation pathways may be controlled by interaction of memory T cells with antigen-presenting cells and environmental cytokine conditions. Such circumstances of memory T cells are very important to properly maintain immunological memory. In the CD4 T-cell systems of A-bomb survivors, there are at least two possibilities that the differentiation from M2 to M1 cells may be insufficient, and that cell transit from M2 and M1 subsets to apoptotic-prone M3 populations may be enhanced. Effects of radiation on cellular and molecular mechanisms controlling the memory T-cell differentiation pathways remain to be investigated. Taken together, our results suggest that function and maintenance of helper T-cell memory in the immune system of A-bomb survivors might have been compromised, after A-bomb irradiation.

Our previous study has shown that proliferative responsiveness of memory CD4 T cells to recall antigens can be enhanced by triggering cell-surface CD43 molecules with HSCA-2 mAb in vitro (Kyoizumi et al. 2004). That suggests that CD43 molecules play a part in certain of the cell signalling events involved in memory T-cell activation. Further, it is likely that CD43 and CD28 mAbs act synergistically to stimulate CD4 T-cell response to TCR cross-linking in vitro, indicating the co-stimulatory function of CD43 in TCR-mediated activation processes (Kyoizumi et al. 2004). It has also been suggested in the mouse immune system that the up-regulation of CD43 expression can have a negative effect on activation-induced cell death of T cells (He and Bevan 1999). A recent study has indicated that CD43 molecules induce a signalling cascade that prolongs the duration of TCR signal-mediated cell proliferation and cytokine secretion,

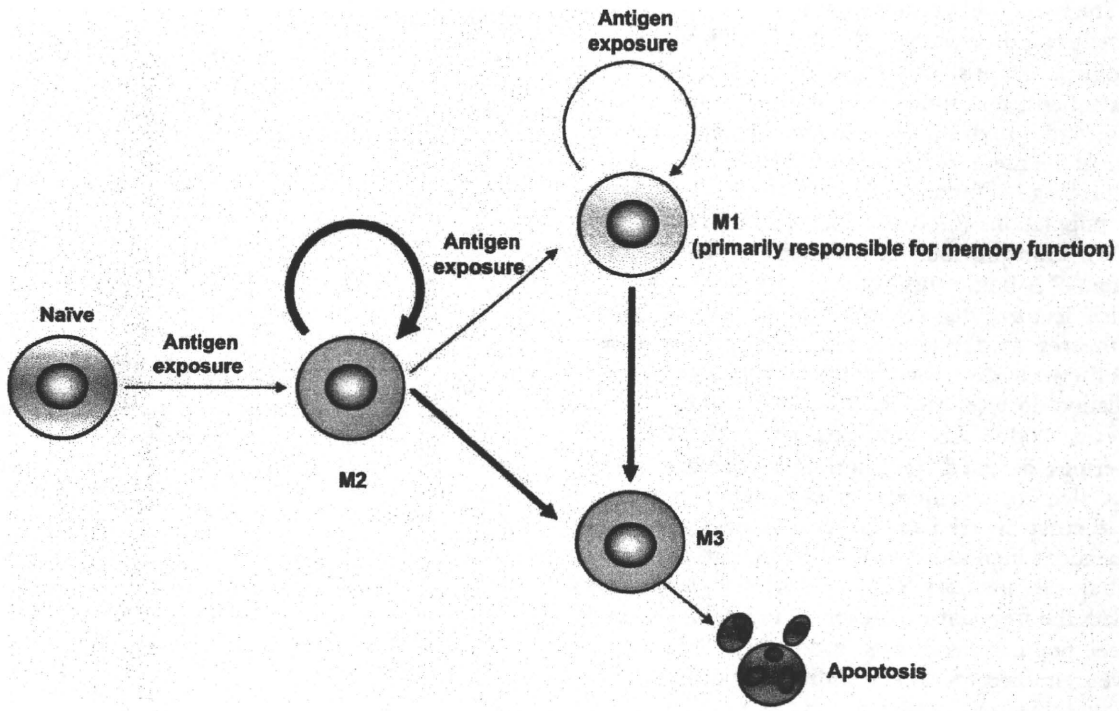

Figure 2. Hypothesised memory CD4 T-cell differentiation pathways for A-bomb survivors. Preferential pathways in the survivors' immune systems are drawn with bold lines.

but that prevents TCR signal-mediated anergy (Fierro et al. 2006). Thus, evidence is accumulating that there are positive effects of CD43-mediated signalling on activation and survival of memory T cells. By contrast, studies that have employed gene disruption techniques have shown that CD43 has either a negative regulatory role (Thurman et al. 1998, Tong et al. 2004), or possibly, plays no significant role in T-cell activation (Carlow et al. 2001). Although the precise mechanism of CD43-dependent regulation of T-cell activation remains to be determined, we have clearly demonstrated that CD43 expression is positively correlated with antigen responsiveness of memory CD4 T cells (Ohara et al. 2002). It is highly likely that the preferential increase in select memory subsets that express lower levels of CD43 (M2 and M3) may be associated with attenuated immune responses to specific pathogens. Levels of immunoglobulin G and A to *Chlamydia pneumoniae* have recently been found to decrease significantly with radiation dose among A-bomb survivors (Hakoda et al. 2006). It would be intriguing to study associations between antigen-specific responses to such ubiquitous pathogens and composition of memory T-cell subsets as defined by the relative level of CD43 expression among A-bomb survivors.

The individual's ability to properly maintain T-cell memory is known to decline with age (Goronzy and Weyand 2005, Weng 2006). This ageing-related immune attenuation is thought to be associated with: (i) The reduction in the size of naïve T-cell pool due to reduced production of new T cells within the involuted thymus, and subsequent, but infrequent entry of antigen-primed cells into the memory T-cell pool, and (ii) divergent antigen recognition repertoire of the memory T-cell pool due to the expansion or shrinkage of functionally incompetent memory T-cell populations (Goronzy and Weyand 2005, Weng 2006). Our previous observations of the immune system of A-bomb survivors are consistent with these typical features that relate to immunological ageing. In this regard, the proportion of naïve CD4 T cells was shown to decrease slightly, but significantly, with radiation dose (Kusunoki et al. 1998, 2002, Yamaoka et al. 2004). Also, the extent to which the TCR repertoire deviated from normal in memory CD4 T cells significantly increased with radiation dose in aged survivors (Kusunoki et al. 2003). An age-dependent increase in the percentage of M1 subset within the memory CD4 T-cell population may reflect the frequent expansion of functional memory T-cell populations in aged individuals. As far as we have examined for several individuals of the present study subjects, clonally expanded populations are largely distributed in M1 subset (Kyoizumi, manuscript in preparation), suggesting that, in aged individuals, only a small population of M1 subset may contribute to recall antigen responses in vivo. The observations in the present study can also be interpreted as an attenuation of helper T-cell memory possibly resulting from radiation-induced perturbation of T-cell homeostasis in A-bomb survivors.

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